Elucidating the mechanisms controlling the carotenoid retention trait in *Capsicum annuum*

Alexandra Claire Holden

This thesis was submitted for the degree of Doctor of Philosophy at Royal Holloway, University of London, August 2019

Declaration of Authorship

I, Alexandra Holden, hereby declare that the work presented in this thesis is the original work of the author unless otherwise stated. Original material used in the production of this thesis has not been previously submitted either in part or whole for a degree of any description from any institution.

Signed: _____

Date: 20th August 2019

Abstract

Fruit colour is a key quality trait in chilli pepper (*Capsicum annuum*), and carotenoids are the major pigments responsible for conferring the red colour observed in pepper fruits. Carotenoids have valuable antioxidant properties. Post-harvest storage of peppers is often required in order for consumer demand to be met, and therefore peppers must retain their quality during storage. An understanding of changes in carotenoid content is essential for high quality carotenoid retaining lines to be bred.

In the present study, the mechanisms underlying the carotenoid retention trait have been investigated. Biochemical profiling, employing both HPLC-PDA and GC-MS has revealed changes in carotenoid content in the fruit of a Doubled Haploid population (375 associated lines), and has revealed that the carotenoid retention trait and intermediary metabolism are not linked. Physiological analysis of fruits has revealed the role of the fruit cuticle in controlling the carotenoid retention phenotype. The fruit cuticle protects fruit from carotenoid degradation by acting as a barrier to harmful oxidative species, which can result in non-enzymatic carotenoid cleavage. Transcriptomic analyses have resulted in the identification of candidate genes underlying the carotenoid retention trait, and a method of Virus Induced Gene Silencing (VIGS) has been established in pepper fruit for functional characterisation of genes, and validated in tomato.

This study sheds light on a previously understudied trait. Understanding the mechanisms underlying carotenoid retention in chilli pepper will not only result in breeding of improved pepper varieties, but this information may also be translated to other crops, which also experience costly post-harvest carotenoid degradation, and consequently, reduced antioxidant properties and nutritional content.

Acknowledgements

First and foremost, I would like to thank Professor Paul Fraser for his supervision of my PhD. Few words can express my gratitude for the opportunities I have been granted whilst working within the Fraser group, and I have learned more than I imagined possible. I am endlessly grateful for the opportunities I have had to develop as a scientist, to meet the international academic community through conferences and collaborations, and to have been given the freedom to explore interesting science. Thank you for always having confidence in me, even when I doubted myself.

I would also like to thank Dr Daniel Rickett, as my supervisor at Syngenta, for the invaluable support and feedback, along with Dr Genny Enfissi and Professor Peter Bramley, for the fascinating discussion that has motivated me to continue exploring new scientific questions.

This project would not have been possible without the support of Syngenta, who have provided both plant material and an array of resources. I am grateful to Dr Chris Stain for his knowledge of, and assistance with microscopy techniques. I would like to thank Dr Aniko Kende for providing the volatile analysis platform, and Dr Julie Green for bioinformatics support of RNAseq analyses.

I am thankful to Professor Asaph Aharoni at the Weizmann Institute, Israel, for hosting me as a visiting student, and to Dr Hagai Cohen, for dedicating time to teaching me the techniques involved in cutin analysis.

I have thoroughly enjoyed the past four years working in the Fraser group, which has always provided a dynamic and entertaining work environment, and I have been fortunate enough to be surrounded by an enthusiastic group of people. I would particularly like to thank Dr Harriet Berry, for establishing pepper research and the basis for this PhD project within the lab, and Diana Donavon for assistance with the VIGS experiments. I would also like to thank Dr Margit Drapal, Dr Laura Perez, and Dr Elliott Price for assistance with metabolomics, and Dr Marilise Noguiera and Dr Juliana Almeida Barros de Silva for teaching me the molecular biology skills that have been essential to this work. I am grateful to all members of the Fraser group, past and present, for the lively discussion and motivation to keep challenging myself. You have not only been great teachers, but also great friends.

To my university friends who I started this academic journey with on a sunny Chapel Quad at Pembroke in 2011; thank you for continuing to bring the same fun, laughter, and adventure, which I have relied on throughout my PhD. Kirsten Goodridge, Kris Blake, Mike Joseph, Emily Allison, Annie Smith, and Ali Marjot: I am always grateful for your friendship. My wonderful house mate and friend, Ellie Warren, deserves a special

thanks for listening to endless evenings of pepper chat, and more so, for being the best rowing pair partner I could hope for. Evenings spent on the river have provided a break from the lab and the opportunity to expel our collective competitive energy.

Mostly, I would like to thank those closest to me. To Richard, thank you for your unending support, encouragement, and understanding in my pursuit of this PhD. To my sister, Charlotte, thank you for always keeping me entertained with laughter when necessary, and for lending a listening ear. This PhD would have been impossible without the support of my extended family. For my Grandparents: Tom and Margaret, whose support of my education and decision to pursue a PhD has been unfaltering, and without whom I wouldn't have achieved as much; and Maureen and George, to whom I promised I would one day become a doctor: I hope I have fulfilled that promise in at least some way. And finally to my parents: Dominic and Dawn, whose constant love, encouragement, and belief in me has always been my driving force in fulfilling my goals.

Contents

List of figures

List of tables

Abbreviations

$^1O_2$	Singlet oxygen
2-MHG	2-mono(10,16-dihydroxyhexadecanoyl)glycerol
3Chl•	Triplet chlorophyll radical
ABA	Abscisic acid
ABC	ATP-binding cassette transporter
AHC	Agglomerative hierarchical clustering
AMD	Age-related macular degeneration
A	Anthesis
AP2	APETELA2
ASTA	American Spice Trade Association
BDG	Bodyguard
BF_3/MeOH	Boron trifluoride methanol
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CAT	Catalase
CCD	Carotenoid cleavage dioxygenase
$CCl_3^•$	Trichloromethyl radical
CCO	Carotenoid cleavage oxygenase
CCS	Capsanthin capsorubin synthase
CD1	Cutin deficient 1
Chl•	Chlorophyll radical
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-associated protein 9
CrtB	Bacterial phytoene synthase
CrtI	Bacterial phytoene desaturase
CS	Capsaicin synthase
cv.	Cultivar
DET1	De-etiolated1
DH	Doubled haploid
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DPA	Days post anthesis
DXP	1-deoxy-D-xylulose 5-phosphate
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
ECR	Trans 2,3-enoyl-CoA reductase
FAE	Fatty acid elongase complex
FAE1	Fatty acid elongation 1
FAS	Fatty acid synthase complex
FDR	False discovery rate
FPP	Farnesyl pyrophosphate
GAP	Glyceraldehyde 3-phosphate
GC-MS	Gas chromatography mass spectrometry
GGPP	Geranylgeranyl pyrophosphate
GGPPS	Geranylgeranyl pyrophosphate synthase
GO	Gene ontology
GOI	Gene of interest
GPAT	Glycerol-3-phosphate acyltransferase
GPP	Geranyl pyrophosphate

GWAS	Genome-wide associated study
H_2O_2	Hydrogen peroxide
HCD	3-hydroxy-acyl-CoA dehydratase
HDR	Hydroxyl-methylbutenyl diphosphate reductase
HDS	Hydroxyl-methylbutenyl diphosphate synthase
HD-Zip IV	Homeodomain-leucine zipper IV
HMBPP	Hydroxyl-methylbutenyl diphosphate
HPLC	High performance liquid chromatography
IPP	Isopentenyl diphosphate
KCR	3-keto-acyl-CoA reductase
KCS	3-keto-acyl-CoA synthase
LACS	Long chain acyl-Coenzyme A
LB	Lysogeny broth
LC-MS	Liquid chromatography mass spectrometry
LCY-b	Lycopene-β-cyclase
LCY-e	Lycopene-ε-cyclase
LOQ	Limit of quantitation
LTP	Lipid transfer protein
MAH1	Midchain alkane hydroxylase
MEcDP	Methylerythritol 2,3-cyclodiphosphate
MeOX	Methoxyamine hydrochloride
MEP	Methylerythritol pathway
MFA	Multiple factor analysis
mQTL	Metabolite quantitative trait loci
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog media
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
MVA	Mevalonic acid pathway
NCED	9-*cis*-epoxycarotenoid dioxygenase
NGS	Next-generation sequencing technology
NIH	National Institute of Health
$O_2^{\bullet-}$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
OR	Orange protein
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PDA	Photodiode array detector
PDS	Phytoene desaturase
PIF	Phytochrome interacting factor
PSY	Phytoene synthase
PTGS	Post-transcriptional gene silencing
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative Polymerase chain reaction
QTL	Quantitative trait loci
rbcL	Ribulose bisphosphate carboxylase large chain subunit
RIN	Ripening inhibitor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
$RS^{\bullet}$	Thiyl radical
SE	Standard error

SEM	Scanning electron microscope
SHN1	Shine1
SHN3	Shine3
siRNA	Short interfering ribonucleic acid
SMRT	Single-molecule real-time sequencing technology
SOB	Super optimal broth
SOC	Super optimal broth with catabolite repression
SOD	Superoxide dismutase
TRPV1	Transient receptor potential cation channel subfamily V member 1
TRV	Tobacco rattle virus
VDE	Violaxanthin deepoxidase
VIGS	Virus Induced Gene Silencing
VLCFA	Very long chain fatty acid
WHO	World Health Organisation
ZDS	ζ-carotene desaturase
ZEP	Zeaxanthin epoxidase
β-CD	β-carotene 15,15'-dioxygenase
β-CO	β-carotene 15,15'-monoxygenase
β-OH	β-carotene hydroxylase

1. Introduction

1.1. *Capsicum annuum*

1.1.1. *Capsicum* genus

The *Capsicum* genus belongs to the Solanaceae family of flowering plants, and is an economically important crop plant. Approximately 20 to 30 species belong to the *Capsicum* genus (Eshbaugh, 1975), which is widely considered to have originated in Bolivia (McLeod et al., 1982, Perry et al., 2007). Of these 20 to 30 *Capsicum* species, five are widely cultivated. These cultivated species are: *C. annuum, C. frutescens L., C. baccatum L., C. pubescens, and C. chinense* (Heiser and Pickersgill, 1969, Perry et al., 2007, Aguilar-Meléndez et al., 2009). The *Capsicum* genus displays a vast array in fruit morphology, with fruits of many shapes, sizes, and colours (Gaur et al., 2016).

1.1.2. Evolution and domestication of *Capsicum*

Whilst the *Capsicum* genus is widely considered to have originated in Bolivia, domestication of *Capsicum* to give rise to the widely cultivated species we see today, is believed to have occurred in central-eastern Mexico (Perry et al., 2007, Kraft et al., 2014). This Mesoamerican region is considered to be one of only two regions in the world, the other being the Fertile Crescent, in which multiple crops originated. Along with *Capsicum,* maize, beans, squash, avocado, and cacao are considered to have originated here (Pickersgill, 2007). *Capsicum* species have been found to have starch grains with unique structures, and therefore these starch grains have been used as microfossils to assist in the determination of domestication site. The oldest unambiguous evidence of domesticated *Capsicum* fruits has been found in caves in the Tehuacán valley in southern-central Mexico, and are believed to date to 5000-6000 BC (Perry et al., 2007). Further evidence to support this location as the site of domestication was found when species distribution modelling, paleobiolinguistics, micro-satellite genetic data, and archaeobotanical data were integrated. These lines of evidence all suggest that the *Capsicum* genus was domesticated in the central-eastern area of Mexico, in which fruit remains were also found in the Tehuacán valley (Kraft et al., 2014).

It is hypothesised that the progenitor of the most common modern cultivated variety: *Capsicum annuum var. annuum*, was the wild variety *Capsicum annuum var. glabriusculum* (Pickersgill, 1971, Pickersgill, 1988). Whilst wild *Capsicum* varieties tend to have small, red, berry-like fruits, which tend to fall from the plant if not eaten by birds, domesticated varieties display a greater variation in fruit shape, size, and flower colour, and will remain attached to the plant even at fruit maturity (Eshbaugh, 1975).

Upon the arrival of Europeans to Mesoamerica and the Caribbean, *Capsicum* was already widespread in this area (Nunn and Qian, 2010). The pepper spread rapidly to

Spain and Africa during the Columbian Exchange of the 15th and 16th centuries, and Columbus noted in his diary in 1493 that there was a lot of chilli present: 'like pepper, but which is worth more than pepper, and everybody does not eat without it, which is very healthy…' (Halikowski Smith, 2015). *Capsicum* peppers then continued to spread to the rest of the Old World, including the East Indies and India by 1542 (Andrews, 1993). *Capsicum* peppers spread across the world in a relatively short space of time: in fewer than 200 years after the discovery of the species by Europeans in Central and South America, peppers were being consumed globally. To date, *Capsicum* peppers are consumed by more than one quarter of the global population each day (Andrews, 1993).

1.1.3. The Solanaceae family

The *Capsicum* genus belongs to the Solanaceae family of angiosperms, which represent one of the most important families of flowering plants. The Solanaceae family includes species such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*), and petunia (*Petunia sps.*), along with the pepper (*Capsicum sps.*). The Solanaceae is a medium sized family, constituting around 3000-4000 species, and 90 genera. A vast array of diversity is observed within this family (Knapp et al., 2004). A molecular phylogeny was created to display the relationship of Solanaceous genera within the Solanaceae family (Särkinen et al., 2013). It is believed the *Capsicum* genus split from the *Solanum* genus, as displayed in the phylogeny, around 19 million years ago (Särkinen et al., 2013).

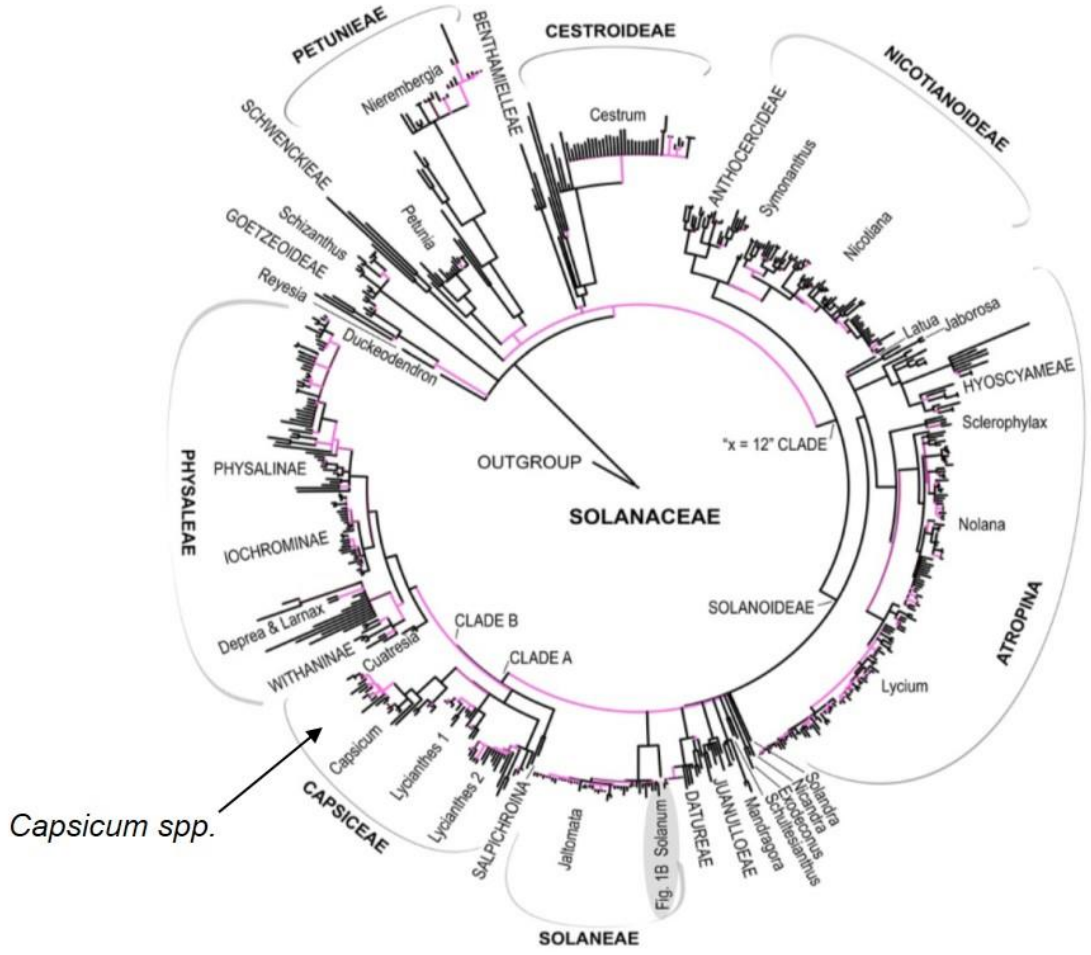


Figure 1-1 Solanaceae phylogeny.

The phylogenetic relationships between major Solanaceae clades, based on Maximum Likelihood analysis of 1076 taxon supermatrix. Clades with low bootstrap support (60-79%) pink, strongly supported clades with high bootstrap support (80-100%) black. Figure adapted from Sarkinen et al 2013.

1.1.4. The use of tomato as a model species for pepper

The tomato is widely regarded to be a model for fleshy fruit species, and whilst the tomato is a close relative of pepper, there are advantages and disadvantages to using tomato as a model for pepper. A comparison of the tomato and pepper genomes revealed an overwhelming conservation in marker order within the genome, however there were some seemingly random interruptions in synteny observed in the pepper (Livingstone et al., 1999), and it is believed that some genomic rearrangement occurred between the pepper and tomato (Rinaldi et al., 2016). Several traits were domesticated in the same way between the pepper and tomato, for example fruit size, in which loci for this trait colocalise between the two traits. Therefore it is thought that similar genes were selected for in the domestication process (Paran and van der Knaap, 2007). However, other traits are species-specific to either the tomato or pepper. The pepper has a pungency trait, which does not exist in the tomato, and there are distinct carotenoid profiles in the fruit of tomato and pepper. This suggests

that whilst the tomato is a useful model species for some pepper traits, it is important to consider that pepper traits may be independent of their tomato counterparts.

1.1.5. Value and importance of *Capsicum* fruit

Chilli peppers are globally the most widely grown spice product (Kraft et al., 2014), and are consumed as both a fresh product, and in the form of a dried powder. Global production of peppers reached 34.6 million tons of fresh fruit, and 3.5 million tons of dried pods in 2011 (Qin et al., 2014). In contrast, 180 million tons of tomato were grown in 2017 (FAOSTAT, 2017). Humans have used wild chillies as a food source since around 8000 BC (Aguilar-Meléndez et al., 2009), and they have become an important component of diverse diets around the world. Large areas of land are devoted to production of pepper, in countries such as Mexico, India, Korea, and China (Crosby, 2008). Along with its use as a food source, *Capsicum* has been noted for its nutraceutical properties. The pepper has been shown to have high levels of phytochemicals, which are regarded to have human health benefits. Peppers contain high levels of antioxidative compounds, including carotenoids, flavonoids, ascorbic acid, and capsaicinoids (Bosland and Votava, 2000). Further to this, pepper fruits have been used for thousands of years for pain relief, and more recently, the pharmaceutical industry has exploited capsaicin, derived from pepper, as an analgesic (Bosland and Votava, 2000).

1.2. Quality traits in pepper and other fruit crops

As pepper is a valuable crop plant, several fruit quality traits determine the value of the fruit to the consumer. These traits include colour, taste, pungency, and appearance.

1.2.1. Colour traits

The wide array of colours, including red, yellow, and orange, observed in pepper fruits is conferred by the presence of the coloured compounds: carotenoids (Curl, 1962). Brightly coloured peppers, with a uniform colour distribution, are considered to be of the highest quality to consumers. Along with the colour profile of freshly harvested fruits, pepper powder colour and the ability of pepper fruits to retain their colour for long periods of time are equally important pepper fruit quality traits. The appearance of peppers is important to consumers, as brighter fruits are more attractive, and this is often the only means by which consumers can distinguish between peppers at the point of purchase.

1.2.2. Taste traits

Whilst the taste of chilli pepper is generally considered to be masked by the ‘spicy’ flavour conferred by capsaicinoids, these compounds are not perceived by odour or

taste receptors, but by the nociceptive pain receptors (Guzman et al., 2011). 64 volatile compounds were identified in fresh bell peppers using gas chromatography-mass spectrometry (GC-MS) (Luning et al., 1994). Using solid phase microextraction (SPME), it was found that volatiles classified as 'sweet' and 'fruity', such as 2,3-butanedione, 3-carene, trans-2-hexanal, and linalool, increased in chilli peppers during ripening. In contrast, hexanal, the 'green aroma' volatile, and 2-isobutyl-3-methoxypyrazine, the 'grassy aroma', decreased during the ripening process of chilli peppers (Mazida et al., 2005).

1.2.3. Pungency traits

The unique 'spiciness', or pungency, of *Capsicum* peppers is conferred by the presence of a family of alkaloids, known as capsaicinoids (Nelson and Dawson, 1923). The presence of capsaicinoids in pepper fruits is believed to have evolved in order to deter frugivory by mammals (Tewksbury and Nabhan, 2001). Capsaicinoids bind nociceptive nerve receptors, known as TRPV1 receptors, in mammals, and cause a burning sensation (Caterina et al., 1997). Eight capsaicinoids have been identified in pepper, however, capsaicin and dihydrocapsaicin are the two major capsaicinoid compounds, constituting over 90 % of all capsaicinoids in pepper fruit (Kozukue et al., 2005). The structures of capsaicin and dihydrocapsaicin are shown in Figure 1-2.

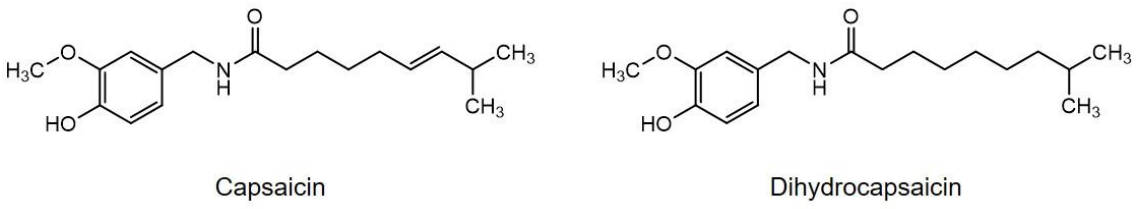


Figure 1-2 Capsaicinoid structures.

Capsaicinoids are synthesised in pepper fruits as a result of a condensation reaction between a molecule of vanillylamine and a branched chain fatty acid. Vanillylamine is derived from phenylalanine, through the phenylpropanoid pathway, and the branched chain fatty acids are derived from either valine or leucine (Curry et al., 1999). Much work has been carried out to determine the biosynthesis of the capsaicinoids, and molecular biology studies have identified the enzymes involved in the synthesis of these compounds (Bennett and Kirby, 1968, Fujiwake et al., 1982, Sukrasno and Yeoman, 1993, Stewart Jr et al., 2005, Mazourek et al., 2009)(Figure 1-3). The *Pun1* locus was identified as involved in capsaicinoid biosynthesis, and is believed to encode an acyltransferase (Stewart Jr et al., 2005).

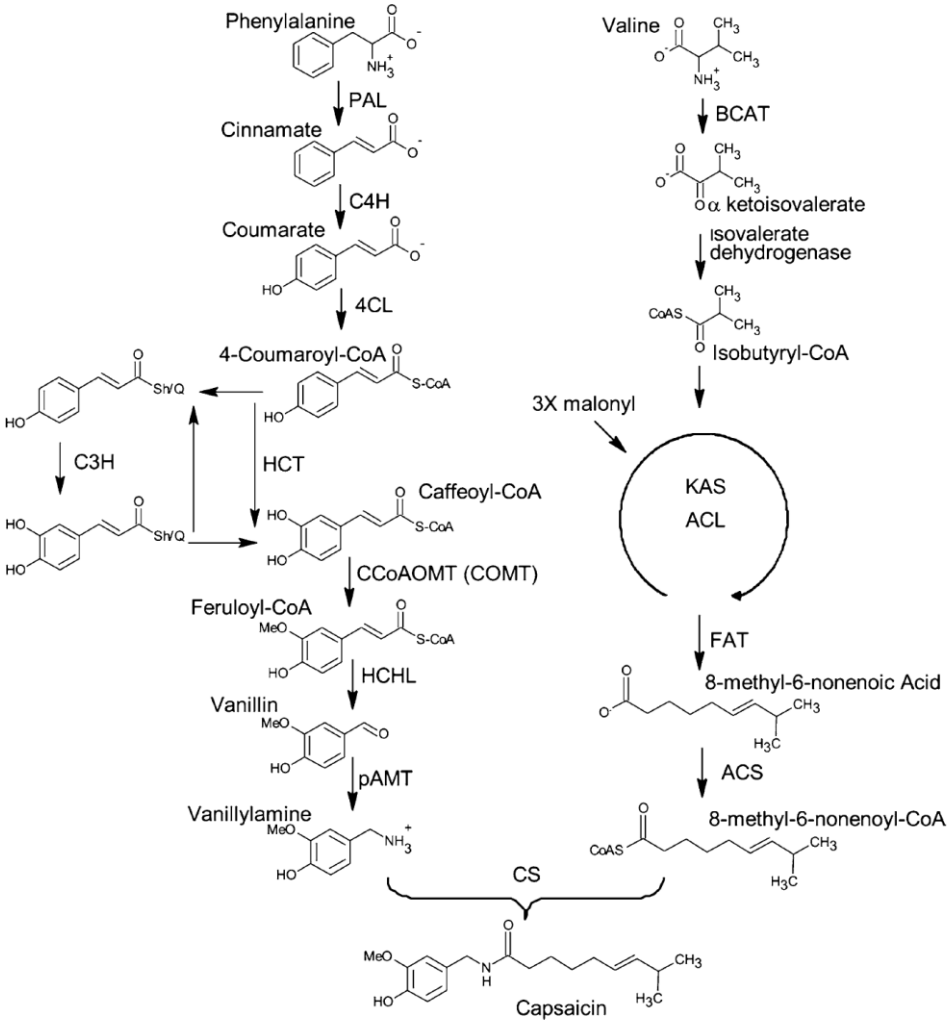


Figure 1-3 Capsaicinoid biosynthesis pathway.

Capsaicinoid biosynthesis is a result of a condensation between vanillylamine and a fatty acid. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; HCT, hydroxycinnamoyl transferase; C3H coumaroyl shikimate/quinate 3-hydroxylase; CCoAOMT, caffeoyl-CoA 3-O-methyltransferase; COMT, caffeic acid O-methyl transferase; HCHL, hydroxycinnamoyl-CoA hydratase/lyase; pAMT, putative aminotransferase; BCAT, branched-chain amino acid transferase; KAS, ketoayl-ACP synthase; ACL, acyl carrier protein; FAT, acyl-ACP thioesterase; ACS, acyl-CoA sythetase; CS, capsaicin or capsaicinoid synthase. Figure adapted from (Aza-González et al., 2011).

Capsaicinoid biosynthesis is known to occur in fruit placental epidermal cells (Suzuki et al., 1980). Pungency is a key quality trait in pepper, as consumers often use peppers as a 'spicy' condiment, and therefore, require fruits with the pungent trait.

1.3. Carotenoids

1.3.1. Carotenoid structure and nomenclature

As stated previously, pepper colour is conferred by the presence of red, yellow, and orange compounds, known as carotenoids (Curl, 1962). Carotenoids are lipophilic compounds, and are classed as tetraterpenes, due to their structure. These tetraterpenes are synthesised by photosynthetic plants, algae, and microorganisms.

Animals are unable to synthesise carotenoids *de novo* and therefore, carotenoids present in animals occur as a result of dietary intake. More than 750 carotenoid structures have been characterised to date (Maoka, 2009), and these display an array of structural modifications (Fraser and Bramley, 2004). Carotenoids are composed of eight isoprenoid units, which are all joined in a head to tail manner, apart from the isoprenoid units at the centre of the carbon chain, in which the double bond order is reversed. This results in the methyl groups 20 and 20' with a 1,6 positional relationship, whilst remaining methyl groups have 1,5 positions (Fraser and Bramley, 2004). The carbon skeleton is symmetrical around the middle, and numbering starts from each end of the molecule, moving towards the centre. The conjugated double bond structure found in carotenoids results in a chromophore, the length of which determines the absorption spectrum of the molecule, and therefore the perceived colour (Fraser and Bramley, 2004) (Figure 1-4).

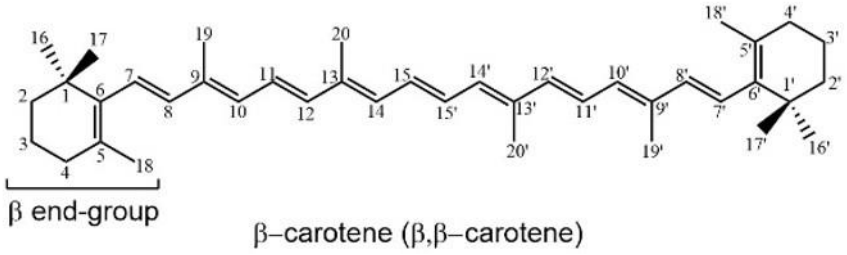


Figure 1-4 Carotenoid nomenclature.

The carotene skeleton dictates carotenoid nomenclature. End groups attached to the skeleton determine the name and nature of the compound. Numbering of the carbon atoms begins at either end of the molecule, and the structure is symmetrical around the centre.

Carotenoids are either classed as carotenes, containing only carbon and hydrogen atoms, and xanthophylls, which contain at least one oxygen atom (Stahl and Sies, 2003). Seven different end groups are observed in carotenoid compounds, although only four end groups are observed in higher plants (β, ε, κ, ψ) (Britton, 1995) (Figure 1-5). *Cis/trans* isomers are observed in carotenoids, as a result of the number of double bonds present, resulting in several *cis/trans* configurations. Carotenoids are lipophilic compounds that accumulate in lipophilic cellular compartments, such as membranes and lipoproteins (Stahl and Sies, 2003).

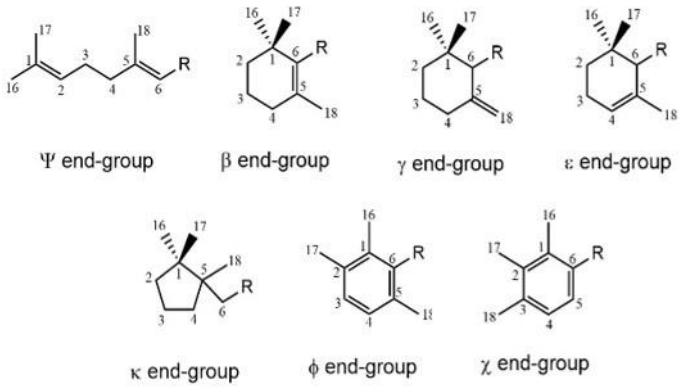


Figure 1-5 Seven possible carotenoid end group structures.

1.3.2. Carotenoid biosynthetic pathway

1.3.2.1. MEP pathway

Carotenoids are synthesised in the plastids of photosynthetic organisms, and are derived from isoprenoids, which are a class of natural products, consisting of more than 55,000 members (Ajikumar et al., 2008, Zhao et al., 2013). Isoprenoids are synthesised from two precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Two biosynthetic pathways have been identified as producing these precursors: the methylerythritol phosphate (MEP) pathway, and the mevalonic acid (MVA) pathway (Zhao et al., 2013). Both pathways are present in higher plants, although they are localised to different cellular compartments. The MEP pathway is localised to the chloroplast, whilst the MVA pathway is found in the cytoplasm of the cell (Hemmerlin et al., 2003). As carotenoids are synthesised and localised to the plastid, plastid-derived IPP, from the MEP pathway, is utilised in the biosynthesis of carotenoids (Rohmer, 1999). For decades, the MVA pathway was believed to be the sole pathway responsible for IPP formation. However, after discrepancies were evident from labelling experiments performed on the MVA pathway, further retrospective labelling led to the discovery of a non-mevalonate pathway for isoprenoid biosynthesis (Eisenreich et al., 2001). Functional characterisation of candidate genes has led to the full elucidation of the plastid IPP and DMAPP forming pathway, which has been termed the 'MEP pathway'. An array of isoprenoids are derived from the substrates: IPP and DMAPP, including monoterpenes, sesquiterpenes, triterpenes, and carotenoids. The synthesis of many of these compounds is partitioned by cellular compartmentalisation (Figure 1-6) (Nogueira et al., 2018).

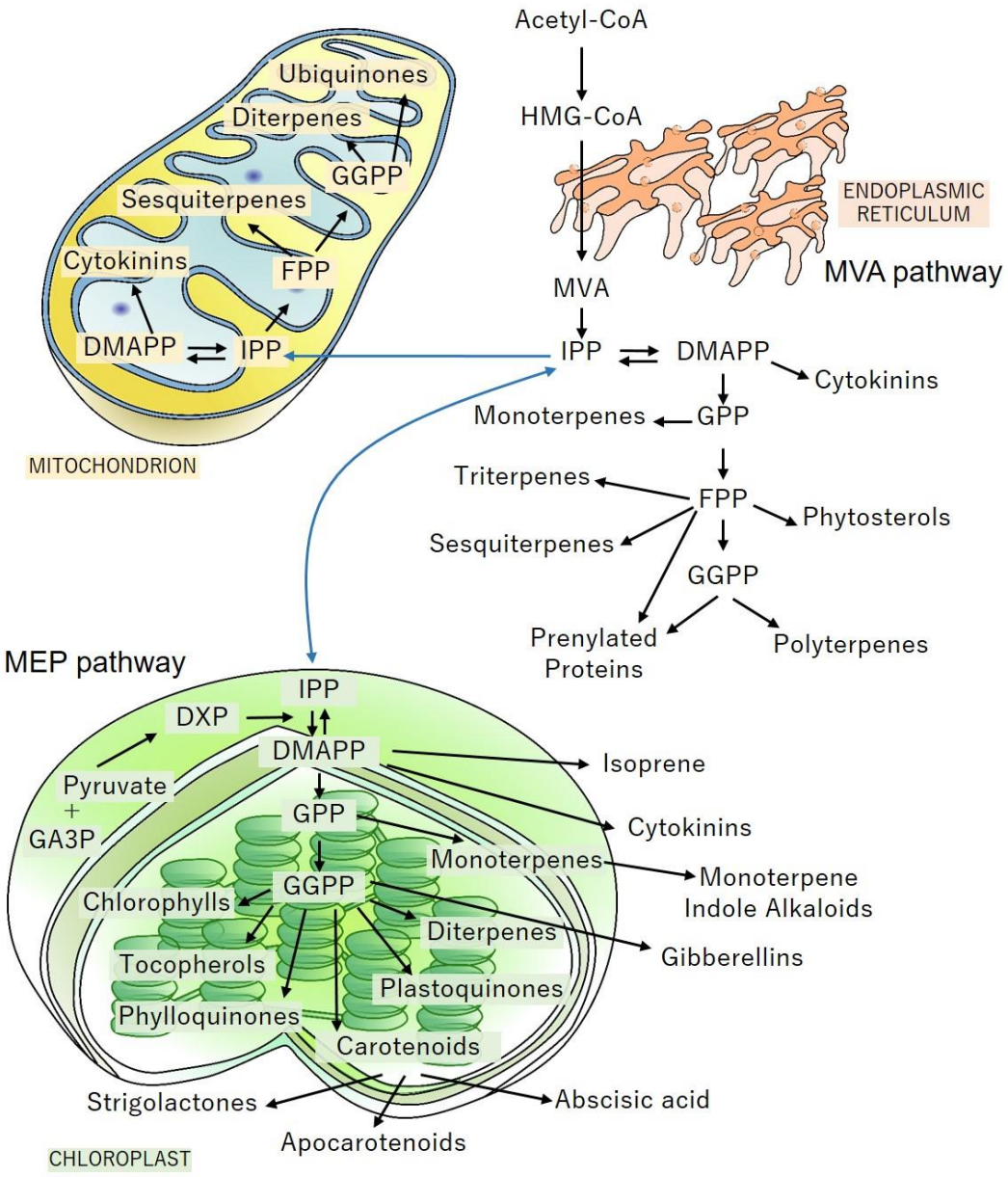


Figure 1-6 MEP and MVA pathways.

The MEP pathway is localised to the chloroplast, whilst the MVA pathway is localised to the cytoplasm of the cell. An array of metabolites are produced, using IPP and DMAPP as precursors. Figure adapted from (Nogueira et al., 2018).

Seven enzymatic steps are present in the MEP pathway, beginning with the synthesis of 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and glyceraldehyde 3-phosphate (GAP), which is catalysed by the enzyme: 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Rohmer et al., 1996, Sprenger et al., 1997). DXP is then converted to MEP by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which is subsequently converted to the cyclic intermediate methylerythritol 2,3-cyclodiphosphate (MEcDP) following three enzymatic steps, including cytidylation (CTP-dependent), phosphorylation (ATP-dependent), and cyclisation. MEcDP is then converted to hydroxyl-methylbutenyl diphosphate (HMBPP) by HMBDP synthase (HDS), and is

finally reduced to IPP and DMAPP by HMBDP reductase (HDR) (Banerjee and Sharkey, 2014) (Figure 1-7).

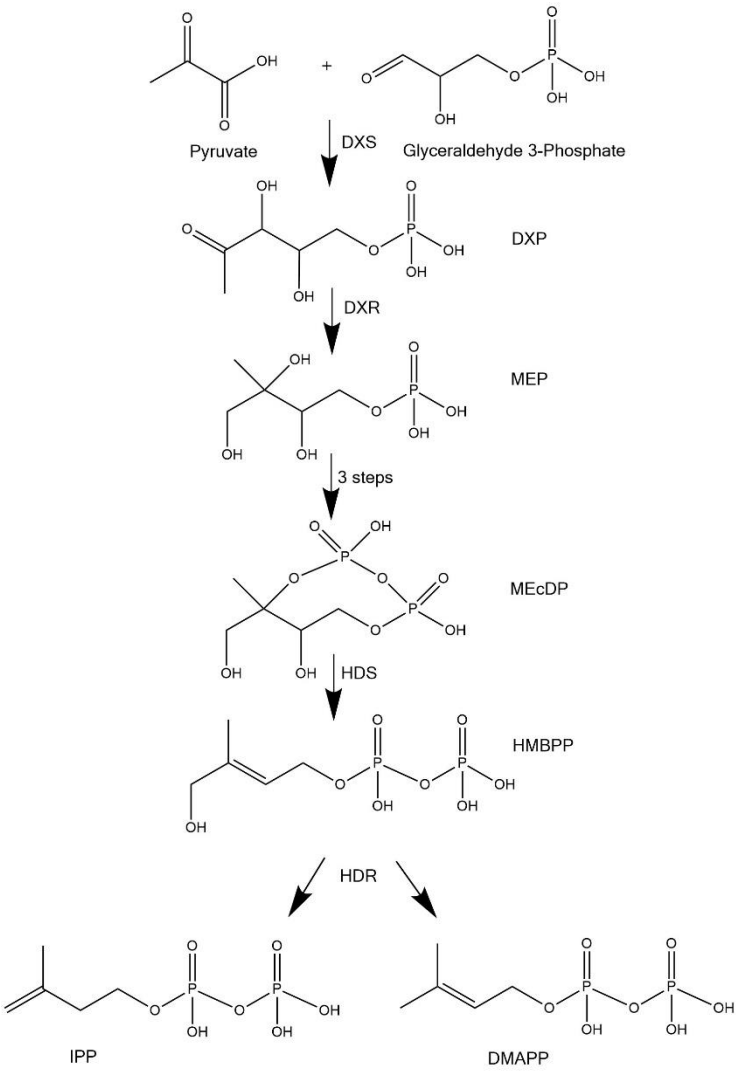


Figure 1-7 MEP pathway.

Biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the MEP pathways from precursors pyruvate and glyceraldehyde 3-phosphate. Abbreviations: DXS = 1-deoxy-D-xylulose-5-phosphate synthase, DXR = 1-deoxy-D-xylulose-5-phosphate reductoisomerase, HDS = hydroxyl-methylbutenyl diphosphate synthase, HDR = hydroxyl-methylbutenyl diphosphate reductase, DXP = 1-deoxy-D-xylulose 5-phosphate, MEP = methylerythritol phosphate, MEcDP = methylerythitol 2,3-cyclodiphosphate, HMBPP = hydroxyl-methylbutenyl diphosphate, IPP = isopentenyl diphosphate, DMAPP = dimethylallyl diphosphate. Figure adapted from (Banerjee and Sharkey, 2014).

1.3.2.2. Carotenoid biosynthesis

From IPP and DMAPP, all isoprenoids are synthesised, including a vast array of plant secondary metabolites, including all terpenes, as well as cytokinins. The immediate precursor of carotenoids is geranylgeranyl pyrophosphate (GGPP). GGPP is synthesised from four C_5 isoprene units, hence making GGPP: a C_{20} compound. IPP and DMAPP condense to form geranyl pyrophosphate (GPP) (C_{10}). Addition of a further IPP molecule to GPP forms farnesyl pyrophosphate (FPP) (C_{15}), and an

additional IPP molecule results in the formation of GGPP, catalysed by GGPP synthase (GGPPS) (Dogbo and Camara, 1987). The condensation of two GGPP molecules results in the formation of phytoene (C_{40}), by action of the phytoene synthase (PSY) enzyme (Romer et al., 1993), which utilises all-*trans* GGPP, and yields predominantly 15-*cis* phytoene (Fraser and Bramley, 2004) (Figure 1-8). This is the first committed step in carotenoid biosynthesis, and yields phytoene, a colourless compound, which contains three conjugated double bonds (Fraser and Bramley, 2004).

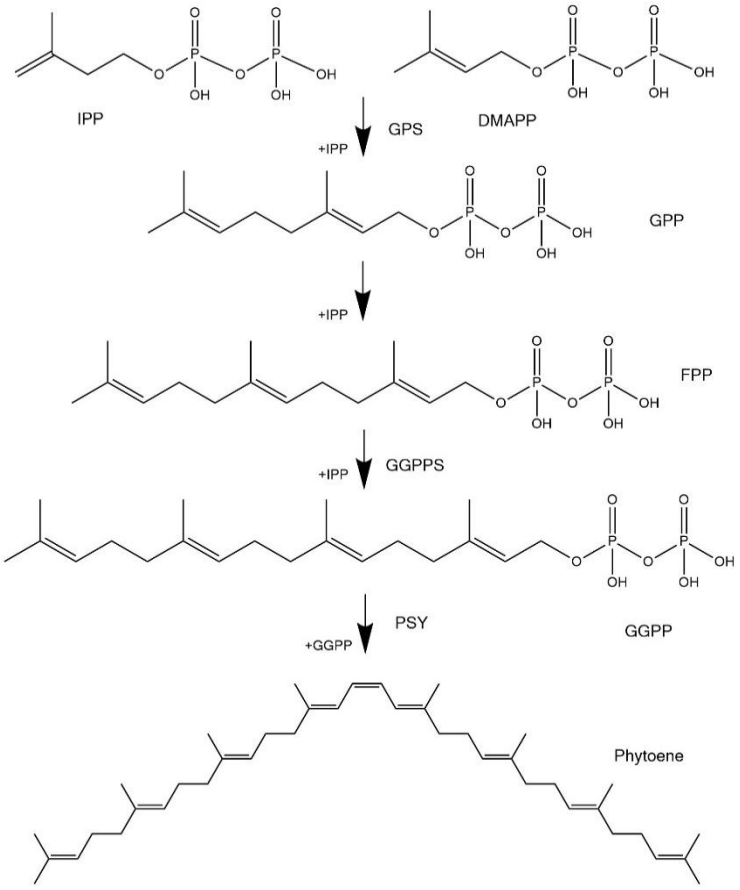


Figure 1-8 Phytoene biosynthesis.

Biosynthesis of 15-cis phytoene from precursors IPP and DMAPP. Abbreviations: GPS = geranyl pyrophosphate synthase, GGPPS = geranylgeranyl pyrophosphate synthase, PSY = phytoene synthase, IPP = isopentenyl diphosphate, DMAPP = dimethylallyl diphosphate, GPP = geranyl pyrophosphate, FPP = farnesyl pyrophosphate, GGPP = geranylgeranyl pyrophosphate. Figure adapted from (Fraser and Bramley, 2004).

The characteristic colour of carotenoids is produced as a result of a series of conjugated double bonds, which modify the basic phytoene structure, creating the chromophore responsible for the observed colour. Desaturation reactions, catalysed by the phytoene desaturase (PDS) enzyme, are responsible for the formation of the conjugated double bonds. Four desaturation reactions occur in pepper, forming phytofluene, ζ-carotene, neurosporene, and lycopene (Fraser and Bramley, 2004). PDS catalyses the desaturation of phytoene to ζ-carotene, via phytofluene (Hugueney

et al., 1992), whilst ζ-carotene desaturase (ZDS) catalyses the desaturation of ζ-carotene to lycopene, via neurosporene (Albrecht et al., 1995).

Further, it was found that isomerisation reactions also contribute to carotenoid biosynthesis in higher plants. In higher plants, phytoene predominantly exists as the 15-*cis* isomer, whilst the predominant geometric isomer of lycopene is all-*trans*. Therefore, isomerisation must occur during the carotenoid biosynthetic pathway. Using map-based cloning, *Crtiso*, an isomerase, was isolated from the *tangerine* tomato mutant, which accumulates prolycopene (7Z,9Z,7'Z,9'Z-tetra-*cis*-lycopene), as opposed to all-*trans* lycopene that is observed in wild type tomato (Isaacson et al., 2002). When *Crtiso* was co-expressed with *PDS* and *ZDS* in *E. coli*, all-*trans* lycopene was observed (Park et al., 2002).

Cyclisation further modifies the carotenoids, and occurs when six-membered rings are added to one, or both ends of the carotenoid precursor. Cyclic end groups may take either the α- or β- form, dependent on the position of the double bond within the cyclohexane ring (Fraser and Bramley, 2004). The type of ring added is dependent on the nature of the cyclase enzyme: lycopene-β-cyclase (LCY-b) results in the introduction of β- rings (Pecker et al., 1996, Hugueney et al., 1995), whilst lycopene-ε-cyclase (LCY-e) results in the introduction of ε- rings (Cunningham and Gantt, 2001). In order for the formation of α-carotene, both LCY-e and LCY-b must act (Figure 1-9).

1.3.2.3. Xanthophyll formation

Further carotenoid modification occurs in the form of hydroxylation and epoxidation reactions, resulting in the formation of xanthophylls. Hydroxylation of α- and β-carotene results in lutein and zeaxanthin synthesis respectively. Hydroxylation of β-carotene to form zeaxanthin through the introduction of hydroxyl moieties is catalysed by a β-carotene hydroxylase enzyme (CrtRB-2) (Bouvier et al., 1998). Zeaxanthin is subsequently epoxidated to violaxanthin, through the mono-epoxidated intermediate antheraxanthin, and this occurs through introduction of 5,6-epoxy groups into the 3-hydroxy-β-rings. This reaction is referred to as the violaxanthin cycle (Yamamoto et al., 1962), as de-epoxidation may convert violaxanthin back to zeaxanthin, and therefore the reaction is reversible. Zeaxanthin epoxidase (ZEP) catalyses the epoxidation of zeaxanthin to violaxanthin (Bouvier et al., 1996), whilst violaxanthin de-epoxidase (VDE) catalyses the de-epoxidation of violaxanthin to zeanxanthin (Rockholm and Yamamoto, 1996). Neoxanthin is formed from violaxanthin, whereby an epoxy group is rearranged to a 5-hydroxy group (Fraser and Bramley, 2004) (Figure 1-9).

1.3.2.4. Pepper carotenoids

Crucial to peppers are the carotenoids: capsanthin and capsorubin, which are responsible for the red colour of chilli peppers. Capsanthin and capsorubin appear to be relatively unique to the ripe fruit of peppers, and are not found in chlorophyll-containing tissues of higher plants. They contain a cyclopentane ring, formed from the 3-hydroxy-5,6-epoxy β-rings of violaxanthin and antheraxanthin. The enzyme catalysing this reaction is capsanthin/capsorubin synthase (CCS), which has been purified from pepper chromoplasts. The *CCS* gene has been shown to resemble the *LCY*-b gene (Bouvier et al., 1994), and has cyclase enzymatic activity (Hugueney et al., 1995) (Figure 1-9).

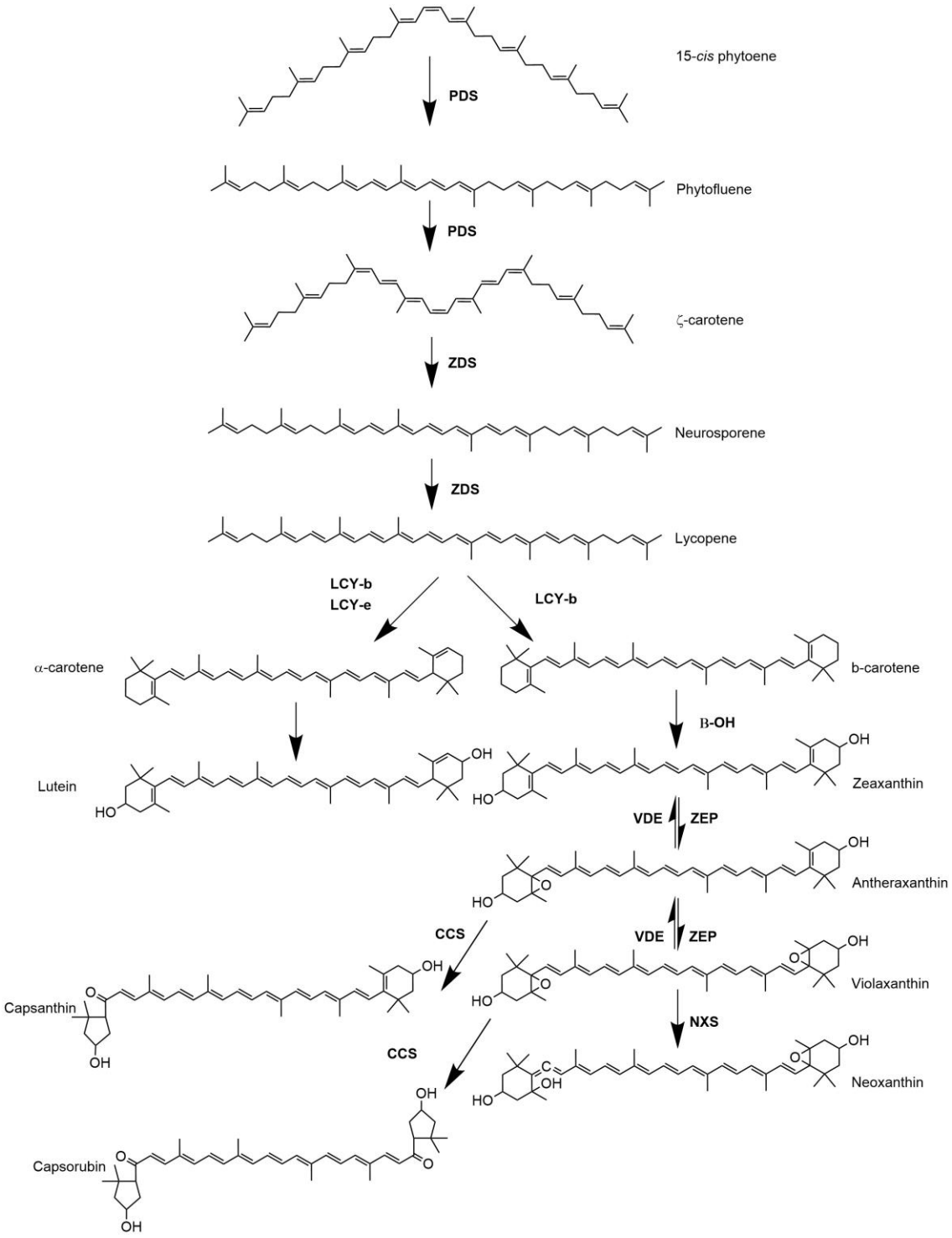


Figure 1-9 Carotenoid and xanthophyll biosynthesis.

Biosynthesis of carotenoids and xanthophylls from precursor 15-cis phytoene. Abrreviations: PDS = phytoene desaturase, ZDS = ζ-carotene desaturase, LCY-b = lycopene-ϐ-cyclase, LCY-e = lycopene-ε-cyclase, ϐ-OH = ϐ-carotene hydroxylase, ZEP = zeaxanthin epoxidase, VDE = violaxanthin de-epoxidase, NXS = neoxanthin synthase, CCS = capsanthin/capsorubin synthase.

1.3.3. Regulation of carotenoid biosynthesis

The regulation of carotenoid biosynthesis in pepper is complex, due to the central role that carotenoids play in plant development and adaptation. This would suggest that the biosynthesis of these compounds is closely linked to other developmental processes

within the plant. Furthermore, as carotenoid biosynthesis mainly occurs during fruit ripening, during which many changes occur within the plant, regulation of the synthesis of these compounds is highly influenced by an array of endogenous and environmental stimuli. Consequently, carotenoid biosynthesis is regulated by an array of mechanisms, including genetics, transcription, post-transcription, metabolite feedback, storage, and degradation (Cazzonelli and Pogson, 2010).

1.3.3.1. Three loci model of pepper fruit colour

Genetic regulation in pepper initially determines carotenoid biosynthesis. A three loci model has been proposed to determine the inheritance of mature fruit colour in pepper (Hurtado-Hernandez and Smith, 1985). Three loci: *Y, C1,* and *C2*, were hypothesised to determine fruit colour, with a genotype of $y^{+}c1^{+}c2^{+}$ resulting in red fruit, and $y^{-}c1^{-}c2^{-}$ resulting in white fruit. The mechanisms underlying this proposed model have been extensively studied, although some questions remain to be answered, and the validity of the model has been questioned due to discrepancies. Studies have shown that, according to the three loci model, the *Y* locus encodes the *CCS* gene (Lefebvre et al., 1998), whilst the *C2* locus encodes the *PSY1* gene (Huh et al., 2001). The third locus of this model, *C1,* remains to be elucidated. The elucidation of these loci has resulted in much research being conducted into explaining the observed variation in pepper fruit colour based on allelic variation in *PSY1* and *CCS*. Several mutations within the *CCS* gene have been identified in yellow-fruited peppers resulting in a premature stop codon (Ha et al., 2007, Popovsky and Paran, 2000). Interestingly, the orange coloured pepper variety, Fogo, displayed early translational termination of *CCS* (Guzman et al., 2010). This suggests the complexity of the control of colour phenotype, as allelic variation in *CCS* did not result in a specific fruit colour (Jeong et al., 2019). Further to this, a splicing mutation in *PSY1* resulted in impaired enzyme activity and the production of orange fruits in the variety *C. chinense* 'Habanero' (Kim et al., 2010). These allelic variations observed in *PSY1* and *CCS* do not fully explain pepper fruit colour variation. Many studies have attempted to elucidate the role of the third locus, *C1*, however, the identity of a candidate gene remains unknown. Consequently, allelic variation in candidate carotenoid biosynthetic genes has been more thoroughly investigated using next-generation sequencing (NGS) technology, known as single-molecule real-time (SMRT) sequencing technology (Jeong et al., 2019). This technology provides read lengths of over 10 kb, which is much longer than previously possible, and has a faster run time. A target gene with a length of less than 10 kb can be sequenced using a single SMRT sequencing run (Rhoads and Au, 2015). 94 pepper accessions were identified, and the coding regions of carotenoid biosynthetic genes: *PSY1, PSY2, Lcyb, CrtZ-2, ZEP,* and *CCS* were analysed within these

accessions using SMRT sequencing. Diverse allelic variation was detected in these genes, including novel allelic variations. Critical mutations, defined as those expected to abolish the functioning of the candidate genes, including non-sense mutations and indels in coding regions resulting in frame-shifts and deletions in the promoter region of genes, were only identified in *PSY1* and *CCS* (Jeong et al., 2019). Clear correlations were shown between carotenoid profile and allelic variation, based on the various combinations of *PSY1* and *CCS.* This finding does support the idea of the three loci model, as *PSY1* and *CCS* clearly play a key role in determining pepper fruit colour, however, the allelic variation controlling this is highly complex. Not only did this study identify novel allelic variations in carotenoid biosynthetic genes, but also highlighted the role that SMRT sequencing may play in rapidly identifying allelic variation in target genes in other germplasm (Jeong et al., 2019). Whilst this study supports the finding that *PSY1* and *CCS* are crucial to determining pepper fruit colour, it has also provided an alternative hypothesis for the genetic variation observed in pepper fruit colour, as a vast array of alleles have been shown to influence the colour phenotype.

1.3.3.2. Transcriptional and post-transcriptional regulation of carotenoid biosynthesis

Regulation of carotenoid biosynthesis and accumulation occurs at many levels, including at the level of transcription and post-transcription. Phytoene synthase is widely regarded as being the most important regulatory enzyme in the carotenoid biosynthesis pathway (Cazzonelli and Pogson, 2010). Indeed, *PSY1* expression was shown to be induced during pepper fruit ripening (Romer et al., 1993), and the *PSY1* gene in pepper has been proposed to be the rate limiting step in carotenoid biosynthesis (Huh et al., 2001). Further to this, *PSY* transcript abundance has been shown to be increased during photomorphogenesis, via a phytochrome-mediated pathway, whereas other key genes in the carotenoid biosynthetic pathway, including *GGPS* and *PDS* retain constant levels of expression (Von Lintig et al., 1997, Welsch et al., 2000). Clearly *PSY* expression plays a crucial role in regulating carotenoid biosynthesis, however *DXS* has also been suggested as a key regulatory gene. A strong correlation was observed between tomato *DXS* mRNA accumulation and carotenoid accumulation (Lois et al., 2000). Overexpression of DXS in *Arabidopsis thaliana* showed an increase in total carotenoid content, whilst plants with suppressed DXS showed decreased total carotenoid content (Estévez et al., 2001). This demonstrates the fact that *DXS* transcript level is another regulatory step in carotenoid biosynthesis.

The *Orange* (*OR*) gene is involved in carotenoid biosynthesis regulation, and the *Or* mutant in cauliflower results in high levels of β-carotene accumulation. Overexpression

of the *Or* gene in *Arabidopsis thaliana* has been shown to significantly increase the amount of active PSY, and therefore, it is suggested that the OR protein acts as a major posttranscriptional regulator of PSY (Zhou et al., 2015). Further to this, the *Or* gene is considered to be the only known molecular switch, initiating the differentiation of chromoplasts from chloroplasts (Lu et al., 2006, Giuliano and Diretto, 2007).

Along with transcriptional changes affecting carotenoid biosynthesis, there is evidence to suggest metabolite feedback regulation modulates the supply of isoprenoid substrates, which in turn affects the accumulation of carotenoids within the plant (Bramley, 2002, Cazzonelli and Pogson, 2010). Increased expression of *PSY,* using the bacterial PSY enzyme (CrtB) as a transgene, showed that upregulation of PSY activity is sufficient to increase the production of carotenoids in etiolated seedlings. Further to this, etiolated seedlings accumulated increased levels of *DXS* mRNA, suggesting a feedback mechanism which is initiated by PSY, and which stimulated the supply of MEP substrates (Rodríguez-Villalón et al., 2009). Therefore, a feedback mechanism is triggered, in which flux-controlling enzymes of the MEP pathway, responsible for synthesising the substrates for PSY activity, are accumulated, following transcription (Rodríguez-Villalón et al., 2009). This highlights the fact that carotenogenesis by PSY is tightly controlled by source and sink metabolites (Cazzonelli and Pogson, 2010).

Evidently, PSY plays a crucial role in carotenoid biosynthesis regulation, and carotenoid content is regulated by a complex array of mechanisms, including feedback and feed-forward mechanisms (Enfissi et al., 2017).

Violaxanthin de-epoxidase activity appears to be modulated post-translation and affects carotenoid composition. Chloroplast pH and ascorbate concentration are critical in determining violaxanthin de-epoxidation, and consequently zeaxanthin levels, during periods of high light. Zeaxanthin absorbs light at longer wavelengths when compared to violaxanthin, and therefore, violaxanthin de-epoxidation is induced by high light, and stimulated by ascorbate level, due to acidification of chloroplasts linked to photosynthetic electron transport (Yamamoto et al., 1972, Rockholm and Yamamoto, 1996).

1.3.3.3. Carotenoid sequestration and storage

The sequestration and storage of carotenoids within plant cells is an important mechanism for regulation of carotenoid biosynthesis, as an efficient storage mechanism acts as a sink within the cell. Carotenoid biosynthesis occurs in plastids of photosynthetic organisms.

Plastids are an essential group of plant cellular organelles, and have evolved to become essential components for plant cell function. Several major metabolic pathways in plants occur within plastids, including lipid biosynthesis and amino acid metabolism (Pyke, 1999). All plastid types are derived from proplastids, which are small, undifferentiated plastids. Proplastids may differentiate into a wide array of plastid types, including amyloplasts for starch storage, chloroplasts for photosynthesis, and chromoplasts for carotenoid storage (Figure 1-10).

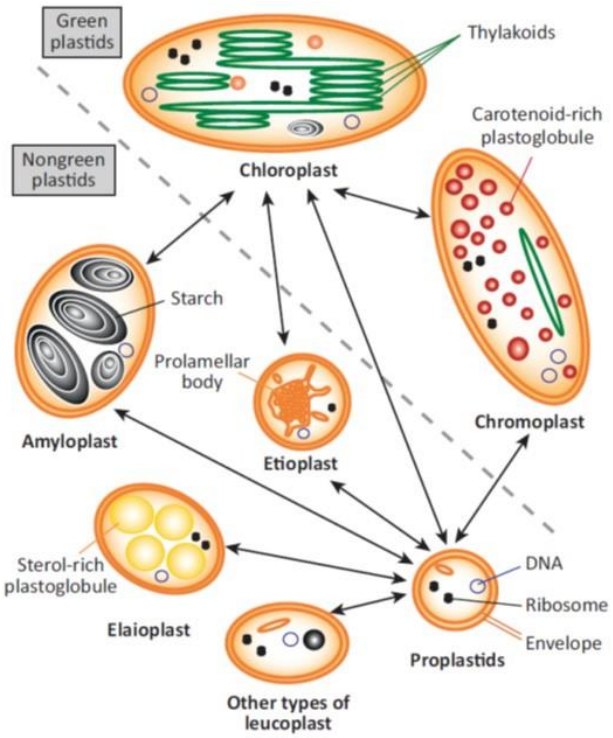


Figure 1-10 Plastid differentiation in angiosperms.

All plastid types are derived from proplastids, which are small, undifferentiated plastids. Amyloplasts are responsible for starch storage, chloroplasts for photosynthesis, chromoplasts for carotenoid biosynthesis and storage, etioplasts, which are plastids that have not been exposed to light, elaioplasts for sterol storage, and other types of leucoplast. Figure adapted from (Botté and Maréchal, 2014).

Chromoplasts are formed as a result of a transition from pre-existing plastids, most commonly chloroplasts, to plastids which synthesise and store large amounts of carotenoid (Egea et al., 2010). The *Orange* (*Or*) gene represents the only known molecular switch to initiate the transition from plastids to chromoplasts (Lu et al., 2006, Giuliano and Diretto, 2007). During the transition from chloroplasts to chromoplasts, the internal membrane system is remodelled. Grana and inter-granal thylakoids are lysed, and new membrane systems are formed (Spurr and Harris, 1968). These newly synthesised membranes are the site for the formation of carotenoid crystals (Simkin et al., 2007) (Figure 1-11).

During the chloroplast to chromoplast transition, carotenoids become localised within plastoglobuli (Steinmuller et al 1985), which are lipoprotein particles (Austin et al., 2006). Plastoglobuli increase in size and number during the chloroplast to chromoplast transition (Spurr and Harris, 1968). Plastoglobules arise as a result of blistering of the stroma-side leaflet of the thylakoid membrane (Austin et al., 2006)(Figure 1-11). Plastoglobuli isolated from chromoplasts contain triacylglycerols, which constitute two thirds of the total content of these plastoglobuli, along with carotenoids (Steinmüller and Tevini, 1985). The colourless carotenoids, phytoene and phytofluene, along with other carotenoids and tocopherols are localised to the plastoglobuli in tomato fruit (Nogueira et al., 2013).

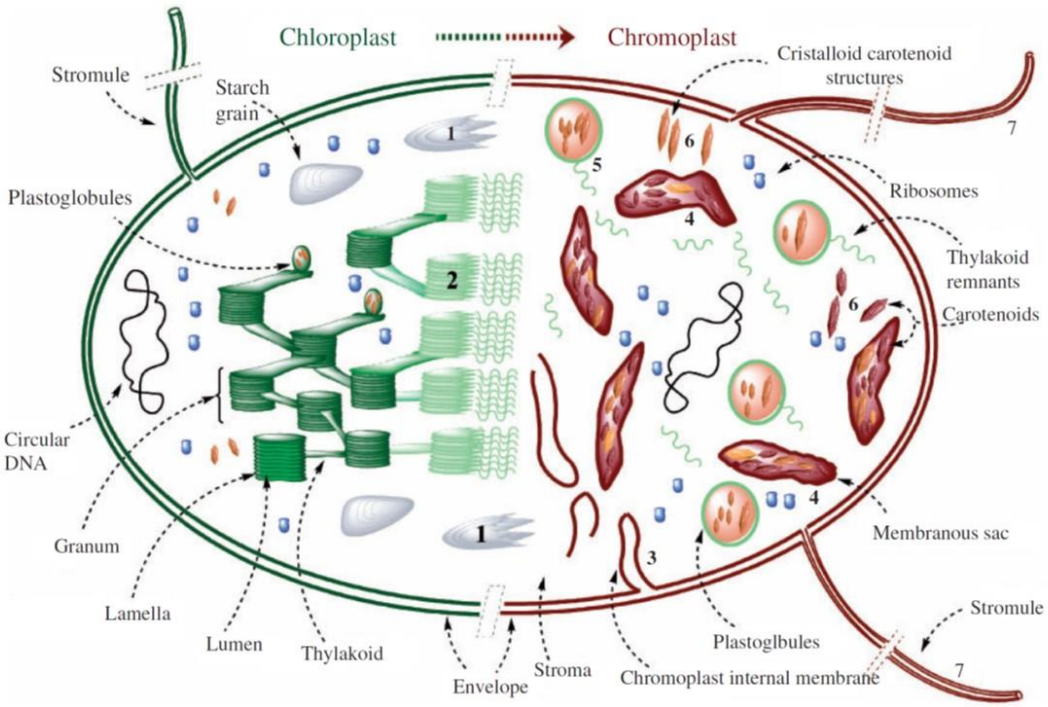


Figure 1-11 Schematic diagram representing the chloroplast to chromoplast transition.

During the chloroplast to chromoplast transition, plastids undergo an array of changes, including the breakdown of starch granules, along with the lysis of grana and thylakoids. New membrane structures are synthesised and form the inner membrane envelope of the plastid. Carotenoid-rich membranous sacs are formed, and plastoglobules increase in size and number. Crystalloid carotenoid-containing structures form, and the number of protrusions from the plastid envelope, known as stromules, increase. Figure adapted from (Egea et al., 2010).

In pepper, carotenoids are associated with fibrillar plastoglobule structures, known as fibrils. 95 % of pepper carotenoid pigments accumulate in lipoprotein fibrils within chromoplasts (Deruère et al., 1994). Along with carotenoids, these fibrillar structures also contain galactolipids, phospholipids, and a 32-kD protein, known as fibrillin. Fibrillin associates with polar lipids and esterified xanthophylls, to form fibrillar structures (Deruère et al., 1994). Xanthophylls of pepper are commonly found to be esterified (Biacs et al., 1989, Minguez-Mosquera and Hornero-Mendez, 1994b), and

esterified carotenoids are more stable than free carotenoids (Biacs et al., 1989). Further to this, carotenoids esterified with saturated fatty acids are more liposoluble, and therefore more easily associated with lipophilic environments, such as the fibrils. Therefore, carotenoid-containing fibrils are commonly observed within pepper chromoplasts. Pepper fibrils contain an array of carotenoid compounds, including free capsanthin, esterified capsanthin, β-carotene, phytoene, and phytofluene (Berry et al., 2019). This sequestration of carotenoid pigments into subchromoplast organelles not only allows their efficient storage and increased stability, but also partitions the carotenoids into compartments away from carotenoid biosynthetic enzymes, which are localised within chromoplast membranes. This form of carotenoid biosynthesis regulation allows ongoing synthesis, as pigments are stored away from the enzymes responsible for producing them (Nogueira et al., 2013).

The storage and sequestration of carotenoids in pepper not only allows the efficient and stable storage of these pigments, but also regulates the biosynthesis by providing a location away from biosynthetic enzymes, in order to facilitate ongoing biosynthesis.

1.3.3.4. Carotenoid degradation

The carotenoid content of a chromoplast is not only regulated at the biosynthetic level, as degradation of carotenoids also plays an important role in determining carotenoid content. Apocarotenoids, the products of carotenoid catabolism, play essential roles in plants, including controlling the rate of carotenoid turnover, contributing to colour and aroma, and as precursors of essential phytohormones: ABA and strigolactones (Hou et al., 2016).

Carotenoid degradation occurs as a result of both enzymatic and non-enzymatic degradative mechanisms, and both mechanisms produce biologically important apocarotenoids (Hou et al., 2016).

1.3.3.4.1. Enzymatic carotenoid cleavage

Carotenoid cleavage oxygenases (CCOs) are a large family of enzymes (Tan et al., 2003) with cleavage activity acting on specific double bonds of carotenoids (Walter and Strack, 2011). CCOs can be split into two classes: carotenoid cleavage dioxygenases (CCDs) and 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), based on their respective substrate, and tend to cleave at different sites, resulting in the production of various apocarotenoids (Walter and Strack, 2011). Eleven members of the *CCO* family have been identified in the pepper genome (Zhang et al., 2016). In *Arabidopsis thaliana*, nine CCOs exist, of which five belong to the NCED family. These include NCED2, NCED3, NCED5, NCED6, and NCED9 (Tan et al., 2003), and are essential for abscisic

acid (ABA) biosynthesis (Nambara and Marion-Poll, 2005). NCEDs cleave the 9-*cis*-isomers of violaxanthin and neoxanthin in order to form a C_{15} product, xanthoxin, which is the precursor for abscisic acid (Schwartz et al., 2001).

The CCD family, which includes CCD1, CCD4, CCD7, and CCD8 have a broad substrate specificity (Hou et al., 2016). CCD1 and CCD4 have a very wide range of substrates, including phytoene and neoxanthin, and produce apocarotenoids including β-ionone, and α-ionone (Schwartz et al., 2001, Simkin et al., 2004, Ibdah et al., 2006). CCD7 and CCD8 are involved in strigolactone biosynthesis from β-carotene (Ruyter-Spira et al., 2013). CCD1 is localised to the cytoplasm (Auldridge et al., 2006), whilst CCD4 is located in the plastoglobules of chromoplasts (Ytterberg et al., 2006, Rottet et al., 2016). CCD4, therefore, is located to the site of carotenoid biosynthesis and storage. CCD4 has been characterised in *Arabidopsis thaliana* (Gonzalez-Jorge et al., 2013), and in potato, down-regulation of CCD4 resulted in tubers with altered morphology and sprouting activity. Further to this, all-*trans*-β-carotene is the likely substrate of CCD4 in potato. This suggests the role of CCD4 in tuber development in potato, which may be a result of the activity of a cleavage product of all-*trans*-β-carotene (Bruno et al., 2015).

CCD1 and CCD4 can cleave β-carotene, and therefore produce β-cyclocitral. β-cyclocitral has recently been shown to be a regulator of root growth. Treatment of an *A. thaliana ccd1ccd4* double mutant, which had significantly fewer root meristematic cells, with the application of β-cyclocitral resulted in recovery of meristematic cell number (Dickinson et al., 2019). This role of root growth regulation has been shown to act independently of reactive oxygen signalling (Dickinson et al., 2019), which has previously been shown to be induced by the formation of β-cyclocitral in response to environmental stress (Ramel et al., 2012). Clearly, apocarotenoids have a wide array of roles within plants.

Whilst the function of CCD4 has not been characterised in pepper to date, these findings suggest that CCD4 may be involved in plant development.

When considering carotenoid degradation in plants, carotenoid cleavage oxygenases clearly play a contributory role. The enzymatic cleavage of carotenoids is essential as the degradation products: apocarotenoids, are required for various role in the plants, including signalling and phytohormone biosynthesis. However, carotenoid degradation also contributes to loss of colour, and photoprotective activity, and therefore, carotenoid degradation by oxidative means must be carefully modulated in plants.

1.3.3.4.2. Non-enzymatic carotenoid cleavage

Alongside enzymatic degradation of carotenoids, non-enzymatic carotenoid cleavage also results in apocarotenoid formation. Oxidative cleavage is a major mechanism causing carotenoid degradation, and therefore colour loss in *Capsicum annuum*. The apocarotenoids formed again may act as signals within the plant, for example, light stress was shown to induce oxidation of β-carotene, resulting in the accumulation of β-cyclocitral. β-cyclocitral was shown to induce changes in gene expression of $^1O_2$ responsive genes, including genes associated with cell rescue and defence being up-regulated, and genes associated with development and biogenesis of cellular components being down-regulated. Consequently, gene expression influenced by β-cyclocitral was associated with increased tolerance to photooxidative stress (Ramel et al., 2012). Evidently, plants respond to environmental stress, including the production of singlet oxygen in chloroplasts under stress conditions, by quenching $^1O_2$. Volatile compounds derived from the oxidation of β-carotene results in the formation of β-apocarotenoids, including β-cyclocitral and β-ionone, which induce gene expression changes, resulting in acclimation to environmental stress (Havaux, 2014).

The products of carotenoid cleavage, the apocarotenoids, are evidently a varied and diverse class of compounds, which can be formed from an array of mechanisms. Evidently, significant work is still to be carried out in order to fully understand apocarotenoid formation and the roles of these compounds, which to date, includes functions such as signalling, photooxidation protection, phytohormone precursors, and aroma (Hou et al., 2016).

1.3.4. Carotenoid function

Carotenoids have a wide array of functions, both in plant development and in benefitting human health, due to their role as antioxidants. Carotenoids are deemed to have antioxidant capacity as their structure permits the scavenging of reactive oxygen species, including singlet molecular oxygen ($^1O_2$) and peroxyl radicals. Upon interaction of carotenoids with $^1O_2$, physical quenching occurs, in which energy is directly transferred between the two molecules. The energy of $^1O_2$ is transferred to the carotenoid and yields ground state oxygen and a triplet excited carotene. The carotenoid then returns to a ground state by dissipating energy through interaction with the surrounding solvent (Stahl and Sies, 2003). This antioxidant capacity is conferred by the conjugated double bond structure observed in carotenoids.

1.3.4.1. The role of carotenoids in plants

Plants must maximise use of light energy during photosynthesis, and therefore have molecules which act as light-harvesting antennae in order to collect light and deliver to

reaction centres. It is at these reaction centres that energy conversion, into the chemical form, occurs (Pascal et al., 2005). Whilst in shade, light is efficiently harvested, however in full sunlight, excess energy is absorbed and must be dissipated to prevent photo-damage of photosynthetic membranes (Horton et al., 1996). This is referred to as non-photochemical energy dissipation. The light-harvesting antenna protein (LHC-II) is composed of specialised membrane-bound light-harvesting pigment-protein complexes and the structure has been determined by X-ray crystallography (Liu et al., 2004). Within these complexes, chlorophylls and carotenoids are organised in a very specific manner (Liu et al., 2004). The xanthophyll cycle plays an important role in non-photochemical energy dissipation. The xanthophyll cycle carotenoids: violaxanthin, antheraxanthin, and zeaxanthin, are associated with LHCs, and plants utilise the xanthophyll cycle to regulate the flow of energy to chlorophyll *a* within photosynthetic proteins. These three xanthophylls are interconverted in the chloroplast membrane during the xanthophyll cycle (Yamamoto et al., 1972). During this process, exposure to excess light beyond what is required for photosynthesis induces violaxanthin de-epoxidation activity, resulting in formation of antheraxanthin and zeaxanthin. Zeaxanthin has 11 conjugated double bonds, and therefore has greater capacity to scavenge excess energy, compared to violaxanthin, which has nine conjugated double bonds. Alternatively, low light levels result in epoxidation of zeaxanthin to form violaxanthin (Frank et al., 1994) (Figure 1-12). Consequently, xanthophylls play a crucial role in non-photochemical quenching in order to protect the plant against photooxidative damage, and to regulate the utilisation of light energy during photosynthesis (Ma et al., 2003). Further to this, carotenoids play an essential role in scavenging reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), in order to protect the photosynthetic components of the cell from oxidative damage (Sharma et al., 2012).

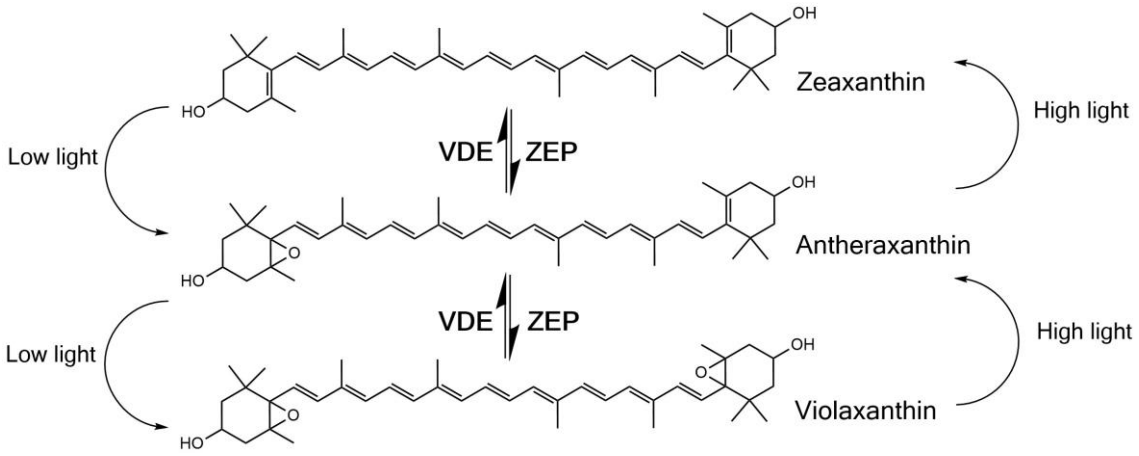


Figure 1-12 Xanthophyll cycle.

The xanthophyll cycle is crucial to non-photochemical quenching in order to dissipate excess light energy, which is not required for photosynthesis. Zeaxanthin is converted to violaxanthin in low light, whereas violaxanthin is converted to zeaxanthin in high light, when photooxidative protection is required. Abbreviations: ZEP = zeaxanthin epoxidase; VDE = violaxanthin de-epoxidase.

1.3.4.2. The role of carotenoids in human health

1.3.4.2.1. Provitamin A activity

Carotenoids are well established to have provitamin A activity, although this function is restricted to carotenoids with β-ring end groups, including β-carotene, zeaxanthin, and β-cryptoxanthin (Fraser and Bramley, 2004). Retinol, retinal, and retinoic acid are all precursors of vitamin A, and are derived from the degradation of β-carotene (Olson, 1964). In mammals, the enzyme catalysing the cleavage of β-carotene to retinal has been found to be a β-carotene 15,15'-dioxygenase (β-CD), which allows the entrance of vitamin A precursors from plant-derived sources, into the mammalian diet (Redmond et al., 2001). The recombinant human enzyme was purified and characterised, although the enzyme is now considered to be a β-carotene 15,15'-monooxygenase (βCO), and high levels of expression were found in the intestine, kidney, and liver (Lindqvist and Andersson, 2002). Vitamin A is essential in the human diet for ocular health along with playing a developmental role, however, vitamin A deficiency is prevalent in many developing countries. Retinoic acid is widely reported to play an essential role in mammalian embryonic development (Rhinn and Dollé, 2012). Therefore, vitamin A supplementation, or pro-vitamin A bio-fortification of foods is necessary (Fraser and Bramley, 2004).

1.3.4.2.2. Disease prevention

The disease prevention attributes of carotenoids are considered to be owed to their antioxidant properties (Rao and Rao, 2007). However, whilst β-carotene has been shown to have antioxidative properties and to quench reactive oxygen species at dietary levels, excessive β-carotene may also act as a pro-oxidant. The

supplementation of β-carotene in smokers resulted in an increased incidence of lung cancer (Alpha-Tocophrol, 1994), and increased mortality from cardiovascular disease (Omenn et al., 1996).

Carotenoids also appear to have a role in protecting against cancer, and have been shown to suppress tumorigenesis in the skin, lung, liver, and colon, amongst others (Nishino et al., 2009). It appears that a combination of carotenoids consumed simultaneously may be more effective in preventing cancer development, as opposed to consuming a single carotenoid alone (Nishino et al., 2009).

1.3.4.2.3. Eye health

Vitamin A deficiency has been shown to result in poor eye health, including age-related eye diseases such as cataracts and age-related macular degeneration (AMD) (Congdon and West, 1999). It is suggested that the lens becomes opaque following damage to the lens enzymes, proteins, and membranes by reactive oxygen species (Taylor et al., 1993). Both lutein and zeaxanthin have been found to be located in the human retina, and specifically within the macula (Bone et al., 1997). Increased intake of dietary carotenoids was shown to be associate with a lower risk of age-related macular degeneration. Specifically, lutein and zeaxanthin, were most correlated with a reduced risk of AMD (Seddon et al., 1994).

1.3.5. Nutritional and industrial uses of carotenoids

As previously stated, carotenoids have high antioxidant capacity and therefore are beneficial to human health. Carotenoids with β-ring groups, including β-carotene, have provitamin A capacity, and therefore, it is from these compounds that vitamin A is derived in humans. According to the American National Institute of Health (NIH), the recommended adult dietary allowance for vitamin A is 700-900 µg per day (National Institutes of Health, 2019), however, the World Health Organisation (WHO) estimates that 250 million preschool children are vitamin A deficient, and that in vitamin A deficient areas, a substantial proportion of pregnant women are vitamin A deficient. Further to this, an estimated 250000 to 500000 vitamin A deficient children become blind each year, with half of these children dying within 1 year of losing their sight (World Health Organization, 2019). Consequently, the demand for carotenoids in human health is evident. Due to these rising health concerns, carotenoids are used by the nutraceutical and pharmaceutical industries, as they are supplemented into food products. This has resulted in an expanding global market for carotenoids, which in 2014 was worth US $ 1.5 billion, and is expected to reach US $ 1.8 billion in 2019, with β-carotene having the highest value at US $ 233 million in 2010, and approximately US $ 209 million in 2018 (Cvetkovic and Nikolic, 2017).

This demand for carotenoids globally has also allowed for bioengineering of the carotenoid biosynthesis pathways. Several crops have been genetically engineered to introduce carotenoid biosynthesis, as a means of introducing provitamin A capacity to widely consumed crops in areas of the world where vitamin A deficiency is prevalent. An example of this is seen in Golden Rice. Phytoene synthase (*PSY*) and lycopene β-cyclase (*β-lcy)* from *Narcissus pseudonarcissus* (daffodil) were transformed into rice endosperm along with the bacterial phytoene desaturase *(CrtI*), although it was subsequently found that *β-lcy* was not required for carotenoid biosynthesis when *PSY* and *CrtI* were present. *β*-carotene was found to be present in rice endosperm of the T_0 generation (Beyer et al., 2002). Although engineering of carotenoids in rice proved successful, several obstacles have prevented this product from being a consumed variety, including carotenoid stability, consumer preference, and legislation.

The heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* naturally produces the red xanthophyll, astaxanthin, which is an industrially important compound in aquaculture, food, and pharmaceutical industries. Consequently, this yeast has been exploited to produce high levels of astaxanthin for industrial use, and this has been carried out by isolating new, high astaxanthin strains of *X. dendrorhous* from the environment, and through selecting mutants following random mutagenesis. Fermentation parameters for *X. dendrorhous* have also been studied in detail, in order to maximise astaxanthin production (Rodríguez-Sáiz et al., 2010). More recently, astaxanthin biosynthesis has been engineered in tomato fruits, and when used in aquaculture feed to colour trout flesh, the tomato-based product was shown to be more efficient in colouring trout flesh, when compared to the synthetic feed (Nogueira et al., 2017).

These studies highlight the industrial demand for carotenoids, and demonstrate the way in which genetic engineering can be used to meet this demand.

1.4. Metabolite biodiversity in pepper fruit

1.4.1. Health-related metabolites

Whilst carotenoids are well documented in explaining the phenotypic variation in colour observed in pepper fruit accessions, variation in other metabolites also explains phenotypic differences in pepper varieties. Major metabolites in pepper fruits, alongside carotenoids, include capsaicinoids, flavonoid glycosides, and vitamins C and E (Wahyuni et al., 2011). A study examining biochemical compounds of 32 *Capsicum* spp. accessions revealed that the composition and amounts of these metabolites in fruits showed great variation between these accessions, and this was independent of species. Vitamin E (α-tocopherol) was found in all 32 accessions analysed in varying

amounts. Vitamin C (ascorbic acid) was also found to be present in all 32 lines, sixteen of which had levels greater than 100 mg/100 g fresh weight pepper (Wahyuni et al., 2011). This represents high vitamin C content. Flavonoids were present as *C*- and *O*-glycosides, and this included quercetin glycosides, luteolin glycosides, and apigenin glycosides. Capsaicinoid content displayed significant variation in the accessions analysed, and this is likely linked to the pungency phenotype of accessions studied (Wahyuni et al., 2011).

The same 32 accessions were further studied using untargeted LC-MS to determine semi-polar metabolite profile, and headspace GC-MS to determine volatile profile. A large proportion of the 88 semi-polar metabolites putatively identified were the pepper flavonoids with various sugar groups. Principal component analysis (PCA) revealed that the major determinant of variation in semi-polar metabolite profile was species driven, as accessions of each species tended to cluster together. In contrast, pungency appeared to be responsible for the variation observed in aroma volatiles (Wahyuni et al., 2013).

1.4.2. Lipid metabolism

Lipid composition is important in pepper fruits, along with all plants, as they are of metabolic and structural significance. Many compounds, including carotenoids, are lipid soluble, and esterification of pepper fruit carotenoids depends on the fatty acid content of the pepper. Esterified xanthophylls are both more liposoluble and more stable to photo- and thermoxidative damage. It has previously been shown that the polyunsaturated fatty acids (PUFA) linoleic (C18:2) and linolenic (C18:3) acid are the most abundant in pepper fruit pericarp. The high abundance of these fatty acids in ripe fruit may be due to the essential role that they play in membrane structure (Pérez-Gálvez et al., 1999). A further study of six *Capsicum* varieties was carried out to determine fatty acid content, and 25 different fatty acids were identified. Again, linoleic acid (C18:2) was found to be amongst the most abundant fatty acids in the pericarp, along with oleic acid (C18:1), and palmitic acid (C16:0). Fatty acid content was found to be consistently lower in pericarp when compared to fatty acid content in seeds. Linolenic acid appears to be absent in seeds, whilst it is one of the most abundant fatty acids in pericarp, although the reason for this is unknown as yet (Sora et al., 2015).

1.4.2.1. Very long chain fatty acids (VLCFAs)

Very long chain fatty acids are fatty acids with acyl chains containing 20 carbons, or more. They are essential biochemical components for all eukaryotes, and can be grouped into four lipid classes: triacylglycerols, waxes, phospholipids, and sphingolipids. Chain length, degree of unsaturation, the type of polar head, and

associated lipids all contribute to the structural and functional diversity observed in fatty acids. Collective changes to waxes, phospholipids, and sphingolipids play crucial roles in plant development. Whilst sphingolipids and phospholipids are essential membrane and signalling components, triacylglycerols are used for seed storage of lipids, and waxes are essential constituents of the cuticle, which protect the plant from biotic and abiotic stress(Bach and Faure, 2010).

Very long chain fatty acids are synthesised by the elongase complex in the endoplasmic reticulum, in which two carbons are sequentially added through enzymatic reactions. The precursor to this reaction is the C_{18} acyl-CoAs, which are produced by the cytosolic fatty acid synthase complex (FAS). In this process, a long chain acyl-CoA condenses with a malonyl-CoA, by action of the 3-keto-acyl-CoA synthase (KCS). The product of this reaction is a 3-keto-acyl-CoA, which is then reduced by a 3-keto-acyl-CoA reductase (KCR) to produce a 3-hydroxy-acyl-CoA. 3-hydroxy-acyl-CoA is dehydrated by 3-hydroxy-acyl-CoA dehydratase (HCD) to form a trans-2,3-enoyl-CoA, which is then reduced by the trans 2,3-enoyl-CoA reductase (ECR) to yield a two-carbon elongated acyl-CoA. Following elongation of the very long chain fatty acids, they can then be incorporated into different lipid classes (Bach and Faure, 2010) (Figure 1-13).

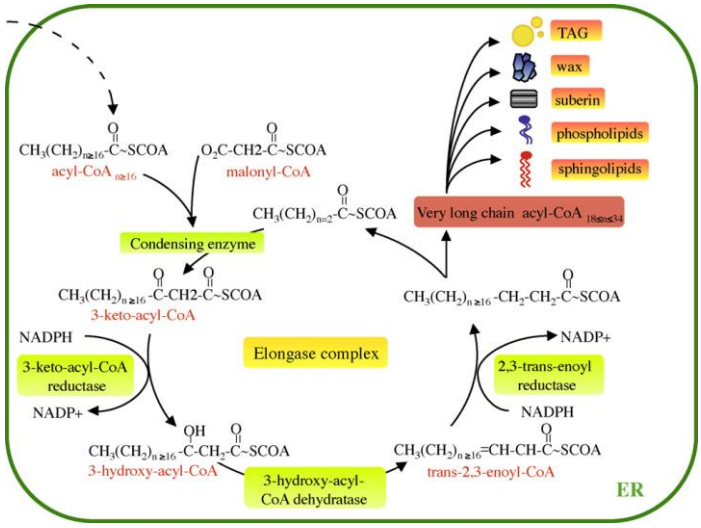


Figure 1-13 Very long chain fatty acid biosynthesis.

Very long chain fatty acids are elongated from long chain acyl-CoA by the endoplasmic reticulum associated elongase protein complex. Addition of two carbons to the acyl-CoA requires four successive enzymatic steps: the condensation of malonyl-CoA with acyl-CoA, reduction of 3-keto-acyl-CoA, dehydration of 3-hydroxyacyl-CoA, reduction of trans-2,3-acyl-CoA. The enoyl-CoA results in the formation of a very long chain acyl-CoA. Figure adapted from (Bach and Faure, 2010).

Amongst others, two condensing (KCS) enzymes were characterised from *Arabidopsis thaliana*: a FATTY ACID ELONGATION 1 (FAE1), which is responsible for the C_{20} and C_{22} fatty acid elongation, involved in seed triacylglycerols (Kunst et al., 1992), along

with CER6, which is responsible for the elongation of fatty acids longer than C_{22} in epidermal cells (Millar et al., 1999). *CER6* was found to be involved in the synthesis of stem wax very long chain fatty acid precursors in *Arabidopsis.* A loss of function in *CER6* resulted in an accumulation of C_{24} chain-length wax components, suggesting *CER6* is responsible for elongating C_{24} very long chain fatty acids (Millar et al., 1999). Subsequently, two *KCS* genes were identified in *Arabidopsis thaliana*, *KCS20* and *KCS2/DAISY*. These genes appeared to be functionally redundant as single mutants of either gene resulted in no change in cuticular wax, whilst the double mutant displayed a significant decrease in the amount of epicuticular wax crystals. Therefore, these genes are functionally redundant in the two-carbon elongation to C_{22} very long chain fatty acid, as a reduction in C_{22} and C_{24} very long chain fatty acids were observed in mutants (Lee et al., 2009). Taken together, these studies reveal that the *KCS* multigene family members are substrate specific, predominantly for chain length (Bach and Faure, 2010).

The 3-ketoacyl-reductase (KCR) protein is encoded by two genes in maize: GL8A and GL8B. *gl8gl8b* double mutant kernels were non-viable as embryos did not develop normally, and substantially lower levels of very long chain fatty acids were accumulated. The KCR protein has been shown to play an essential role in cuticular wax biosynthesis. As the double mutant was lethal, this demonstrates the essential function of very long chain fatty acids in plant development (Dietrich et al., 2005). In *Arabidopsis,* two orthologs of the yeast *KCR* gene *YBR159w* were identified and designated *AtKCR1* and *AtKCR2*. Complementation of the yeast mutant *ybr159Δ* with AtKCR1 and AtKCR2 demonstrated that only AtKCR1 was able to recover the elongase activity of the yeast mutant, suggesting that AtKCR1 catalyses the reduction of 3-ketoacyl-CoA. Further to this, suppression of *AtKCR1* resulted in embryo lethality (Beaudoin et al., 2009).

Two *Arabidopsis thaliana* homologs were identified for the yeast 3-hydroxy-acyl-CoA dehydratase (*PHS1*) gene. However, again only one of these homologs: *PASTICCINO2* (*PAS2*) was able to recover the defects in cytokinesis and sphingolipid long chain base overaccumulation observed in the yeast *phs1* mutant. Further to this, *PHS1* expression complemented the developmental defects observed in the *pas2* mutant. Complete loss of *PAS2* was lethal to the embryo (Bach et al., 2008).

The final reaction in the elongase complex is the reduction of trans-2,3-enoyl-CoA to a two-carbon elongated acyl-CoA, catalysed by a trans 2,3-enoyl-CoA reductase. In *Arabidopsis thaliana*, this gene has been identified as *CER10,* the suppression of

which resulted in decreased cuticular wax load and affected the very long chain fatty acid composition of seed triacylglycerols and sphingolipids (Zheng et al., 2005).

Together, these findings display the diverse and essential roles of very long chain fatty acids in plant development.

1.5. Fruit cuticle structure and function

The plant cuticle provides a protective barrier over the aerial epidermis of all land plants, against the external environment. The evolution of the plant cuticle was instrumental in the transition of plants from an aquatic environment to the terrestrial environment, as plants had to protect themselves against dessication on land, and have more a more rigid structure in order to stand upright (Edwards, 1993). The cuticle is a hydrophobic layer, formed of two major constituents: a cutin polymer, and cuticular waxes, which form a macromolecular scaffold. The cuticle biosynthetic pathway has been elucidated, although this is controlled by a complex regulatory network, which has not yet been fully elucidated. Although the primary role of the cuticle is to protect the plant against dessication and environmental stresses, it seems that the cuticle plays other roles, including development and interaction with microbes (Yeats and Rose, 2013).

The cuticle is considered to be independent of the underlying epidermal layer, however the two layers are physically associated with one another. The cuticle is often split into two domains, with a cutin-rich section, which is referred to as the ‘cuticular layer’, and an overlaying section rich in waxes, which is referred to as the ‘cuticle proper’. Waxes may be either embedded within the cutin matrix, and known as intracuticular waxes, or may accumulate on the surface as crystals or films, and are known as epicuticular wax crystals and films. Epicuticular wax crystals tend to result in a dull appearance of the plant surface, whilst epicuticular films result in a shiny appearance observed in many fruits (Figure 1-14) (Yeats and Rose, 2013).

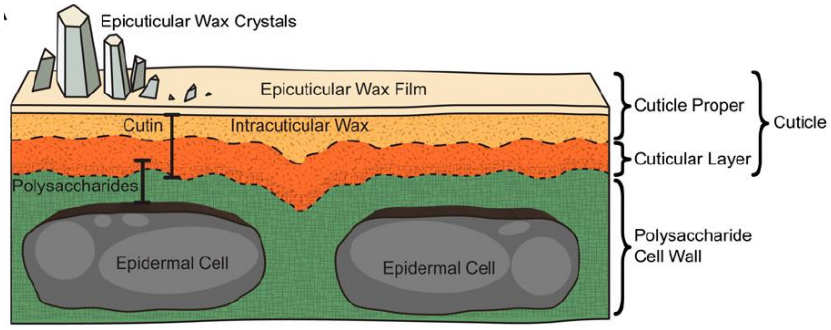


Figure 1-14 Plant cuticle structure.

Schematic figure displaying structure of plant cuticle, highlighting structural features of the cuticle and underlying epidermal cell layer. Not drawn to scale. Figure adapted from (Yeats and Rose, 2013).

1.5.1. Cuticle wax biosynthesis

Cuticular waxes are derived from very long chain fatty acids (VLCFAs) with carbon chain lengths ranging from C_{20} to C_{34}. The compounds comprising cuticular wax include alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. Other lipophilic secondary metabolites, including triterpenoids, flavonoids, and tocopherols may also be associated with the cuticular waxes (Riederer and Muller, 2008).

The biosynthesis of cuticular wax begins with the synthesis of C_{16} or C_{18} fatty acids in epidermal cell plastids. These fatty acids are then converted to CoA thioesters by the action of a long chain acyl-coenzyme A synthase (LACS) isozyme, and are then transported to the endoplasmic reticulum. C_{16} acyl-CoA can then be used as a substrate for the fatty acid elongase (FAE) complex, which has previously been discussed (Section 1.4.2.1), to ultimately produce very long chain fatty acids. In order to elongate fatty acids to longer than C_{28}, a family of proteins including CER2, CER26, and CER26-like is required (Haslam et al., 2012, Pascal et al., 2013). These enzymes are characterised as BAHD acyltransferases. Very long chain fatty acid-CoA may then become primary alcohols by the action of fatty acyl-CoA reductase, which is encoded by *CER4* in *Arabidopsis thaliana* (Rowland et al., 2006). Primary alcohols may occur in the cuticle wax, or may be esterified to a fatty acid to form a wax ester, in which case, the alcohol is coupled to an acyl group, catalysed by a member of the wax synthase/diacylglycerol acyltransferase family: WSD1 (Li et al., 2008). Aldehydes and alkanes are also formed. LACS1 appears to have a role in both C_{16} cutin monomer biosynthesis, along with in C_{30} very long chain fatty acid biosynthesis. It appears that the conversion of an intracellular pool of free very long chain fatty acids back to very long chain fatty acid-CoA can ultimately result in the formation of aldehydes and alkanes (Lü et al., 2009). Evidence suggests that CER1 and CER3, along with cytochrome b_5, as an electron donor, catalyse the reduction and decarbonylation of

very long chain fatty acids to cuticular alkanes (Bernard et al., 2012). Alkanes may then undergo further modifications in order to form secondary alcohols and ketones. Both oxidation reactions are performed by the cytochrome P450 enzyme MAH1, which is a midchain alkane hydroxylase, in *Arabidopsis* (Greer et al., 2007).

1.5.2. Cutin monomer biosynthesis

Cutin monomers comprise the second component of the plant cuticle structure. Cutin monomers are derived from C_{16} and C_{18} fatty acids, and include various oxygenated fatty acid-glycerol esters, known as monoacylglycerols. Fatty acids are conjugated to coenzyme A, terminal and/or midchain carbons are oxidised, and the fatty acid is transferred from CoA to glycerol (Fich et al., 2016). Hydroxy fatty acids of cutin tend to be ω-hydroxy fatty acids, often with one or two additional midchain hydroxyl or epoxy groups (Yeats and Rose, 2013). Following *de novo* fatty acid biosynthesis in the plastid of epidermal cells, an ω-hydroxylation reaction, and a midchain hydroxylation reaction occur, followed by the esterification of CoA to the fatty acid, producing acyl-CoA. This reaction is carried out by a long-chain acyl-CoA synthetase (LACS) protein. As previously discussed, it appears that *LACS1* has function in the production of both waxes and cutin (Lü et al., 2009). The ω-hydroxylase appears to be encoded by a cytochrome P450: *CYP86A4* in *Arabidopsis* flowers, and the midchain hydroxylase is encoded by *CYP77A6* in *Arabidopsis* flowers (Li-Beisson et al., 2009). ω-dicarboxylic fatty acids are thought to be produced as a result of the activity of the ACE/HTH (ADHESION OF CALYX EDGES/HOTHEAD) protein, which acts to oxidise long chain ω-hydroxy fatty acids to ω-oxo fatty acids (Kurdyukov et al., 2006b). *CYP86A7* has also been suggested to be related to cutin biosynthesis, as expression of this gene is induced by the transcription factor: SHINE1 (SHN1), which is a known regulator of cuticle formation (Kannangara et al., 2007). Monoacylglycerol cutin monomers are then produced upon the transfer of the acyl group from acyl-CoA to glycerol-3-phosphate, by glycerol-3-phosphate acyltransferase (GPAT) enzymes. *Arabidopsis GPAT4* and *GPAT8* have been shown to contribute to stem cutin formation (Li et al., 2007), whilst *GPAT6* is responsible for petal cutin synthesis (Li-Beisson et al., 2009). Interestingly, these GPAT enzymes have been shown to preferentially transfer the acyl group to the *sn*-2 glycerol position, resulting in the synthesis of 2-monoacylglycerol (Yang et al., 2010).

1.5.3. Cuticle precursor transport

Following the synthesis of wax and cutin precursors in the endoplasmic reticulum, they must be transported across the plasma membrane, and trafficked through the polysaccharide cell wall, to the cuticular membrane, and site of polymerisation. Whilst

transport of cuticle precursors is not entirely understood, the role of ATP-binding cassette (ABC) transporters has been shown. The half transporters *CER5/ABCG12* and *ABCG11* have been shown to be involved in wax transport in *Arabidopsis* (Pighin et al., 2004, Bird et al., 2007). ABCG32 has been shown to be involved in cutin deposition in *Arabidopsis* (Bessire et al., 2011), and ABCG13 has also been shown to play a role in cuticular wax transport (Panikashvili et al., 2011).

The export of hydrophobic cutin monomers through the hydrophilic polysaccharide wall, in order to reach the site of cuticle synthesis, is poorly understood. Lipid transfer proteins (LTPs) are postulated to play a transport role in this process (Yeats and Rose, 2008), although genetic or biochemical evidence is still lacking. LTPG1 and LTPG2 have been demonstrated to play a role in transport of cuticular waxes (DeBono et al., 2009, Kim et al., 2012).

1.5.4. Cutin polymerisation

Cutin polymerisation is the final step of cuticle synthesis, as cutin monomers must be incorporated into the cutin polymer. However, the process of polymerisation has not been fully elucidated. Studies of cutin polymerisation in tomato fruit have revealed the role of a member of the GDSL-motif lipase/esterase (GDSL) superfamily in cutin synthesis. Characterisation of a *cutin deficient 1* (*cd1*) mutant showed a decrease of more than 95 % of polymeric cutin, but which accumulated the cutin monomer 2-mono(10,16-dihydroxyhexadecanoyl)glycerol (2-MHG). *In vitro* incubation of recombinant CD1 with 2-MHG resulted in the production of linear cutin oligomers, which were composed of terminal ester-linked fatty acids. This suggested the role of CD1 in transferring the hydroxyacyl group from one molecule of 2-MHG to the terminus of the chain, and releasing glycerol in the process (Yeats et al., 2012). Further to this, RNA interference was used to suppress the *GDSL* gene, which resulted in reduced cuticle thickness and cutin monomer content ((Girard et al., 2012). However, whilst the tomato fruit mutant *cd1* displayed significantly decreased levels in cutin polymer, it was not entirely absent, and this may suggest the role of other cutin synthase genes in this polymerisation process. A further enzyme, which may play a role in cutin synthesis is BODYGUARD (BDG), which is a member of α/β-hydrolase family, and is localised to the outer epidermal cell wall. An *Arabidopsis thaliana bdg* mutant displayed a disorganised cuticle, but also had increased levels of cutin polymer, which suggests the enzyme may not be involved in polymerisation, but in another stage of cutin synthesis (Kurdyukov et al., 2006a).

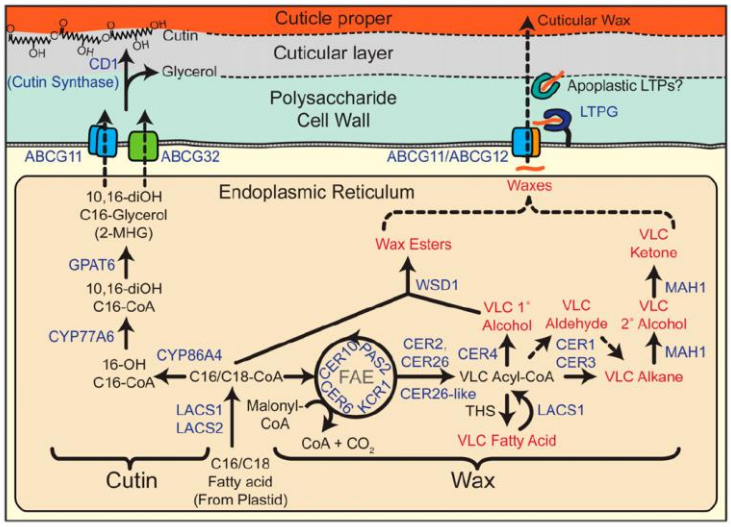


Figure 1-15 Cutin and wax biosynthetic pathways.

Genes involved in the biosynthesis of cutin and wax components of the plant cuticle. Figure adapted from (Yeats and Rose, 2013).

1.5.5. Cuticle biosynthesis regulation

Cuticle biosynthesis is regulated by a complex signalling network, which is associated with response to environmental stress, pathogen attack, and plant development. Whilst many studies have elucidated the regulation of cuticle biosynthesis in *Arabidopsis,* this does not provide the best model for considering fleshy fruit cuticle biosynthesis and regulation, as fleshy fruit cuticles tend to be much thicker than that of *Arabidopsis,* and do not contain stomata (Hen-Avivi et al., 2014). Consequently, studies performed in fleshy fruits have been considered where possible. Drought, sodium chloride concentration, and abscisic acid (ABA) all appear to induce wax synthesis, whereas cutin biosynthesis only appears to be induced by water deficit in *Arabidopsis* (Kosma et al., 2009).

Several transcription factors have been shown to play a role in regulating cuticle biosynthesis. *WAX INDUCER1/SHINE1* (*WIN1/SHN1*), an APETELA2 (AP2) transcription factor family member, has been shown to regulate cuticle biosynthesis, as overexpression in *Arabidopsis* resulted in glossy leaves and increased wax levels, although this may have been linked to decreased stomata density (Aharoni et al., 2004). Further to this, cutin content increased with the overexpression of *WIN1/SHN1* (Kannangara et al., 2007). Silencing of the three genes which comprise the *WIN1/SHN1* family in *Arabidopsis* resulted in a reduction in cutin content, but not in wax content (Shi et al., 2011). The related transcription factor *SHN3* in tomato has also been shown to be involved in regulation of fruit cuticle formation. Silencing of

SlSHN3 in tomato resulted in significant reduction in cuticle formation, as cutin and wax in the tomato cuticle were reduced (Shi et al., 2013).

MYB transcription factors may also play a role in cuticular wax accumulation regulation. An R2R3-MYB transcription factor has been identified as a potential regulator of wax accumulation in grapevine. Overexpression of this gene in tomato resulted in a glossy fruit appearance, and a decrease in total amyrin amount (Mahjoub et al., 2009). Amyrins, amongst other triterpenes, may be found within cuticle structures. MIXTA is another MYB-related protein, and this has been suggested to be involved in transcriptional control of epidermal cell shape and pigmentation in *Antirrhinum* petals (Noda et al., 1994). The MIXTA-like MYB transcription factors: MYB106 and MYB16, have been shown to regulate cuticle development coordinately with WIN1/SHN1 in *Arabidopsis* (Oshima et al., 2013). The MIXTA-like transcription factor in tomato has also been shown to regulate epidermal conical cell shape, and cutin biosynthesis (Lashbrooke et al., 2015). This demonstrates the tightly coordinated regulation of epidermal cell development and cuticle biosynthesis in fleshy fruits.

Ripening associated transcription factors may also be involved in regulation of cuticle biosynthesis. Tomato homologs of the *Arabidopsis* MADS domain transcription factor FRUITFULL: FUL1 and FUL2, are known to interact with the ripening regulator RIPENING INHIBITOR (RIN), and are expressed during fruit ripening. Analysis of the tomato double mutant showed that this mutant resulted in a decrease in lycopene levels, along with increased water loss. Genes related to cutin and fatty acid synthesis were also down-regulated in the double mutant, suggesting the FUL1 and FUL2 transcription factors also regulate cuticle formation (Bemer et al., 2012).

The homeodomain-leucine zipper IV (HD-Zip IV) member: CUTIN DEFICIENT 2 (CD2) has also been reported to regulate cutin monomer biosynthesis (Isaacson et al. 2009). The tomato *cd2* mutant had a decrease of 98 % in cutin content, and substantially affected the protection of tissues from microbial infection. *CD2* was revealed to encode a homeodomain protein, which has been proposed as a key regulator of tomato fruit cutin biosynthesis (Isaacson et al., 2009).

Evidently, regulation of cuticle biosynthesis is highly complex, and is linked to other essential processes such as fruit development and ripening, which further complicates the process. Whilst the regulatory mechanisms discussed here display some of the ways in which cuticle biosynthesis is controlled, there are likely to be many more regulatory mechanisms which play a role in regulating the synthesis of this important structure.

1.6. Reactive oxygen species-mediated signalling and damage

Oxidative damage has been discussed as a potential mechanism controlling the degradation of carotenoids during post-harvest storage. This includes processes such as lipid peroxidation. Carotenoids may protect essential lipids from peroxidation, but in the process, become degraded themselves.

The production of free radical species, and specifically reactive oxygen species (ROS) as a result of many cellular processes (Sharma et al., 2012) leads to lipid peroxidation of membranes, and ultimately cell damage. Cell damage from high ROS level results in phytotoxic levels of oxidants attacking cellular components, causing membrane leakage and cell lysis (Van Breusegem and Dat, 2006). Therefore, an understanding of the processes resulting in the production of ROS is required.

Molecules with, or capable of independent existence with, one or more unpaired electrons in their orbitals are referred to as free radicals (Halliwell and Chirico, 1993). Some examples of free radical species include superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$), both of which are oxygen centred radicals, along with thiyl ($RS^{\bullet}$) and trichloromethyl ($CCl_3^{\bullet}$). Radicals react with other molecules: if two radicals meet, their unpaired electrons may combine in order to form a covalent bond (Halliwell and Chirico, 1993).

As a result of metabolic activity, organisms produce an array of ROS and free radicals. This is a result of the activity of oxidase enzymes. Although ROS are often regarded as toxic to the cell, they have also been shown to play regulatory roles within cells (Laloi et al., 2004). As organisms evolved defence mechanisms against the harmful effects of ROS, which occur as a result of aerobic metabolism, ROS have also subsequently become key regulators of growth and development (Mittler et al., 2004). Gene transcription is altered dependent on changes in ROS concentration. For example, stress-response genes were activated in response to singlet oxygen in the *fluorescent* mutant of Arabidopsis. The *fluorescent* mutant can be characterised by the release of singlet oxygen in a controlled manner as a result of accumulation of protochlorophyllide, which releases singlet oxygen upon excitation by light. Following a dark-to-light shift, singlet oxygen was released, resulting in stress-responses in the plants: plants stopped growing and developed necrotic lesions. This was shown to be as a result of peroxidation of chloroplast membrane lipids, and the accumulation of hydroxyoctadecatrieonic acid due to the enzymatic oxidation of linolenic acid. Other distinct gene sets were activated by superoxide and hydrogen peroxide. ROS, therefore, can act as a signal which activates several stress-response pathways (op den Camp et al., 2003). ROS have subsequently been demonstrated to induce post-transcriptional, translation, and post-translational modifications, and therefore influence

the plant's proteome (Van Ruyskensvelde et al., 2018). Clearly, ROS have become important regulators and signalling molecules within a plants growth and development cycle, and this suggests that plants have evolved a high degree of control over the mechanisms producing toxic ROS.

A large gene network is required to allow toxic reactive oxygen molecules to be protected against, whilst reactive oxygen species, such as H_2O_2 and $O_2^{\bullet-}$, are used as signalling molecules. In *Arabidopsis thaliana*, this gene network has been shown to include at least 152 genes (Mittler et al., 2004). Clearly, an intricate balance between the regulatory properties of ROS and their damaging properties needs to be achieved in order for the plant to survive and thrive. This balance is dependent on the extent of ROS production, the cell target and the capability and activity of antioxidant defences (Halliwell and Chirico, 1993). H_2O_2 is required to trigger an array of plant responses, and is produced in a genetically controlled manner by membrane-bound NADPH oxidases (Foreman et al., 2003). H_2O_2 is used as a signal molecule in response to a variety of abiotic and biotic stresses, including causing defence reactions against pathogens or herbivores (Mittler, 2002), forcing the closure of stomata (Kwak et al., 2003), and regulating the expansion and development of plant cells (Foreman et al., 2003).

Despite the essential role of ROS as signalling molecules, their harmful oxidative activity can have a detrimental effect on cell structure and function, for example, DNA strand breakage, damage to membrane ion transporters and other proteins, and lipid peroxidation (Halliwell and Chirico, 1993).

1.6.1.1. Lipid peroxidation

Reactive oxygen species, such as hydroxyl radicals ($OH^{\bullet}$) may attack biological molecules, including lipids, during lipid peroxidation. This process is initiated when a fatty acid or fatty acyl side chain is attacked, and a hydrogen atom is abstracted. Fatty acids with increased double bonds are more susceptible to hydrogen abstraction, as removal of a hydrogen atom is easier, and therefore, polyunsaturated fatty acids are particularly prone to lipid peroxidation. The removal of the hydrogen atom leaves behind an unpaired electron on the carbon atom where it originated from. In aerobic cells, the lipid radical can then react with O_2 to produce a peroxyl radical. Peroxyl radicals may have several fates: they can combine with each other, attack membrane proteins, or are also capable of abstracting hydrogen from fatty acid side chains in the membrane. By this process, a chain reaction is established, and the lipid peroxidation reaction is propagated. Consequently, a single initiation reaction may subsequently result in the peroxidation of hundreds of fatty acids. The number of peroxidation

reactions can be determined by the lipid-protein ratio of the membrane, as the chance of a radical attacking a membrane protein increases as the protein content of the membrane increases, along with fatty acid composition, oxygen concentration, and the presence of chain-breaking antioxidant molecules within the membrane (Halliwell and Chirico, 1993).

$$\text{L-H} + \text{OH}^{\bullet} \rightarrow \text{H}_2\text{O} + \text{L}^{\bullet}$$

As polyunsaturated fatty acids are particularly susceptible to lipid peroxidation, this explains why pepper carotenoids are often esterified with saturated fatty acids, as they are less prone to oxidation, and therefore are more stable (Schweiggert et al., 2005).

1.6.2. Antioxidative mechanisms

As discussed, oxidative damage can have major detrimental effects on cell function, and therefore antioxidative mechanisms are required to mitigate these harmful effects. Scavenging of harmful ROS needs to be very carefully regulated, in order to ensure that the signalling roles of ROS can still be fulfilled, whilst cellular damage is limited (Sharma et al., 2012). Examples of antioxidative mechanisms utilised by the plant in order to limit oxidative damage include enzymatic components, such as catalase and superoxide dismutase, along with non-enzymatic components: metabolites, including carotenoids, tocopherols, and phenolic compounds.

1.6.2.1. Non-enzymatic antioxidative mechanisms

Carotenoids are degraded by oxidative processes such as lipid peroxidation because they are playing a protective, antioxidative role. Carotenoids scavenge singlet oxygen ($^1O_2$) and quench excited chlorophyll (Chl$^{\bullet}$ and 3Chl$^{\bullet}$) in order to protect the photosynthetic components of the cell from oxidative damage (Sharma et al., 2012). Overexpression of the β-carotene hydroxylase gene in *Arabidopsis thaliana* resulted in increased xanthophyll production, and increased tolerance to stress conditions, as shown by a reduction in leaf necrosis, and a reduction in lipid peroxidation (Davison et al., 2002). Oxidation of β-carotene in *Arabidopsis thaliana* leaves revealed the presence of $^1O_2$-specific endoperoxides when plants were grown under low light conditions, suggesting the chronic oxidation of carotenoids by $^1O_2$. β-carotene endoperoxide was rapidly accumulated during high-light stress (Ramel et al., 2012). This metabolite provides a marker for the extent of $^1O_2$ production in leaves, and further, scavenges these reactive oxygen species before the oxidation of fatty acids can occur. Carotenoid structures account for their ability to scavenge radical species and protect cells against oxidative damage: their isoprene residue chains, with

numerous conjugated double bonds, allows for energy uptake from excited molecules, and the dissipation of excess energy as heat (Sharma et al., 2012).

Other secondary metabolites also play essential antioxidant roles in protecting against oxidative damage, though carotenoids are focused on in this study due to their role in conferring the colour phenotype.

1.6.2.2. Enzymatic antioxidative mechanisms

Enzymatic components of the antioxidative defence system include several antioxidant enzymes, for example superoxide dismutase (SOD) and catalase (CAT). Such enzymes act in different cellular compartments, and may react simultaneously when the cell is under oxidative stress.

Superoxide dismutase (SOD) plays a crucial role in all aerobic organisms in defence against oxidative stress (Scandalios, 1993). SOD has three isozymes, including copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and iron SOD (Fe-SOD) (Fridovich, 1989), and these isozymes are specific to different subcellular compartments in plant cells (Fridovich, 1989). Mn-SOD is localised to the mitochondria, Fe-SOD is found in the chloroplasts, and Cu/Zn-SOD is found in the cytosol, chloroplasts, peroxisome, and mitochondria (Alscher et al., 2002). SOD activity has been shown to increase in response to various environmental stresses in plants, such as drought and metal toxicity (Sharma and Dubey, 2005, Mishra et al., 2011).

Superoxide dismutases protect aerobic organisms against oxidative damage by scavenging superoxide radicals in the following reaction (Scandalios, 1993):

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

Previous studies have identified four SOD isoenzymes present in chilli pepper. In pepper, two Cu/Zn-SOD isozymes were identified. SOD activity was qualitatively analysed using a native protein gel based assay in fresh and dry fruit (Berry, 2015). Such isoforms have also previously been identified in cassava (Reilly et al., 2003, Xu et al., 2013). Of the identified SOD isozymes in pepper, a Cu/Zn-SOD isoform, identified by a band on the protein gel, showed the most intense activity, and this was thought to be due to the fact that the chloroplasts are a site of high ROS generation, and therefore demand higher levels of enzymatic antioxidants (Berry, 2015). Interestingly, when SOD activity was compared between dried lines in a colour diversity panel of chilli peppers displaying differences in colour retention phenotype, low colour retention lines

showed the most intense SOD activity when compared to high retention lines (Berry, 2015). This suggests that SOD activity alone is not sufficient to confer protection against the degradation of carotenoids following fruit drying. This may be caused by low colour retention lines producing high levels of ROS, and therefore causing increased expression and activity of SOD in order to protect the chloroplasts and chromoplasts from oxidative damage (Apel and Hirt, 2004). Low carotenoid retention lines may have higher levels of SOD activity as the additional antioxidative capacity was required to compensate for the fact that carotenoids had already been degraded (Berry, 2015). As the pepper fruits were dried prior to analysis, it is possible that carotenoid degradation had already occurred in the low carotenoid retention varieties, and therefore increased SOD activity was required to compensate for the decreased carotenoid antioxidant capacity.

However, it is also important to note the potential toxic activity of SOD. High SOD levels, when accompanied by micromolar concentrations of Fe^{2+} have been found to increase the production of hydroxyl radicals. This was due to the Fenton reaction (Mao et al., 1993). It has been suggested that the balance between SOD activity and catalase activity is of greater importance for sensitivity to oxidative stress, than simply the level of SOD alone. Therefore, an increase in SOD concentration, and the toxic effects accompanied with this, may be due to increased levels of hydrogen peroxide. Without adequate quantities of catalase, and in the presence of small amounts of iron, hydrogen peroxide may undergo conversion to the highly toxic hydroxyl radicals, due to a Fenton-type reaction (Mao et al., 1993).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$$

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet$$

As discussed, catalase (CAT) is another key enzyme involved in the antioxidative scavenging of harmful reactive oxygen species. Catalase is a tetrameric heme-containing enzyme, responsible for catalysing the dismutation of two H_2O_2 molecules and converting them into water and oxygen. Catalase has a high specificity for H_2O_2. H_2O_2 is produced in response to many stress conditions, therefore when H_2O_2 is produced via catabolic processes in large quantities, CAT is required to degrade H_2O_2 efficiently (Mallick and Mohn, 2000). The reaction mechanism of catalase is as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

It is clear therefore, that both SOD and CAT must work simultaneously in order to ensure that ROS are quenched when produced, otherwise inefficient enzymatic activity may lead to further ROS production via H_2O_2 degradation to $OH^{\bullet}$.

Reactive oxygen species are an inevitable component of cell metabolism, and are required for cell signalling, however, it is important that cells detoxify excessive ROS, in order that detrimental processes such as lipid peroxidation can be protected against. Carotenoids may quench reactive oxygen species in order to protect cells from damage by ROS, however, this leads to their degradation. Other cellular antioxidative processes include the activity of enzymes such as superoxide dismutase and catalase, which quench ROS. SOD and CAT activities may influence the rate of carotenoid degradation through ROS quenching, as increased enzyme activity may mean that lower levels of carotenoid scavenging activity is required for efficient ROS detoxification. This, therefore, results in less carotenoid degradation.

1.7. Post-harvest storage of the pepper crop

The pepper crop is in demand from consumers year-round, however, due to monsoon seasons in pepper growing regions, the pepper crop cannot be grown and harvested throughout the year. Therefore, storage of dry peppers for several months following harvest is essential to ensure that consumer demand can be met throughout the year. However, pepper fruits must retain their quality during the post-harvest storage period in order that they are still valuable to growers. Consequently, the key quality traits in pepper previously discussed, including colour, taste, and pungency, must be retained at a high level for several months once fruits have been removed from the plant. Understanding fruit changes during post-harvest storage is essential in order that quality retaining pepper varieties can be bred and grown. This is particularly essential in the face of drastic global climate change, and a growing global population, as demands on food sources become even greater.

Pepper fruits grown for the dried powder market are harvested from plants, and sun-dried for two to three weeks, before being stored in cold, dark conditions (Syngenta, personal communication). Therefore, changes to the fruit during these drying and storage processes should be understood in order to limit losses.

Post-harvest storage losses are not only observed in fruit crops, such as pepper, but in a wide array of important crops, including cereals, such as rice, wheat, and maize (Kumar and Kalita, 2017), along with tuberous crops, such as potato (Prusky, 2011). Whilst some of the major losses to grain crops during storage have been reported as

due to pest infestation, including insect, rodent, and moulds (Tefera, 2012, Kumar and Kalita, 2017), major losses are also experienced to the nutritional quality of crops.

Carotenoid losses during post-harvest storage are well reported in a variety of crop species (Bechoff et al., 2011, Burt et al., 2010), and this affects both the colour quality and nutritional quality. Interestingly, storage of biofortified orange maize in low O_2 environments enhanced carotenoid retention, and therefore resulted in increased provitamin A capacity (Taleon et al., 2017). Further to this, increased β-carotene level in cassava roots was correlated with decreased post-harvest physiological deterioration (Chavez et al., 2000). A study examining the effect of drying methods on carotenoid retention revealed that sun drying cassava was more detrimental to provitamin A capacity compared to oven or shade drying, whereas no difference was observed to provitamin A capacity between drying methods in sweet potato (De Moura et al., 2015).

In contrast, an increase in lutein and β-carotene content was observed in pumpkins, which were stored following harvest (Jaswir et al., 2014). Several studies have examined the response of carotenoids in chilli pepper to post-harvest storage. Decreases in carotenoid content were observed in red peppers following post-harvest storage (Schweiggert et al., 2007), whilst another study demonstrated that carotenoid content increased during the drying process in some pepper varieties (Minguez-Mosquera et al., 2000). However, it should be noted that these studies were performed on different pepper varieties and in different environmental conditions.

Studies carried out previously have attempted to uncover the mechanisms controlling carotenoid retention during post-harvest storage, although this trait appears to be complex. Expression of the cauliflower *Or* transgene in potato promoted the retention of β-carotene in potato tubers, and further stimulated β-carotene accumulation during post-harvest cold storage (Li et al., 2012). The *Or* gene is the only known gene that may act as a molecular switch to trigger the chloroplast to chromoplast transition (Lu et al., 2006), and therefore, an increase in expression of this gene may trigger the formation of increased chromoplasts, which are the site for carotenoid biosynthesis and sequestration. Autoxidation has also been suggested as a mechanism for controlling the rate of carotenoid degradation, with compounds less susceptible to oxidation degrading slower (Pérez-Gálvez and Mínguez-Mosquera, 2001, Pénicaud et al., 2011). Evidently, several potential mechanisms exist that could potentially explain the carotenoid retention trait.

1.8. Resources and tools for breeding improved plant traits

1.8.1. Plant breeding

Plant breeding is used to develop improved cultivars for the needs of farmers and consumers, using methods to create, select, and fixate superior plant phenotypes. Plant breeding is often using to target quality traits with commercial value, including yield, nutrition, and resistance. On the global scale, the development of high yielding maize, wheat, and rice varieties resulted in the Green Revolution, in which agricultural productivity was increased on a dramatic scale (Borlaug, 1983). The earliest examples of plant breeding could be said to be the prehistoric selection of plant phenotypes which increased productivity, and resulted in the domestication of the first crop varieties. Today, advances in plant biology, genetic variation analysis, quantitative genetics, molecular biology, and genomics, have all contributed to modern plant breeding (Moose and Mumm, 2008).

Conventional plant breeding relies on a process known as recurrent backcrossing. In this process, two varieties, one of which contains a trait of interest, for example high carotenoid content, are crossed. Individuals within the progeny (F_1 generation) also displaying this trait are selected and backcrossed against the parent. Recurrent backcrossing is carried out for several generations, until the resulting progeny are phenotypically similar to the parent, except for the fact that they now carry the trait of interest (Smith et al., 2009). Selfing is used in order to achieve homozygosity, which is important for studying traits of interested. However, several selfing events are required in order to reach homozygosity. Interestingly, it has been found that the progeny of such crosses may present a stronger phenotype for the trait of interest, than that of either parent. This is known as hybrid vigour, or heterosis.

1.8.1.1. Doubled Haploid (DH) populations

Doubled haploid (DH) populations are useful in plant breeding, and have advantages over conventional breeding methods. A major advantage of DH populations is the fact that homozygous progeny can be produced from heterozygous parents, in just one generation. Evidently, this saves a significant amount of time, when compared to conventional breeding, in which reaching homozygous lines takes many generations. Generally, in producing doubled haploid systems, an F_1 hybrid is produced from the cross of two independent varieties. Haploids are subsequently produced, followed by a chromosome doubling event using colchicine (Kasha, 2005), in order to produce a population of doubled haploids (Snape, 1981). Doubled haploid populations are particularly useful in studying the inheritance of quantitative traits, in quantitative trait loci (QTL) analysis (Hussain et al., 2012).

1.8.2. Quantitative Trait Loci (QTL) mapping

Many agronomic traits, including quality, yield, drought tolerance, and resistance, are controlled by multiple genes, and are known as polygenic or quantitative. Consequently, multiple genes contribute to the trait, in a quantitative manner. Regions within the genome in which genes associated with the quantitative trait of interest are located, are called quantitative trait loci (QTLs) (Hussain et al., 2012). Molecular markers are also located throughout the genome. Markers which associate with a QTL which influences the trait of interest will segregate more frequently with the phenotype of the trait of interest, due to being closely located in the genome, whereas, markers not associated with QTLs will not show significant association with the trait of interest. QTL mapping is therefore a statistical technique, to determine which markers are significantly associated with the trait of interest. The location of these genetic markers on the molecular map isolates the region in which the QTL for a trait is located (Young, 1996). Candidate genes for the trait of interest can be identified by narrowing down the genes located within the QTL region.

1.8.3. Use of metabolomics to identify novel QTLs

Metabolomics can be defined as the analysis of the complete composition of metabolites within cells and organisms, which collectively is known as the 'metabolome' (Price, 2016). Integration of metabolomics data with genomics, transcriptomics, and proteomics allows for a systems biology approach, which may be necessary to answer complex biological questions (Hall, 2006). Metabolomic studies may encompass a variety of approaches, including untargeted metabolite profiling to identify known and unknown metabolites, along with targeted metabolite profiling, and absolute quantification of known metabolites. Determination of the most relevant approach relies on several factors, including the plant phenotype being studied, whether a specific class of compounds is known to be associated with the phenotype, and whether a broad profile of metabolites or accurate quantification is required.

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are two major tools used in metabolite detection. Mass spectrometry is frequently coupled to a chromatography system: commonly gas chromatography (GC), or liquid chromatography (LC) (Okazaki and Saito, 2012). Gas chromatography and high performance liquid chromatography (HPLC), coupled to detection systems such as flame ionisation (FID), or photodiode array (PDA) detectors, are particularly useful for targeted analysis of metabolites (Price, 2016) as these systems provide quantitative measurements of the compounds of interest.

Characterisation of the metabolome of an array of plant species has been useful in determining QTL regions underlying biochemical pathways of interest. Metabolomic analysis using LC-MS of a wheat doubled haploid population resulted in the identification of 558 metabolic features, and genomic loci were identified associated with several metabolite classes, such as organic acids (Hill et al., 2015). Non-targeted metabolite profiling of tomato leaves was carried out to assess components of leaf metabolism, and QTL mapping was performed to identify genome regions associated with regulation of leaf primary carbon and nitrogen metabolism (Nunes-Nesi et al., 2019). This was carried out to compare against metabolic quantitative trait loci (mQTLs) identified through metabolite profiling of tomato fruits (Alseekh et al., 2015). Evidently, the integration of metabolomic platforms with genomic tools is valuable in identifying genes underlying metabolites of interest, and can be highly useful in breeding plant varieties with valuable agronomic and metabolic traits. In addition, in the future, genome-wide association studies (GWAS) coupled to untargeted metabolomics could potentially be used to annotate metabolites underlying traits of interest.

1.8.4. Gene functional characterisation

Once a gene of interest has been identified, functional characterisation of the gene is required in order to determine its role within the organism. Stable transformation of pepper has been unsuccessful, due to a lack of a protocol which has reliably shown this to be possible (Wang et al., 2013). This is in stark contrast to tomato, which is very amenable to stable transformation. Whilst metabolic engineering has been successful in tomato using stable transformation (Enfissi et al., 2005, Gutensohn and Dudareva, 2016, Nogueira et al., 2017, Enfissi et al., 2019), the same techniques have not proven possible in pepper, and therefore metabolic engineering of pepper has not been reported.

As pepper is an economically valuable crop, characterisation of gene function is essential in order to target the breeding of valuable traits. Consequently, virus-induced gene silencing, a transient method of gene functional characterisation, has been used widely in pepper to determine gene function. For example, *Phytoene desaturase* (*PDS*) and *Ribulose-1,5-bisphosphate carboxylase small subunit* (*rbcS*) genes were silenced in pepper leaves, and displayed bleached and pale yellow phenotypes, respectively (Chung et al., 2004). A reporter gene system has been developed in pepper to allow the identification of silenced regions within pepper plants, and this relies on co-silencing the gene of interest with genes that have a visible phenotype, such as those involved in anthocyanin biosynthesis. Silencing of *Capsaicin synthase* (*CS*) in purple fruits, in tandem with the silencing of the *An2* MYB transcription factor,

which is a genetic determinant of anthocyanin-rich pepper fruits, resulted in decreased capsaicin levels in fruit tissue devoid of purple pigmentation (Kim et al., 2017). Evidently, this is a useful tool for analysing gene function.

The method of gene silencing using VIGS relies on the plant's innate immune system of post-transcriptional gene silencing (PTGS) against intracellular viral proliferation. The viral genomes, which are modified to contain part of the plant's target gene, are transformed into the plant using *Agrobacterium tumefaciens.* Upon transformation, transgenic RNA is transcribed and replicated by an endogenous RNA-dependent RNA polymerase (RDRP), in order to produce double stranded RNA. This triggers post-transcriptional gene silencing. Double stranded RNA molecules are recognised by the DICER-like proteins, which cleave double stranded RNA into short interfering RNA (siRNA). These short RNA molecules are then recognised by the RISC complex, which converts double stranded siRNAs into single stranded siRNA. The RISC complex targets any sequences complementary to these siRNA sequences, and degrades them. These target sequence-specific siRNAs are amplified and then transported throughout the plant, resulting in systematic gene silencing throughout the plant (Lange et al., 2013) (Figure 1-16). The *Tobacco rattle virus* (*TRV*) has been widely used as the viral vector for use in VIGS, although, other RNA viruses may also be used in this system (Lange et al., 2013).

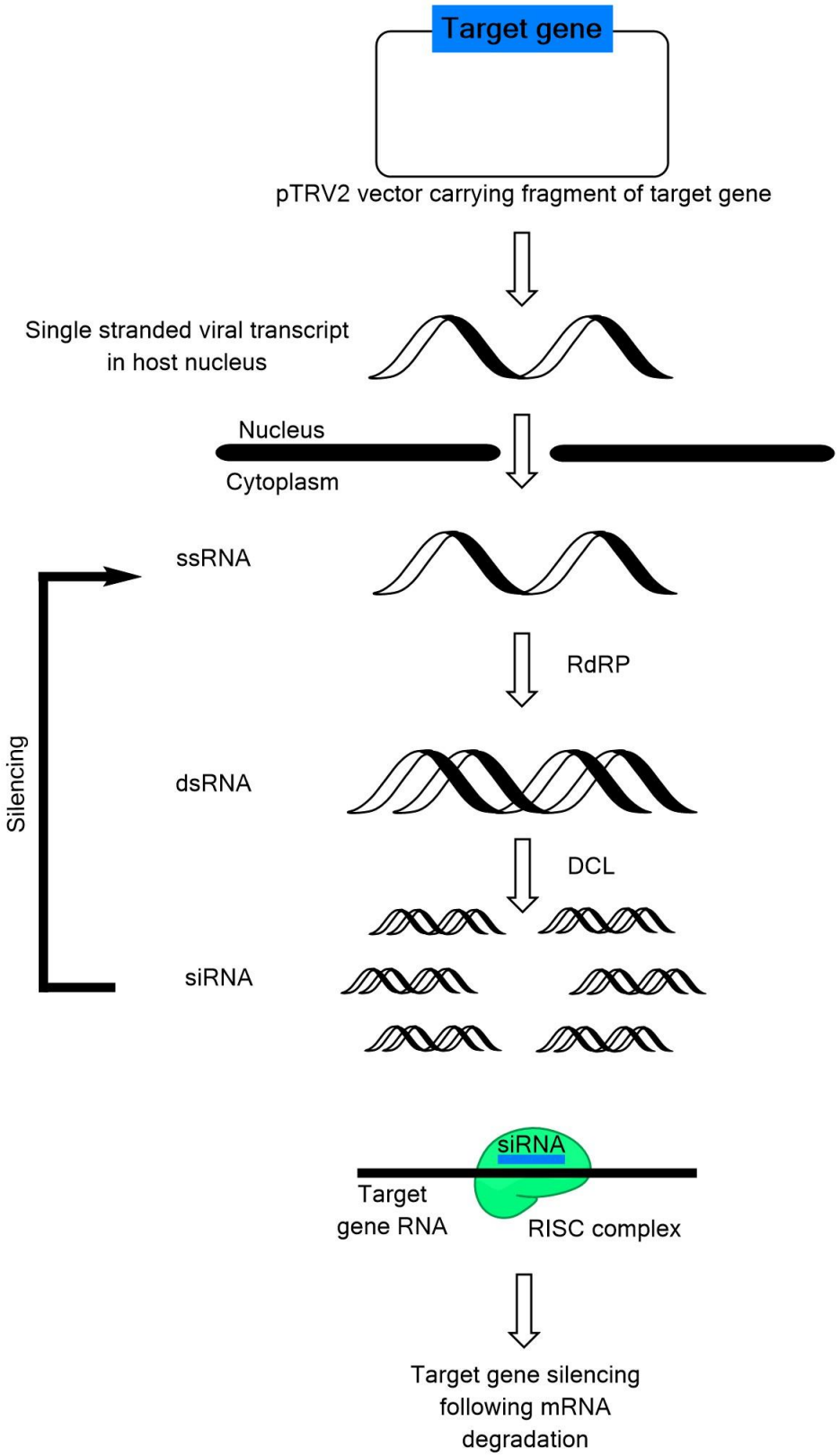


Figure 1-16 Virus-Induced Gene Silencing mechanism

VIGS uses the plants innate immune system of post-transcriptional gene silencing against viral replication in order to silence genes. Following transformation of the plant using Agrobacterium tumefaciens, transgenic RNA is transcribed and replicated by an endogenous RNA-dependent RNA polymerase (RdRP) resulting in double stranded RNA (dsRNA). Double stranded RNA is recognised by DICER-like protein (DCL) and cleaved into short interfering RNA (siRNA). siRNAs are recongised by the RISC complex and converted into single stranded siRNAs. The RISC complex targets endogenous sequences complementary to the siRNA, and degrades them, resulting in target gene silencing. Target sequence-specific siRNAs are amplified and transported throughout the plant, resulting in systematic gene silencing.

1.9. Aims and objectives

The aim of the work presented here is to understand the mechanisms controlling carotenoid retention in *Capsicum annuum* during post-harvest fruit storage. Through gaining an understanding of the biochemical, molecular, and physiological mechanisms associated with this key quality trait, breeders should be able to develop pepper varieties which retain high colour quality throughout post-harvest storage.

Several objectives were defined for the work, in order to ensure a systematic approach was used in investigating these mechanisms.

Objective one: Understand the biochemical mechanisms controlling carotenoid retention in pepper.

- Determine the carotenoid profile of the fresh fruit of a doubled haploid (DH) population displaying variation in the colour retention phenotype, using an initial screen.

- Determine the carotenoid profile of the post-harvest dried and stored fruit of this DH population, in an initial screen.

- Calculate the carotenoid retention value for each accession within the DH population, to ultimately be used in QTL mapping.

- Profile fruit metabolites for the fresh DH population, using GC-MS analysis, to determine whether other fruit metabolites correlate with carotenoid retention phenotype. This data may be used for metabolite QTL mapping.

- Select accessions from these profiling screens, which display an extreme high or low carotenoid retention phenotype, to form a sub-population. Detailed carotenoid profiling and metabolite profiling of these lines will be performed.

- Determine changes in volatile profile of accessions within the sub-population during post-harvest storage.

Objective two: Understand the changes in carotenoid profile during a period of post-harvest storage, in greater detail.

- Determine changes in carotenoid profile at time points throughout post-harvest storage, on a carotenoid retention diversity panel to determine biochemical and physiological changes to pepper fruits during storage.

- Determine whether changes in pepper fruit structure alter carotenoid retention phenotype.

Objective three: Determine whether physiological changes to the pepper fruit contribute to carotenoid retention phenotype.

- Characterise the physical structure of the cuticle of pepper fruits, displaying variation in carotenoid retention, using microscopy techniques.

- Determine the biochemical components of the fruit cuticle, and how this is associated with the carotenoid retention phenotype.

- Analyse carotenoids associated with pepper fruit epidermis and cuticle, and determine whether pepper fruits have a difference in number of epidermal cell chromoplasts, dependent on the carotenoid retention phenotype.

- Analyse gene expression changes in high and low carotenoid retention epidermal cells.

Objective four: Understand the changes in gene expression between high and low carotenoid retention pepper varieties, and develop a method for functional analysis of pepper genes.

- Analyse changes in gene expression between high and low carotenoid retention varieties through ripening, and determine how this may contribute to the carotenoid retention phenotype.

- Develop VIGS as a method for characterising gene function in pepper fruits.

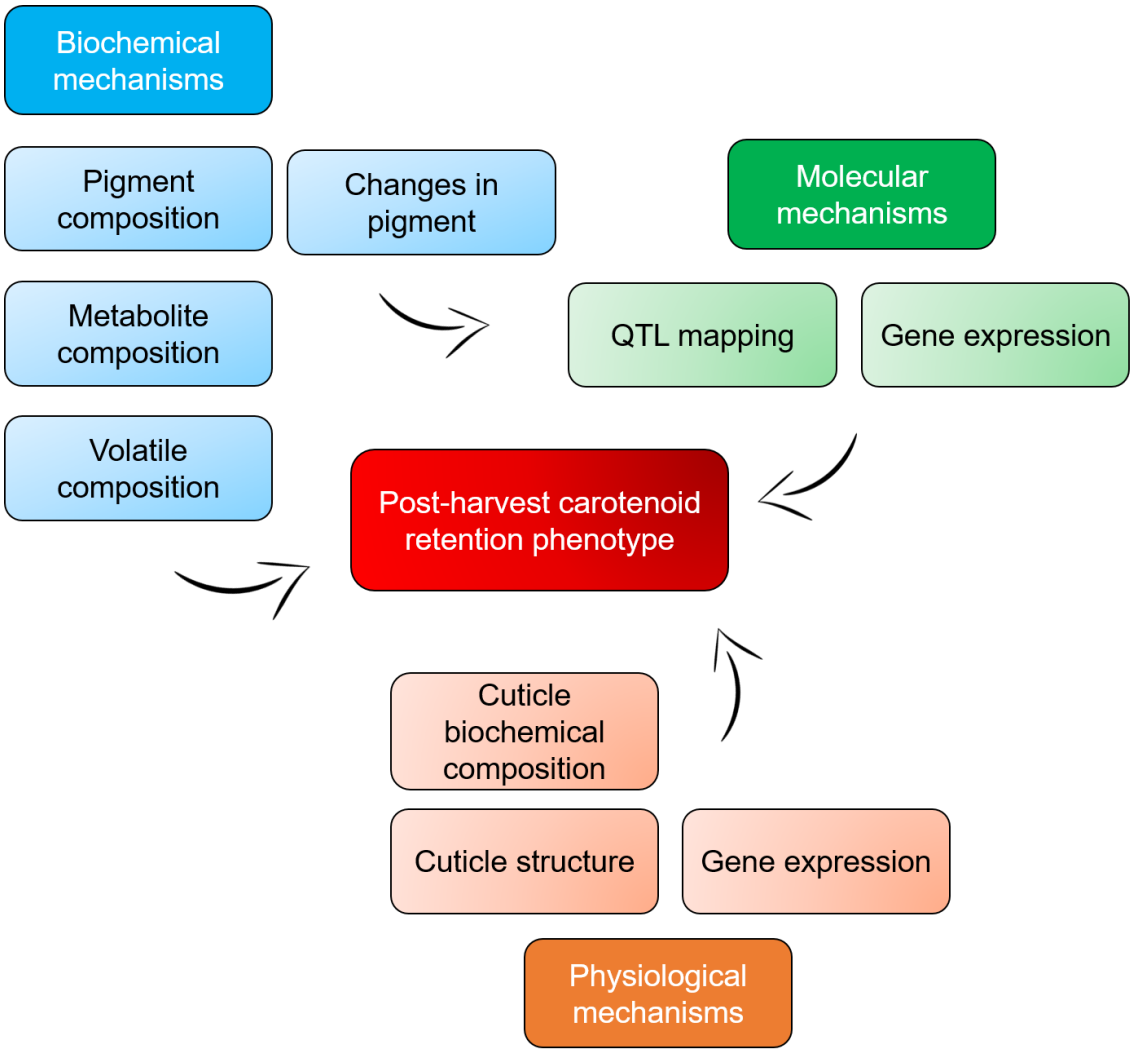


Figure 1-17 Experimental strategy to address the aims of this study.

Investigations performed to determine biochemical mechanisms contributing to pepper carotenoid retention, including carotenoid, metabolite, and volatile profiling. Molecular mechanisms investigated using QTL mapping, and RNAseq to study gene expression. Physiological mechanisms investigated using microscopy to study cuticle structure, cuticle composition analysis, and gene expression analysis.

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich, UK, unless stated otherwise. All primers were purchased from Eurofins MWG Operon, UK.

2.1.1. Diversity panel

A panel of 12 chilli pepper lines displaying diversity in colour intensity and retention phenotypes was provided by Syngenta. *Capsicum annuum* L. cv. CM334 (Criollo de Morelos 334), a landrace from the Mexican state of Morelos, which has been used extensively in hot pepper research, was sourced from INRA (Institut National de la Recherche Agronomique), France. The diversity panel was grown in glasshouses (25 °C; 16/8 h light/dark) at Royal Holloway, University of London. John Innes 3 compost was used to grow peppers.

2.1.2. Double Haploid (DH) population

A Double Haploid (DH) population consisting of 375 lines displaying variation in colour retention phenotype was created by breeders at Syngenta, by crossing a line characterised as low retention with a line characterised as high retention.

Seeds were sown in transplanting trays containing coco peat, and seedlings were transferred to 12 cm plastic pots four weeks after sowing. Seedlings were grown in a growth chamber with 25/20 °C (day/night) under a 16 hour light photoperiod. Flower buds were collected at the stage in which anthers had uninucleate microspores, for anther culture. Flower buds were surface sterilised using 10 % sodium hypochlorite for 15 minutes, and then rinsed 3-4 times with sterile distilled water. Anthers were removed without filaments and placed in 30 mm petri dishes under aseptic conditions. MS medium (30 g/L sucrose, 5 mg/L $AgNO_3$) was used for anther culture. Anthers were incubated at 35 °C in the dark for seven days, and then transferred to 25 °C with 16 hour/8 hour light/dark photoperiod. Illumination was provided by white fluorescent lamps (3000 lux). After two weeks anthers were subcultured into MS media without hormones and $AgNO_3$, and with sucrose (30 g/L) and plates were incubated under the same conditions. Embryos with a torpedo shape were transferred to hormone-free MS media (30 g/L sucrose) and cultured at 25 °C, with the same photoperiod for germination.

Ploidy level of plants was detected using a flow cytometer (Partec, Germany). Young leaves of plants obtained from anther culture were taken *in vitro* and put into petri dishes containing 400 mL extraction buffer (Partec CyStain UV Precise P), and chopped using a razor blade. Samples were filtered using a Partec 40 µm CellTrics

disposable filter. The filtrate was combined with 1.6 mL staining buffer, and analysed in the flow cytometer.

All plants evaluated as diploid (spontaneously doubled), were hardened directly. Plants evaluated as haploid were treated with colchicine, by complete dipping of plantlets in aqueous colchicine solution (0.1 g/L), for 24 hours. Part of the leaves were maintained outside of the liquid in order to avoid asphyxia and plant stress, but all axillary buds were dipped in the colchicine solution. Without rinsing, plantlets were transferred into soil in a humidity saturated environment for several days, and then transferred into normal humidity conditions.

The DH population (375 lines) was grown in Aurangabad, India by Syngenta, and fruits were harvested and sent for analysis at Royal Holloway. Fruits from each line in the population were sent from two post-harvest time points: immediately post-harvest (0 days post-harvest), and at 7 months post-harvest (following drying in the sun for 15 days, and cold storage).

2.1.3. Tissue preparation

Following harvesting, seeds were removed from fruit tissue, pericarp was cut into small pieces (1 cm^2) and flash frozen in liquid nitrogen before being lyophilised. In the case of the DH population, which was grown in India, fruits were shipped to Royal Holloway on ice, and then lyophilised at Royal Holloway.

2.2. Metabolite profiling methods

2.2.1. High Pressure Liquid Chromatography (HPLC) with on-line photodiode array (PDA) detection

2.2.1.1. Carotenoid extraction for High Pressure Liquid Chromatography

Lyophilised and homogenised chilli powder (10 mg) was weighed and placed in a microcentrifuge tube (2 ml). HPLC-grade methanol (250 µl) and chloroform (500 µl) were added, and subsequently vortexed (10 seconds). The suspension was incubated on ice, in the dark (20 minutes). HPLC-grade water (250 µl) was added, and the sample was again vortexed (10 seconds). The sample was centrifuged (12000 rpm; 5 minutes) in order to achieve a phase separation, and the chloroform layer was then removed and placed in a fresh microcentrifuge tube (2 ml). Chloroform (500 µl) was again added to the original tube, and the sample was vortexed and incubated on ice, in the dark (20 minutes). The sample was centrifuged (12000 rpm; 5 minutes), and the chloroform fraction was again removed and pooled along with the first chloroform

fraction. Chloroform was evaporated from the sample using a rotary evaporator (GeneVac Ez-2 plus; 30 minutes), and the residue was stored (-20 °C) prior to analyses by HPLC.

2.2.1.2. Preparation of carotenoid extracts for HPLC-PDA analysis

HPLC coupled to photodiode array (HPLC-PDA) was used to quantify carotenoids. Samples were resuspended in ethyl acetate (200 µl; HPLC grade), vortexed, and centrifuged (12000 rpm; 5 minutes). The sample (50 µl) was transferred to a glass insert within a glass vial, and loaded onto the HPLC.

A Waters Alliance system (separation module 2695) with photodiode array detector (Waters 996) was used for HPLC analysis. A reverse phase C_{30} column (5 µm; 250 x 4.6 mm) with accompanying guard (YMC Inc., USA) was used. Column temperature was maintained at constant temperature (25 °C) with a column chiller (Jones Chromatography 7955). The sample was injected (5 µl), and the solvent flow rate was 1 ml/minute.

Solvent system:

Solvent	Solvent proportion – 1	Solvent proportion – 2	Linear gradient to:
A – Methanol (HPLC grade)	95%	80%	30%
B – Ammonium acetate (0.2%)	5%	5%	5%
C – Tert-methyl butyl ether (MTBE; HPLC grade)	-	15%	65%

Waters Empower software (Waters Alliance) was used to analyse data.

2.2.2. Gas Chromatography Mass Spectrometry (GC-MS)

2.2.2.1. Metabolite extraction for Gas Chromatography Mass Spectrometry

Lyophilised and homogenised chiili powder (10 mg) was weighed and placed in a microcentrifuge tube (2 ml). HPLC-grade methanol (400 µl) and distilled water (400 µl) were added to the tubes, which were subsequently vortexed (10 seconds). The suspension was inverted, in the dark (room temperature; 1 hour). Chloroform (800 µl) was added to the sample; the sample was vortexed and centrifuged (12000 rpm; 5

minutes), to allow phase separation. The polar phase was removed and transferred to a fresh microcentrifuge tube. The non-polar phase was removed and transferred to a clean glass vial.

2.2.2.2. Preparation of extracts for Gas Chromatography Mass Spectrometry

The polar sample (20 µl) was transferred to a glass insert within a glass vial, and spiked with deuterated succinic acid (10 µg) as internal standard. The non-polar sample was spiked with deuterated myristic acid (10 µg) as internal standard. Samples were stored (-20 °C) until derivitisation.

Methoxyamine hydrochloride (MeOX; 20 mg/ml) was dissolved in pyridine anhydrous. MeOX (30 µl) was added to the sample, vortexed, and incubated (40 °C; 1 hour). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; 70 µl) was added to the sample, vortexed, and incubated (40 °C; 2 hours). The sample was transferred to a glass insert, if not done previously, and loaded onto GC-MS.

2.2.2.3. Gas Chromatography Mass Spectrometry data analysis

Gas Chromatography Mass Spectrometry analysis was performed on an Agilent Technologies 7890B gas chromatograph with a 5977A MSD. Samples (1 µl) were injected with a split/splitless injector at 290 °C in splitless mode. Metabolites were separated on a DB-5MS 30 m x 250 µm x 0.25 µm column (J&W Scientific, Folsom, California, US), equipped with a 10 m guard column. Retention time was locked to the internal standard. Oven temperature was as follows.

Temperature	Time
70 °C	4 minutes
Ramp to 310 °C	5 °C/minute
310 °C	10 minutes

Mass spectrometry was performed in full scan mode using 79 eV EI+ and scanned from 10 to 800 D. Chromatogram components were identified in the pepper profiles using a mass spectral (MS) library constructed from in-house standards, alongside the NIST 2.0 (National Institute of Standards and Technology, USA) MS library. Using retention indices and MS, identification was performed by comparison with the MS library. Response factor was calculated relative to the internal standard.

GC-MS data was analysed using various software. Component peak identification and spectral deconvolution were performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS, v2.71), NIST, Excel, SIMCA v15 (Umetrics AB), MetaboDrift (Thonusin et al., 2017), and XLStat (Addinsoft 2019).

2.2.3. Semi-volatile analysis

2.2.3.1. Sample preparation for semi-volatile analysis

Volatile analysis performed on material from Double Haploid population was stored dry at -20 °C, and therefore needed to be rehydrated prior to analysis. Material was weighed (500 mg) and distilled water (2 mL) was added to rehydrate the material. This was calculated based on previous experiments which suggest that peppers are comprised of 80 % water, as approximately pepper weight was decreased by 80 % during drying process. Therefore, water was added to the dry material to make back up to the relevant 'fresh' weight. Further distilled water (7.5 mL) was then added in order to create a 3:1 ratio (water : material). Samples were vortexed well. Aliquots (1.5 mL) were removed and placed into cryo tubes, and subsequently flash frozen in liquid nitrogen.

2.2.3.2. Semi-volatile extraction

Volatile extraction and analysis was carried out according to Kende et al. 2019 (Kende et al., 2019). Homogenised juice (1.5 mL) was agitated with an adsorbent rod. The rod was rinsed with water, dried, and thermally desorbed (260 °C; 5 minutes).

2.2.3.3. Semi-volatile analysis by Gas Chromatography Mass Spectrometry

Volatile components were analysed using an Agilent 7890B gas chromatograph and 5977A MSD. The inlet used was a Gerstel Thermal Desorption Unit (TDU).

Volatile results were calculated in ng/mL using a 7 point calibration curve. Known amounts of each standard were spiked into water (1.5 mL) containing a rod and internal standard (1,4-dichlorobenzene; 250 ng/ml). The limit of quantitation (LOQ) was defined as the lowest standard for which a detectable peak was observed. Volatile results were calculated in ng/mL using a 7-point calibration curve.

2.3. Pepper storage methods

2.3.1. Drying and storage of pepper material

Peppers were harvested when ripe and dried in a drying oven (30-40 °C) under a 12/12 hour light cycle. Peppers were dried for 2 weeks before being stored in hessian bags

(4 °C) for a period of between 2 – 12 weeks. These conditions were selected in order to replicate the industrial drying and storage process that occurs during commercial growing and storage of chilli peppers (these conditions are used by Syngenta commercially).

Upon removal from cold storage, samples were stored at -80 °C. Samples were lyophilised to ensure all moisture was removed from peppers. Samples were then processed for HPLC, GC-MS, or volatile analysis.

2.4. Fruit surface structure analysis methods

2.4.1. Fruit cuticle microscopy

2.4.1.1. Light microscopy

Light microscopy was carried out at Syngenta Ltd., Jealotts Hill, Bracknell, UK, with the assistance of Dr Chris Stain. Fresh chilli pepper fruits were harvested immediately prior to slide preparation. Pepper fruits were cut in half, and the hand sectioning method was used to cut very fine cross sections from the middle of the fruit. Sections were collected in distilled water. A solution of Nile Red (0.5 mg/ml) was prepared in acetone, and Fast Green solution (0.5 %) in ethanol (90 %). Sections were washed in acetone (1 minute) before being submerged in the Nile Red solution (5 minutes). Samples were washed in ethanol (90 %; 1 minute), and were then submerged in Fast Green solution (1 minute). Samples were again washed in ethanol (90 %; 1 minute), and then in distilled water (1 minute). Samples were mounted on glass slides in distilled water and covered by a glass cover slip, before being examined under the microscope immediately.

A Leica DM 500 compound light microscope was used, with 10x and 20x objective lenses. The software used to capture images was IMS (Imagic, Imaging Ltd.).

2.4.1.2. Scanning Electron Microscopy (SEM)

SEM was carried out at Syngenta Ltd., Jealotts Hill, Bracknell, UK, with the assistance of Dr Chris Stain. Samples were mounted on aluminium pin stubs (SEM sample supports), using adhesive carbon tabs. The samples were not coated. Microscopy was carried out using a Zeiss Evo LS15 scanning electron microscope. Sample imaging was carried out using the microscope's variable pressure mode (to allow for the imaging of non-conductive uncoated samples), and images were captured via the instrument's C2D detector.

2.4.2. Exocarp thickness measurements

Exocarp thickness was measured using ImageJ software. Exocarp thickness was measured using light microscopy images. Exocarp was defined as area stained pink following Nile Red staining. Three images per genotype were measured, and six measurements per image were recorded. ANOVA was used to determine statistical significance.

2.4.3. Isolation of fruit exocarp by enzymatic digestion

Fruits were harvested from plants immediately prior to wax exocarp removal. Fruits were washed with tap water and dried gently. A cork-borer was used to cut circles (1 cm diameter) of fruit pericarp tissue, which was incubated whilst gently shaking in pectinase (1.5 % w/v), cellulase (0.1 % w/v), in citrate buffer (0.2 mM, pH 3.7), with sodium azide (1 mM) to prevent microbial growth. Samples were incubated in the dark, whilst shaking (35 °C; 4 days; 100 rpm). After incubation, samples were washed three times in acetone with butylated hydroxytoluene (50 mg/L) as an antioxidant. Samples were allowed to air dry.

2.4.4. Cutin monomer analysis

2.4.4.1. Cutin monomer extraction

Cuticles were refluxed with chloroform and methanol (1:1) in conical flasks (1 week) to remove lipids from the wax discs. Transesterifcation was then carried out by adding boron trifluoride methanol solution (BF_3/MeOH; 4 mL) and incubated (16 h; 70 °C) in sealed glass vials. Vials were then removed from incubation and allowed to cool to room temperature. C_{32} alkane (100 µg) was added as internal standard and vortexed. The transesterification reaction was stopped by transferring the transesterification solution to a vial containing water saturated sodium bicarbonate ($NaHCO_3$/H_2O; 2 ml). In order to extract cutin monomers, chloroform (3 mL) was added to the transesterified sample and phase separation was allowed to occur. The chloroform fraction containing the cutin monomers was transferred to a clean glass vial, and chloroform (3 mL) was again added to the transesterified sample to extract further monomers. Phase separation was again allowed to occur, and the chloroform layer was removed and pooled with the first chloroform fraction. Water (1 mL; HPLC grade) was added to chloroform extracts. Phase separation again occurred, and the upper polar phase was discarded. Chloroform was dried by adding anhydrous sodium sulphate (1.5 g) in order to eliminate all traces of water. Chloroform extracts were transferred into a reaction vial and chloroform was evaporated under nitrogen. Monomer extracts were then resuspended in fresh chloroform (100 µL). Samples were derivitised by adding pyridine (20 µL) and N,O-Bis(trimethlysilyl)trifluoroacetamide (BSTFA; 20 µL).

Samples were vortexed thoroughly and incubated (70 °C; 1 h). Samples were cooled to room temperature, vortexed, and transferred to GC-MS autosampler vials for GC-MS analysis.

2.4.4.2. Cutin monomer analysis by Gas Chromatography Mass Spectrometry

Gas Chromatography Mass Spectrometry was performed on an Agilent Technologies 7890A gas chromatograph. Samples (1 µL) were injected in splitless mode at a temperature of 200 °C. The column used had dimensions of 30 m x 250 µm x 0.1 µm (J+W 122-1131), and the flow rate was 1.2 mL/minute. Oven temperature was as follows:

Temperature	Time
70 °C	2 minutes
Ramp to 150 °C	10 °C/minute
Ramp to 310 °C	3 °C/minute
310 °C	20 minutes

Mass spectrometry was performed in full scan mode. Chromatogram components were identified in the cutin profiles by comparing sample spectra to literature reported spectra, alongside the NIST 2.0 MS library. Cutin monomer quantities were calculated as relative to the internal standard.

2.4.5. Cuticle wax analysis

2.4.5.1. Cuticle wax extraction

Fruits were harvested from plants immediately prior to cuticle wax analysis. Discs (1 cm diameter) were cut from fresh fruits with a cork borer. Fruit discs were dipped in chloroform (10 mL; 10 seconds) with cuticle side facing downwards into solvent in order to extract cuticle bound lipids and waxes. Deuterated myristic acid (10 µg) was added as internal standard to the chloroform. 10 discs were dipped per replicate. Following cuticle wax extraction, the cuticle extract was transferred to a cleaned glass vial (15 mL) and dried under nitrogen in order to evaporate chloroform. Extracts were resuspended in fresh chloroform (500 µL) and vortexed, before being transferred to a glass GC-MS vial. Extracts were again dried under nitrogen, and then derivitised for GC-MS analysis using pyridine (30 µL) and N-Methyl-N-(trimethylsilyl)

trifluoroacetamide (MSTFA; 70 µl). Samples were incubated (40 °C; 1 h) and then transferred to a glass insert within a vial for GC-MS analysis.

2.4.5.2. Cuticle wax analysis by Gas Chromatography Mass Spectrometry

Gas Chromatography Mass Spectrometry analysis was performed on an Agilent Technologies 7890B gas chromatograph with a 5977A MSD. Samples (1 µl) were injected with a split/splitless injector at 290 °C in splitless mode. Metabolites were separated on a DB-5MS 30 m x 250 µm x 0.25 µm column (J&W Scientific, Folsom, California, US), equipped with a 10 m guard column. Retention time was locked to the internal standard. Oven temperature was as follows.

Temperature	Time
70 °C	4 minutes
Ramp to 310 °C	5 °C/minute
310 °C	10 minutes

2.4.6. Plastid/Genome ratio

Plastid number was calculated per epidermal cell and expressed as a plastid:genome ratio. Fruit epidermal cells were isolated using sandpaper, and DNA was extracted from these cells using the Qiagen DNAeasy Plant Mini kit, according to the manufacturer's instructions. Quantitative PCR (qPCR) was used to determine gene copy number of *Phytoene desaturase* (*PDS*), as a nuclear encoded gene, and *Ribulose bisphosphate carboxylase large chain subunit* (*rbcL*), as a plastid encoded gene, using primers in Table 2-1. Gene copy number was calculated, and the plastid gene number was expressed as a ratio to the nuclear gene number, to represent the number of plastids per cell.

2.4.7. Isolation of carotenoid from exocarp

Pepper fruits discs (1 cm diameter) were dissected, and exocarp discs were isolated according to Section 2.4.3. Exocarp discs were washed for 72 hours in chloroform and methanol (1:1 ratio, 10 mL), in dark conditions on a rotator. Chloroform/methanol wash solution was replaced every 24 hours, and all solvent used for extraction was pooled and evaporated. Exocarp carotenoids were analysed by HPLC-PDA, according to section 2.2.1.

2.4.8. Cuticle gene expression analysis

2.4.8.1. RNA extraction from fruit epidermal cells

Fruit epidermal cells were isolated using an abrasion method. Sterile sandpaper was used to remove epidermal cells from fruits.

At least three chilli pepper fruits were harvested per plant, per time point. Each plant was treated as an independent biological replicate. RNA was extracted immediately following harvest in order to prevent degradation, using Trizol (Invitrogen) and following an adapted PureLink RNA Minikit (ThermoFisher Scientific, Hemel Hempsted, Bucks, UK) protocol. Sterile sandpaper was used to remove epidermal cells from the fruit, and these cells were collected in sterile Falcon tubes (15 mL). Trizol (1 mL; Invitrogen) was immediately added to the cells, and samples were then vortexed vigorously. Samples were centrifuged (4 °C; 4000 rpm, 3 minutes) to remove any contaminating sand. Samples were transferred to microcentrifuge tubes (1.5 mL) leaving behind any sand, and vortexed well. Samples were again centrifuged (4 °C; 12000 rpm; 5 minutes) and supernatant was transferred to a fresh tube. Chloroform (0.2 mL) was added and incubated at room temperature (2-3 minutes). Samples were centrifuged (4 °C; 12000 x *g*, 15 minutes) resulting in a phase separating, leaving a lower red phenol-chloroform phase, and a colourless upper aqueous phase. The upper aqueous phase (600 µL), containing the RNA, was transferred to a fresh tube. An equal volume of ethanol (70 %) was added and vortexed vigorously. The tube was inverted to disperse any precipitate that formed following the addition of ethanol. The sample (700 µL) was transferred to a PureLink RNA Minikit spin cartridge, and centrifuged (12000 x *g*; 15 seconds). The flow through was discarded and the spin cartridge reinserted into the same collection tube. The remaining sample was transferred to the spin cartridge and centrifugation repeated. Wash Buffer I (700 µL) was added to the spin cartridge, and centrifuged (12000 x *g*, 15 seconds). Flow-through was discarded, the spin cartridge was placed back in the collection tube, and Wash Buffer II (500 µL) was added to the spin cartridge. Centrifugation (12000 x *g*, 15 seconds) was carried out, and flow through discarded. The wash step with Wash Buffer II was repeated once. The spin cartridge was then centrifuged (12000 x *g*, 1 minute) to dry the membrane, and then inserted into a fresh microcentrifuge (1.5 mL) tube. RNA was eluted from the membrane in water (40 µL) using centrifugation (12000 x *g*; 2 minutes). The eluted sample was pipetted back onto the spin cartridge membrane in order to elute any remaining RNA, and centrifugation was repeated.

2.4.8.2. DNase treatment

Extracted RNA was treated with TURBO™ DNase (ThermoFisher Scientific) by adding 10X Reaction Buffer (4.5 µL) and TURBO™ DNase (2 µL) to the RNA sample (40 µL), and incubated (37 °C; 25 minutes). In order to inactivate the DNase, the DNase Inactivation Reagent (8 µL) was added and incubated (room temperature; 5 minutes). Samples were centrifuged (12000 x *g*, 1 minute) and the supernatant transferred to a fresh tube. RNA quality was assessed on an agarose gel (1 % w/v) and concentration was measured using a NanoDrop. Samples were then stored at -80 °C, until further analysis.

2.4.8.3. Preparation of RNA for RNAseq analysis

Samples were shipped to Syngenta, Research Triangle Park, USA, for analysis, and therefore needed to be prepared for shipping. Samples were aliquoted (3 µg) into fresh RNA free tubes, and RNAstable (20 µL; Biomatrica) was added. Samples were dried in a rotary evaporator (GeneVac Ez-2 plus; 1 h), before being packaged in a moisture free bag, and then shipped.

2.4.8.4. RNAseq analysis (this work was performed by Syngenta)

The quality of the RNA was tested using a TapeStation. PolyA selection and isolation was carried out by using 500-1000 ng of total RNA (checked by quality control) and polyA isolation was performed using NEBNext® Poly(A) mRNA Magnetic Isolation Module. The mRNA library was prepared using NEBNext® Ultra II RNA Library Prep with sample purification beads and in-house 8 base pair indexes. Libraries were then sequenced, typically 6-8 per lane on a HiSeq.

2.4.8.5. RNAseq bioinformatics analysis (this work was performed by Syngenta)

The R package EDASeq v2.16.3 (Risso, 2013) was used to normalise gene count to correct for within sample biases, which may have been due to gene length or GC content, and between sample biases, which may have been due to differences in sequencing depth. Genes with low read counts were filtered out of the dataset to decrease the likelihood of detecting false positives during differential expression analysis. Gene set enrichment analysis of normalised gene count data was performed using ROAST (Wu et al., 2010). Gene sets were derived from CANNUUMCYC 1.0. Pepper gene and Gene Ontology (GO) term mappings were obtained by running the pepper CM334 gene models through InterProScan. Only gene sets that mapped to at least two or four genes in the transcriptomic dataset (for pathways and GO terms, respectively), were included for ROAST enrichment analyses. Gene sets were considered enriched at FDR < 0.05.

2.5. Gene expression analysis (RNAseq) methods

2.5.1. Extraction of RNA

Fruits were harvested from the plant and immediately flash frozen in liquid nitrogen. At least three fruits per replicate were harvested. The material was processed and homogenised using a TissueLyser II (Qiagen) to a very fine powder, whilst still frozen. 100 mg of frozen material was weighed and placed into microcentrifuge tubes. RNA extraction was carried out using the Plant RNAeasy Minikit (Qiagen) according to the manufacturer's protocol. Briefly, a lysis buffer was added to the tissue to lyse cells. The lysate was transferred to a QIAshredder spin column and centrifuged. The supernatant of the flow-through was used in subsequent steps. Ethanol was added, and the sample was immediately transferred to an RNeasy Mini spin column before being centrifuged. A series of wash steps were performed with buffers provided. An additional ethanol wash step was performed on pepper material in order to remove pigment from the spin column membrane. The RNeasy spin column was placed in a fresh 1.5 mL Eppendorf tube, RNase-free water (40 µL) was added and the sample was centrifuged in order to elute the RNA. Genomic DNA contamination was removed using RNase-Free DNase (Qiagen) according to manufacturer's protocol. Briefly, the RNA was added to Buffer RDD (10 µL) and DNase I stock solution (2.5 µL). Samples were incubated at room temperature (20-25 °C; 10 minutes). RNA was then cleaned up using the wash steps described above. In samples where genomic DNA contamination appeared to be very high, or genomic DNA was resistant to this method of DNA removal, TURBO™ DNase (ThermoFisher Scientific) was used, according to manufacturer's protocol. Briefly, 0.1 volume 10X TURBO DNase buffer and 1 µL TURBO DNase were added to the RNA, and mixed gently. Samples were incubated (37 °C; 20-30 minutes). The DNase was then inactivated by adding the DNase Inactivation reagent (2 µL), and was mixed well. The sample was incubated at room temperature (5 minutes); samples were then centrifuged (10000 x *g*, 1.5 minutes) and the supernatant, containing the RNA, was removed to a fresh tube. RNA quality was assessed on an agarose gel (1 %) and concentration was measured using a NanoDrop. Samples were then stored at -80 °C, until further analysis.

2.5.2. Preparation of RNA for RNAseq analysis

Samples were shipped to IGA Technology Services, Italy, for analysis, and therefore needed to be prepared for shipping. Samples were aliquoted (4 µg) into fresh RNA free tubes, and RNAstable (20 µL; Biomatrica) was added. Samples were dried in a rotary evaporator (GeneVac Ez-2 plus; 1 h), before being packaged in a moisture free bag, and then shipped.

2.5.3. RNAseq analysis (this work was performed at IGA Technology Services, Italy)

Library preparation was carried out using TruSeq Stranded mRNA Sample Prep kit (Illumina, San Diego, CA) according to the manufacturer's instructions, using good quality RNA (1-2 µg; R.I.N >7). The poly-A mRNA was fragmented (94 °C; 3 minutes), and purification was carried out using 1X Agencourt AMPure XP beads.

RNA samples and final libraries were quantified using the Qubit 2.0 Flurometer (Invitrogen, Carlsbad, CA) and quality tested using Agilent 2100 Bioanalyzer RNA Nano assay (Agilent Technologies, Santa Clara, CA). Libraries were processed with Illumina cBot for cluster generation on the flowcell, following the manufacturer's protocol, and sequenced on paired-end mode at the multiplexing level requested on HiSeq2500 (Illumina, San Diego, CA). The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data for both format conversion and de-multiplexing.

2.5.4. RNAseq bioinformatics analysis

Low quality bases and adapter sequences were removed from data files using ERNE and Cutadapt (Martin, 2011) softwares. Sequence alignments were then made to the reference genome (CM334) using TopHat2 (Kim et al., 2013). TopHat aligns RNAseq reads to genomes using the ultra-high throughput short read aligner Bowtie, and analyses the mapping results to identify splice junctions between exons. Transcripts were counted using Cufflinks (Trapnell et al., 2012), using default parameters. Cufflinks assembles transcripts and estimates their abundances in RNAseq samples. Alignments are assembled into a parsimonious set of transcripts, ignoring alignments which are not structurally compatible with the reference transcript provided. The relative abundances of these transcripts are then estimated, based on how many reads supports each one. Pair-wise differential expression analysis was carried out using Cuffdiff (Trapnell et al., 2012). Cuffdiff compares expression levels between genes and transcripts, and provides information on which genes are up- or down-regulated between two or more conditions.

2.6. Virus Induced Gene Silencing (VIGS) of carotenoid biosynthesis genes in pepper

2.6.1. Materials for molecular biology

2.6.1.1. Primers

Table 2-1 Primers used in this study.

Primer Name	Target	Sequence (5'-3')
CaPDSgDNA_F	Ca*PDS*	GCTTTGATTTCCCCGAAGCTTT
CaPDSgDNA_R	Ca*PDS*	AAGGTAAGCGAAGTTCCAGAGG
CaRbclgDNA_F	Ca*RuBisCO*	ACCTTAGGACATCCTTGGGGTA
CaRbclgDNA_R	Ca*RuBisCO*	GCAAGATCGCGTCCTTCATTAC
CaPDS_gibson_F	Ca*PDS*	AGAAGGCCTCCATGGGGATCATGCCCCAAATTGGACTTGT
CaPDS_gibson_R	Ca*PDS*	GAGACGCGTGAGCTCGGTACTGATGATGATAAGAATGCAGC
CaPSY1_gibson_F	Ca*PSY1*	AGAAGGCCTCCATGGGGATCATGTCTGTTGCCTTGTTATG
CaPSY1_gibson_R	Ca*PSY1*	GAGACGCGTGAGCTCGGTACCTGCCTCAAAACCACATCAT
CaCCS_gibson_F	Ca*CCS*	CCATGGGGATCCATGGAAACCCTTCTAAAGC
CaCCS_gibson_R	Ca*CCS*	GAGCTCGGTACCAACTTGTTCAGCTAGCCG
pTRV2_sequencing_F	pTRV2 plasmid with GOI insert	GTTCTTGTGTGTCAACAAAGATGG
pTRV2_sequencing_R	pTRV2 plasmid with GOI insert	GTCGAGAATGTCAATCTCGTAGG
pTRV1_sequencing_F	pTRV1	TAGGCGCCTCAATGTGGAAGAA
pTRV1_sequencing_R	pTRV1	CCAAGCGCCAATCTCAAACAGT
CaPDS_qPCR_F	Ca*PDS*	ATGTTGAAGCTCAAGACGGGAT
CaPDS_qPCR_R	Ca*PDS*	CACTGCATCGAAAGCTCATCAG
CaPSY1_qPCR_F	Ca*PSY1*	GTCTCAAACGGGACAGGATTCT
CaPSY1_qPCR_R	Ca*PSY1*	ACTCCACCTTTGTTTTCCACCT
CaCCS_qPCR_F	Ca*CCS*	CGTGATCATCATTGGAACTGGC
CaCCS_qPCR_R	Ca*CCS*	GTGAAGGGTCAACGCAACATAC
SlPDS_qPCR_F	Sl*PDS*	ATGTTGAAGCTCAAGATGGGAT
SlPDS_qPCR_R	Sl*PDS*	CACTGCATTGAAAGTTCGTCAG

2.6.1.2. Media

2.6.1.2.1. LB

Lysogeny broth (LB) (20 g) was added to water (to 1 L) and autoclaved to create sterile LB liquid media.

LB (20 g) was added to agar (16 g), and made up with water (to 1 L) and autoclaved. 25 mL was poured per Petri dish to make LB agar plates.

2.6.1.2.2. SOB

Super Optimal Broth (SOB) was made by adding tryptone (15 g), yeast extract (3.75 g), NaCl (0.375 g), KCl (0.140 g) to de-ionised water (750 mL). pH was adjusted to 7.5, using NaOH. Media was autoclaved, and 1 % (v/v) $MgCl_2$ (1 M) and 1 % (v/v) $MgSO_4$ (1 M) was added respectively. Media was stored at 4 °C.

2.6.1.2.3. SOC

Super Optimal Broth with Catabolite repression (SOC) was made by adding SOB (49 mL) to Glucose (1 M; 1 mL).

2.6.1.2.4. MS media

Murashige and Skoog media (MS media) was prepared by adding MS with B5 vitamin (2.2 g), sucrose (10 g), Phyto agar (8 g) to water (to 1 L) and autoclaved. Media was melted and poured into Magenta boxes under sterile conditions.

2.6.1.3. Antibiotic

Antibiotic stocks (1000X) were prepared by dissolving into deionised water. Solutions were filter sterilised using 0.2 µm Filtropur filters, and were then stored at -20 °C. An exception to this was in the preparation of Rifampicin. Rifampicin stock (50 mg/mL) was prepared with 100 % DMSO, and filter sterilised.

2.6.1.4. Sequencing

Sequencing reactions were performed by Eurofins MWG Operon Ltd. Primers used for sequencing are listed in Table 2-1.

2.6.2. Vector design

The Sol Genomics Network (https://solgenomics.net/) VIGS tool was used to identify gene regions suitable to be targeted using VIGS. This tool uses an algorithm to simulate *in silico* the mechanism of VIGS within the plant cell. All possible 21-nucleotide fragments of a target gene are processed, to create the short sequences equivalent to the siRNAs produced by Dicer-like ribonucleases within the cell. The siRNAs are mapped to the transcriptome using Bowtie (Langmead, 2010), and the positions of targets and off-targets are obtained. Score values are assigned based on the number of targets, and this information is used to identify a 300-nucleotide window

within the gene, with the minimum number of off-target matches and the maximum percentage of target gene coverage (Fernandez-Pozo et al., 2015).

2.6.3. Vector construction

2.6.3.1. Tobacco rattle virus (TRV) vectors

Tobacco rattle virus (TRV)-based VIGS vector have been shown to be efficient in their use in VIGS to study gene function (Liu et al 2002). Therefore, pTRV1 and pTRV2 vectors were sourced from Tair. The pTRV2 vector is 9663 nucleotide base pairs long, and contains a multiple cloning site.

2.6.3.2. Cloning of target sequences into pTRV2 to create silencing vectors

2.6.3.2.1. RNA extraction from pepper fruit for cloning

RNA was extracted from pepper fruits as described in section 2.5.1.

2.6.3.2.2. cDNA synthesis from plant RNA for cloning

cDNA was synthesised from extracted RNA using the QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturer's protocol. Briefly, 1 µg template RNA was incubated with gDNA Wipeout buffer 7X (2 µL; 42 °C; 2 minutes) then placed on ice immediately. For first strand cDNA synthesis, template RNA was then incubated with reverse-transcription master mix (1 µL), Quantiscript RT buffer 5X (4 µL) and RT primer mix (1 µL) (42 °C; 30 minutes). The reaction was then incubated (95 °C; 3 minutes) to inactivate the Reverse Transcriptase. cDNA was stored at -20 °C until ready to be used.

2.6.3.2.3. Polymerase Chain Reaction (PCR)

The target gene sequence (300 bp) was amplified by polymerase chain reaction (PCR) from *C. annuum* fruit cDNA, using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific), as detailed.

Reagent	Quantity
5X Phusion HF Buffer	10 µL
dNTPs (10 mM)	1 µL
Forward primer (10 µM)	2.5 µL
Reverse primer (10 µM)	2.5 µL
Template DNA	2 µL
Phusion DNA polymerase	0.5 µL
Water	31.5 µL
Total	50 µL

Primers for Gibson Assembly were designed with 20 nucleotide base pair overhangs, complementary with the pTRV2 vector, when linearised with restriction enzymes: KPN1 and BamH1. These primers required a high melting temperature, and therefore, a two-step PCR program was used.

Temperature	Time	Cycles
98 °C	30 seconds	-
98 °C	10 seconds	34 cycles
72 °C	30 seconds	
72 °C	5 minutes	-

2.6.3.2.4. Gel purification

The PCR product was analysed by gel electrophoresis to check the product size, and purified from an agarose gel (1 %), using the Zymoclean Gel DNA Recovery kit (Zymo Research), according to manufacturer's instructions. Briefly, the DNA fragment was cut out of the agarose gel using a scalpel and transferred to a 1.5 mL microcentrifuge tube. ADB was added in a 3:1 ratio to the mass of the gel slice, and this was incubated (55 °C; 10 minutes) until the gel slice had completely dissolved. The melted agarose solution was transferred to a Zymo-spin column in a collection tube, and centrifuged (30 seconds). DNA wash buffer was added (200 µL) and centrifuged (30 seconds). The flow-through was discarded and the wash step repeated. DNA was eluted by adding 10 µL water, and centrifuging (1 minute).

2.6.3.2.5. Vector linearization and purification

The pTRV2 vector was linearised using BamH1 and KPN1 restriction enzymes (ThermoFisher Scientific). The pTRV2 vector (> 3 µg) was incubated with buffer BamH1, BamH1 (4 µL), KPN1 (8 µL), and water (40 µL) using a thermocycler (37 °C; 2 hours) for linearisation, followed by enzyme inactivation (80 °C; 20 minutes). The linearised vector was analysed by gel electrophoresis on a 1% agarose gel and the Zymoclean Gel DNA Recovery kit (Zymo Research) was used to gel purify linearised vector, according to manufacturer's protocol.

2.6.3.2.6. Gibson assembly

Linearised vector and gene fragment with attached overhangs were assembled using the Gibson Assembly kit (New England Biolabs). In order for efficient assembly to occur, 115 ng vector and 33 ng PCR product were required. This was calculated based on the length of the fragments to be assembled and the number of fragments. The appropriate amounts of vector and PCR product were added to the Gibson mastermix (15 µL), and made up to 20 µL with water. The mix was incubated using a thermocycler (50 °C; 45 minutes).

2.6.3.2.7. Preparation of chemically competent *Escherichia coli*

A glycerol stock of *Escherichia. coli* (strain DH5α) was used, and bacteria were spread onto an LB agar media plate and incubated (37 °C; 16 hours). A single colony was picked and used to inoculate LB liquid media (5 mL) in a 50 mL Falcon tube, and incubated whilst shaking (37 °C; 16 hours; 200 rpm). From the starter culture, 500 µL was used to inoculate 50 mL LB liquid media in a 250 mL flask. This was incubated (37 °C; 180 rpm) until an optical density (OD_{600}) of 0.6-0.8 was reached. The culture was transferred to a pre-chilled 50 mL Falcon tube, and the cells were incubated on ice (10 minutes). Cells were centrifuged (4 °C; 10 minutes; 2700 *x g*). The supernatant was removed and cells were resuspended in ice cold $CaCl_2$/glycerol solution (10 mL; $CaCl_2$ 0.1 M, glycerol 10 %, filter sterilised 0.2 µm). Cells were again incubated on ice (at least 15 minutes). Cells were centrifuged (4 °C; 10 minutes; 2700 *x g*), and the supernatant was removed. Cells were again resuspended in ice cold $CaCl_2$/glycerol solution (1 mL) and aliquoted (50 µL) into pre-chilled sterile 1.5 mL Eppendorf tubes. Cells were flash frozen in liquid nitrogen and stored at -80 °C.

2.6.3.2.8. Chemical transformation of *E. coli*

An aliquot of *E. coli* cells was removed from -80 °C and placed immediately on ice. Gibson Assembly mix (3 µL) was added to 50 µL chemically competent *E. coli* cells, mixed gently and placed on ice (30 minutes). Cells were heated to 42 °C (30 seconds), in order to induce chemical transformation of the plasmid into *E. coli* cells. Cells were

placed on ice immediately following heat shock (5 minutes). The cells were incubated with SOC medium (250 µL) following transformation (37 °C; 1 hour; shaking). Cells were then spread onto pre-warmed LB agar plates, containing kanamycin (50 µg/mL) as antibiotic selection marker. Colonies were allowed to grow overnight (37 °C, 16 hours).

2.6.3.2.9. Plasmid extraction

Bacterial colonies were picked and grown in LB media (5 mL), with kanamycin selection (50 µg/mL), overnight (37 °C; 16 hours; shaking), and the plasmid was extracted using the Wizard Plus SV Minipreps DNA Purification System kit (Promega), according to the manufacturer's protocol. Briefly, liquid culture (5 mL) was pelleted by centrifugation (4000 rpm; 5 minutes). Pellets were resuspended in cell resuspension solution (250 µL) and transferred to a microcentrifuge tube (1.5 mL). Cell lysis solution (250 µL) was added to lyse cells, and samples were inverted four times, in order to mix. Alkaline protease solution (10 µL), and again samples were inverted four times to mix. Samples were incubated at room temperature (5 minutes). Neutralisation solution (350 µL) was added, and inverted four times to mix. This was then centrifuged at top speed (10 minutes; room temperature). Cell lysate was bound to the membrane of a spin column by decanting the cleared lysate into the column and centrifuging (20000 x *g*, 1 minute, room temperature). The plasmid DNA was washed using a serious of wash steps, and the plasmid was then eluted into a sterile 1.5 mL microcentrifuge tube by adding 80 µL water to the spin column and centrifuging (20000 x *g;* 1 minute; room temperature). Plasmids were stored at -20°C until ready to be used. Plasmids were sent for sequencing to check the inserted gene fragment was correct. pTRV2 specific primers were used for sequencing.

2.6.3.2.10. Glycerol stock solutions of transformed *E. coli*

Sterile glycerol (50 %; 500 µL) was added to *E. coli* culture (500 µL), mixed thoroughly by repeated inversion, frozen, and stored at -80 °C.

2.6.3.2.11. Preparation of chemically competent *Agrobacterium tumefaciens* cells

A glycerol stock of *Agrobacterium tumefaciens* (strain: GV3101) was used to streak out colonies onto an LB agar media plate, containing rifampicin (50 µg/mL) and gentamycin (25 µg/mL) as antibiotic selection markers. Cells were incubated (28 °C; 2 days). A single colony was then used to inoculate SOB liquid media (5 mL), with rifampicin (50 µg/mL) and gentamycin (25 µg/mL) as antibiotic resistance selection. Cultures were grown overnight (28 °C; 24 hours; shaking 100 rpm). 200 µL starter culture was then used to inoculate SOB (100 mL) with rifampicin (50 µg/mL) and gentamycin (25 µg/mL)

and incubated (28 °C; 16 hours; shaking 200 rpm) in a 250 mL flask. Once cultures reached an OD_{600} of 0.6-0.8, cells were chilled on ice (10-15 minutes). Cultures were centrifuged (4 °C; 10 minutes; 2500 *x g*), and supernatant was discarded. Cells were resuspended in ice cold $CaCl_2$ (20 mM) and glycerol (10 %). Cells were aliquoted (100 µL) into pre-chilled Eppendorf (1.5 mL) tubes, and cells were then stored at -80 °C until ready to be used.

2.6.3.2.12. Transformation of plasmid into *A. tumefaciens*

An aliquot of *A. tumefaciens* was thawed on ice, and 1 µg plasmid was added to 100 µL cells. Cells were incubated on ice (30 minutes), before being frozen in liquid nitrogen (1 minute). Cells were thawed (37 °C; 5 minutes) and SOC (500 µL) was added. Cells were incubated on a roller drum (28 °C; 2 hours), before being centrifuged (2 minutes; 8000 rpm). All but 100 µL SOC was removed, and cells were gently resuspended in the remaining media. Cells were plated onto antibiotic selection plates (LB agar + rifampicin (50 µg/mL); gentamycin (25 µg/mL); kanamycin (50 µg/mL)) and incubated (28 °C; 2 days).

Colonies were then selected and used to inoculate liquid LB + rifampicin (50 µg/mL); gentamycin (25 µg/mL); kanamycin (50 µg/mL), and were grown overnight (28 °C).

Glycerol stocks of the culture were stored, and the plasmid was harvested according to section 2.6.3.2.9.

2.6.3.3. Preparation of plants for transient transformation

C. annuum CM334 seeds were sown on ½ MS10 B5 media, following seed sterilisation (washed in ethanol (70 %; 5 minutes), washed in 0.8 % sodium hypochlorite (commercial bleach (90 mL), Tween 20 (8 drops), sterile water (410 mL)), rinsed with sterile water (5 times)). Seeds were sown on solidified media under sterile conditions and allowed to grow in dark conditions (25 °C; 4 days), followed by in the light (25 °C; 4 days; 16/8 h light cycle; 60-100 $\mu mol^{-2}\ s^{-1}$. Seeds germinated on sterile media until the point at which two cotyledons had developed. Seedlings were then transferred to compost (John Innes 3) in plug trays, and were grown in a glasshouse until the first two true leaves had developed.

Solanum lycopersicum cv. MoneyMaker seeds were sown on compost (F2, Scotts Levington, UK) and grown for two weeks in a glasshouse until the point at which the first two cotyledons and the first two true leaves had developed.

2.6.3.4. Preparation of *A. tumefaciens* liquid culture for transformation

pTRV2:GOI glycerol stocks, which had been cloned, along with the pTRV1 stock were streaked out onto antibiotic selection plates (LB agar + rifampicin (50 µg/mL), gentamycin (25 µg/mL), kanamycin (50 µg/mL)). Colonies were allowed to grow (28 °C; 2 days). Starter cultures were prepared by inoculating LB liquid media and appropriate antibiotic selection with a single colony, which was allowed to grow overnight (28 °C; 16 hours). 200 µL starter culture was used to inoculate 50 mL LB liquid media with appropriate antibiotic selection, and allowed to grow (28 °C) until an OD_{600} of 0.6-0.8 was reached. Cultures were centrifuged (20 °C; 15 minutes; 4000 rpm) to pellet cells.

The inoculation buffer (MES 10mM, $MgCl_2$ 10 mM, Acetosyringone 200 µM) was prepared and cells were resuspended in this buffer to an OD_{600} value of 0.7. Cells were left to incubate in this buffer (22 °C; 1 hour).

pTRV2:GOI cell suspension was mixed with pTRV1 cell suspension at a ratio of 1:1. An empty vector control (pTRV2:00) was prepared and used throughout this experiment.

2.6.3.5. Inoculation of plants with VIGS vectors

Both tomato and pepper seedlings were inoculated with the same constructs. 50 pepper seedlings were inoculated, whilst 20 tomato seedlings were inoculated.

A 1 mL syringe was used to inoculate the cotyledons and first two true leaves with culture. The syringe tip was placed on the abaxial side of the leaf, rotated whilst applying gentle pressure to the leaf, and culture was then infiltrated into the plant. Culture was inoculated into the leaf until the leaf appeared to be saturated with culture.

Plug trays were covered once all seedlings had been inoculated, and trays were placed in the dark (21 °C; 24 hours) to allow the plants to recover from inoculation. Trays were then transferred to a glasshouse and monitored closely. Plants were transferred into larger pots when necessary. Tomato plants were transferred to M3 compost (Scotts Levington, UK) once they were large enough to be transferred to bigger pots.

2.6.4. Biochemical analysis of material

Leaf and fruit material was harvested from plants and flash frozen in liquid nitrogen. Material was stored at -80 °C until ready to be processed. Carotenoids were extracted and analysed as described in section 2.2.1. Three biological replicates per vector were

analysed, and pTRV2:00 inoculated plants were treated as the control. Carotenoids were identified following comparison against an analytical standard.

2.6.5. Genetic analysis of material

Leaf and fruit material was harvested from plants and flash frozen in liquid nitrogen. Material was stored at -80 °C until ready to be processed. RNA was extracted from plant material using the RNeasy Minikit (Qiagen) as described in section 2.5.1. cDNA was synthesised according to section 2.6.3.2.2.

Real-time qPCR was used to determine the level of gene expression of genes silenced by VIGS. qPCR was performed on a Rotor-Gene Q (Qiagen) machine using the Rotor-Gene SYBR green PCR kit (Qiagen). Reactions were performed in a total volume of 20 µL, and consisted of Rotor-Gene SYBR green PCR master mix (10 µL), a normalised concentration of template cDNA, and forward and reverse primers (300 nM). The qPCR programme was as follows:

Temperature	Time	Cycles
95 °C	10 minutes	-
95 °C	10 seconds	40 cycles
58 °C	15 seconds	
72 °C	20 seconds	

For each qPCR experiment, cDNA made from three biological replicates of pTRV2:00 plants and three replicates from pTRV2:GOI was used, and qPCR reactions were performed with technical triplicates.

Relative quantification of gene expression was calculated using the ΔΔCq method, using the ATPa gene (in the case of pepper), and actin (in the case of tomato) were used as reference genes.

Primer pairs were optimised using standard PCR, to ensure that only one target was amplified. *Taq* was used in this reaction, as detailed below.

Reagent	Quantity
AllTaq master mix, 4X	5 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
H_2O	13 µL
Template DNA	1 µl
Total volume	20 µL

Temperature	Time	Cycles
95 °C	2 minutes	-
95 °C	5 seconds	35 cycles
55 °C	15 seconds	
72 °C	10 seconds	
72 °C	5 minutes	-

For each primer pair, including both reference gene primers and gene of interest primers, a standard curve was generated to ensure primers amplified in a linear manner, and that both reference gene and gene of interest amplified with similar efficiencies. A pool of cDNA was created, using cDNA from all samples RNA was extracted from. A serial dilution of this pool was created to generate concentrations of: 1:5, 1:10, 1:100, 1:1000, 1:5000. qPCR reactions were performed in technical triplicate, and it was ensured that primer pairs had a linear regression (R^2) value of >0.99, and the reaction efficiency was between 0.90-1.10. The standard curve was used to determine an appropriate starting concentration for sample cDNA, to ensure that the Cq value for each gene occurred between 20-30 cycles.

3. Metabolite profiling of a hot pepper population with variation in colour retention

3.1. Introduction

Fruit colour is a key quality trait in chilli pepper, and therefore, an understanding of the biochemistry of this trait may facilitate breeding for specific pepper colour requirements, including carotenoid content, composition, and stability. Alongside colour, various other metabolic traits are desirable in pepper, and also need to be understood, in order for pepper varieties to be bred towards these traits. Such metabolic traits include Brix content, phenolic volatile composition, and vitamin E content, which are major constituents of flavour, aroma, and nutritional profile, respectively. These attributes are often called consumer or quality traits, and have been detailed in Chapter 1. Specifically in pepper, capsaicinoid content is a crucial trait as pungency is determined by the amount of capsaicin present. Clearly, breeders must be aware of the capsaicinoid profile of varieties in order to breed for either pungent or non-pungent pepper varieties.

QTL mapping for specific metabolic traits (mQTL mapping) may reveal molecular markers for individual metabolites, which can be used to direct and augment breeding programmes. Metabolite QTL analysis has previously been performed on the *Solanum pennellii* introgression lines in order to identify genomic loci associated with secondary metabolisms within the tomato fruit pericarp tissue. 670 mQTLs were identified for secondary metabolites, and these included compounds such as flavonoids, glycoalkaloids, and hydroxycinnamates (Alseekh et al., 2015). This demonstrates the potential for identifying genomic regions associated with secondary metabolites, which are important to understand when breeding for high quality fruit varieties. Similar studies have not previously been performed in pepper fruit, and therefore, the potential for mQTLs in pepper remains undiscovered to date.

The composition of carotenoids vastly differs in pepper varieties, and this explains the variation and spectrum of colours observed. Furthermore, the change in carotenoid content during post-harvest storage differs between pepper varieties. As peppers may often be stored following harvest for periods of up to one year before being consumed, the change in carotenoid profile is also a key quality trait. Whilst carotenoid content in pepper varieties has been widely reported, understanding the change in carotenoid profile has been largely neglected by previous studies.

For these reasons, the carotenoid profile of a DH pepper population, described in Chapter 2, has been characterised, both following harvest, and following seven months of post-harvest storage in industrially relevant cold conditions (Figure 3-1). Using this data, the carotenoid ‘retention’ profile has been calculated to indicate the change in

carotenoid content that occurs during post-harvest storage. This data has subsequently been used for QTL mapping, in order to identify genomic regions associated with the carotenoid retention trait.

Further to this, the DH population has been analysed immediately following harvest to determine the profile of other primary and secondary metabolites (Figure 3-1). This data has also been analysed using QTL mapping to determine genetic regions associated with individual metabolites. Additionally, these data have been used to study whether the carotenoid profile of the DH population correlates with any of the other metabolites analysed.

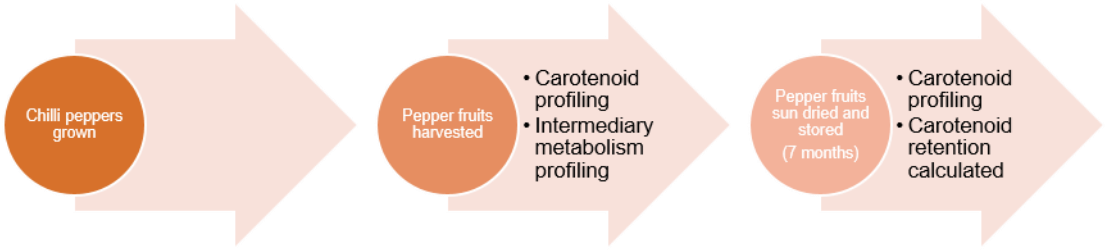


Figure 3-1 Post-harvest storage processing of chilli peppers of DH population.

Chilli peppers of the DH population were grown in the field in India, before being harvested, sun-dried for two weeks, and subsequently stored in cold, dark conditions for seven months. Pepper fruits were analysed immediately following harvest (termed 'fresh'), and following the seven month storage period (termed 'stored').

3.2. Results

3.2.1. Carotenoid profiling of *Capsicum annuum*

High Pressure Liquid Chromatography (HPLC) using a C_{30} reverse phase column is a well-established method for analysing carotenoid profiles of plant samples (Fraser et al., 2000). HPLC chromatograms of extracted ripe pepper carotenoids revealed a complex profile of this class of compound when analysed at the wavelength corresponding with carotenoid chromophore absorption (450 nm), particularly when compared to the relatively simple profile observed in tomato fruit, in which fewer carotenoid compounds, are found (Nogueira et al., 2013). Up to 40 components were observed in pepper samples; the complexity seen in the pepper carotenoid profile is owing to the fact that pepper carotenoids are often esterified (Biacs et al., 1989). Therefore, the 40 peaks observed were a result of the on-column separation of free carotenoids, carotenoid mono-esters, and carotenoid di-esters (Figure 3-2, Table 3-1).

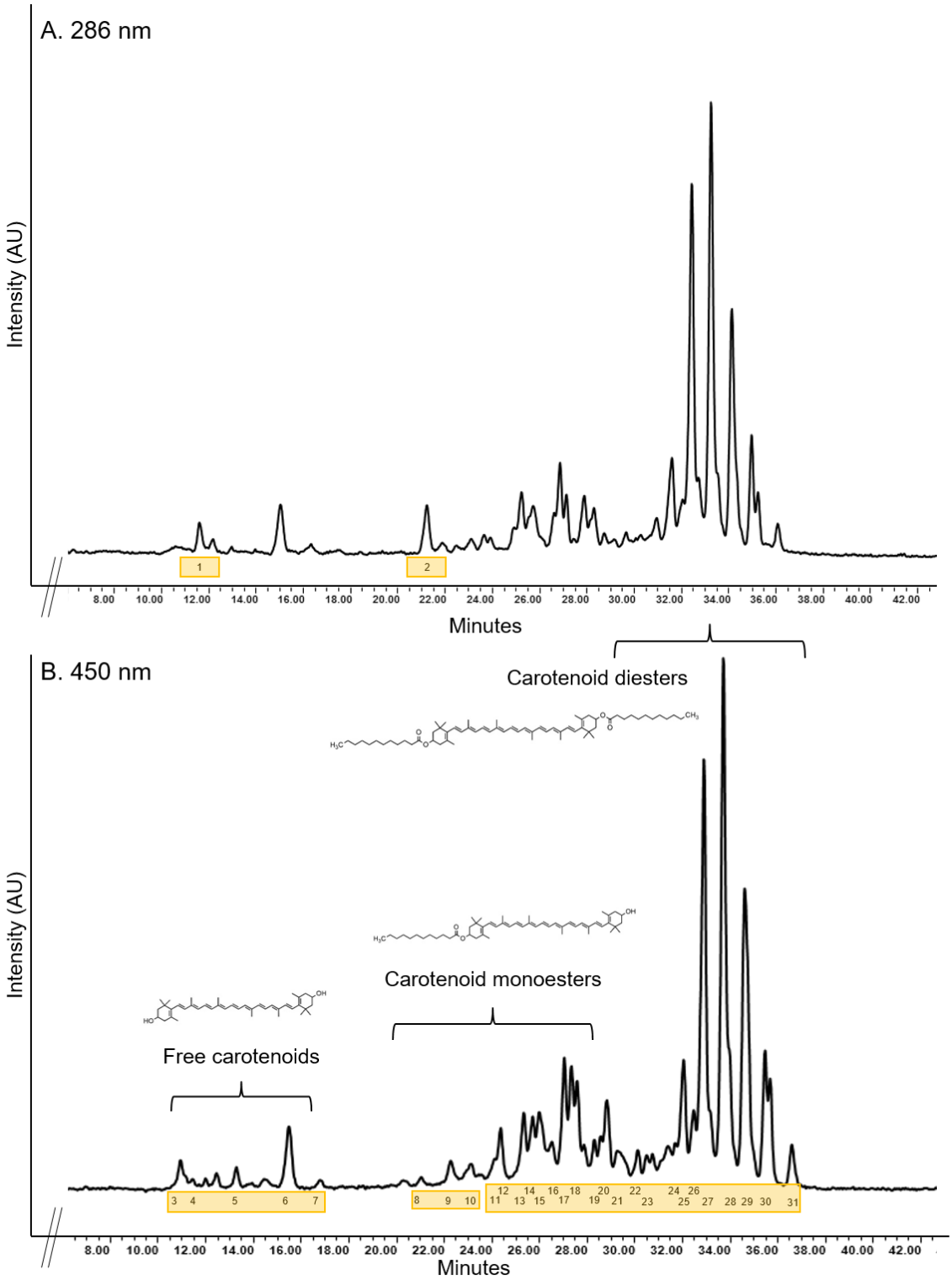
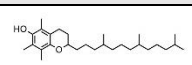
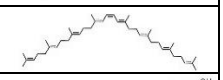
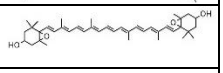
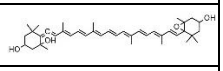
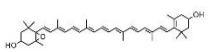
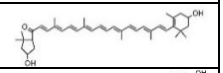
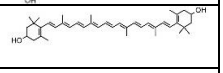
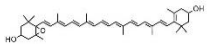
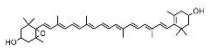
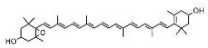
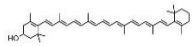
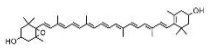
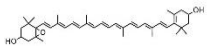
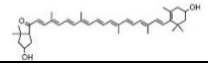
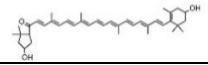
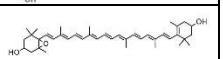
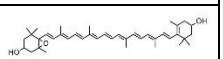
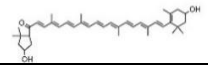
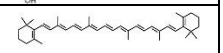
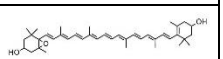
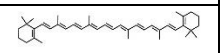
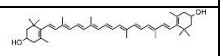
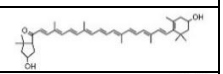
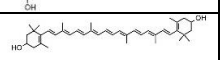
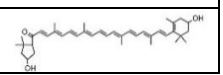


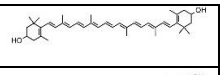
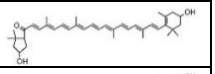
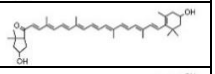
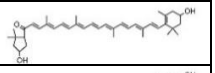
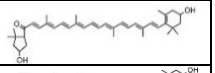
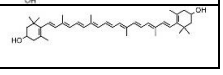
Figure 3-2 Annotated HPLC chromatogram of red ripe pepper carotenoid profile.

A reverse phase C_{30} column was used, with solvents methanol and methyl tert- butyl ether in a 60 minute run, to separate carotenoids extracted from chilli pepper fruits. Peak numbers correspond to identified carotenoids (Table 3-1). (A) wavelength 286nm, (B) wavelength 450nm. Phytoene and α-Tocopherol were separated at 286 nm; free carotenoids, carotenoid monoesters, and carotenoid diesters were separated at 450 nm.

Table 3-1 Identified pepper carotenoids.

Pepper carotenoids, as identified by HPLC-PDA. Peak number corresponds to Figure 3-2. Retention time is presented in minutes. Compounds were identified based on comparison with authentic standard, or against known samples (Berry et al. 2019). Carotenoid structure is presented, although esterified structures are not presented here.

Peak Number	Retention time (minutes)	Carotenoid identification	Spectral characteristics (450 nm)	Identification level	Carotenoid structure
1	11.8	α-Tocopherol	293.5	Authentic standard	
2	21.7	15-cis phytoene	286.4	Authentic standard	
3	10.9	Violaxanthin	441.5, 469.3	Authentic standard	
4	11.3	Neoxanthin	437.9, 469.3	Berry *et al.* 2019	
5	13.2	Antheraxanthin	443.9, 472.9	Berry *et al.* 2019	
6	15.6	Capsanthin	475.3	Authentic standard	
7	16.9	Zeaxanthin	452.4, 477.8	Authentic standard	
8	21.1	Antheraxanthin monoester	442.7, 469.3	Berry *et al.* 2019	
9	22.3	Antheraxanthin monoester	447.5, 476.6	Berry *et al.* 2019	
10	23.2	Antheraxanthin monoester	440.3, 470.5	Berry *et al.* 2019	
11	24.1	β-cryptoxanthin	450.0, 481.4	Berry *et al.* 2019	
12	24.4	Antheraxanthin monoester	447.5, 472.9	Berry *et al.* 2019	
13	25.3	Antheraxanthin monoester	446.3, 474.1	Berry *et al.* 2019	
14	25.8	Capsanthin monoester	468.1	Berry *et al.* 2019	
15	26.0	Capsanthin monoester	454.8	Berry *et al.* 2019	
16	26.6	Antheraxanthin monoester	446.3, 474.1	Berry *et al.* 2019	
17	27.1	Antheraxanthin monoester	447.5, 475.3	Berry *et al.* 2019	
18	27.4	Capsanthin monoester	471.7	Berry *et al.* 2019	
19	28.3	β-carotene isomer	452.4, 479.0	Authentic standard	
20	28.7	Antheraxanthin diester	446.3, 472.9	Berry *et al.* 2019	
21	29.3	β-carotene isomer	453.6, 469.3	Authentic standard	
22	30.2	Zeaxanthin diester	451.2	Berry *et al.* 2019	
23	30.7	Capsanthin diester	474.1	Berry *et al.* 2019	
24	31.5	Zeaxanthin diester	453.6, 479.0	Berry *et al.* 2019	
25	32.1	Capsanthin diester	479.0	Berry *et al.* 2019	

26	32.5	Zeaxanthin diester	453.6, 479.0	Berry *et al.* 2019	
27	33.0	Capsanthin diester	472.9	Berry *et al.* 2019	
28	33.8	Capsanthin diester	474.1	Berry *et al.* 2019	
29	34.7	Capsanthin diester	472.9	Berry *et al.* 2019	
30	35.6	Capsanthin diester	472.9	Berry *et al.* 2019	
31	36.7	Zeaxanthin diester	453.6, 479.0	Berry *et al.* 2019	

3.2.2. Carotenoid profiling screen of Double Haploid population following harvest

A screen for carotenoid profile was carried out on the DH pepper population, whereby carotenoids were extracted from each sample, and analysed by HPLC. Total carotenoid content and carotenoid composition was calculated for each line within the population, for samples immediately following harvest (Figure 3-3). Total carotenoid amounts were scaled (0-100) in order to make direct comparisons between values for freshly harvested samples, and values for samples following post-harvest storage (Section 3.2.3). Absolute values can be found in Supplementary Table 1. Interestingly, the population parent lines (high and low retention parents), have very similar total carotenoid values, with both lines at the high extreme compared to the resulting population. Also interesting to note is the fact that total carotenoid content does not correlate with the value for any specific carotenoid.

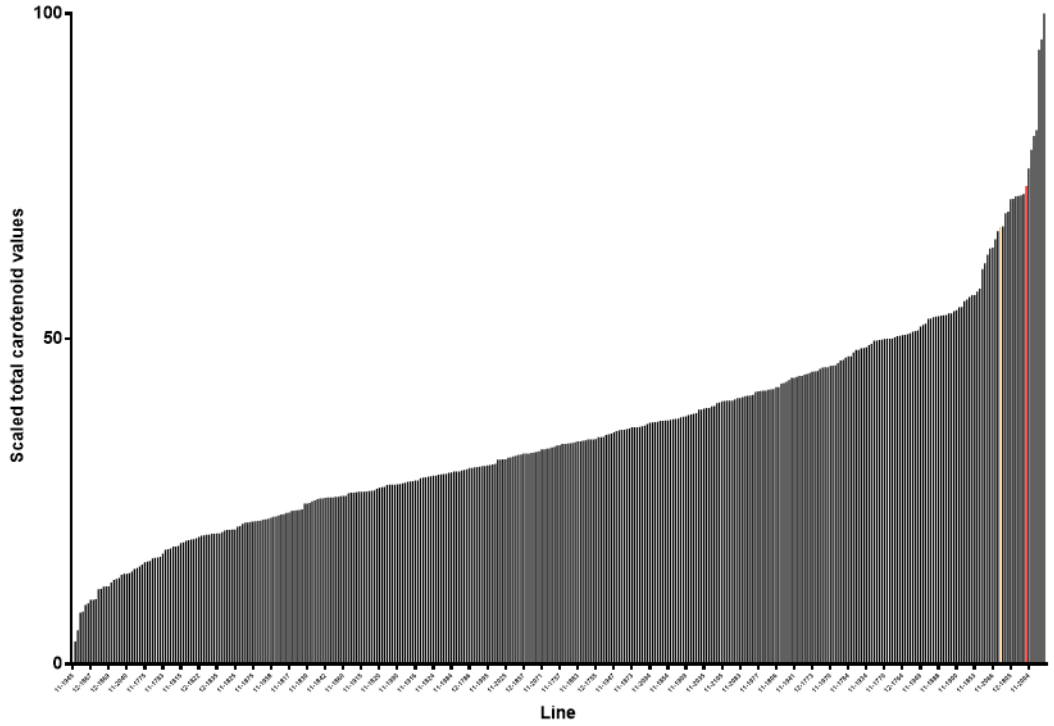


Figure 3-3 Scaled total carotenoid values for fresh pepper DH population.

The fruits of the 375 DH population lines were screened at the fresh time point by HPLC-PDA. Individual bars represent each line within the population. Parent lines are coloured: red (high retention parent), yellow (low retention parent). Carotenoid values were scaled (0-100) to allow comparisons to be made, and lines were ordered according to total carotenoid value (n = 1). Scaled carotenoid value and absolute carotenoid value can be found in supplementary table 1.

3.2.3. Carotenoid profiling screen of Double Haploid population following long term post-harvest storage

A carotenoid profile screen was subsequently carried out on DH population samples which had been stored following harvest, in order to compare against carotenoid profile immediately following harvest. Total carotenoid content and carotenoid composition was calculated for each line within the population, at the stored time point, and total carotenoid amounts were scaled (0-100) to allow for direct comparisons of lines at different time points to be made (Figure 3-4). Absolute values can be found in Supplementary table 2. As observed at the freshly harvested time point, the population parents have very similar total carotenoid values, and the resulting offspring display greater variation compared to the values obtained for the parents.

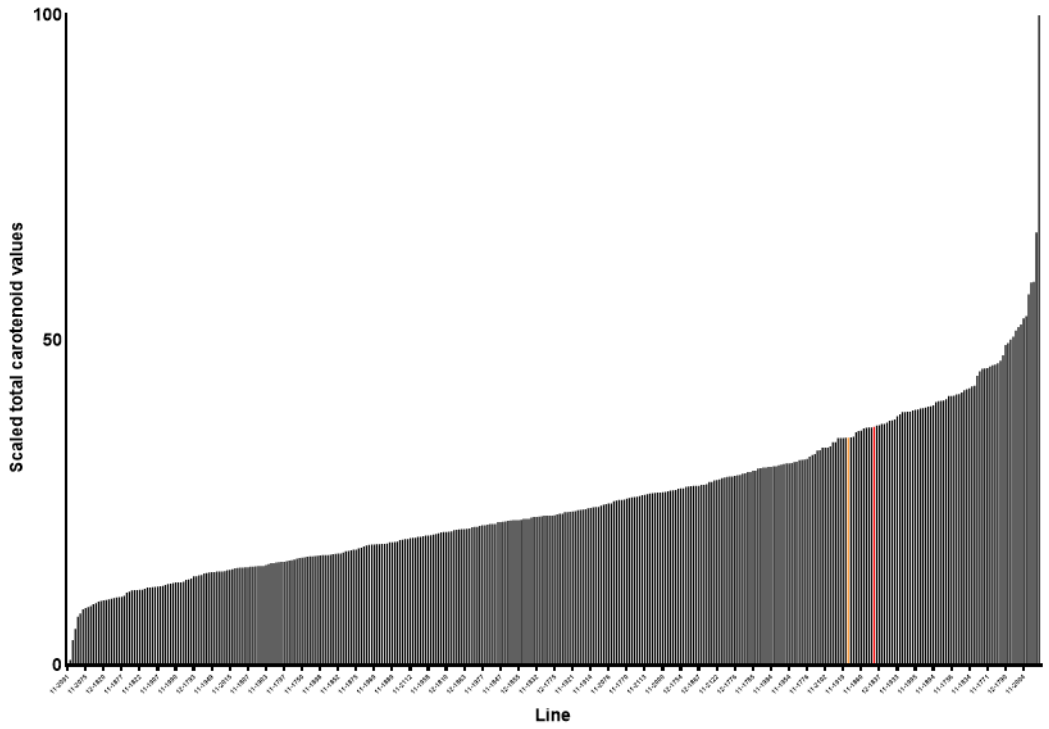


Figure 3-4 Scaled total carotenoid values for stored pepper DH population.

The fruits of the 375 DH population lines were screened following post-harvest storage by HPLC-PDA. Individual bars represent each line within the population. Parent lines are coloured: red (high retention parent), yellow (low retention parent). Carotenoid values were scaled (0-100) to allow comparisons to be made, and lines were ordered according to total carotenoid value (n = 1). Scaled carotenoid value and absolute carotenoid value can be found in supplementary table 2.

3.2.4. Characterising the carotenoid retention profile of the Double Haploid population

Using the data from the carotenoid profile screens carried out at both the fresh harvest time point and following post-harvest storage, the change in carotenoid content was calculated for each line within the DH population, and expressed as a percentage. This value has been designated the 'carotenoid retention' value. Carotenoid retention was calculated according to the following equation:

$$Carotenoid\ retention = \frac{Stored\ total\ carotenoid\ content - Fresh\ total\ carotenoid\ content}{Fresh\ total\ carotenoid\ content} \times 100$$

Principal component analysis (PCA) was carried out on the retention value dataset and identified the line 11-2053 as an extreme outlier. Subsequently, this line was removed from the carotenoid retention screen. A large degree of variation was observed in the DH population for carotenoid retention values. A large proportion of the population was clustered around the centre of the PCA, and extreme outliers were forced to the edges of the PCA (Figure 3-5A). Lines identified as high or low extreme carotenoid retention

were identified based on their total carotenoid retention values, and these lines were characterised in a more in-depth study (Section 3.2.5).

Lines identified as extreme high or low retention phenotypes were designated as such based on their total carotenoid retention value. These lines were separated by PCA and forced to the edges of the plot. The PCA scores plot was based on all 13 identified carotenoid retention values, therefore it was possible to determine which specific carotenoid compounds were forcing the separation of high and low retention lines (Figure 3-5B). Free capsanthin, capsanthin monoesters, and capsanthin diesters forced the separation of high retention lines, as these lines clustered in the plot region in which capsanthin and its esters were located. Capsorubin diesters and β-carotene forced the separation of low retention lines. However, whilst these lines were separated to opposite sides of the PCA scores plot, and the separation was driven by the compounds discussed, clearly the retention phenotype was not purely determined by these compounds. Other lines not designated as extreme high retention phenotypes were located with the identified lines, suggesting that these lines also had high capsanthin retention values. Therefore, another variable must have differentiated these lines from those identified as high retention. Further to this, only 17.7% of the variation in the dataset was explained by principal component 1, and 11.5% of the variation was explained by principal component 2. Evidently, there was a large amount of variation in this dataset that was explained by variables other than those plotted in this analysis.

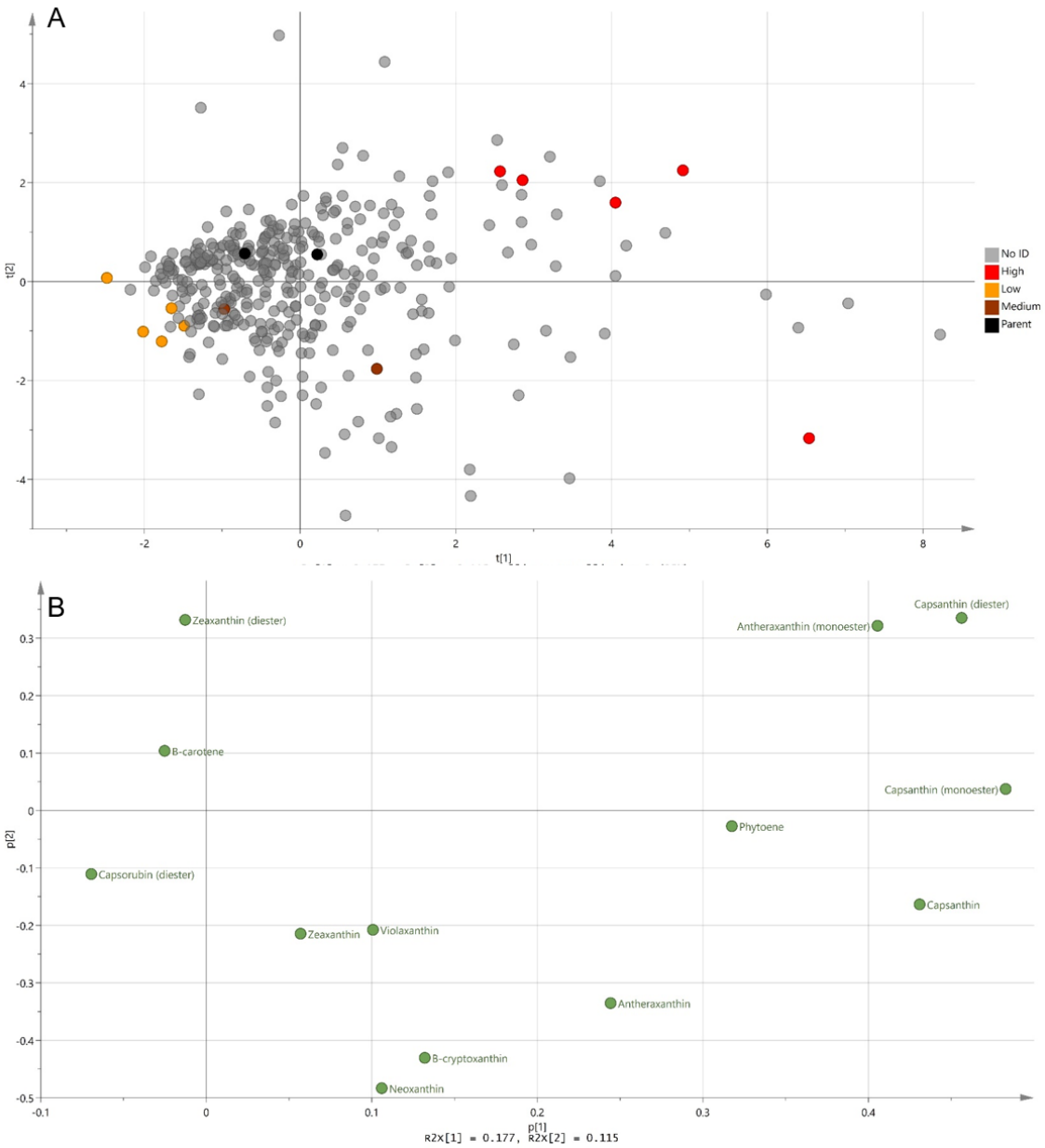


Figure 3-5 PCA plots of carotenoid retention values for DH population.

Principal component analysis was used to determine the variation in the carotenoid retention values for the population. A: DH population carotenoid screen with line 11-2053 removed (high retention lines: red, low retention lines: yellow, medium retention lines: brown, population parents: black, all other lines: grey), B: Loadings plot displaying separation of carotenoid retention values, driving variation in pepper lines.

Carotenoid retention values were scaled (0-100) in order to make direct comparisons against the carotenoid screens from which this data was calculated (Figure 3-3, Figure 3-4). Of 375 DH population lines, including two parents, 367 lines showed an increase in carotenoid content during post-harvest storage. These lines have a scaled carotenoid retention value of greater than 7.8. Interestingly, only 8 lines of the population showed a decrease in total carotenoid content during post-harvest storage, despite the fact that it was hypothesised that the majority of lines would show a decrease in total carotenoid content during storage.

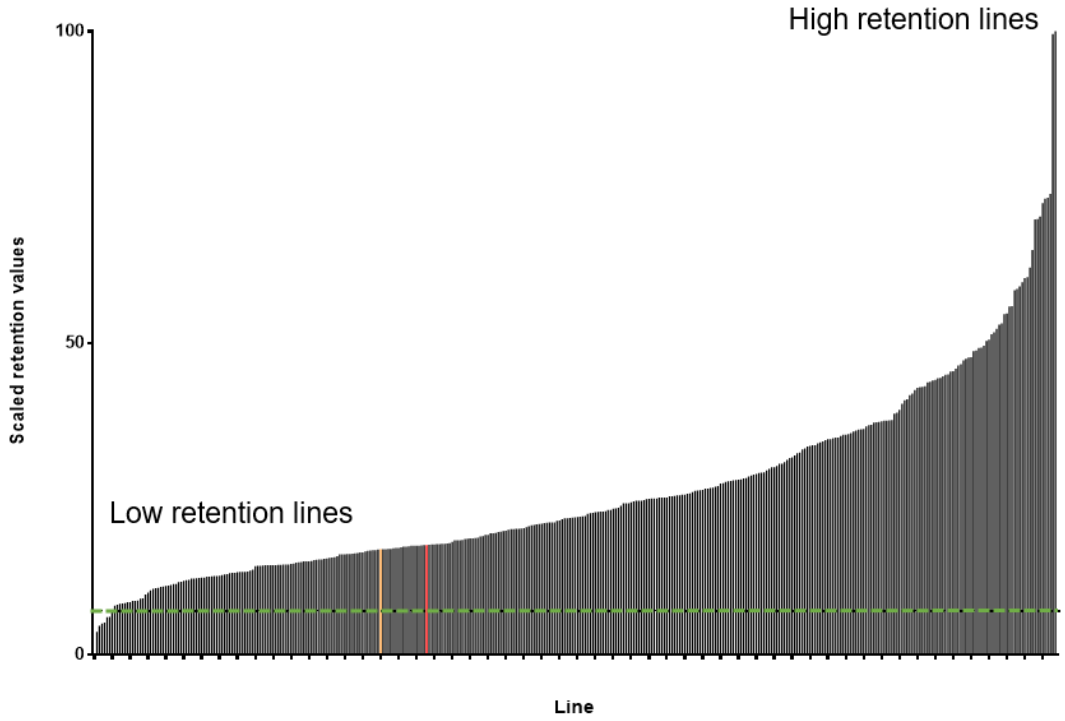


Figure 3-6 Scaled carotenoid retention values for pepper DH population.

Carotenoid retention values were calculated using the fresh and stored carotenoid data for the DH population. Parent lines are coloured: red (high retention parent), yellow (low retention parent). Carotenoid retention values for the 375 lines of the DH population were scaled (0 to 100) to allow comparisons to be made. Each bar represents a line within the DH population (n = 1). Dotted line represents the change in carotenoid content value equal to 0. Carotenoid retention values can be found in supplementary table 3.

Agglomerative hierarchical clustering (AHC) using Spearman dissimilarity correlations displayed the clustering of population lines into groups, based on their retention profile (Figure 3-7). The DH population clustered into three distinct groups. Both population parents (lines 11-1175 and 11-1179) grouped into the same cluster within the dendrogram, indicating their similarity. Clusters were not designated as high or low total carotenoid retention, as lines characterised as high and low retention were grouped into each cluster. Lines clustered into the groups according to their overall carotenoid retention profile, based on the retention values for the 13 identified carotenoid compounds. Therefore, the lines within the clusters were determined to be similar based on the overall carotenoid retention profile, accounting for retention values of the 13 carotenoid compounds. There was no single carotenoid which could be deemed responsible for the total carotenoid retention phenotype.

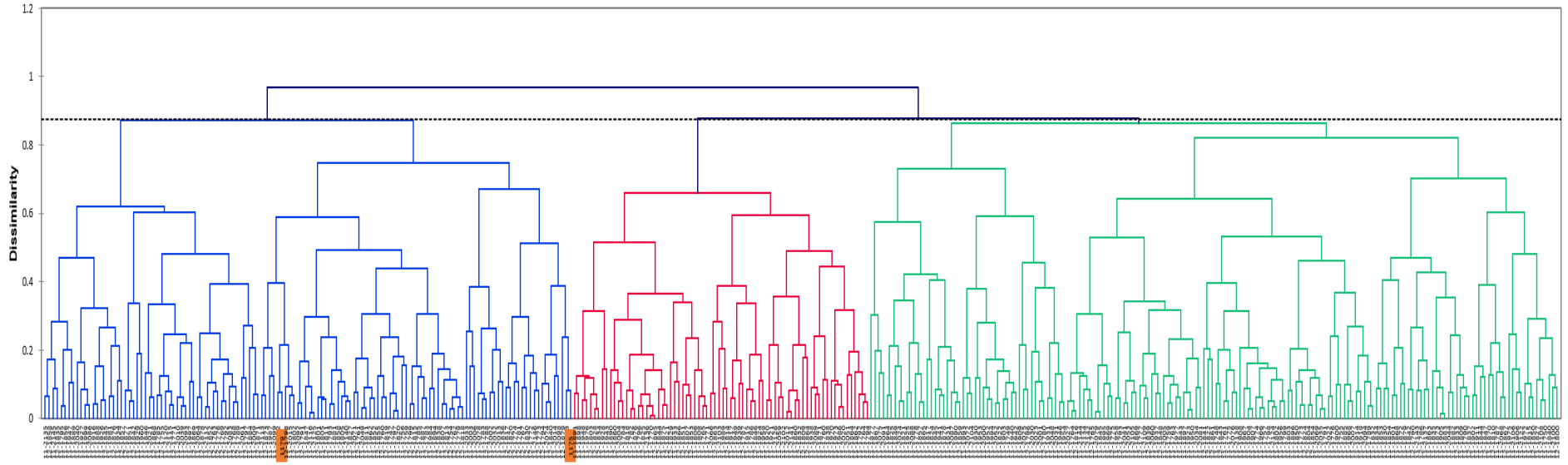


Figure 3-7 Dendrogram displaying clustering of pepper population based on carotenoid retention phenotype.

Agglomerative hierarchical clustering (AHC) using Spearman dissimilarity correlations was used to group DH population lines into clusters, based on their retention value. Three distinct clusters were identified. AHC was carried out using retention values for 13 identified carotenoids, therefore clustering is based on carotenoid retention profile, as opposed to total retention value. Clusters identified did not represent high, medium, and low retention, but rather reflect the carotenoid profile of lines within each cluster. Carotenoid retention value, classification, and assigned cluster can be found in appendix 9.1. Green cluster = cluster 1, red cluster = cluster 2, blue cluster = cluster 3.

3.2.5. Carotenoid retention subpopulation characterisation

Screening of the DH population for carotenoid retention phenotype identified lines within the population with extreme high or low carotenoid retention phenotypes. Lines characterised as extreme were selected and formed a sub-population. Five low retention lines, six high retention lines, two medium retention lines, and the two population parent lines were selected in order to create the carotenoid retention sub-population. Carotenoid profile of this sub-population was characterised in greater detail than was performed at the screening stage. Carotenoid profile was analysed at the freshly harvested time point, along with the post-harvest stored time point, and the carotenoid retention profile was calculated. This analysis was performed in triplicate.

Lines were selected to create the subpopulation based on retention phenotype, as opposed to total carotenoid content at any one time point.

Table 3-2 Carotenoid retention subpopulation carotenoid amounts (µg/g DW) from freshly harvested fruit.

Lines characterised as either extreme high or extreme low carotenoid retention from the DH population were further analysed by HPLC-PDA. Total carotenoid amounts were calculated using a standard curve. Total amounts are presented, along with standard error (±SE). Three technical replicates per line were analysed (n = 3).

Line	Phytoene	Violaxanthin	Neoxanthin	β-carotene	β-cryptoxanthin	Antheraxanthin	Antheraxanthin (monoester)	Capsanthin	Capsanthin (monoester)	Capsanthin (diester)	Capsorubin (diester)	Zeaxanthin	Zeaxanthin (diester)	Total
11-1764	310.9	110.4	71.8	271.7	63.0	23.3	402.4	560.7	887.7	2526.7	242.5	49.9	447.5	5968.4
SE (±)	5.0	3.0	35.9	56.2	38.7	0.8	105.4	11.4	15.1	248.6	242.5	3.5	94.4	575.0
11-1802	52.7	76.3	0.0	198.2	32.0	18.5	257.1	505.2	713.4	2315.2	226.1	20.3	324.4	4739.6
SE (±)	1.2	38.2	0.0	13.2	16.0	1.0	64.5	4.2	6.8	244.2	226.1	2.4	50.1	301.3
11-1870	63.9	115.0	36.9	266.5	36.7	20.8	252.0	521.4	769.6	2226.3	168.7	78.0	656.1	5211.8
SE (±)	1.9	2.5	36.9	37.5	18.8	0.0	9.0	12.4	15.2	351.9	168.7	56.7	132.5	398.3
11-1937	128.6	121.6	36.3	462.0	50.9	25.7	408.1	548.2	1052.4	3360.1	0.0	35.7	1058.3	7287.9
SE (±)	5.6	0.8	36.3	98.6	26.1	0.7	20.8	4.6	43.6	167.0	0.0	2.9	68.2	208.3
11-1967	167.7	117.3	38.0	283.4	13.7	31.0	324.4	609.2	1170.4	2212.8	267.9	40.2	343.1	5619.2
SE (±)	14.6	5.9	38.0	31.6	13.7	1.3	58.9	15.9	132.9	447.6	267.9	5.5	45.8	707.5
11-2041	53.6	109.2	37.0	70.8	44.2	24.7	119.2	562.8	611.8	1398.2	176.7	33.0	87.6	3328.8
SE (±)	2.3	2.7	37.0	11.4	3.8	2.1	23.4	7.6	7.8	114.0	176.7	2.0	18.8	132.9
11-2053	88.1	80.0	0.0	203.0	59.6	25.2	238.1	563.3	854.8	2415.4	0.0	26.7	103.9	4658.1
SE (±)	1.7	40.0	0.0	13.8	3.9	1.2	15.2	10.9	46.6	212.6	0.0	2.3	46.3	231.0
11-2075	26.1	131.6	0.0	194.7	67.0	48.1	322.0	564.2	1320.7	3187.3	605.4	20.2	352.0	6839.3
SE (±)	1.1	2.9	0.0	30.9	36.8	5.1	10.1	14.2	6.4	490.6	40.2	0.9	218.6	276.6
11-2091	44.8	111.3	34.9	134.1	30.5	25.2	193.8	532.6	844.8	2058.0	210.9	22.6	233.8	4477.2
SE (±)	4.6	3.1	34.9	41.9	15.2	1.2	8.0	5.0	55.8	284.2	210.9	1.9	95.1	238.1
12-1809	23.5	110.2	36.1	111.6	60.1	25.0	141.5	529.8	841.8	2066.1	423.1	22.3	66.8	4457.9
SE (±)	0.4	1.8	36.1	7.5	6.1	0.7	4.6	12.5	60.6	185.0	221.1	4.8	50.9	316.3
12-1821	71.3	104.2	0.0	153.1	41.3	22.2	109.0	496.6	624.5	1596.1	0.0	25.5	109.7	3353.4
SE (±)	5.7	0.6	0.0	16.5	3.3	0.0	3.6	0.9	13.5	61.2	0.0	2.8	54.9	153.9
12-1852	51.6	115.6	0.0	111.4	52.2	38.3	109.3	610.5	751.9	1751.3	170.2	24.2	27.5	3814.1
SE (±)	15.0	1.9	0.0	14.6	3.9	6.3	6.7	12.5	15.5	161.2	170.2	1.3	5.2	387.4
12-1865	90.3	114.9	34.5	122.5	41.7	34.0	149.4	617.5	755.8	1797.2	0.0	28.4	61.4	3847.7
SE (±)	5.7	1.5	34.5	14.4	3.1	2.9	20.7	7.4	37.1	199.4	0.0	1.4	21.4	161.0
11-1175	82.7	79.6	62.5	245.6	39.1	30.4	181.6	350.7	838.3	1890.9	0.0	25.6	151.0	3977.9
SE (±)	8.1	4.4	2.3	10.0	2.5	0.7	32.9	19.8	77.8	114.5	0.0	1.8	17.4	231.3
11-1179	133.7	67.9	59.2	10.0	46.5	18.5	220.5	332.9	700.4	2047.5	156.3	24.1	394.5	4426.4
SE (±)	6.5	3.1	1.4	10.0	5.8	1.0	9.0	9.2	35.3	278.1	78.2	1.9	113.7	266.4

Table 3-3 Carotenoid retention subpopulation carotenoid amounts (µg/g DW) following post-harvest storage.

Lines characterised as either extreme high or extreme low carotenoid retention from the DH population were further analysed by HPLC-PDA. Total carotenoid amounts were calculated using a standard curve. Total amounts are presented, along with standard error (±SE). Three technical replicates per line were analysed (n = 3).

Line	Phytoene	Violaxanthin	Neoxanthin	β-carotene	β-cryptoxanthin	Antheraxanthin	Antheraxanthin (monoester)	Capsanthin	Capsanthin (monoester)	Capsanthin (diester)	Capsorubin (diester)	Zeaxanthin	Zeaxanthin (diester)	Total
11-1764	155.5	113.0	0.0	256.9	91.3	40.6	260.0	573.7	1358.9	1755.8	375.2	22.0	458.0	5460.9
SE (±)	10.0	5.4	0.0	29.7	19.6	2.0	56.7	9.9	145.6	36.5	188.4	2.5	44.8	362.5
11-1802	72.9	112.6	39.6	159.2	62.0	22.2	215.1	483.7	942.8	2280.3	190.5	16.5	726.4	5323.6
SE (±)	4.2	1.6	39.6	22.4	5.8	0.3	8.1	9.4	70.0	92.2	190.5	1.4	57.3	285.9
11-1870	19.0	122.3	35.7	219.2	37.7	34.2	214.2	582.0	1021.8	1792.8	536.1	21.0	266.2	4902.2
SE (±)	2.7	2.0	35.7	52.7	19.3	2.2	45.1	15.3	106.5	312.9	9.9	3.5	121.9	298.4
11-1937	64.4	126.3	78.1	371.6	124.6	32.2	198.1	536.3	1167.7	2717.5	590.6	29.0	675.5	6711.9
SE (±)	4.1	1.2	39.3	56.9	50.8	2.7	33.8	5.5	122.4	407.5	22.3	1.7	102.0	403.8
11-1967	86.3	113.2	34.4	201.1	78.8	35.9	180.7	620.1	1054.3	1491.5	0.0	36.1	370.9	4303.2
SE (±)	11.1	0.6	34.4	43.6	18.1	1.4	36.9	20.9	62.7	67.0	0.0	4.2	38.8	258.9
11-2041	76.4	118.3	0.0	96.9	58.2	36.9	147.7	577.4	1042.8	1819.8	361.2	20.1	402.7	4758.3
SE (±)	0.9	0.7	0.0	17.1	4.5	3.8	10.4	4.3	99.7	256.9	181.3	2.7	174.0	383.6
11-2053	66.3	131.2	34.6	410.5	107.5	40.4	371.8	550.6	1505.4	3454.2	244.2	31.6	660.7	7609.1
SE (±)	0.1	12.0	34.6	208.5	33.4	4.8	121.5	13.9	372.3	1323.9	244.2	3.7	253.6	1970.9
11-2075	26.8	118.8	38.1	121.4	83.7	29.0	180.1	534.6	1159.5	2519.6	546.2	18.1	209.7	5585.5
SE (±)	3.2	5.4	38.1	33.6	31.9	0.7	2.2	8.5	90.7	495.1	28.3	1.0	171.2	276.7
11-2091	54.7	120.9	36.2	264.1	39.1	30.4	305.5	515.3	1105.9	3397.9	162.7	25.1	418.7	6476.5
SE (±)	2.5	2.6	36.2	45.8	19.6	1.3	13.1	9.8	102.8	410.9	162.7	1.0	227.8	167.6
12-1809	41.2	121.8	72.1	182.1	85.7	33.8	267.6	529.9	1381.7	3468.6	592.8	17.2	436.3	7230.9
SE (±)	1.3	5.6	36.1	42.3	22.2	3.2	16.0	10.9	92.9	747.7	19.1	0.3	356.5	404.5
12-1821	187.8	117.6	107.3	276.8	38.2	31.9	267.2	523.5	1081.2	2969.7	327.2	29.0	351.3	6308.5
SE (±)	3.2	0.7	0.4	23.6	20.3	1.2	12.7	0.9	60.0	443.0	164.2	3.3	104.9	346.2
12-1852	192.8	128.9	74.7	307.2	37.8	36.1	293.6	557.1	1076.1	3107.4	0.0	25.7	308.7	6146.2
SE (±)	9.3	2.5	37.4	15.3	20.3	2.8	38.0	6.5	52.8	304.9	0.0	2.4	159.5	243.1
12-1865	1053.5	124.5	71.0	361.5	23.7	37.7	305.2	535.9	992.9	3341.3	0.0	19.1	310.6	7176.8
SE (±)	165.2	4.8	35.6	9.6	23.7	3.3	25.1	22.0	13.6	560.2	0.0	1.8	131.2	622.2
11-1175	86.9	96.4	64.5	136.6	87.1	26.7	272.2	332.1	1301.7	2994.2	101.4	19.5	142.5	5661.6
SE (±)	6.5	4.2	2.7	19.9	20.4	8.0	16.1	34.7	111.9	110.8	101.4	1.6	14.5	373.7
11-1179	120.8	78.2	65.0	235.6	45.5	31.7	288.3	362.5	998.1	3168.5	96.4	29.9	248.0	5768.3
SE (±)	8.9	2.7	1.4	37.5	0.4	4.6	12.4	10.1	20.8	52.4	96.4	6.8	10.6	157.8

Amongst the sub-population, in which lines showed extreme high or low carotenoid retention phenotypes, all lines identified in the population screen as high retention except 11-2053 showed a significant increase in total carotenoid content at the post-storage time point, compared to immediately following harvest (Figure 3-8). This highlights that the further characterisation of these lines supported the initial screen. Further to this, most lines identified in the screen as low retention did not show significant differences in total carotenoid content between the freshly harvested and stored time points. This again supported the finding that carotenoid content did not significantly decrease during post-harvest storage, even within the low retention lines, but rather stayed at a consistent level through storage. Exceptions to this were lines 11-2075 and 11-2091, both of which were identified in the original carotenoid retention screen as low retention. In the case of 11-2075, a significant decrease in carotenoid content during post-harvest storage was observed, suggesting that this line was an extreme low retention phenotype line. In the case of 11-2091, a significant increase in carotenoid content was observed during post-harvest storage, possibly suggesting that the identification of this line as low retention during the initial screen was an inaccurate observation.

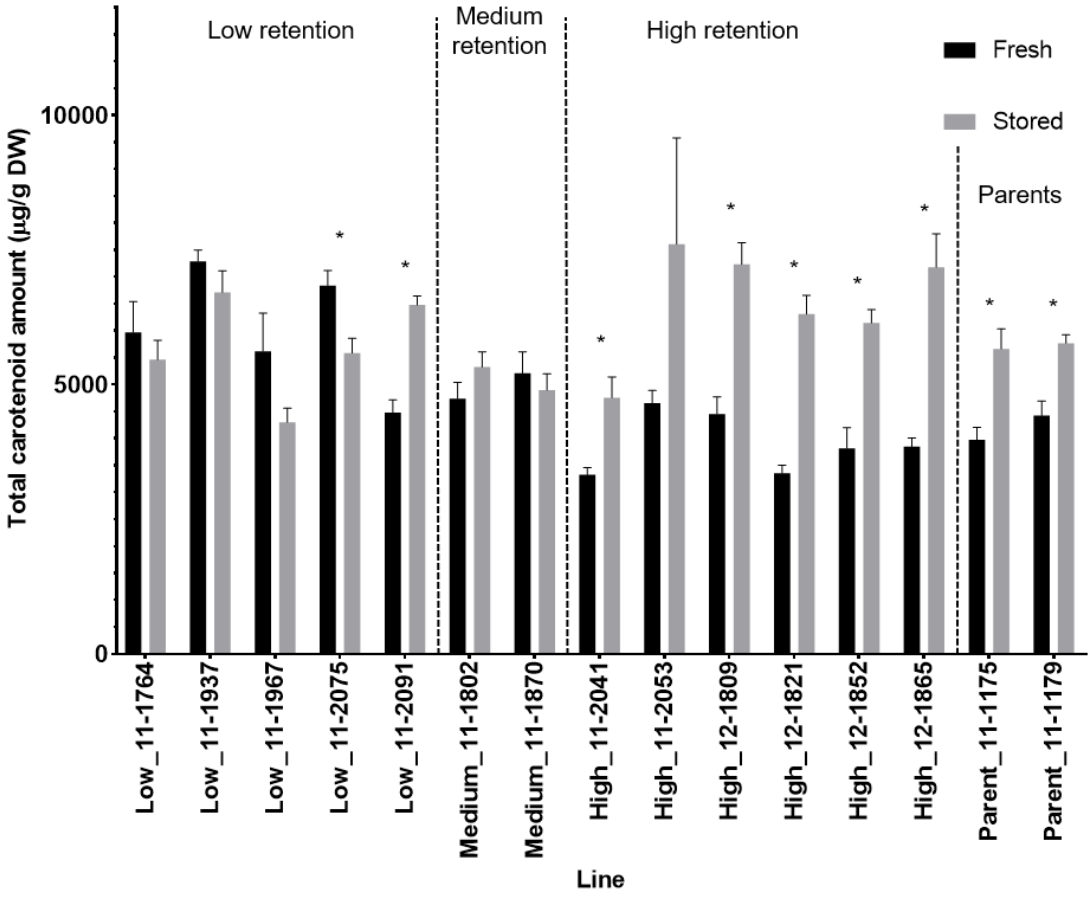


Figure 3-8 Total carotenoid content at fresh and stored time points of the carotenoid retention sub-population.

Lines identified as extreme high or extreme low carotenoid retention, along with two medium retention lines, and the two DH population parent lines, were further analysed as part of the carotenoid retention sub-population. Fresh and stored fruits were analysed by HPLC-PDA. Three technical replicates per sample were analysed (n = 3). T-tests were carried out to determine the significance between fresh and stored values for each line ($p < 0.05$). Error bars represent ± SE.

Carotenoid retention was again calculated as the change between fresh and stored samples, and expressed as a percentage. Values were not recorded for all fresh samples in the cases of compounds: neoxanthin, β-cryptoxanthin, and capsorubin diester, but were recorded in stored samples. This appeared as an infinite increase in the compound in question, however, it is more likely that the compound was below the limit of detection in some fresh samples. In samples where this observation was made in all three replicates, the retention value for these compounds has not been reported, and instead a symbol (*) has been recorded in Table 3-4. In cases where the compounds in question were observed at the fresh and stored time point in at least one replicate, these values alone were used to calculate the average retention of the compound, and infinite values were discounted. Therefore, in some cases, one replicate alone was used to calculate the average retention, hence explaining why a standard error value was not reported.

Table 3-4 Carotenoid retention subpopulation changes in carotenoid content (%).

Lines characterised as extreme high or extreme low carotenoid retention from the DH population were further analysed by HPLC-PDA. Total carotenoid amounts were calculated using a standard curve, and carotenoid retention values were calculated and expressed as a percentage, along with standard error (±SE). Three technical replicates were analysed (n = 3). The symbol (*) represents where an infinite increase was observed for a compound.

Line	Phytoene	Violaxanthin	Neoxanthin	β-carotene	β-cryptoxanthin	Antheraxanthin	Antheraxanthin (monoester)	Capsanthin	Capsanthin (monoester)	Capsanthin (diester)	Capsorubin (diester)	Zeaxanthin	Zeaxanthin (diester)	Total
11-1764	-49.9	2.7	-66.7	6.7	-11.7	74.3	-33.4	2.4	52.6	-29.3	-9.2	-55.3	11.8	-7.9
SE (±)	3.8	6.9	33.3	30.4	29.1	5.1	9.3	2.7	14.0	6.1	9.2	6.6	25.8	3.4
11-1802	38.1	-1.1	0.0	-17.7	41.1	20.7	-7.7	-4.3	32.2	0.4	-5.3	-16.7	137.5	12.6
SE (±)	5.0	1.0	0.0	15.5	2.1	7.5	17.5	1.3	9.9	9.4	5.3	11.3	50.3	3.8
11-1870	-69.9	6.6	-1.1	-10.2	4.0	64.2	-13.6	11.9	33.0	-8.9	9.2	-26.1	-50.3	-5.4
SE (±)	5.2	4.0	1.1	27.9	15.0	11.0	21.4	5.4	14.9	33.5		32.8	23.4	6.2
11-1937	-49.7	3.9	0.8	-8.7	100.9	26.1	-50.8	-2.2	12.2	-18.7	*	-18.3	-36.8	-7.5
SE (±)	4.5	1.0	0.8	25.7	59.3	13.2	10.0	1.4	16.4	12.1	*	2.8	5.9	7.7
11-1967	-48.3	-3.0	-3.2	-27.6	179.6	16.3	-44.3	1.8	-8.6	-27.2	-33.3	-5.8	15.0	-21.4
SE (±)	5.5	5.1	3.2	15.5		8.1	7.1	2.9	6.4	14.2	33.3	19.1	27.7	8.8
11-2041	43.0	8.5	-33.3	46.7	32.5	49.5	29.4	2.7	70.5	31.2	3.8	-38.8	452.8	42.7
SE (±)	5.5	3.0	33.3	35.9	8.1	10.5	14.1	2.2	16.4	17.6	3.8	8.9	288.8	8.7
11-2053	-24.7	19.2	0.0	112.4	85.6	63.0	63.4	-2.1	81.1	51.1	0.0	19.8	593.9	68.1
SE (±)	1.3	0.1	0.0	118.9	60.2	26.0	57.5	4.3	50.1	61.4	0.0	14.3	88.7	48.3
11-2075	2.2	-9.7	0.0	-39.8	17.5	-38.7	-43.9	-5.1	-12.2	-13.8	-9.0	-10.1	-2.8	-18.0
SE (±)	9.6	3.6	0.0	8.0	80.9	5.2	2.1	3.8	6.8	26.3	7.0	5.7	49.2	5.9
11-2091	26.5	9.0	1.3	176.4	-34.2	20.9	58.4	-3.2	30.5	68.3	-50.0	12.1	93.3	45.3
SE (±)	20.6	5.1	1.3	144.1	65.8	7.5	11.1	2.6	3.4	22.9	50.0	5.1	72.6	6.3
12-1809	75.2	10.5	-2.0	59.4	46.3	34.8	88.8	0.2	64.4	64.6	-0.4	-16.9	469.2	64.3
SE (±)	2.4	4.5	2.0	29.4	38.3	9.1	5.5	4.0	6.4	27.1	19.0	14.6	151.2	17.0
12-1821	167.9	12.8	*	84.9	-12.4	43.4	146.5	5.4	73.4	88.5	0.0	15.8	290.2	89.8
SE (±)	27.8	0.2	*	25.6	44.5	5.6	19.8	0.0	11.3	34.0		17.6	69.9	18.8
12-1852	391.9	11.6	0.0	181.5	-27.6	-3.1	172.8	-8.7	43.5	80.4	-33.3	6.8	935.8	64.7
SE (±)	203.8	1.5		22.5	36.8	8.4	47.1	0.8	9.8	25.4	33.3	11.0	364.4	19.0
12-1865	1057.7	8.4	-0.4	201.3	-50.6	10.8	110.9	-13.2	32.1	83.6	0.0	-31.8	378.9	85.9
SE (±)	125.1	5.3	0.4	26.8	49.4	3.7	28.9	3.8	7.5	11.2	0.0	9.7	39.5	9.0
11-1175	6.5	21.5	3.4	-44.3	121.0	-11.3	63.9	-5.6	58.2	59.3	0.0	-22.4	-4.1	42.8
SE (±)	11.1	4.6	5.5	7.9	43.4	27.5	38.7	5.6	19.7	9.3	0.0	10.9	10.1	8.9
11-1179	-9.9	16.0	10.0	5.3	1.6	72.0	31.2	9.2	43.6	60.5	-24.5	24.5	-22.2	31.6
SE (±)	3.1	8.7	4.9	16.6	15.0	23.2	8.5	6.0	10.7	21.3	38.5	25.3	26.5	10.5

3.2.6. Metabolite profiling of the Double Haploid population immediately post-harvest

A metabolite profiling screen of the DH population was carried out to characterise intermediary metabolism of chilli pepper. Metabolite profiling was used to determine metabolic features associated with key quality traits within pepper, and to determine whether any metabolic features correlated with the carotenoid retention phenotype.

The metabolite screen was carried out in order for the resulting data to be used in metabolite QTL (mQTL) analysis, ultimately to identify genomic regions associated with these metabolites.

Important to note is the fact that the data presented in this study represents semi-quantitative values for each metabolite measured. Values were calculated as relative to an internal standard, and therefore these semi-quantitative values for each compound may be compared across samples, but compound values should not be compared to one another within the same sample.

Principal component analysis of the DH population shows the broad variation in general metabolism across these pepper lines. Lines were coloured in the PCA scores plot according to their designated carotenoid retention status. As the distribution of high, low, and medium retention lines was evenly distributed across the scores plot, and no clustering was evident, clearly carotenoid retention phenotype did not influence the intermediary metabolism of the population (Figure 3-9A). Different classes of compounds forced the separation of some extreme lines, for example the fatty acids, amino acids, and sterols. Other compound classes, such as the sugars and organic acids clustered towards the centre of the PCA loadings plot (Figure 3-9B), therefore meaning that these compounds did not drive the variation in intermediary metabolism across the DH population.

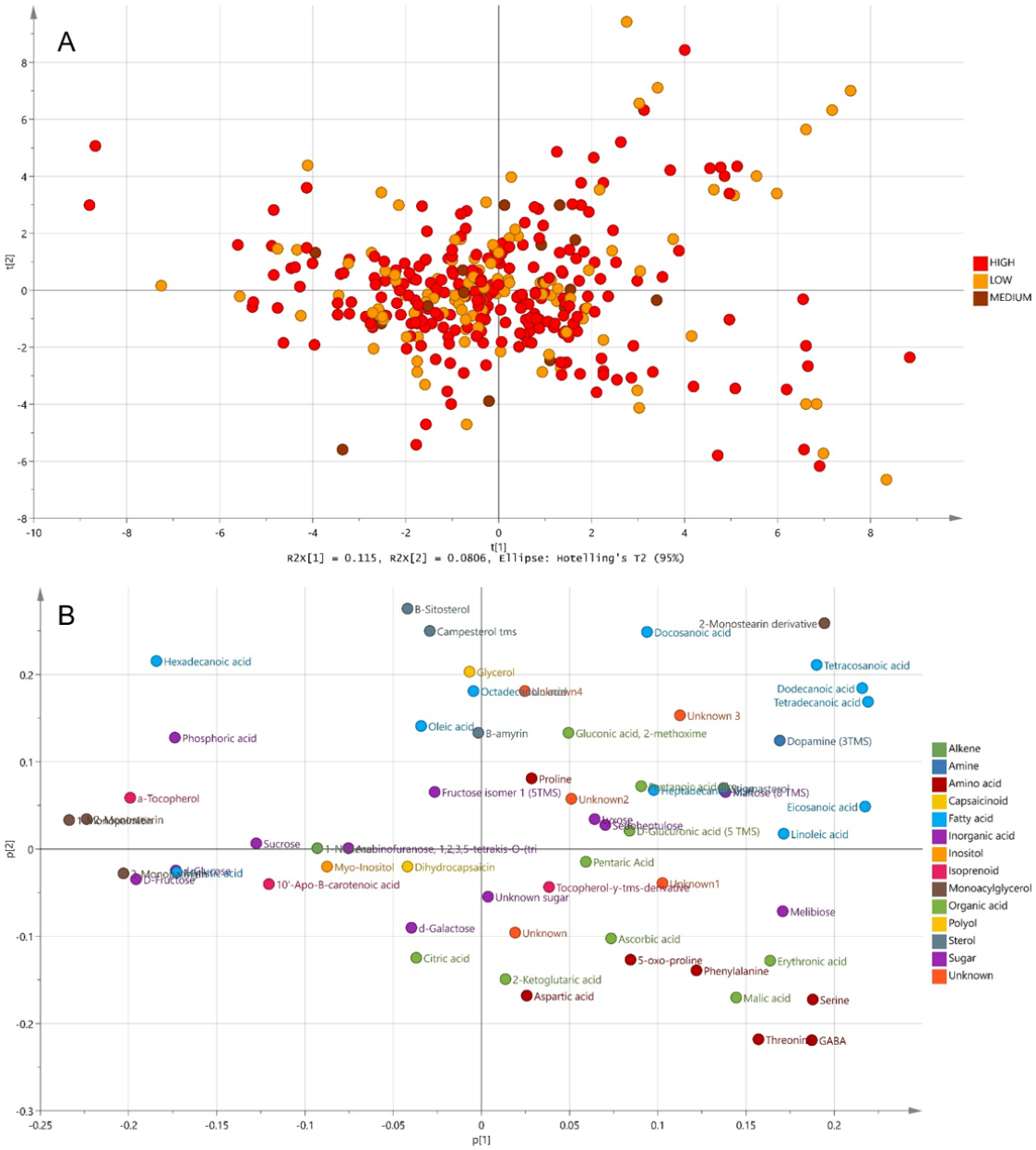


Figure 3-9 PCA plots displaying separation of DH population lines based on metabolite profiling.

Metabolite profiling was performed on fresh fruits of the DH population, using GC-MS. Principal component analysis was used to display the separation of DH population lines, based on the compounds identified in this screen. (A) Scores plot, lines coloured according to carotenoid retention phenotype; high retention lines = red, medium retention lines = brown, low retention lines = yellow; (B) Loadings plot displaying separation of compounds, compounds coloured according to compound class. Relative amounts of each compounds for each line analysed can be found in supplementary table 4.

3.2.7. The influence of intermediary metabolism on specialised metabolism

Using multiple factor analysis (MFA) to analyse both the carotenoid retention dataset and the GC-MS metabolite dataset allowed the relationship between these two sets of variables to be studied. As these two datasets were comprised of different numbers of variables, and values were on vastly different scales, MFA was deemed to be an appropriate method for analysing these two datasets simultaneously. MFA accounts

for datasets of different sizes, and does not place more weighting on a larger data set. This analysis was performed using the MFA function within XLStat. In the case of this analysis, the carotenoid retention dataset separated from the metabolite dataset, again suggesting that these two sets of variables were not influenced by one another (Figure 3-10). Further to this, the RV coefficient was calculated for these two datasets, in which the similarity between two matrices can be compared. The RV coefficient for this analysis was calculated as 0.002, with a p value of 1. This means that the carotenoid retention dataset and the metabolite dataset were not significantly similar to one another. This again provides evidence to suggest that carotenoid retention and the composition of intermediary metabolites present in the population were not influenced by one another, as these two datasets showed no similarity to one another.

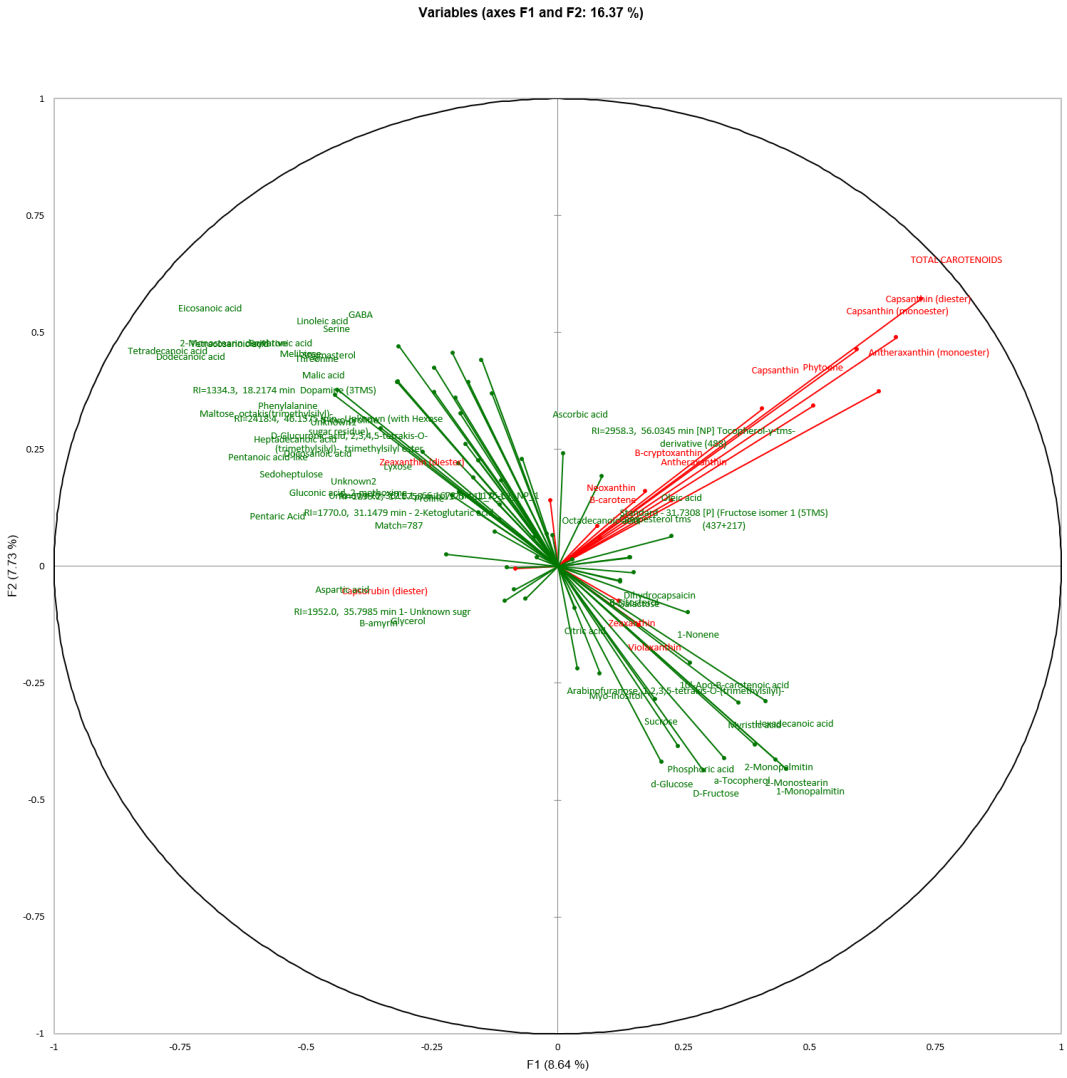


Figure 3-10 Multiple factor analysis of DH population carotenoid retention and metabolite datasets.

Data from DH population carotenoid retention and metabolite profiling screens were collated, and multiple factor analysis (MFA) was performed to determine whether a relationship existed between these two datasets. MFA was carried out using XLstat software. Plot is coloured according to datasets: red = carotenoid retention values, green = intermediary metabolism relative values.

3.2.8. Detailed metabolite profiling of lines identified as retention phenotype extremes

Metabolite profiling was used to determine differences in intermediary metabolism at the fresh and post-storage time points for the retention subpopulation, which was composed of lines characterised as extreme high or low carotenoid retention. Principal component analysis revealed that samples stored following harvest were distinctly separated from freshly harvested samples (Figure 3-11A). According to the loading plot, stored samples were separated based on amino acid content, whereas the separation of fresh samples was driven by some fatty acids and monoacylglycerols (Figure 3-11B). The unsaturated fatty acids: oleic acid and linoleic acid also contributed to the separation of the fresh samples from the stored samples. The abundant saturated fatty acids: hexadecanoic acid (C_{16}) and octadecanoic acid (C_{18}), which are amongst the most abundant fatty acids found in plants, both contributed to the separation of the fresh samples from the stored samples, although they were located more centrally on the PCA compared to unsaturated fatty acids such as linoleic acid. This suggests differences in saturated fatty acids were less responsible for forcing the separation of fresh and stored samples, compared to differences observed in unsaturated fatty acids.

These findings demonstrate that some major changes occurred in intermediary metabolism during post-harvest storage as fresh and stored samples were separated from one another, and these differences were greater than any differences between lines at the same time point.

Principal component analysis of subpopulation lines at the fresh time point shows that there was no separation of high retention lines from low retention lines, based on metabolites analysed by GC-MS (Figure 3-12A). This observation was also made when comparing high and low retention lines at the stored time point (Figure 3-12B). This suggests that there was no major difference in intermediary metabolism between lines identified as high or low carotenoid retention. However, major changes in intermediary metabolism did occur within lines during post-harvest storage.

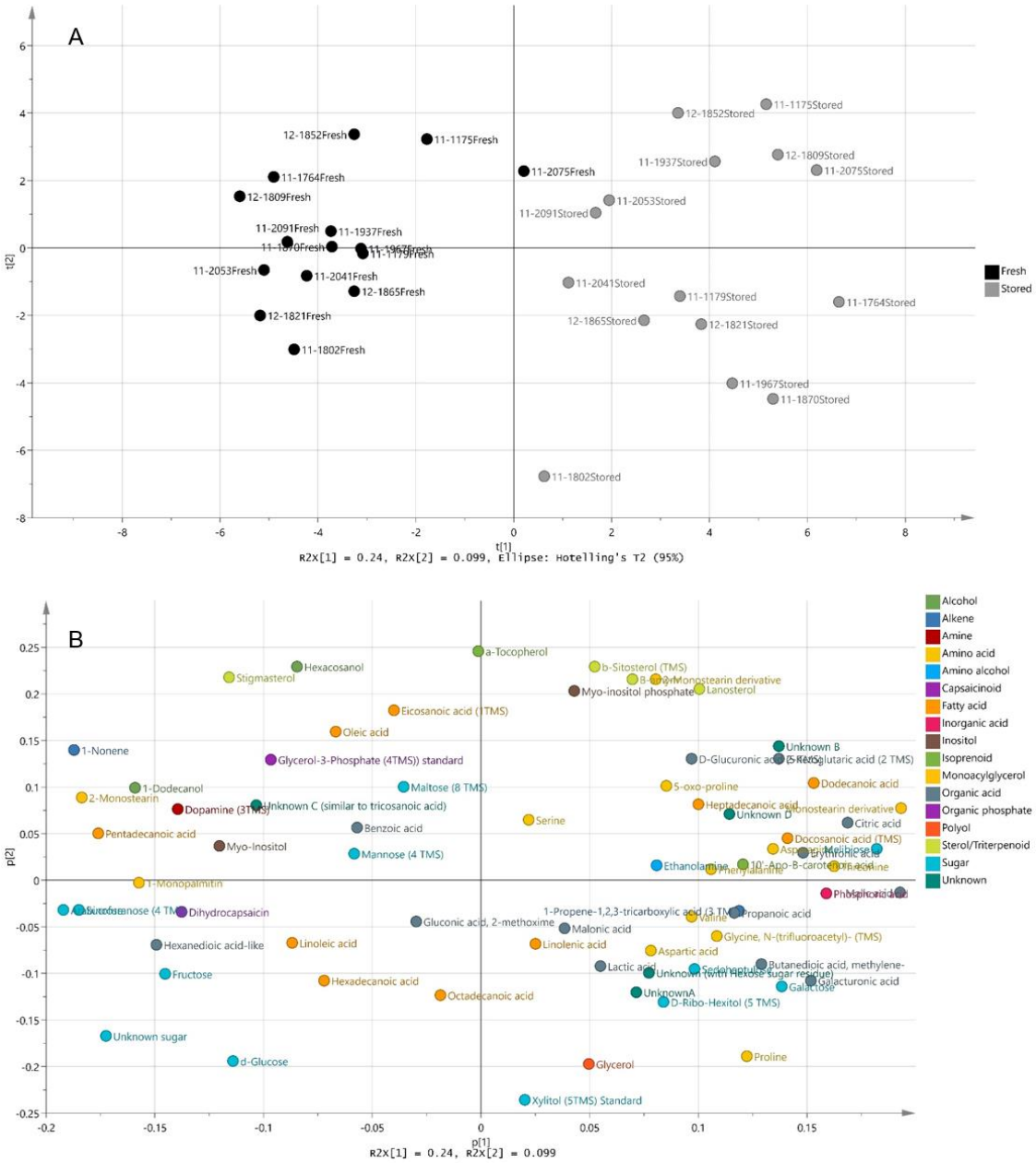


Figure 3-11 PCA plots displaying the separation of pepper subpopulation lines by metabolite profile.

Metabolite profiling was performed on the extreme high and low carotenoid retention lines in the retention sub-population, which were identified from the DH population carotenoid profiling screen. Samples were analysed at both the fresh and stored time points. Three technical replicates per sample were performed (n = 3). (A) Scores plot displaying pepper lines and storage time point (fresh, black; stored, grey), (B) Loadings plot, compound classes identified by colour.

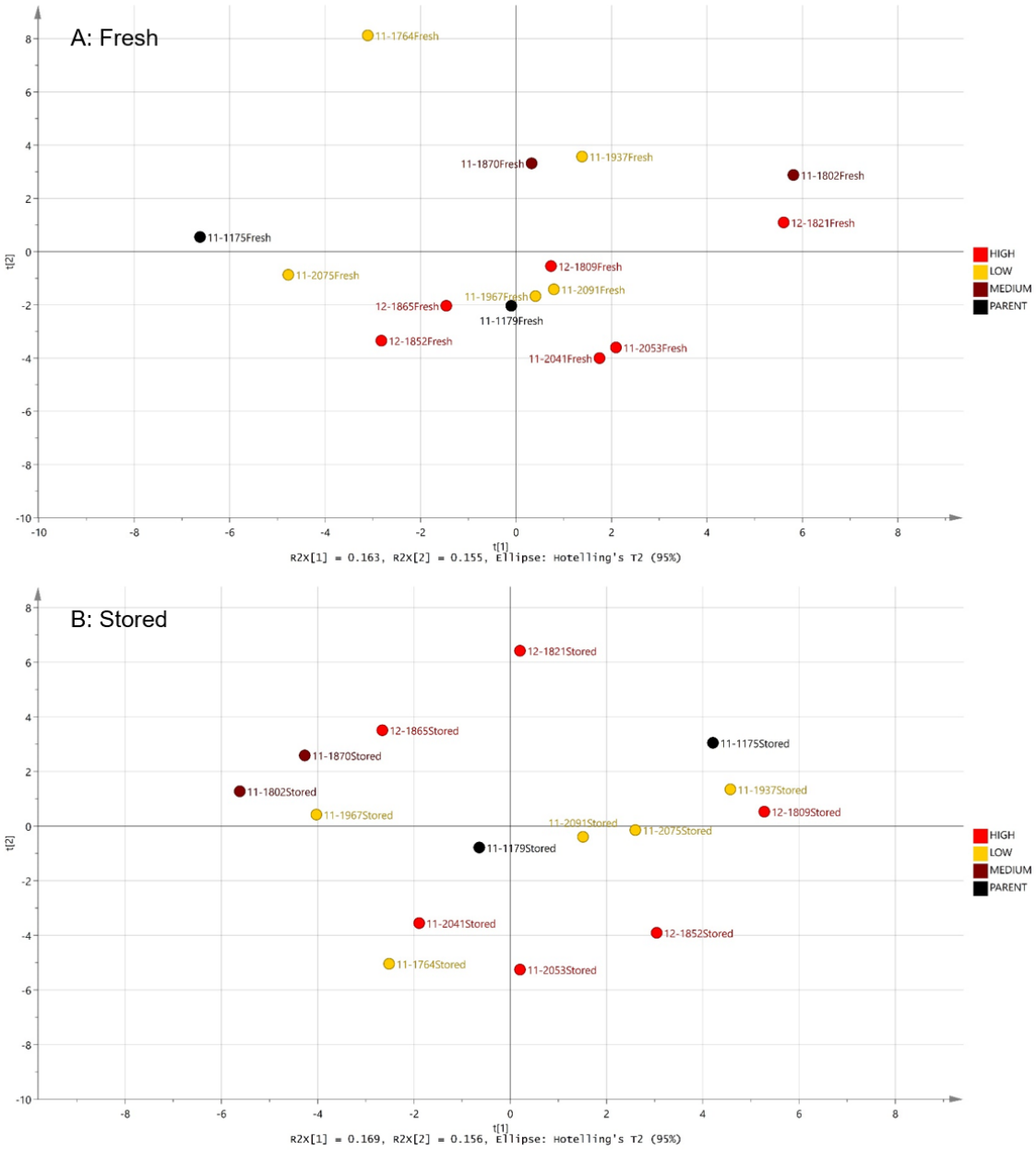


Figure 3-12 PCA scores plots displaying separation of high and low retention lines, based on metabolite profile.

Extreme high and low carotenoid retention lines were subjected to metabolite profiling by GC-MS. Principal component analysis was used to display the variation in high and low carotenoid retention lines both at the fresh time point (A), and at the stored time point (B). Three technical replicates were performed per sample (n = 3). High retention lines = red, medium retention lines = brown, low retention lines = yellow, DH population parents = black.

3.2.9. Semi-volatile analysis of lines identified as retention phenotype extremes

Lines within the DH population characterised as a retention phenotype extreme were analysed by GC-MS for their semi-volatile profile. Volatiles of these pepper lines were analysed both immediately following harvest, and after post-harvest storage. Principal component analysis (PCA) was used to assess variation in semi-volatile profile between freshly harvested and stored pepper lines. Fresh samples clustered in the left panel of the PCA, whereas stored samples clustered on the right, and displayed a

greater spread when compared to fresh samples (Figure 3-13A). The loadings plot (Figure 3-13B) indicates that carotenoid-derived volatiles, such as β-ionone, β-ionone epoxide, β-cyclocitral, and 6-methyl-5-hepten-2-one forced the separation of the stored pepper samples, to the right of the PCA, therefore driving the separation of stored samples. Total detected volatiles are displayed in Table 3-5.

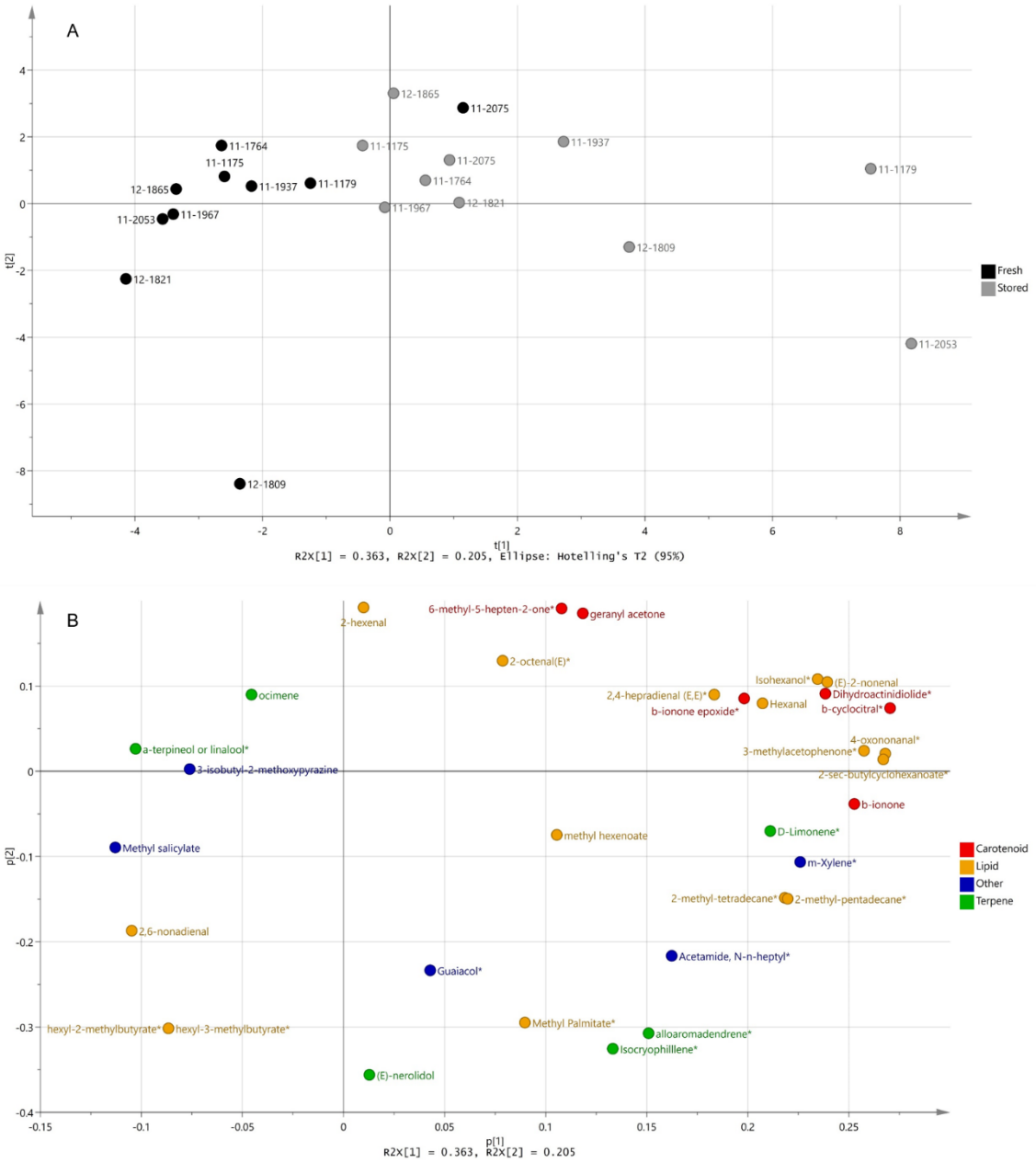


Figure 3-13 PCA plots displaying variation in volatile profile between fresh and stored carotenoid retention sub-population.

Volatile analysis was performed using GC-MS to determine the volatile profile of lines within the carotenoid retention sub-population. Analysis was performed on both fresh and stored fruit material. (A) Scores plot displaying variation between fresh and stored lines; Fresh samples = black, Stored samples = grey. (B) Loadings plot displaying variation in compounds; Carotenoid derived volatiles = red, Lipid-derived volatiles = orange, Terpene derived volatiles = green, volatiles derived from other compounds = blue. Three technical replicates were analysed per sample (n = 3).

In all ten lines analysed by volatile profiling, except line 11-2075, the total detected amount of volatiles was greater in stored samples than in fresh samples. Further to this, whilst the total amount of volatiles in fresh samples across all ten lines showed a small amount of variation, ranging from 2642 ng/mL to 6093 ng/mL, the total amount of volatiles in stored samples across the ten lines showed much greater variation, from 4613 ng/mL to 14724 ng/mL. In all lines measured except the low carotenoid retention line 11-2075, the amounts of the β-carotene-derived volatiles: β-cyclocitral and β-ionone epoxide increased significantly during storage. In the case of β-ionone, a significant increase was observed during storage in all samples apart from the low retention line 11-2075 and the high retention line 12-1809. Dihydroactinidiolide is also derived from β-carotene, and increases were observed in stored samples compared to fresh samples in all lines except 11-2075 and 11-1175 (Figure 3-14). β-cyclocitral and β-ionone amounts both showed a greater increase in high retention lines when compared to low retention lines.

The acyclic carotene-derived volatile, geranyl acetone, also displayed significant increases in stored samples compared to fresh samples in all lines except 11-2075 (Figure 3-14). Evidently, carotenoid-derived volatiles increased significantly during post-harvest storage in the majority of pepper varieties analysed.

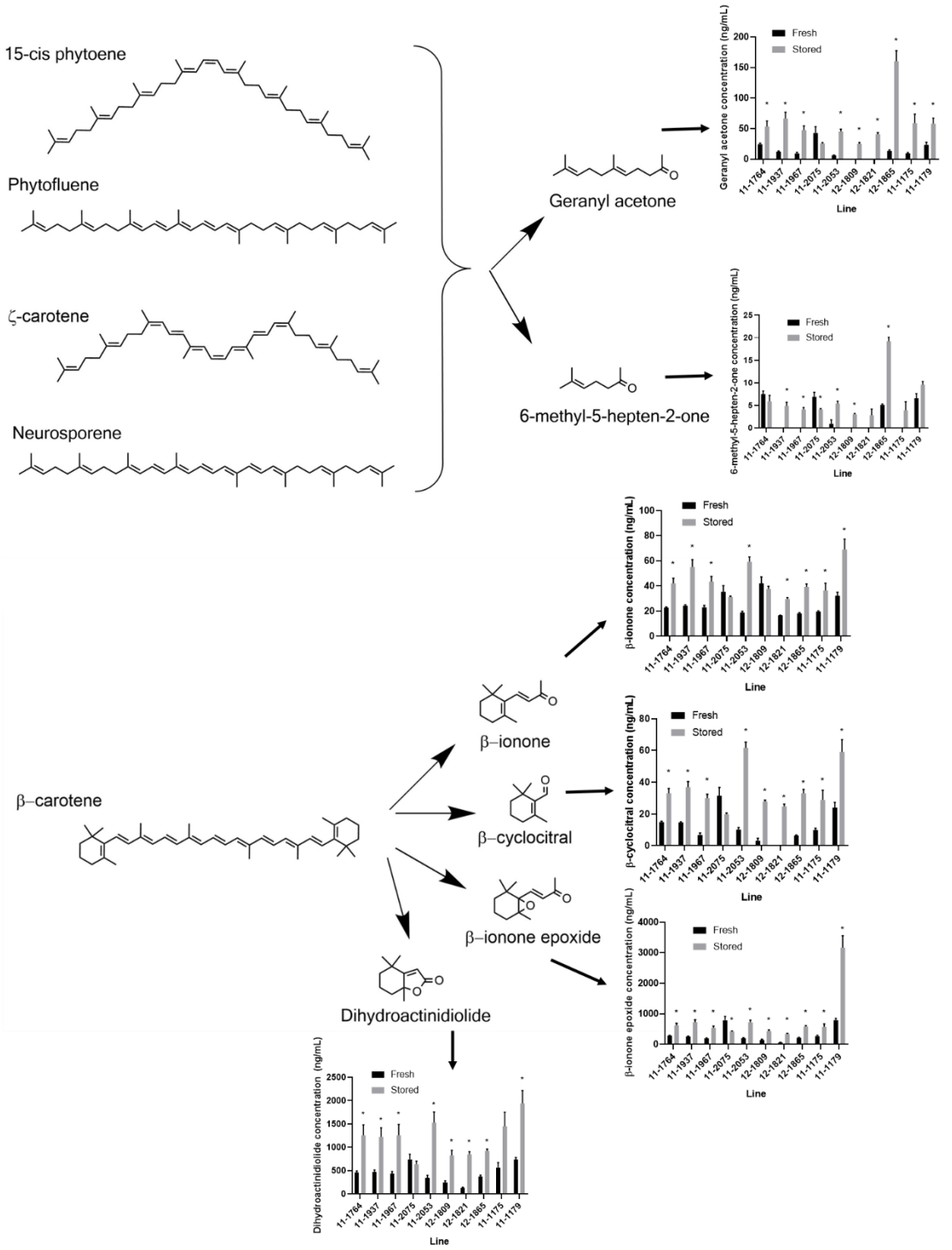


Figure 3-14 Carotenoid derived volatile concentrations in fresh and stored fruits.

Carotenoid-derived volatiles are deemed to be those volatiles produced as a result of carotenoid cleavage. Acyclic carotenoid-derived volatiles: geranyl acetone and 6-methyl-5-hepten-2-one, were measured. β-carotene derived volatiles: β-ionone, β-cyclocitral, β-ionone epoxide, and dihydroactinidiolide, were measured. Pepper lines deemed to be extreme high or low carotenoid retention, along with DH population parents, were analysed. High carotenoid retention: 11-2053, 12-1809, 12-1821, 12-1865. Low carotenoid retention: 11-1764, 11-1937, 11-1967, 11-2075. DH population parent lines: 11-1175, 11-1179. Three technical replicates per sample were analysed (n = 3). Error bars ± SE. Student's T-test was used to determine significant differences between fresh and stored samples (p < 0.05).

A heat map was used to display changes in volatile component during post-harvest storage (Figure 3-15). Carotenoid-derived volatiles, including β-cyclocitral and β-

ionone, tended to show increases in content during post-harvest storage, in both high and low carotenoid retention lines. This is unsurprising in the low retention lines, as a decrease in carotenoid content was observed during post-harvest storage. However, this increase in carotenoid-derived volatiles during post-harvest storage in the high retention line may be interesting, as carotenoid content was shown to increase (Figure 3-8). This suggests that whilst carotenoid synthesis is ongoing during post-harvest storage, carotenoid degradation also occurs.

Lipid-derived volatiles, including 2-hexenal, methyl hexenoate, and 2-octenal, tended to display decreases during post-harvest storage, particularly in low carotenoid retention lines. Both oleic and linoleic acid were shown to drive the separation of the fresh samples from the stored samples when compared using PCA (Figure 3-11) due to increased levels. Therefore, increased fatty acid concentration at the fresh time point, explains the increase in the volatiles derived from these compounds. This decrease in lipid derived volatiles is more prevalent in low carotenoid retention lines compared to high carotenoid retention lines. Increased lipid peroxidation may have occurred in fresh fruit or early in storage of low carotenoid retention lines, due to the decreased availability of carotenoids to protect against lipid peroxidation. Therefore, lipid-derived volatiles may be at low levels in the low retention line following storage as many lipid-derived volatiles had already been produced. Consequently, these volatiles were not detected. However, the decrease in lipid-derived volatiles was not as stark in the high carotenoid retention lines, as carotenoids could protect fatty acids from lipid peroxidation early in storage, and so lipid-derived volatiles could still be detected at the point of analysis. For example, 2-hexenal has been shown to be derived as an oxidation product from oleic acid (Frankel, 1983). Oleic acid levels were shown to be higher in fresh samples compared to stored samples. 2-hexenal levels showed a greater decrease during post-harvest storage in low carotenoid retention lines than in high retention lines. This may have been due to 2-hexenal being produced from the oxidation of oleic acid in fresh fruit, or very early in storage, due to the reduced ability of these low retention lines to protect against lipid peroxidation. Consequently, low levels of 2-hexenal were detected at analysis. However, the high carotenoid retention line had a greater ability to protect against lipid peroxidation of oleic acid during storage. Therefore, increased levels of 2-hexenal were detected as the degradation of oleic acid to 2-hexenal was slower, and therefore this volatile was still being produced at the point of analysis (Table 3-5, Figure 3-15).

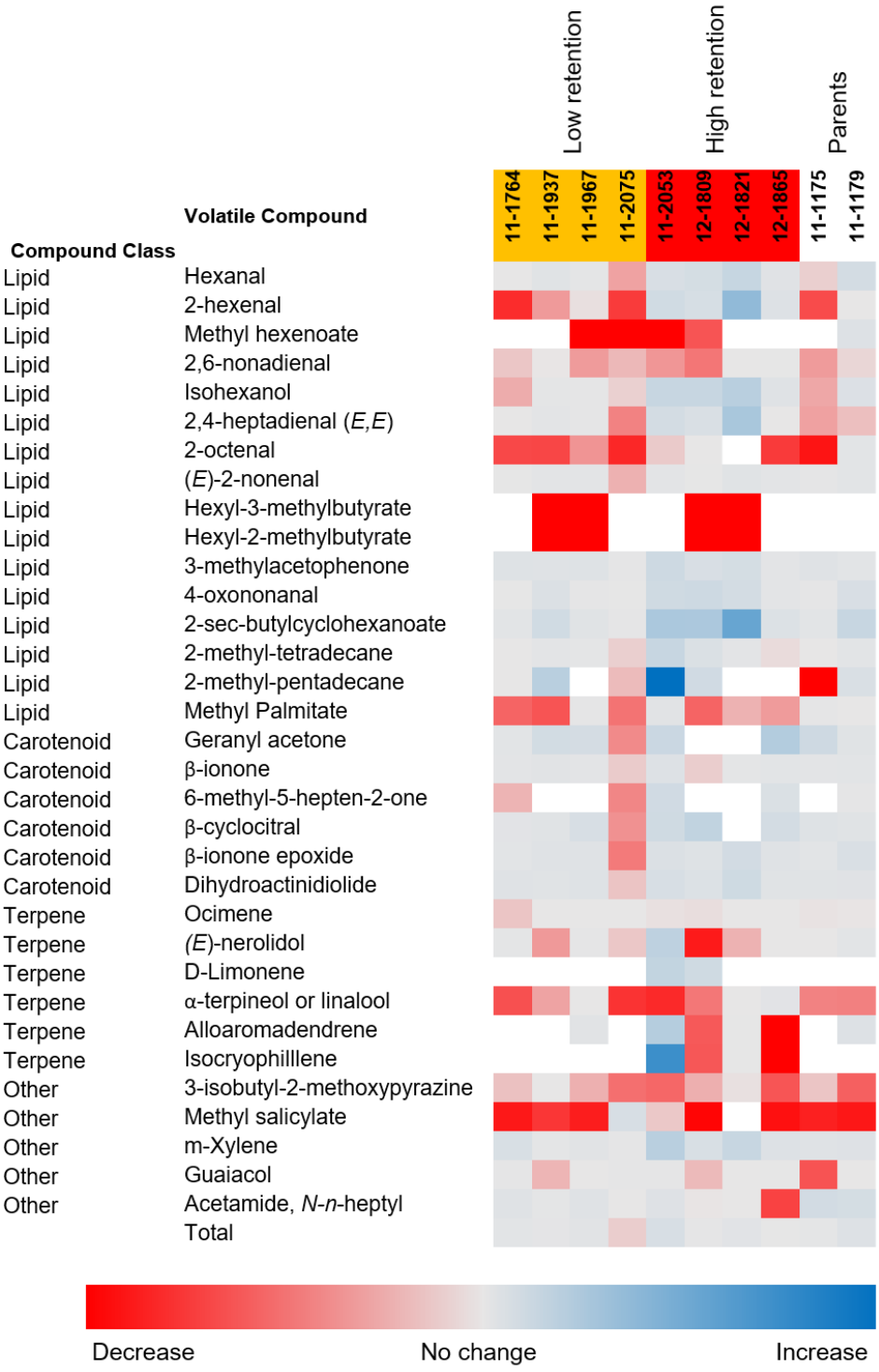


Figure 3-15 Heat map displaying change in semi-volatile content during post-harvest storage.

Changes in semi-volatile content during post-harvest storage were calculated and expressed as a percentage for members of the carotenoid retention sub-population. A heat map has been used to display the increases and decreases of each volatile for high (red), and low (yellow) retention lines. The parents of the DH population were also included in this analysis. Within the heat map, red blocks display a decrease in compound during post-harvest storage, whereas blue blocks display an increase in compound. The more intense the colour is, the greater the increase or decrease was observed.

Table 3-5 Carotenoid retention subpopulation semi-volatile amounts (ng/mL).

Semi-volatiles were quantified by GC-MS for fresh and stored subpopulation samples. Low retention lines (orange); high retention lines (red); parent lines (grey). Standard error (± SE) reported; values highlighted in bold significantly different when comparing fresh vs stored within same pepper line (n = 3; p < 0.05).

	11-1764		11-1937		11-1967		11-2075		11-2053		12-1809		12-1821		12-1865		11-1175		11-1179	
	Fresh	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**
Hexanal	92.1	106.9	**105.7**	**270.5**	**38.5**	**64.9**	371.4	262.7	**55.6**	**236.9**	**48.4**	**241.3**	**21.2**	**166.0**	**56.7**	**148.4**	84.3	76.0	**210.1**	**1094.8**
SE (±)	2.2	14.7	**1.3**	**19.5**	**1.6**	**2.7**	40.2	9.2	**6.5**	**27.3**	**2.1**	**8.4**	**1.0**	**12.1**	**2.2**	**11.6**	7.8	15.0	**20.8**	**141.8**
2-hexenal	**278.3**	**54.2**	**384.3**	**258.4**	77.0	74.8	**504.4**	**131.4**	**27.0**	**156.6**	**41.2**	**187.1**	**11.0**	**206.1**	**136.5**	**443.6**	**467.0**	**153.6**	**65.5**	**76.4**
SE (±)	**14.3**	**9.1**	**4.5**	**23.7**	4.1	5.3	**33.3**	**4.9**	**2.8**	**8.6**	**3.1**	**9.6**	**0.6**	**14.2**	**3.4**	**23.0**	**19.8**	**27.2**	**1.9**	**2.4**
Methyl hexenoate	<LOQ	0.1	<LOQ	0.2	0.2	<LOQ	0.2	<LOQ	0.2	<LOQ	0.5	0.2	<LOQ	0.1	<LOQ	<LOQ	<LOQ	<LOQ	**0.3**	**0.9**
SE (±)		0.1		0.1	0.2		0.1	0.0	0.2		0.5	0.1		0.1					**0.0**	**0.1**
Ocimene	**6.7**	**5.8**	**6.1**	**7.0**	**5.9**	**6.1**	5.8	5.8	5.9	5.8	6.0	5.8	6.5	6.7	**6.1**	**7.1**	**5.9**	**5.8**	5.8	5.8
SE (±)	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	0.0	0.0	0.1	0.0	0.2	0.0	0.1	0.1	**0.0**	**0.2**	**0.0**	**0.0**	0.0	0.0
3-isobutyl-2-methoxypyrazine	**0.8**	**0.6**	0.7	0.7	0.8	0.6	**1.4**	**0.7**	**1.6**	**0.7**	1.0	0.7	0.7	0.7	**1.6**	**0.6**	**0.7**	**0.6**	**2.0**	**0.8**
SE (±)	**0.0**	**0.0**	0.0	0.1	0.1	0.0	**0.1**	**0.0**	**0.1**	**0.0**	0.2	0.0	0.0	0.1	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**
2,6-nonadienal	**6.7**	**5.8**	6.4	6.4	8.9	6.0	8.1	6.5	9.7	6.3	12.3	6.3	**5.8**	**6.7**	**6.1**	**7.4**	9.1	6.1	6.5	6.0
SE (±)	**0.2**	**0.1**	0.2	0.1	2.4	0.1	0.6	0.1	2.3	0.1	2.2	0.1	**0.1**	**0.3**	**0.1**	**0.2**	2.4	0.1	0.3	0.0
Methyl salicylate	**19.6**	**2.1**	**9.4**	**2.2**	**13.4**	**1.7**	**0.5**	**2.3**	6.9	6.0	**31.4**	**0.8**	**<LOQ**	**4.5**	**62.9**	**4.6**	**7.2**	**1.0**	**29.4**	**2.9**
SE (±)	**1.0**	**0.3**	**0.9**	**0.3**	**2.5**	**0.1**	**0.3**	**0.2**	0.3	0.3	**3.6**	**0.1**		**0.8**	**2.5**	**0.5**	**0.5**	**0.5**	**3.7**	**0.6**
Geranyl acetone	**24.6**	**53.6**	**12.4**	**66.2**	**9.3**	**47.6**	42.9	25.8	**6.3**	**45.2**	**<LOQ**	**25.1**	**<LOQ**	**40.7**	**13.6**	**160.3**	**9.4**	**59.0**	**23.5**	**58.1**
SE (±)	**1.4**	**8.8**	**1.0**	**10.6**	**1.9**	**6.8**	10.4	1.3	**0.4**	**3.8**		**2.1**		**2.9**	**1.3**	**17.3**	**1.5**	**14.7**	**4.2**	**9.0**
β-ionone	**22.8**	**42.0**	**24.3**	**55.1**	**23.0**	**43.5**	35.4	31.3	**18.9**	**59.2**	42.0	37.6	**16.6**	**29.6**	**18.2**	**39.2**	**19.6**	**36.4**	**32.2**	**69.0**
SE (±)	**0.6**	**4.1**	**0.7**	**5.9**	**1.5**	**4.0**	4.9	0.6	**0.9**	**3.9**	5.2	2.1	**0.1**	**1.2**	**0.5**	**2.4**	**0.6**	**5.8**	**2.8**	**8.3**
(E)-nerolidol	**7.5**	**12.7**	**12.5**	**8.4**	23.2	35.3	6.9	6.0	**10.3**	**99.3**	**228.7**	**26.2**	63.0	48.8	7.8	9.0	6.5	7.9	**10.7**	**21.9**
SE (±)	**0.3**	**1.5**	**0.6**	**0.8**	2.5	4.3	0.6	0.3	**0.8**	**9.1**	**49.4**	**2.2**	1.5	6.4	0.4	0.5	0.3	1.0	**1.1**	**2.9**
Isohexanol*	123.8	92.9	**179.4**	**316.9**	**50.2**	**76.8**	320.5	291.1	**44.3**	**341.7**	**43.5**	**330.5**	**21.8**	**231.8**	**86.8**	**233.5**	199.2	145.0	**92.3**	**319.3**
SE (±)	12.2	11.7	**4.0**	**18.9**	**2.3**	**3.3**	32.3	6.3	**4.8**	**33.8**	**1.6**	**12.1**	**0.6**	**22.9**	**0.6**	**9.2**	3.5	24.4	**7.3**	**35.0**
m-Xylene*	**178.5**	**753.1**	**408.0**	**668.0**	**209.3**	**491.1**	**554.2**	**825.3**	**377.8**	**3954.9**	**275.3**	**1153.2**	**134.7**	**1042.1**	**204.5**	**645.7**	**216.3**	**589.5**	**326.2**	**984.3**
SE (±)	**2.3**	**90.0**	**20.6**	**25.2**	**33.8**	**39.4**	**21.0**	**21.2**	**71.0**	**881.7**	**5.4**	**11.5**	**6.1**	**116.0**	**10.6**	**30.5**	**17.5**	**128.1**	**38.1**	**85.1**
6-methyl-5-hepten-2-one*	7.5	5.9	**<LOQ**	**4.9**	**<LOQ**	**4.1**	**6.9**	**4.1**	**0.9**	**5.4**	**<LOQ**	**3.0**	<LOQ	2.8	**5.1**	**19.2**	<LOQ	3.9	6.6	9.6
SE (±)	0.7	1.3		**0.8**		**0.4**	**1.0**	**0.2**	**0.9**	**0.5**		**0.2**		1.4	**0.2**	**0.9**		1.9	1.0	0.7
D-Limonene*	<LOQ	2.6	<LOQ	2.3	<LOQ	<LOQ	**<LOQ**	**5.8**	**0.9**	**7.6**	**0.9**	**5.6**	**<LOQ**	**7.7**	<LOQ	0.9	<LOQ	1.2	**<LOQ**	**3.9**
SE (±)		1.3		1.2				**0.3**	**0.9**	**1.2**	**0.9**	**0.3**		**0.3**	<LOQ	0.9		1.2		**0.4**

2,4-heptadienal (E,E)*	73.7	79.9	**72.4**	**146.7**	**38.8**	**62.3**	**204.6**	**116.2**	**40.3**	**208.2**	**31.5**	**124.3**	**7.2**	**100.1**	69.2	75.0	143.7	100.7	102.2	84.9
SE (±)	6.2	7.9	**3.0**	**11.0**	**2.9**	**2.9**	**22.6**	**5.3**	**2.0**	**15.2**	**1.6**	**3.4**	**0.2**	**13.0**	1.5	4.7	6.0	15.9	10.9	7.4
2-octenal(E)*	**19.6**	**6.1**	**12.1**	**3.7**	4.9	3.1	**58.3**	**9.9**	8.5	7.5	3.8	3.9	<LOQ	1.8	**14.1**	**3.6**	**20.1**	**1.8**	**23.7**	**49.9**
SE (±)	**1.0**	**0.9**	**1.1**	**0.4**	1.0	0.3	**9.2**	**0.6**	0.9	0.7	0.3	0.2		0.9	**0.6**	**0.5**	**1.0**	**0.9**	**3.4**	**5.6**
Guaiacol*	101.1	152.6	203.5	160.5	157.3	164.7	101.5	156.5	186.2	300.5	279.3	226.2	197.5	206.5	144.6	182.5	342.5	124.1	155.3	172.5
SE (±)	24.8	44.9	40.9	39.8	45.5	52.7	19.6	50.1	90.4	69.6	130.4	96.4	38.1	56.8	32.2	47.1	158.0	25.5	30.5	32.6
(E)-2-nonenal	93.9	118.2	**85.2**	**161.3**	58.8	109.9	167.7	129.3	69.8	130.8	85.8	116.2	51.2	129.3	59.3	113.1	69.2	98.7	110.0	236.7
SE (±)	11.6	23.3	**11.5**	**24.1**	7.7	18.7	31.7	18.7	4.0	30.1	17.8	14.1	10.4	27.8	4.8	19.5	5.6	15.9	19.4	51.2
Hexyl-3-methylbutyrate*	<LOQ	<LOQ	**4.0**	**<LOQ**	1.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**46.4**	**<LOQ**	**24.7**	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
SE (±)			**0.3**		1.4						**6.8**		**3.3**							
Hexyl-2-methylbutyrate*	<LOQ	<LOQ	**4.0**	**<LOQ**	1.5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**46.5**	**<LOQ**	**24.8**	**<LOQ**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
SE (±)			**0.3**		1.5						**6.8**		**3.3**							
α-terpineol or linalool*	**61.9**	**21.7**	19.4	13.9	19.4	19.8	**32.9**	**7.3**	**88.4**	**16.1**	**26.2**	**13.6**	16.3	16.7	**9.7**	**21.4**	**38.7**	**21.9**	39.1	21.8
SE (±)	**1.2**	**1.9**	1.2	1.6	1.7	1.5	**4.3**	**0.4**	**5.4**	**1.6**	**1.9**	**0.5**	1.0	1.7	**0.6**	**2.2**	**3.7**	**4.3**	5.8	3.7
3-methylacetophenone*	**41.4**	**123.9**	**51.3**	**143.4**	**36.0**	**107.0**	**71.2**	**101.8**	**28.6**	**184.9**	**26.9**	**114.8**	**19.8**	**97.5**	**32.1**	**61.2**	**36.6**	**97.4**	**59.0**	**120.9**
SE (±)	**2.0**	**16.5**	**3.4**	**14.9**	**4.2**	**11.3**	**8.9**	**4.4**	**4.8**	**19.6**	**2.3**	**4.1**	**0.4**	**2.7**	**3.2**	**5.7**	**9.4**	**19.2**	**4.7**	**13.5**
β-cyclocitral*	**14.8**	**33.0**	**14.6**	**36.8**	**6.7**	**30.0**	31.5	19.9	**10.2**	**61.7**	**3.1**	**27.9**	**<LOQ**	**24.7**	**6.4**	**33.0**	**9.9**	**28.8**	**24.0**	**59.1**
SE (±)	**0.6**	**3.1**	**0.4**	**3.6**	**1.4**	**2.4**	5.2	0.7	**1.3**	**3.6**	**1.6**	**0.8**		**1.5**	**0.3**	**2.5**	**1.1**	**6.1**	**3.3**	**7.7**
4-oxononanal*	55.7	75.1	**68.0**	**238.9**	**35.3**	**54.1**	161.6	225.9	**49.0**	**297.3**	**46.0**	**295.0**	**31.8**	**168.6**	**56.1**	**107.0**	**48.3**	**72.9**	**92.5**	**394.0**
SE (±)	9.9	3.2	**4.1**	**5.3**	**3.8**	**2.4**	32.9	22.5	**2.2**	**34.8**	**5.0**	**6.2**	**2.9**	**35.2**	**2.0**	**10.7**	**5.3**	**6.5**	**20.1**	**43.1**
2-sec-butylcyclohexanoate*	**45.4**	**98.6**	**72.3**	**419.2**	**20.5**	**51.5**	242.9	364.3	**38.8**	**523.9**	**41.4**	**548.3**	**12.5**	**344.0**	**47.4**	**159.2**	**45.2**	**96.1**	**87.1**	**666.5**
SE (±)	**9.1**	**6.0**	**6.1**	**19.4**	**2.7**	**3.1**	67.9	55.8	**0.3**	**110.4**	**5.5**	**39.3**	**0.5**	**92.7**	**2.3**	**28.5**	**7.9**	**10.9**	**28.2**	**138.0**
2-methyl-tetradecane*	43.3	50.1	**48.0**	**94.7**	**37.0**	**60.0**	76.5	69.1	**48.5**	**373.0**	**82.7**	**310.8**	**42.1**	**85.2**	45.9	43.9	**42.4**	**48.3**	61.8	127.5
SE (±)	6.1	1.6	**3.0**	**7.6**	**3.7**	**1.6**	14.5	4.5	**4.8**	**12.2**	**5.6**	**27.8**	**3.9**	**11.3**	2.8	3.6	**5.8**	**1.5**	9.3	16.0
Alloaromadendrene*	**<LOQ**	**17.2**	**<LOQ**	**18.1**	**11.0**	**26.8**	<LOQ	<LOQ	**7.2**	**81.8**	**62.3**	**24.2**	**11.5**	**15.6**	7.5	<LOQ	<LOQ	<LOQ	**8.2**	**26.2**
SE (±)		**0.5**		**0.8**	**0.2**	**0.9**			**3.6**	**2.5**	**4.9**	**0.4**	**0.1**	**0.7**	3.7				**4.1**	**2.4**
Isocryophilllene*	<LOQ	<LOQ	<LOQ	<LOQ	**<LOQ**	**64.1**	<LOQ	<LOQ	**5.7**	**208.3**	**207.9**	**79.3**	26.9	34.8	11.4	<LOQ	<LOQ	<LOQ	**<LOQ**	**77.3**
SE (±)						**2.1**			**5.7**	**6.9**	**26.5**	**2.3**	1.5	5.8	5.7					**9.5**
2-methyl-pentadecane*	0.9	0.9	**0.9**	**9.6**	**<LOQ**	**3.9**	6.6	5.4	**1.1**	**55.3**	**7.6**	**45.1**	**<LOQ**	**8.0**	<LOQ	<LOQ	0.9	<LOQ	**3.7**	**15.0**
SE (±)	0.9	0.9	**0.9**	**1.2**		**0.3**	2.4	0.7	**1.1**	**2.0**	**0.9**	**4.6**		**1.9**			0.9		**2.0**	**2.6**
β-ionone epoxide*	**281.3**	**620.0**	**257.8**	**725.2**	**199.1**	**540.5**	**789.6**	**418.9**	**202.6**	**721.2**	**145.0**	**435.8**	**62.0**	**341.5**	**215.1**	**584.7**	**266.8**	**574.5**	**790.6**	**3167.2**
SE (±)	**12.2**	**68.6**	**11.6**	**80.7**	**11.7**	**59.1**	**124.9**	**18.4**	**19.2**	**71.7**	**20.5**	**36.7**	**2.3**	**19.0**	**15.0**	**27.6**	**27.1**	**97.7**	**61.0**	**391.7**

Dihydroactinidiolide*	**458.1**	**1260.1**	**472.8**	**1226.3**	**438.4**	**1256.4**	745.7	636.8	**344.7**	**1530.1**	**244.8**	**826.4**	**138.1**	**851.1**	**367.3**	**926.0**	566.7	1451.2	**742.6**	**1940.4**
SE (±)	**37.2**	**222.8**	**45.3**	**193.1**	**42.9**	**236.8**	106.4	68.7	**57.4**	**227.8**	**37.5**	**111.7**	**9.8**	**57.0**	**37.2**	**38.4**	109.9	302.7	**40.1**	**276.2**
Methyl Palmitate*	**106.8**	**46.6**	**210.2**	**77.0**	115.0	170.6	**73.3**	**36.7**	**174.5**	**413.2**	456.8	199.5	239.2	185.4	**81.8**	**55.1**	31.7	49.6	326.7	338.6
SE (±)	**8.2**	**11.2**	**27.7**	**13.9**	15.5	26.5	**7.6**	**5.4**	**42.2**	**67.5**	116.1	22.0	20.7	17.0	**9.2**	**2.3**	5.0	15.6	48.9	45.2
Acetamide, *N-n*-heptyl*	1422.4	4043.0	1005.9	1643.3	**1001.6**	**2852.3**	1469.5	1555.8	**1547.3**	**4685.0**	3289.1	3270.0	1517.9	1674.8	**1827.5**	**528.1**	**203.5**	**1118.9**	**499.4**	**2484.6**
SE (±)	393.5	867.6	180.7	298.3	**224.7**	**392.5**	381.8	159.2	**301.5**	**777.9**	1192.7	402.0	127.0	563.1	**251.3**	**92.8**	**49.2**	**325.9**	**54.3**	**434.9**
Total	**3589.0**	**7885.5**	**3751.5**	**6785.9**	**2642.4**	**6429.6**	6093.2	5452.6	**3418.2**	**14724.6**	5864.4	8684.8	**2704.6**	**6080.1**	3601.3	4613.3	2891.3	4971.0	**3936.8**	**12637.2**
SE (±)	**400.6**	**1286.8**	**276.4**	**661.0**	**318.9**	**806.6**	628.8	187.9	**585.4**	**1967.6**	919.2	593.9	**129.8**	**395.4**	344.7	212.0	372.0	1020.6	**259.9**	**1424.1**

In the semi-volatile analysis, 15 significant differences were observed between the low and high retention parent line fresh samples, and 18 significant differences were observed between the parent line stored samples. Total semi-volatile amounts were significantly different between the parent line stored samples (Figure 3-16B), whilst no significant difference was observed between total semi-volatile amounts in fresh parent line samples (Figure 3-16A), suggesting that there was a greater variation in parent lines once the samples had been stored, as opposed to immediately following harvest. This reflects what was observed in other pepper lines, as stored samples displayed greater variations in volatile amounts compared to fresh samples.

Increased lipid-derived volatiles were detected in the low retention parent (11-1175) line compared to the high retention parent in fresh fruit (Figure 3-16A). This supports the hypothesis that lipid-derived volatiles may be produced to a greater extent in low retention line fresh fruit than their high retention counterparts. Carotenoid scavenging of reactive oxygen species may be less efficient in low carotenoid retention fruits, therefore resulting in increased lipid peroxidation and production of lipid-derived volatiles earlier in the post-harvest storage period. However, in stored fruit, lipid-derived volatiles were increased in the high carotenoid retention parent (11-1179) compared to the low retention parent, suggesting that these volatiles were still being produced at this time point (Figure 3-16B). The decrease in these lipid-derived volatiles in the low retention parent in stored fruit compared to fresh fruit suggests that these lipid-derived volatiles were produced early in storage, and could be detected to a lesser extent at the point of analysis of stored fruit.

Evidence from the sub-population and the parent lines of the DH population indicates significant changes during post-harvest storage in semi-volatile profile. This further demonstrates the change to metabolite profile, which occurs during post-harvest storage.

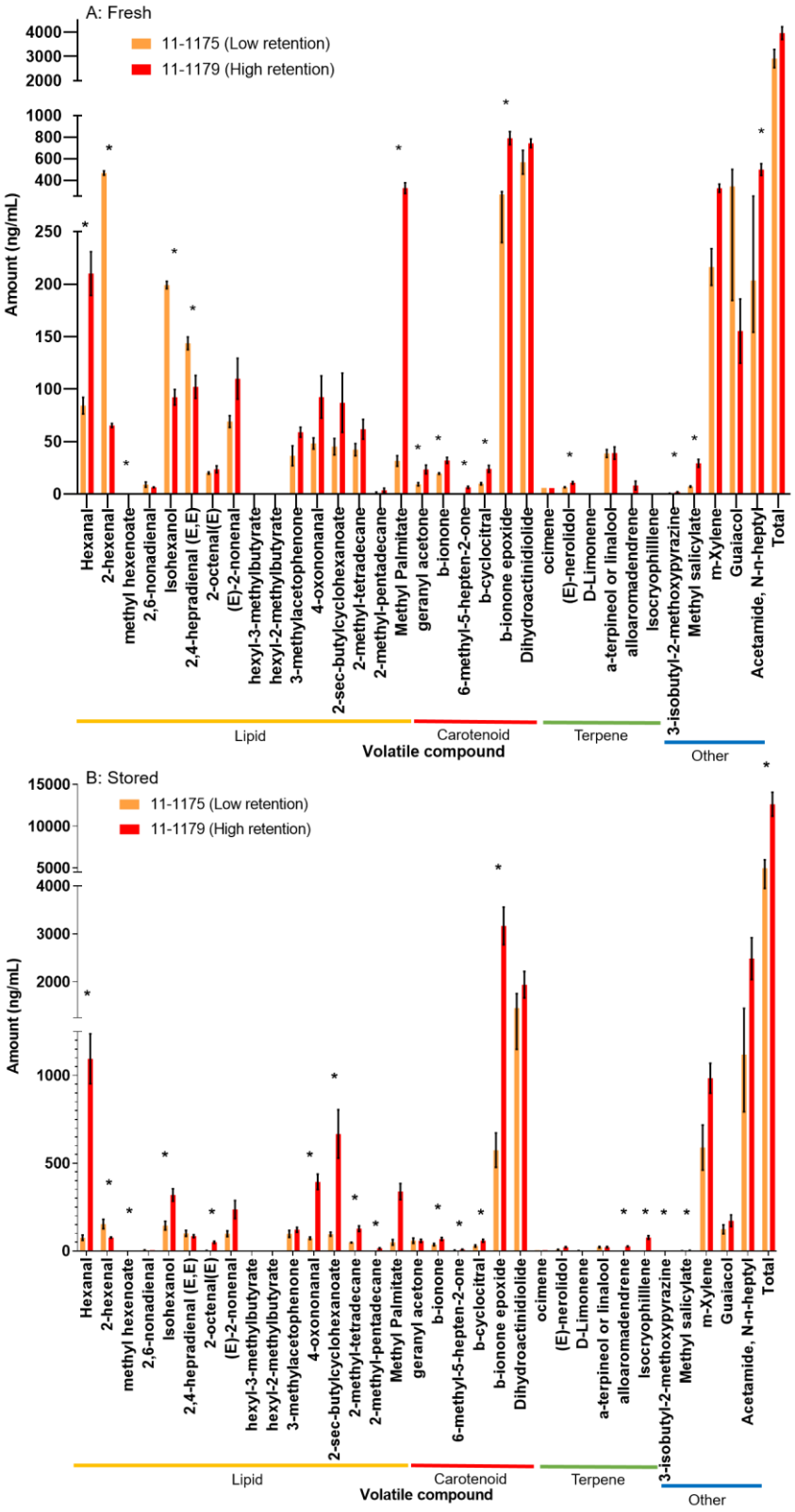


Figure 3-16 Comparison of semi-volatile compound quantities between high and low retention parent lines.

Semi-volatile analysis was performed on the high (red) and low (orange) carotenoid retention parents. (A) Fresh parent samples; (B) Stored parent samples. Volatile compounds were grouped based on the compounds from which they were derived: lipid = yellow, carotenoid = red, terpene = green, other = blue. T-tests were performed between high and low retention parent for each identified volatile compound ($n = 3$, $p < 0.05$).

Analysis of the semi-volatile profile within fresh samples displayed a uniform distribution of low and high carotenoid retention lines (Figure 3-17A) with no lines displaying significant clustering. In contrast, within the stored samples analysed, lines identified as low retention displayed clustering to the centre of the PCA plot with little variation, whereas high retention lines showed a greater variation in volatile profile, forcing separation across the PCA plot (Figure 3-17B). This suggests that there were no major differences between high and low retention lines at the fresh time point. At the stored time point, this uniform distribution was not observed. Low retention lines at the stored time point displayed little variation, suggesting their similarity to one another. However, high retention lines displayed much greater spread across the PCA, suggesting major differences in their semi-volatile profile at this time point. This indicates that the same trend between high and low carotenoid retention was not observed at the fresh and stored time point, and major changes occurred in the high retention samples during post-harvest storage to explain the greater variation in semi-volatile profile at this time point.

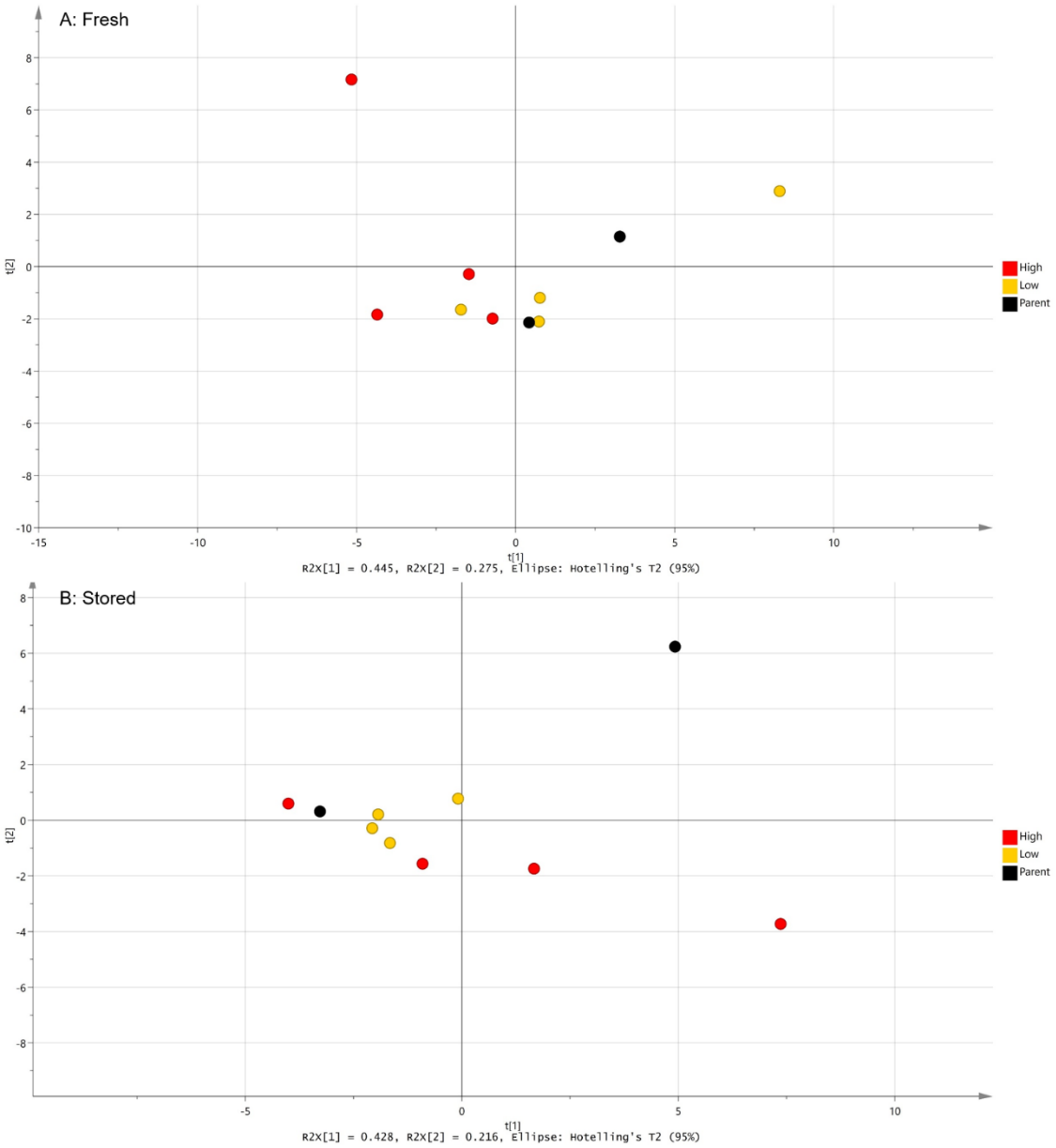


Figure 3-17 PCA plots displaying variation in semi-volatile profile of carotenoid retention sub-population at fresh and stored time points.

Semi-volatile analysis was performed on carotenoid retention sub-population, at fresh and stored time points. (A) PCA scores plot for fresh pepper samples, (B) PCA scores plot for stored pepper samples. High carotenoid retention lines (red), low carotenoid retention lines (yellow), parent lines (black).

3.3. Discussion

Understanding the metabolome of fruit species is important in order to direct breeding for key quality traits. Carotenoids are essential in pepper in conferring the observed red, yellow, and orange colours, and broader metabolism is crucial as the metabolites measured are essential for pepper taste, pungency, and nutritional properties.

Whilst pepper fruit colour is important as a quality trait immediately following harvest from the plant, pepper fruits may be dried and stored for several months, and therefore, pepper colour following post-harvest storage is an equally important quality trait. Several studies have characterised the carotenoid profiles of various pepper varieties, and the general carotenoid composition of red chilli peppers is well established. The major carotenoid conferring the red colour in chilli peppers is capsanthin, whilst other xanthophylls such as violaxanthin, neoxanthin, and antheraxanthin, along with β-carotene are also present in fruits (Minguez-Mosquera and Hornero-Mendez, 1994b, Hornero-Méndez et al., 2000, Berry et al., 2019). Carotenoids are commonly found in an esterified form in pepper fruit, and esterified carotenoids comprise the majority of all carotenoids present (Camara and Monéger, 1978). Fewer studies, however, have assessed the change in carotenoid content during post-harvest storage of pepper fruits. The majority of those studies that have looked at this change, have studied paprika, which is the dried, ground fruit material (Minguez-Mosquera and Hornero-Mendez, 1994a, Minguez-Mosquera et al., 2000, Topuz and Ozdemir, 2004), as opposed to whole intact fruits. Therefore, when comparing these paprika post-harvest storage studies to the present studies, it is difficult to draw direct comparisons, due to the different nature of the material. Those studies which have analysed the change in carotenoid content during post-harvest storage in pepper fruits have previously only analysed a small number of pepper varieties (Minguez-Mosquera and Hornero-Mendez, 1994a). Therefore, although specific conclusions have been drawn regarding changes in carotenoid content of these varieties, it would be impossible to draw general conclusions regarding the mechanisms underlying the carotenoid retention phenotype.

This study has analysed the carotenoid content of freshly harvested pepper fruits and the change in carotenoid content during post-harvest storage of an entire double haploid pepper population, comprised of 375 lines. Therefore, the scale of this study has allowed more general conclusions to be drawn regarding the mechanisms underlying carotenoid retention.

3.3.1. Heterosis is observed in the resulting progeny when parent lines with similar phenotypes are crossed

The parent lines used in this study to produce the DH population were selected in order that the resulting population would display variation in carotenoid retention. Although these lines were designated as high (11-1179) carotenoid retention and low (11-1175) carotenoid retention, analysis carried out during this study suggested that the two lines have very similar carotenoid retention phenotypes. In the three carotenoid profiling screens performed on the DH population, the two parent lines were ranked very close to one another when compared to the rest of the population. It was hypothesised that these two parent lines would be at the extremes of the population, and the resulting variation in the population lines would fall between the two parents, however this clearly didn't occur for the phenotypes studied. Consequently, it can be concluded that the progeny produced when creating a DH population from these two parents display heterosis. Heterosis can be defined as the phenomenon observed when the progeny of a cross display more extreme phenotypes than those seen in the parents (Birchler et al., 2010).

It is suggested that heterosis can be caused by two distinct processes: recessive alleles at different loci complementing one another in the hybrid, or interactions occuring between different alleles in the hybrid, therefore leading to a more extreme phenotype (Charlesworth and Willis, 2009, Birchler et al., 2010).

Breeders may have purposefully selected two parent lines which appear to have similar carotenoid retention phenotypes, as other quality traits must be considered when breeders are producing new progeny. Although two lines may have drastically different phenotypes for the trait being studied, if these lines are poor candidates for other important traits, such as flavour, yield, or disease resistance, breeders may choose to select varieties which are more similar to one another, if they are also good candidates for other traits. Breeders must select lines which have commercial value, including for traits beyond the one which is being directly studied.

3.3.2. Total carotenoid content does not influence carotenoid retention

The carotenoid profile analysis of 375 pepper lines at both the fresh and post-harvest storage time points allowed the comparison of carotenoid intensity, being the total carotenoid content at the fresh time point, against the carotenoid retention phenotype. Lines which were found to have a high total carotenoid content immediately following harvest (Figure 3-3) were not necessarily the lines that were then found to have the highest carotenoid content following post-harvest storage (Figure 3-4), and subsequently, were not deemed to be the highest retention lines (Figure 3-6). This

observation was also made when considering the parent lines of the population. Whilst the parent lines had some of the highest carotenoid content values following harvest, they were not as high in the ranking amongst the population following post-harvest storage, and their ranking based on carotenoid retention was significantly lower than many other lines within the population. Regarding the carotenoid retention subpopulation, in which lines were deemed to have extreme high or low carotenoid retention values, high carotenoid retention lines had lower total carotenoid amounts at the freshly harvested time point as opposed to low retention lines (Figure 3-8). These observations suggest that fresh total carotenoid content and carotenoid retention are two independent phenotypes. A high fresh total carotenoid content does not predict a high carotenoid retention phenotype, and therefore these phenotypes should be considered as separate from one another.

Whilst some studies have been carried out previously to determine changes in carotenoid content in pepper fruit during post-harvest storage (Minguez-Mosquera and Hornero-Mendez, 1994a), such studies have not analysed enough pepper varieties to determine correlations between these two distinct traits.

The fact that carotenoid content does not influence the retention phenotype suggests that the mechanisms controlling the carotenoid retention phenotype are not linked to carotenoid biosynthesis, as it may be expected that these two phenotypes would show the same trend if the underlying mechanism was associated with the synthesis of these compounds. Instead, the retention phenotype may be influenced by an array of diverse factors, including carotenoid storage and sequestration, carotenoid degradation, and other fruit physiological and cellular mechanisms. It has been shown that carotenoid accumulation is influenced by sequestration in chilli pepper fruits, and that carotenoid biosynthesis and accumulation may independently contribute to the total carotenoid amount in fresh fruit (Berry et al., 2019). Carotenoid biosynthesis, accumulation and sequestration, and degradation may not only contribute to carotenoid content in fresh fruit, but further may influence how the carotenoid content changes in fruit during post-harvest storage. Indeed, it has been shown that carotenoids may accumulate during post-harvest storage in other species when carotenoid sequestration is promoted. Expression of the cauliflower *Orange* (*Or*) gene in potato tubers resulted in the retention of β-carotene, and stimulated its accumulation during post-harvest cold storage (Li et al., 2012). The *Or* gene from cauliflower is known to initiate the differentiation of plastids into chromoplasts (Lu et al., 2006), and this therefore provides evidence for a non-biosynthesis linked mechanism promoting retention of carotenoids during post-harvest storage.

3.3.3. Carotenoid content increases during post-harvest storage

Whilst a decrease in carotenoid content in paprika has been observed during post-harvest storage (Topuz and Ozdemir, 2004), the study presented here clearly shows that carotenoid content increases during storage in whole fruit under the discussed drying and storage conditions. An increase in carotenoid content following harvest was observed, despite it being hypothesised that carotenoids would degrade, and therefore decrease in quantity during storage. A similar phenomenon has been reported in other studies examining the change in carotenoid content during post-harvest storage, in which the *Bola* variety of peppers displayed an increase in red carotenoid pigments (Minguez-Mosquera and Hornero-Mendez, 1994b). This was described as a 'transformation' of pigments, as the total carotenoid content did not significantly increase. However, this does indicate that fruits were still metabolically active during the drying process in order for the enzymatic conversion of yellow pigments to red pigments. Ongoing metabolic activity during post-harvest drying and storage was also observed in the *Jaranda* variety of pepper, in which carotenoid content increased during storage (Minguez-Mosquera et al., 2000). In this case, not only did ongoing metabolic activity during the dehydration process result in a transformation of carotenoid pigments, but an increase in total carotenoid content was observed, meaning that carotenoid biosynthesis *de novo* was occurring. This reflects what was observed in the carotenoid profiling of the DH population in this study, as an increase in total carotenoid content was observed in many lines.

Interestingly, the carotenoid content of Navelina oranges was found to increase during post-harvest storage at 12 °C for a period of seven weeks (Carmona et al., 2012). This demonstrates that carotenoid biosynthesis may be stimulated in species other than pepper during periods of post-harvest cold storage.

3.3.4. Carotenoid retention is influenced by changes to the entire carotenoid profile

Despite the fact that carotenoid content increases during post-harvest storage in many lines, it does not appear that this increase in total carotenoid content is driven by an increase in just one carotenoid compound. The retention values for the subpopulation show that whilst lines may be characterised as high retention, some carotenoids decrease as others increase. When comparing lines which have been characterised as the same retention phenotype, the carotenoids showing fluctuations during storage are different dependent on line. In contrast, the colour intensity of pepper fruits, conferred by the presence of carotenoids, was previously shown to be controlled predominantly by the presence of capsanthin diesters (Berry et al., 2019). However, in the case of the carotenoid retention phenotype, this study shows that carotenoid

retention is not predominantly controlled by one carotenoid, but by the profile of all carotenoids within the fruit.

Comparison of retention values for the entire population by hierarchical clustering demonstrated that lines cluster on similarity of carotenoid profile as opposed to total carotenoid retention value. This provides further evidence to suggest that retention is the product of an array of changes in carotenoid profile, as opposed to the content of one single carotenoid changing. Three distinct clusters are observed when data is analysed by hierarchical clustering, and this suggests that retention is driven by at least three mechanisms. These mechanisms may independently control the increase or decrease in different carotenoids, resulting in different carotenoid profiles being observed in lines designated with the same retention phenotype.

3.3.5. Carotenoid retention does not influence broader fruit metabolism

Analysis of broader fruit metabolism demonstrated that carotenoid retention phenotype and broader fruit metabolism are not associated with one another at the fresh time point. These datasets were not significantly similar to one another, suggesting that intermediary fruit metabolism is not linked to carotenoid retention (Figure 3-10). Changes in intermediary fruit metabolism during post-harvest storage were measured for the sub-population. Fruit metabolism did change during post-harvest storage, however, high and low retention lines could not be distinguished based on these changes (Figure 3-11, Figure 3-12). Biochemical changes have previously been reported between fresh and processed fruit (Loizzo et al., 2015), however, changes following post-harvest storage have not been reported. Comparison of metabolites in tomato fruit during pre- and post-harvest showed that hexose sugars, including glucose and fructose, and cell wall components such as galacturonic acid were correlated with on-vine fruit ripening, whilst compounds such as mannose and gluconic acid were correlated with post-harvest storage (Oms-Oliu et al., 2011). In pepper, glucose and fructose were correlated with the freshly harvested time point, however, mannose was also shown to be correlated with this time point. Whilst gluconic acid was shown to be correlated with the post-harvest storage time point in tomato, in pepper, gluconic acid was correlated with the fresh time point. This suggests that metabolic changes occurring during post-harvest storage in pepper and tomato are different from one another.

The effect of post-harvest storage on paprika was measured, and this showed that there was no significant change in unsaturated fatty acids, such as oleic, linoleic, and linolenic acids during storage (Pérez-Gálvez et al., 2009). However, this study suggests that unsaturated fatty acids were responsible for forcing the separation of

fresh fruit samples from their stored counterparts (Figure 3-11). This demonstrates that studies performed on paprika samples following post-harvest storage do not necessarily reflect changes occurring in whole fruit. Increased unsaturated fatty acids were associated with the fresh fruit samples, and this may be due to the fact that unsaturated fatty acids are more susceptible to lipid peroxidation than saturated fatty acids (Halliwell and Chirico, 1993). If polyunsaturated fatty acids are subjected to lipid peroxidation during the process of post-harvest storage, lower levels of these unsaturated fatty acids will be observed in stored fruit, as was the case in this study. Further supporting this hypothesis, was the finding that saturated fatty acids were not responsible for forcing the separation of the fresh and stored sub-population metabolite analyses. Saturated fatty acids, including hexadecanoic acid, and octadecanoic acid, were located towards the centre of the PCA plot (Figure 3-11), implying that saturated fatty acid content did not significantly change during post-harvest storage of pepper fruits. If saturated fatty acids are less susceptible to oxidative damage than unsaturated fatty acids, this may explain why a decrease was observed in unsaturated fatty acids following post-harvest storage, but no change was observed in saturated fatty acids.

Finally, unsaturated fatty acids are also key constituents of cell membranes (Crombie, 1958, Pérez-Gálvez et al., 1999). Therefore, the physiological changes occurring during fruit drying may influence the cell membrane structure, resulting in these membrane-associated unsaturated fatty acids being more susceptible to oxidative damage. This may also have contributed to the decrease in unsaturated fatty acid content observed in pepper fruits following post-harvest storage.

3.3.6. Semi-volatile profile is influenced by carotenoid retention phenotype

Fresh and stored subpopulation pepper samples show differences in their semi-volatile profiles, providing further evidence for metabolic changes occurring during post-harvest storage (Figure 3-13). Increased carotenoid derived volatiles in stored samples are a driving force behind the differences observed. This increase in carotenoid-derived volatiles in all lines, demonstrates that carotenoid degradation is occurring during post-harvest storage (Figure 3-14). Carotenoid content has been shown to increase during storage in high retention lines, and yet, carotenoid-derived volatiles also increase. This confirms that the carotenoid retention trait is the product of both carotenoid biosynthesis and carotenoid degradation mechanisms. In low carotenoid retention lines, carotenoid content was not shown to increase significantly, but increases in carotenoid-derived volatiles are prevalent. This demonstrates that carotenoid

degradation mechanisms may be influencing carotenoid content to a greater extent than carotenoid biosynthesis mechanisms are, in these low retention lines.

Carotenoid-derived volatiles display a greater increase in high retention lines compared to low lines during post-harvest storage. It has been shown that high retention lines show a dramatic increase in carotenoid content during post-harvest storage, and this would therefore explain why the increase in carotenoid-derived volatiles is greater in high retention lines compared to low. High retention lines have greater carotenoid content following storage, and therefore there is more carotenoid available to be degraded to produce carotenoid derived volatiles. It could be hypothesised that the increase in carotenoid-derived volatiles acts as a feedback mechanism to initiate further carotenoid biosynthesis, hence explaining why an increase in carotenoids is observed in these lines.

The formation of these carotenoid-derived volatiles may have occurred as a result of either enzymatic or non-enzymatic degradation of carotenoids. The carotenoid cleavage dioxygenase enzyme family plays an important role in the degradation of carotenoids, forming carotenoid-derived volatiles, or apocarotenoids. CCD1 and CCD4 are known to have a wide range of substrates, and therefore may be involved in the cleavage of both acyclic carotenes, along with β-carotene (Schwartz et al., 2001, Simkin et al., 2004, Ibdah et al., 2006, Huang et al., 2009). The activity of these enzymes may have contributed to the resulting carotenoid-derived volatiles observed in high and low carotenoid retention lines. The apocarotenoids measured in this study may also have been produced as a result of non-enzymatic cleavage of carotenoids. Light stress has previously been shown to induce the oxidation of β-carotene, forming β-cyclocitral (Ramel et al., 2012). Therefore, the carotenoid-derived volatiles measured in this study are likely to have been formed as a result of an array of both enzymatic and non-enzymatic carotenoid cleavage processes. Whilst it is not possible to determine the contribution that each of these mechanisms makes to the resulting carotenoid-derived volatile profile, it is clear that significant changes occur in the fruit during post-harvest storage, resulting in the degradation of carotenoids and the formation of a variety of carotenoid-derived volatiles.

During the process of post-harvest storage, lipid-derived volatiles were produced as a result of fatty acid degradation. An increase in lipid-derived volatiles in high carotenoid retention lines suggests that lipid degradation is occurring throughout the seven month period of post-harvest storage. Carotenoids in high retention lines protect lipids from degradation throughout storage to a greater extent than in low retention lines, meaning that following seven months of storage, lipids may still be susceptible to degradation in

high retention lines. However, a decrease in lipid-derived volatiles in low carotenoid retention lines implies that lipid degradation was no longer occurring in these fruits. A decreased protective capacity of carotenoids in low retention lines may have resulted in extensive lipid degradation during post-harvest storage. As unsaturated fatty acids forced the separation of fresh samples from stored samples using PCA, this implies that fatty acids were degraded during the post-harvest storage period. A storage period of seven months is a considerable amount of time for storage of fruits, and therefore it is hypothesised that lipid degradation occurred early in the post-harvest storage period, resulting in lipid-derived volatiles. At the point of analysis, after seven months of post-harvest storage, these lipid-derived volatiles were no longer detected. The low carotenoid retention lines have a low capacity for carotenoids to scavenge ROS, which can ultimately result in lipid peroxidation (Sharma et al., 2012), and therefore, lipid-derived volatiles are produced very early in the post-harvest storage period. A detailed study of changes throughout the post-harvest storage period may provide further evidence to support this hypothesis.

A similar trend has previously been reported, in which low carotenoid retention pepper fruits display a greater decrease in lipid-derived volatiles compared to high retention fruits, when fruits are dried (Berry, 2015). Whilst this change was observed in dried fruit, as opposed to fruits dried and stored post-harvest, this does imply that lipid-derived volatiles decrease more rapidly in low carotenoid retention lines, as opposed to their high carotenoid retention counterparts. Further to this, it was previously hypothesised that the rapid decrease in lipid-derived volatiles in low retention lines may be due to the degradation of chloroplast and chromoplast membranes (Berry, 2015). Linolenic acid is a major constituent of the chloroplast membrane (Crombie, 1958), and therefore the decrease in linolenic-derived volatiles suggests a rapid decrease in chloroplast and chromoplast membranes. This would result in increased carotenoid degradation, as the integrity of the chloroplast and chromoplast structures is compromised. In the study presented here, 2-hexenal, a linolenic acid-derived volatile, decreased significantly from 467 ng/mL to 154 ng/mL in the low carotenoid retention parent line (11-1175), whereas a slight increase was observed in 2-hexenal content in the high retention parent (11-1179). This may suggest a rapid degradation of the precursor to 2-hexenal: linolenic acid, due to degradation of chromoplast membranes. Therefore, a decrease in lipid-derived volatiles implies chromoplast membranes are degraded, resulting in carotenoid degradation in low carotenoid retention lines.

Whilst little variation is seen between high and low retention lines in fresh total detected volatile amounts, much more variation is observed in stored samples. Lipid-derived volatiles and carotenoid-derived volatiles are increased in high retention lines following

post-harvest storage. This adds further evidence to suggest that high retention lines are more metabolically active than low lines during post-harvest storage. Carotenoid content, and carotenoid-derived volatiles, along with lipid derived volatiles all increase during post-harvest storage in high carotenoid retention lines. In low retention lines, carotenoid-derived volatiles increase, whilst carotenoid content remains constant, and lipid-derived volatiles decrease. This suggests that high retention lines have higher levels of metabolic activity during storage, whereas low retention lines are less metabolically active, with less carotenoids synthesised, and a decrease in lipid-derived volatiles.

3.4. Conclusions

The pepper fruit carotenoid retention trait is evidently a complex trait, influenced by an array of metabolic changes in the fruit during post-harvest storage. The carotenoid retention trait has been defined as the change in carotenoid content from fresh fruit to dried and stored fruit. Absolute carotenoid content in fresh fruit stage is independent of the carotenoid retention trait, as an increase in fresh fruit carotenoid content does not correlate with an increased carotenoid retention phenotype. Carotenoid retention is evidently the product of the dynamic changes occurring due to carotenoid biosynthesis and degradation, as carotenoid content has been shown to increase in pepper lines defined as high retention, although this is concurrent with an increase in carotenoid-derived volatiles.

Intermediary metabolism and the carotenoid retention trait are not linked as the retention trait does not correlate with specific changes to the metabolic profile, and this may be beneficial to breeders, as selecting for the high carotenoid retention trait should not influence other important metabolic quality traits. However, it is important to note that volatiles derived from intermediary metabolism, for example lipid-derived volatiles, are linked to the carotenoid retention trait. Lipid-derived volatiles are produced in response to cellular changes, and carotenoids are responsible for mitigating the harmful effects of some of these cellular changes. Consequently, volatiles derived from intermediary metabolism are linked to carotenoid retention, as changes in carotenoid profile may occur in response to harmful cellular processes, resulting in volatile production.

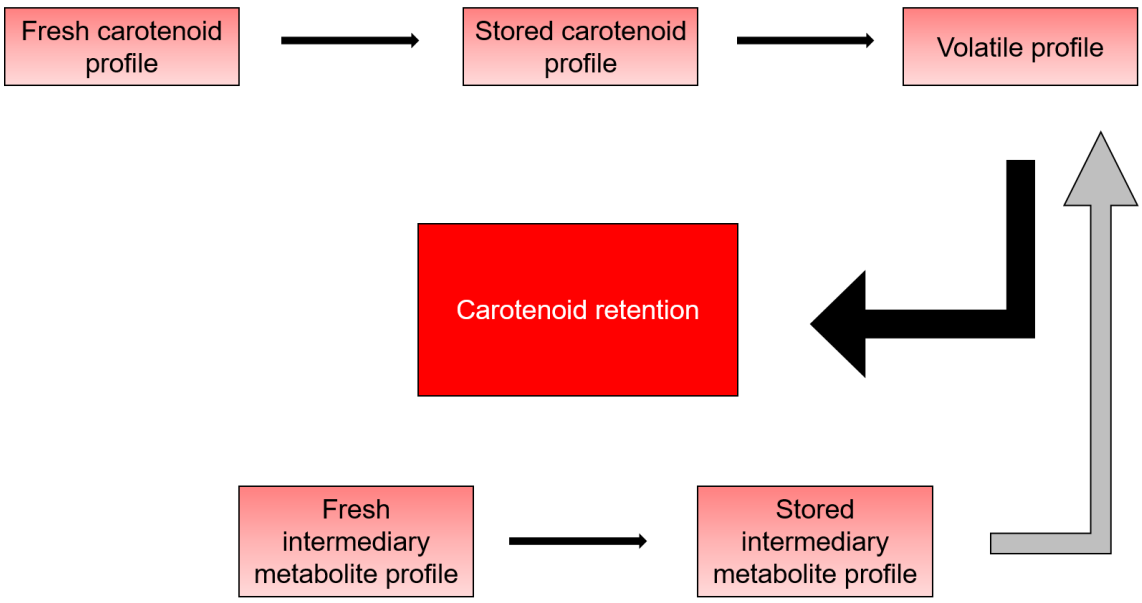


Figure 3-18 Schematic representation of metabolic changes occurring in pepper fruit, influencing the carotenoid retention phenotype.

4. Pepper fruit carotenoid content increases during post-harvest storage

4.1. Introduction

Pepper carotenoid content was unexpectedly shown to increase during post-harvest storage in Chapter 3. Post-harvest storage is essential for many crop species, as consumer demand must be met throughout the year. Crops must therefore be stored in order to meet this year-round demand. However, crop quality deterioration is a major problem faced by growers once the crop is stored, as these crops undergo changes during storage. A major change observed in some plant species is a degradation of carotenoid, resulting in the fading colour of crops. This may be due to either enzymatic degradation by carotenoid cleavage enzymes (CCDs) or non-enzymatic degradation, following processes such as lipid peroxidation, in which carotenoids are degraded in order to quench the activity of harmful free radical species. Post-harvest carotenoid losses have been observed in maize kernels, wheat, and cassava (Ortiz et al., 2016, Hidalgo and Brandolini, 2008, Nascimento et al., 2009). Furthermore, whilst increased provitamin A capacity has been engineered in rice, resulting in 'Golden rice', maintaining these increased nutritional properties of the carotenoid enriched variety has proved challenging, as carotenoid degradation occurs upon post-harvest storage (Gayen et al., 2015).

Carotenoids are widely regarded as the first line of defence in plants against singlet oxygen toxicity (Ramel et al., 2012), due to their ability to scavenge and quench these harmful free radical species. Hydrogen peroxide has been previously used in studies to understand oxidant-induced changes, in which hydrogen peroxide is added either directly or in-directly to plants. Hydrogen peroxide may subsequently be reduced to hydroxyl radical by superoxide, which are highly reactive, and can rapidly lead to oxidative damage within cells (Apel and Hirt, 2004). Carotenoids may then scavenge these harmful reactive oxygen species (ROS) (Edge et al., 1997), in order to limit the extent of oxidative damage within the cell. As pepper fruits may be stored post-harvest, it would be expected that they are susceptible to oxidative damage, as they are exposed to both atmospheric and intracellular reactive oxygen species for long periods of time. For this reason, it would be expected that carotenoid content would decrease in pepper fruits during post-harvest storage, as carotenoids act as antioxidants to scavenge reactive oxygen species.

Therefore, the observed increase in carotenoid content of pepper fruits within the DH population during post-harvest storage, discussed in Chapter 3, was not anticipated. A decrease in carotenoid content was expected, as it was thought that carotenoids would be degraded by either enzymatic or non-enzymatic mechanisms. Therefore, the aim of

this chapter is to uncover the mechanisms underlying the unanticipated increase in carotenoid content in pepper fruits during post-harvest storage.

As was shown in Chapter 3, it is hypothesised that high carotenoid retention varieties will show an increase in carotenoid content during post-harvest storage, whereas low retention varieties will either show a stable level or a decrease in carotenoid content.

4.2. Results

A diversity panel, supplied by Syngenta, containing twelve pepper genotypes was grown, oven dried, and stored, in a manner to replicate commercial conditions. A thirteenth line, CM334, was also analysed in this way. Pepper fruits were harvested from plants, dried in an oven (30-40 °C) for two weeks, and then stored in cold conditions (4 °C) for up to twelve weeks. Fruits were removed for analysis at four week intervals. The changes in fruit appearances was starkly different between the fruit genotypes. Whilst some pepper fruits, such as genotypes R5 and R6, appeared darker in colour after twelve weeks of storage, others, such as R3, appeared lighter (Figure 4-1). All thirteen pepper lines showed a drastic decrease in weight, presumably due to fruit water loss during the oven drying process. A stark contrast was also observed between genotypes in the pepper fruit texture. Whilst some pepper fruits, such as R1, R6, and R8, retained the smooth, waxy surface texture once oven dried that all the genotypes had at the fresh time point, other lines, such as R3, R4, and R7, displayed significant surface cracking, in which the fruit surface wrinkled upon fruit drying. Therefore, all lines were characterised as having either a smooth cuticle surface, or a cracked cuticle surface (Figure 4-1).

4.2.1. Diverse pepper genotypes show differences during post-harvest storage

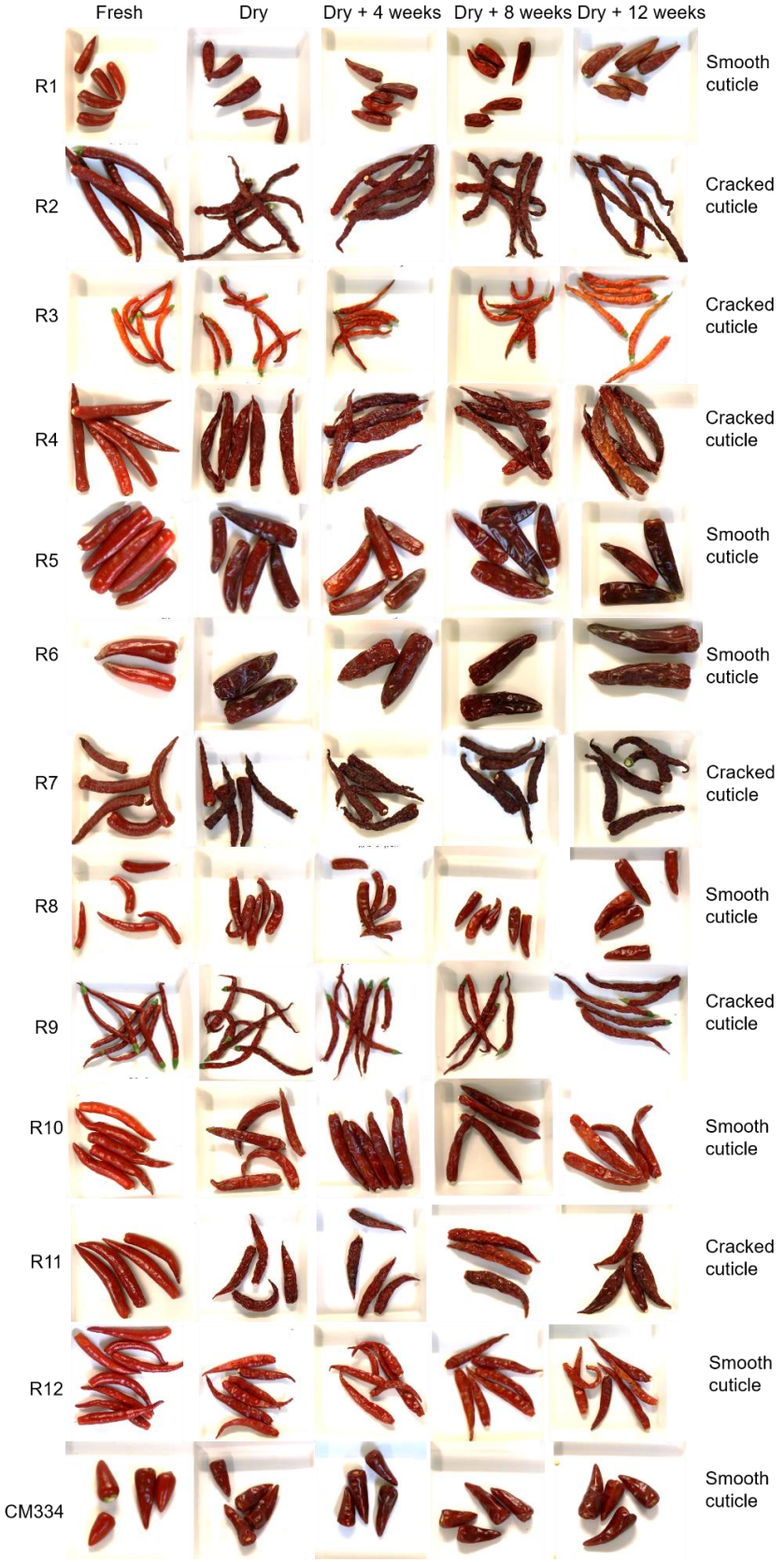


Figure 4-1. Diverse pepper fruit genotypes display physiological differences in post-harvest storage.

Pepper fruits were harvested from plants grown in a glasshouse, dried for two weeks in an oven (30-40 °C), and stored for up to 12 weeks in refrigerated (4 °C), dark conditions. Fruits were removed from

storage for analysis at four week intervals. A minimum of three fruits per plant were pooled for analysis at each time point, and three biological replicates per genotype were analysed (n = 3).

4.2.2. Classification of retention phenotypes by Syngenta breeders

As the twelve line diversity panel used in this study represents an industrially relevant resource for studying carotenoid-associated phenotypes for Syngenta, the carotenoid retention phenotypes of the panel were previously classified by breeders visually determining retention phenotype following post-harvest storage (Table 4-1).

Table 4-1 Retention phenotype classification as determined by Syngenta breeders, by eye.

Line	Syngenta retention phenotype
R1	High
R2	Low
R3	Low
R4	Low
R5	Medium
R6	High
R7	Medium
R8	High
R9	Low
R10	High
R11	High
R12	Low
CM334	N/A

However, this method of analysis did not quantify total carotenoid content during post-harvest storage, and therefore the carotenoid profile of all 13 lines was analysed by HPLC to quantify total carotenoid content, and to quantify how carotenoid content changes during post-harvest storage.

4.2.3. Diverse pepper genotypes show differences in carotenoid content during post-harvest storage

Whilst significant differences were observed between the appearance and texture of the 13 pepper varieties studied in the post-harvest storage experiment described, differences were also observed in the change in total carotenoid content during post-harvest storage. Evidently, the 13 lines studied showed varying levels of carotenoids. Line R3 contained only 2.5 mg/g dry weight of carotenoids at the fresh fruit stage, whereas line R7 contained more than 18 mg/g dry weight of carotenoids at the same

fruit stage (Figure 4-2). Clearly, there was a vast difference in total carotenoid content in this diversity panel. Furthermore, the change in carotenoid content during the twelve week post-harvest storage period differed between the 13 pepper varieties analysed. Some lines displayed very little change in total carotenoid content during post-harvest storage, such as lines R1 and R12, whereas other lines showed significant increases (R5) or decreases (R7) in total carotenoid content.

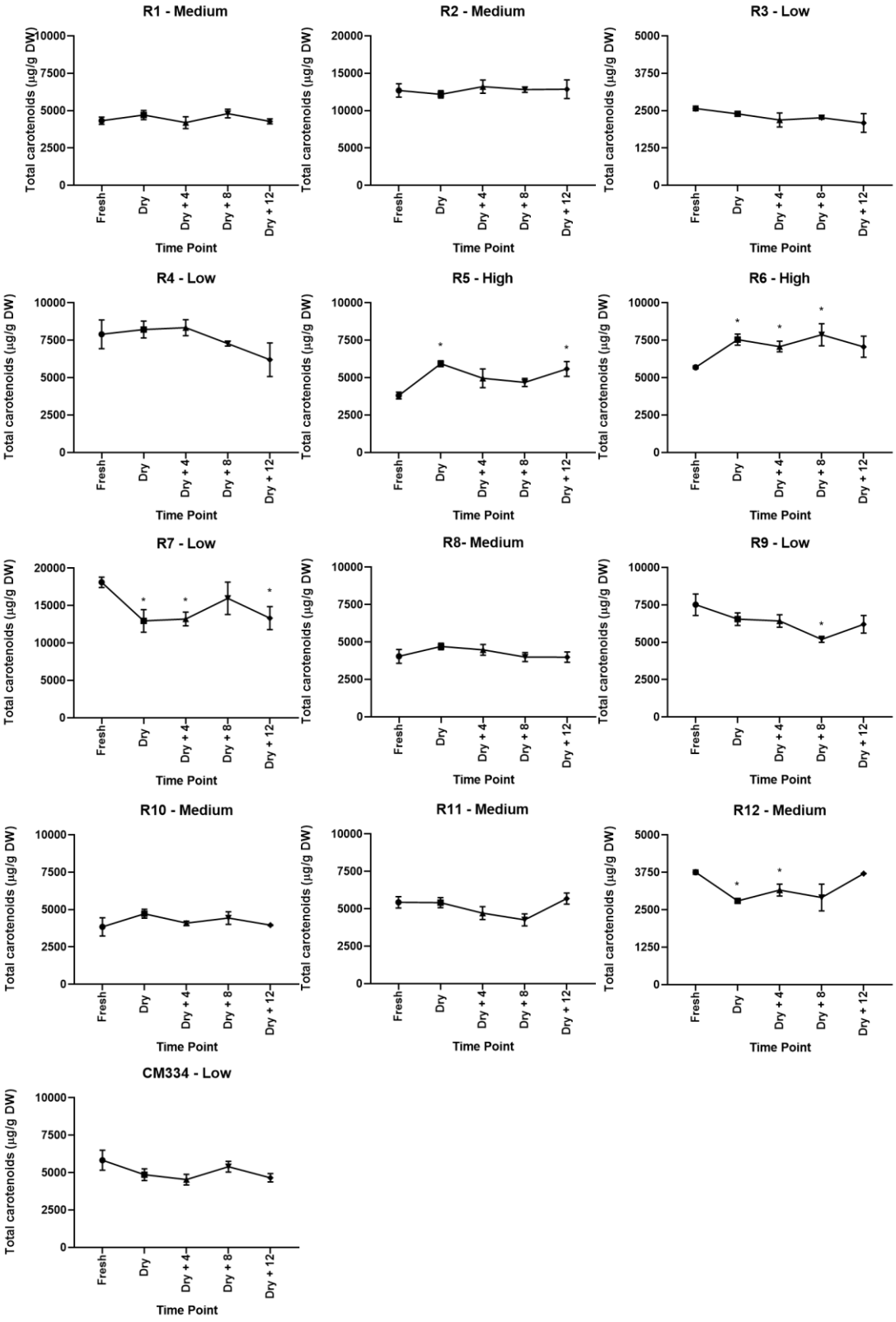


Figure 4-2 Variation in total carotenoid content during post-harvest storage of diverse pepper genotypes.

Pepper fruits of 13 different varieties were harvested at the ripe fruit stage from plants grown in a glasshouse, dried in an oven for two weeks (30-40 °C), and stored in refrigerated, dark conditions (4 °C) for up to 12 weeks. Fruits were removed from storage conditions at four week intervals for analysis by HPLC. At least three fruit per plant were analysed at each time point, and three biological replicates were analysed (n = 3). Error bars ± SE. Fruits were freeze-dried, homogenised, and carotenoids were extracted. Student's T-test was used to determine significant differences between the fresh time point and time points during storage ($p < 0.05$).

4.2.4. Diverse pepper genotypes show differences in carotenoid retention during post-harvest storage

Carotenoid retention was calculated as the change in carotenoid content between the fresh fruit time point, and following 12 weeks of post-harvest storage. Whilst several lines showed increases in carotenoid content during storage, such as R5 and R6, other lines showed decreases in carotenoid content, such as R3 and R7. Other lines showed little change in total carotenoid content during post-harvest storage, for example R1 and R2 (Figure 4-3).

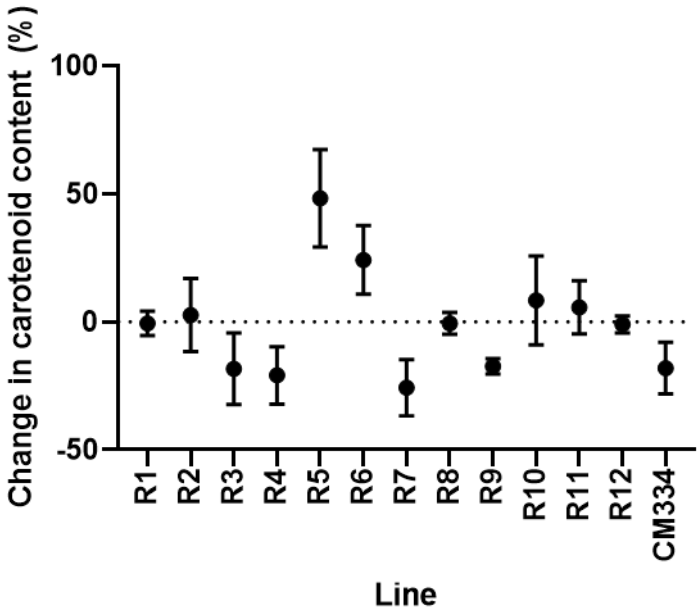


Figure 4-3 Change in carotenoid content during post-harvest storage of pepper diversity panel.

Carotenoid profiles of pepper fruits stored following harvest were analysed by HPLC. Change in carotenoid content between fruits harvested at the fresh time point, and following 12 weeks post-harvest storage was calculated and expressed as a percentage. At least three fruits were pooled per plant, and three biological replicates were analysed (n = 3). Error bars ± SE.

Pepper varieties were characterised as low, medium, or high carotenoid retention dependent on the change in carotenoid content during 12 weeks post-harvest storage. Lines which decreased in carotenoid content by more than 10% were deemed to be low retention. Lines which increased in carotenoid content by more than 10% were characterised as high retention. Lines which showed a change in carotenoid content between -10% and 10% were deemed to be medium carotenoid retention (Table 4-2). Interestingly, characterisation of carotenoid retention phenotypes of pepper lines using the visual method by Syngenta breeders (Table 4-1) was not always consistent with the carotenoid quantification method (Table 4-2). For example, whilst R1 was characterised as high carotenoid retention using the visual method, this line was characterised as medium carotenoid retention when using the carotenoid quantification

method. This may be due to several reasons. Characterisation of the carotenoid retention phenotype by eye is a very subjective method of determining phenotype, as environmental differences may influence characterisation, and other fruit traits, such as level of shine on the fruit surface, may also influence the carotenoid retention phenotype. As these methods are inherently different in their method of measuring colour, it is not surprising that discrepancies appeared. Further to this, pepper fruits were grown in different locations, at different times, and stored in different locations between the visual phenotype allocation and carotenoid quantification experiments. These factors may all have contributed to the fact that carotenoid retention characterisation differed between the two methods. However, as this study is focused on determining the mechanisms underlying carotenoid retention in pepper fruits, the retention classification determined using carotenoid quantification will primarily be used. In the case of lines of particular commercial interest, such as R8, retention phenotype classification will be based on both the visual classification and carotenoid content classification. Consequently, this line will be referred to as medium/high retention.

Interestingly, visual inspection of the fruits indicated that lines identified as either high or medium carotenoid retention tended to have smooth cuticles upon fruit drying and storage, when compared to low retention lines, which tended to possess a cracked cuticle (Figure 4-1; Table 4-2).

Table 4-2 Carotenoid retention phenotypes of pepper diversity panel.

Carotenoid profiles of pepper fruits stored following harvest were analysed by HPLC. Change in carotenoid content between fruits harvested at fresh, and following 12 weeks storage, was calculated and expressed as a percentage. At least three fruits were pooled per plant, and three biological replicates were analysed (n = 3). Carotenoid retention phenotype was determined to be low, medium, or high, dependent on the change in carotenoid content.

Line	Change in carotenoid content (%)	Carotenoid retention
R1	-0.55	Medium
R2	2.69	Medium
R3	-18.28	Low
R4	-20.86	Low
R5	48.34	High
R6	24.21	High
R7	-25.68	Low
R8	-0.55	Medium
R9	-17.34	Low
R10	8.46	Medium
R11	5.65	Medium
R12	-0.98	Medium
CM334	-18.02	Low

Principal component analysis (PCA) displayed the variation in the pepper diversity panel based on change in carotenoid content during the post-harvest storage (Figure 4-4). Lines identified as high carotenoid retention, based on change in carotenoid content, clustered closely with one another at the centre of the PCA, suggesting that there was little variation in carotenoid profile of these lines. Lines R5 and R6 showed little separation relative to other lines in the PCA plot, even when comparing fresh samples to those stored for 12 weeks. In contrast, low carotenoid retention lines displayed a greater spread in variation, when comparing lines and time points. Line R7, which was identified as the lowest retention line (Table 4-2), displayed a large spread in variation in the PCA plot when analysing samples stored for differing lengths of time, suggesting that significant changes were observed in carotenoid profile during post-harvest storage. Some low retention lines, such as R3, were clustered separately from other low retention lines. This again suggests that greater variation was observed in change in carotenoid content in low retention lines when compared to high retention lines. Whilst medium carotenoid retention lines showed greater variation in change in carotenoid content when compared to high retention lines, they showed less spread

across the PCA plot compared to low retention lines. This therefore suggests that less change was observed in carotenoid content in medium retention lines compared to low retention lines. This trend is unsurprising, as low retention lines, in which the greatest loss in carotenoid content was observed during post-harvest storage, showed the greatest variation in carotenoid content.

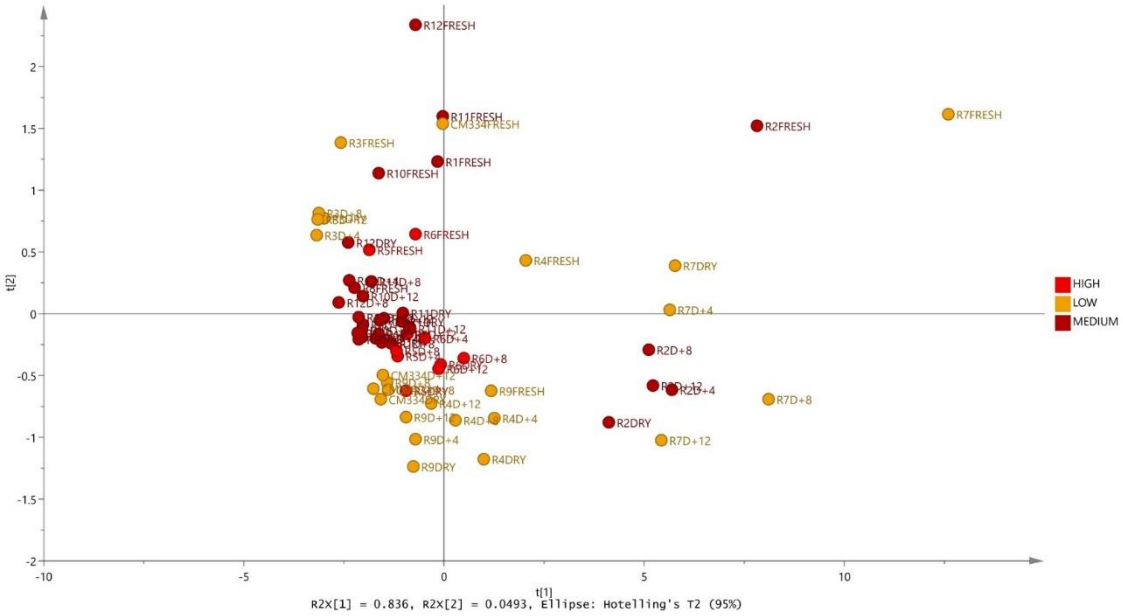


Figure 4-4 PCA plot displaying variation in carotenoid content during post-harvest storage of pepper diversity panel.

Principal component analysis to display separation in diverse pepper varieties, based on changes in carotenoid content during post-harvest storage. Carotenoid profile of 13 diverse pepper varieties was measured, over five time points: fresh fruit, oven dried fruit, dry + 4 weeks, dry + 8 weeks, dry + 12 weeks. This included lines identified as high, medium, and low carotenoid retention, based on the change in carotenoid content during post-harvest storage: High retention (red), medium retention (burgundy), low retention (orange). Twelve compounds were analysed: violaxanthin, neoxanthin, antheraxanthin, capsanthin, zeaxanthin, β-cryptoxanthin, β-carotene, antheraxanthin monoesters, capsanthin monoesters, capsanthin diesters, zeaxanthin diesters, capsorubin diesters, along with the total carotenoid content. At least three fruits per plant were pooled, and three biological replicates per pepper variety were analysed (n = 3).

4.2.5. High retention peppers stored as homogenised powder show greater decreases in carotenoid content compared to in whole fruit

The carotenoid profile of pepper fruits oven dried and stored post-harvest in the form of a homogenous powder were analysed to determine whether there is a difference in carotenoid retention, dependent on the state in which the pepper is stored. As high and medium carotenoid retention pepper varieties tended to have a smooth cuticle, it was hypothesised that an absence of cracking, and therefore a more protective and less permeable cuticle, may protect carotenoids from degradation to a greater extent in high retention lines. Pepper fruits were harvested at ripe, and then dried in an oven (30-40 °C) for two weeks. Pepper fruits were then de-seeded, and homogenised to a powder. Pepper powder was stored in cold conditions (4 °C) in covered petri dishes for

up to eight weeks. Samples were removed and analysed at four week intervals during this storage process. A period of eight weeks post-harvest storage was measured in this experiment, which was shorter than the period of post-harvest storage used in industry, due to time constraints. All samples were lyophilised to ensure uniformity, and carotenoids were extracted and analysed by HPLC.

A decrease in carotenoid content was observed during post-harvest storage of powdered pepper fruits in the low retention line, R4, however, little change was observed in carotenoid content of low retention line, R3. An increase in carotenoid content was observed in high retention line, R6, whilst little change was observed in the medium/high retention line, R8 (Figure 4-5).

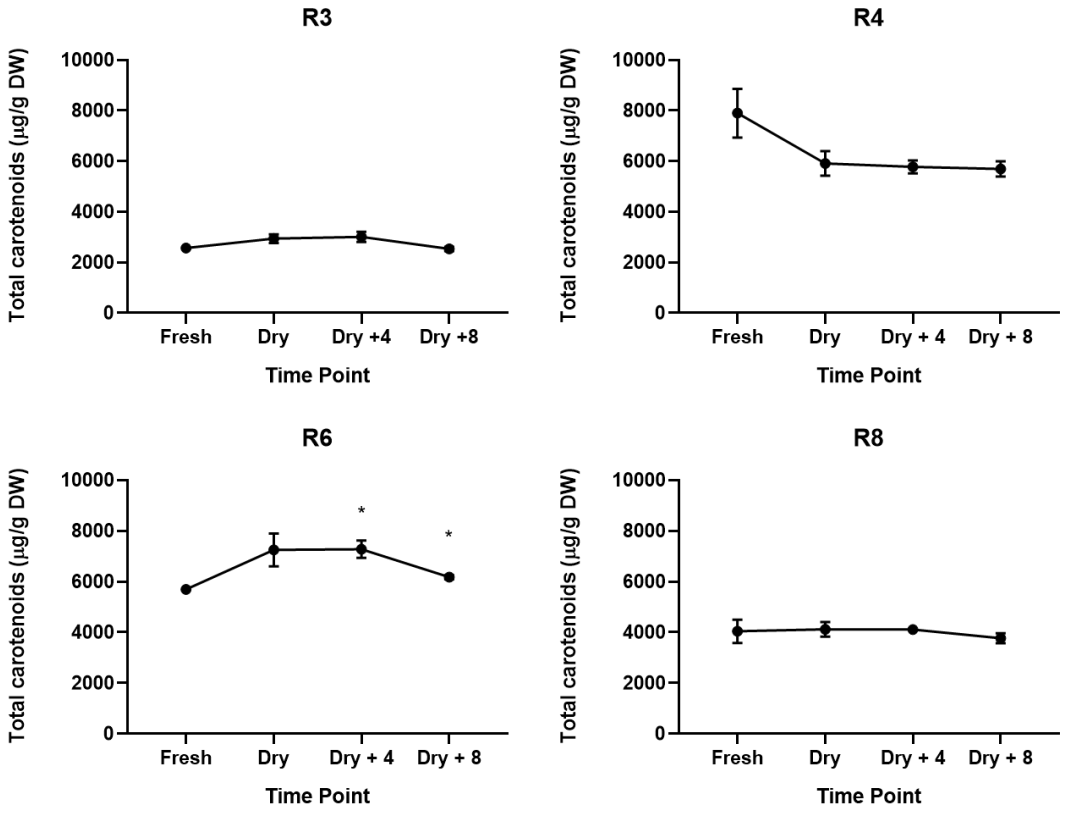


Figure 4-5 Variation in carotenoid content during post-harvest storage of powdered pepper fruits.

Pepper fruits of four varieties were harvested at the fresh time point. Fruits were dried in an oven (30-40 °C) for two weeks. Seeds were removed from all fruits and fruits were then homogenised using a blender, to a powder state. Four pepper varieties were analysed: R3 (low retention), R4 (low retention), R6 (high retention), R8 (high/medium retention), and fruits were analysed at the following time points: fresh, oven dried, dry + 4 weeks, dry + 8 weeks. At least three fruits per plant were pooled, and three biological replicates were analysed per variety (n = 3). Student's T test was used to determine significant differences between fresh fruit and stored fruit. Error bars = ± SE.

The low retention line, R4, when stored as a powder, showed similar levels of carotenoid degradation over an eight week period as to when stored as whole fruits over 12 weeks. Over an eight week storage period stored as powder, 25% of total carotenoids were lost (Table 4-3), compared to a 20% decrease over a 12 week period

when stored as whole fruit (Table 4-2). This suggests that carotenoid degradation was increased in this line when stored as a powder, as greater losses were observed over a shorter time period. In contrast, low retention line, R3, showed less of a decrease in carotenoid content when stored as a powder for eight weeks, when compared to whole fruits stored for a 12 week period. A 1.5% decrease in carotenoid content was observed in powdered fruits stored for eight weeks (Table 4-3), whilst an 18% decrease in carotenoid content was observed when fruits were stored whole for 12 weeks (Table 4-2). This difference may have been caused by several factors. Whilst whole fruits were stored for 12 weeks, powdered fruits were only stored for eight weeks due to time constraints. It would be interesting to observe whether the decrease in carotenoid content occurred at a steady rate, or if a sudden decrease would be observed in powdered fruit, if stored for a longer period. In addition, whilst the conditions for storage were kept as similar as possible in the two experiments, powdered fruits were stored in petri dishes, whilst whole fruits were stored in hessian bags. Therefore, it is possible that air flow to powdered samples was restricted, therefore resulting in less oxidative degradation of carotenoids in this form.

Both lines R6 (high retention) and R8 (high/medium retention) had lower levels of carotenoids when stored as a powder for eight weeks, compared to when stored as whole fruits for 12 weeks. Carotenoid content in R6 increased by 24% in whole fruits stored for 12 weeks (Table 4-2), but only increased by 8.5% when powdered fruits were stored for eight weeks (Table 4-3). Whilst carotenoid increases occurred during the oven drying process of these fruits (Figure 4-5), as has been observed previously, storage of this line in powdered form led to a decrease in carotenoid content compared to when stored as whole fruit. Whilst carotenoid content still increased, the increase observed in powdered fruit was less than that observed in whole fruit.

Further to this, the medium/high retention line, R8, showed a slight decrease in carotenoid content when stored as a powder for eight weeks, compared to when stored as whole fruit for 12 weeks. Carotenoid content remained almost constant during storage as whole fruits (Table 4-2), whereas storage as powder resulted in a 3% decrease in total carotenoids (Table 4-3). This again suggests that carotenoid losses were greater when fruits were stored as powder.

Table 4-3 Carotenoid retention values of powdered pepper fruits.

Carotenoid profiles of pepper fruits, stored as powder, were analysed by HPLC. Change in carotenoid content, between the fresh time point and following eight weeks of post-harvest storage, was calculated and expressed as a percentage. At least three fruits per plant were pooled, and three biological replicates per genotype were analysed (n = 3).

Line	Carotenoid retention phenotype	Change in carotenoid content (%)
R3	Low	-1.54
R4	Low	-25.41
R6	High	8.45
R8	Medium	-3.25

Interestingly, lines identified as high or medium/high retention showed greater decreases in carotenoid content when stored as powder as opposed to whole fruit, compared to their low carotenoid retention counterparts. Both R6 and R8 have smooth cuticles upon drying, with minimal cuticle cracks observed. This would suggest that the smooth cuticle is a more protective barrier preventing oxidative degradation of carotenoids in these genotypes. Upon disruption of the smooth cuticle, through fruit homogenisation, greater decreases were observed in carotenoid content (Figure 4-6). In contrast, the low carotenoid retention lines tended to have cracked cuticles, in which the barrier to oxidative degradation of carotenoids is compromised. Therefore, little difference was observed in carotenoid content of these low retention lines when stored as either a powder or whole fruit, as the cuticle was compromised in both environments. Line R3 showed less degradation of carotenoids when stored as a powder compared to when stored as whole fruit, and this may have been due to the different environments, in which there may have been reduced air flow, and therefore oxidative species, to cause oxidative degradation of carotenoids in powdered samples.

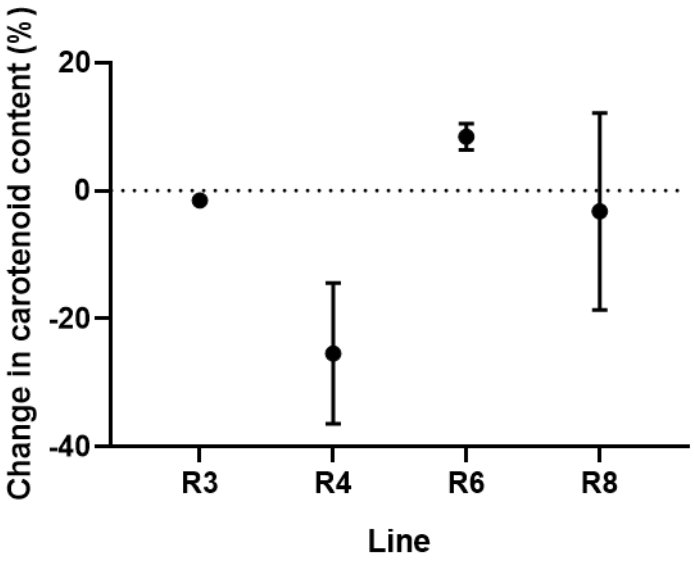


Figure 4-6 Change in carotenoid content during post-harvest storage of pepper varieties stored in powder form.

Carotenoid profiles of pepper fruits, stored as powder, were analysed by HPLC. Change in carotenoid content, between the fresh time point and following eight weeks of post-harvest storage, was calculated and expressed as a percentage. At least three fruits per plant were pooled, and three biological replicates per genotype were analysed (n = 3). Error bars ± SE.

4.2.6. Initiation of carotenoid degradation by hydrogen peroxide reveals the crucial carotenoid-protecting role of the cuticle

As greater decreases in carotenoid content were observed in high carotenoid retention lines when stored as powder compared to when stored as whole fruit (Figure 4-3, Figure 4-6), along with the fact that these lines retain a smooth cuticle when dried (Figure 4-1), it was hypothesised that the fruit cuticle protects against carotenoid degradation. This may be due to the fact the cracks do not exist on smooth cuticle fruits, and therefore the cuticle provides a protective barrier against oxidative species, resulting in less oxidative degradation of carotenoids. In contrast, low retention lines tended to have cuticles which cracked upon fruit drying (Figure 4-1). These cracks may allow the entrance of oxygen, creating an environment for oxidation in the fruit. This resulted in increased oxidative degradation of carotenoids. As little difference was observed in change in carotenoid content in either powdered or whole fruit (Figure 4-3, Figure 4-6), it suggests that the fruit cuticle was compromised to oxidative species even in whole fruits.

In order to determine the role of the fruit cuticle in protecting against carotenoid degradation, pepper fruits were treated with hydrogen peroxide, as an oxidative agent. R3 (low retention) and R8 (medium/high retention) fruits were analysed. Fruits were harvested at ripe. Control fruits were treated with varying concentrations of hydrogen peroxide (0 mM, 0.2 mM, 2 mM) immediately following harvest, and were then oven dried, and stored for four weeks. Other fruits were allowed to oven dry, and then were

treated with hydrogen peroxide after drying, and stored for four weeks. Carotenoids were extracted and analysed by HPLC.

Following post-harvest storage, a decrease in total carotenoid content was observed in R3 (low retention) peppers treated with 2 mM H_2O_2 before drying (Figure 4-7), at which point, the cuticle remained smooth and not exhibiting any cracks. No decrease was observed in peppers treated with 0.2 mM H_2O_2 before storage. In contrast, R3 peppers treated with both 0.2 mM and 2 mM H_2O_2 after fruit drying showed decreases in total carotenoid content (Figure 4-7). Fruits treated with H_2O_2 following drying were more susceptible to carotenoid degradation, when a lower concentration (0.2 mM) H_2O_2 was used. As the fruit cuticle showed cracking after drying, this structural alteration may have allowed the entrance of oxidative species into the fruit and promoted carotenoid degradation.

Line R8, which had a smooth cuticle in both fresh and dried fruit, showed no difference in total carotenoid content, regardless of concentration of H_2O_2 used (Figure 4-7). This was observed in fruits treated both before and after fruit drying. As the fruit cuticle remained smooth and intact following drying, a protective barrier was in place to bar the entry of oxidative species into the fruit. Consequently, no difference in carotenoid content was observed, even when relatively high concentrations of H_2O_2 (2 mM) were used.

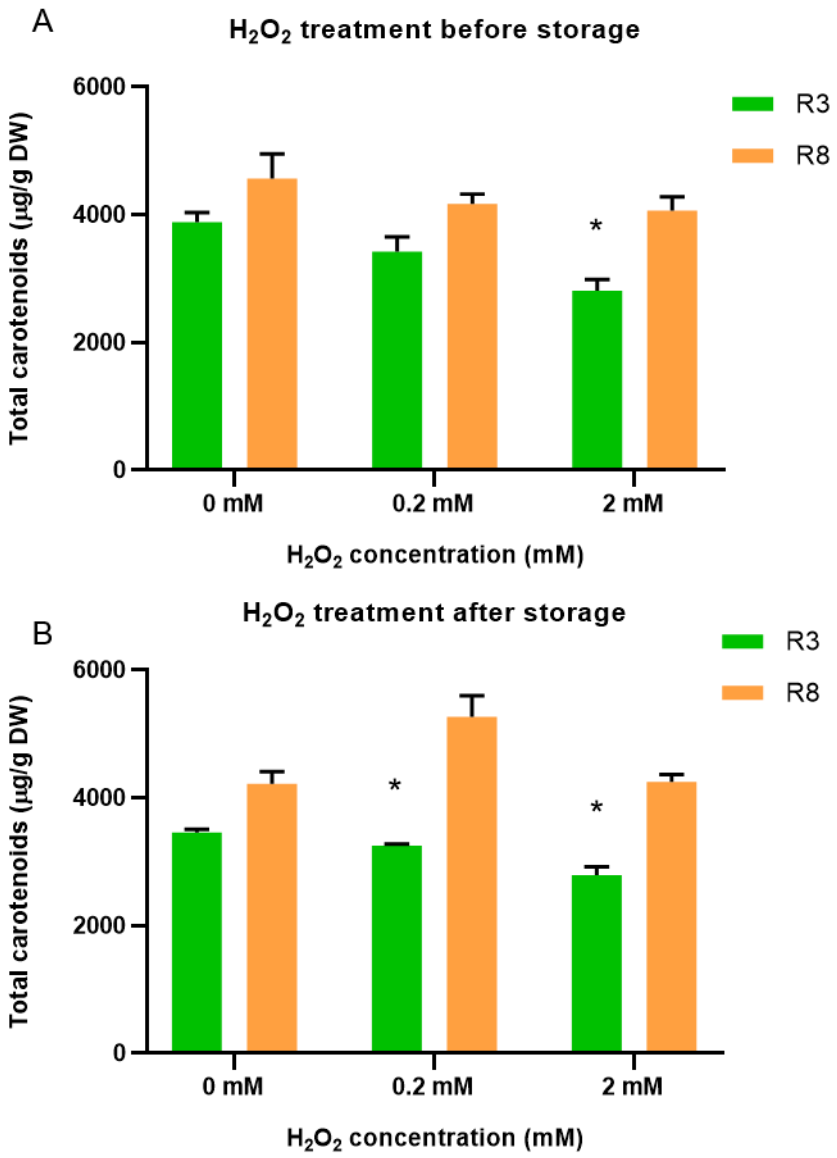


Figure 4-7 Carotenoid content of pepper fruits after post-harvest storage, following treatment with hydrogen peroxide.

Pepper fruits were harvested at ripe. Control fruits were treated with H_2O_2 whilst fresh, and were then oven dried and stored in cold conditions (4 °C). Other fruits were oven dried and then treated with H_2O_2, before storage in cold conditions (4 °C). Three concentrations of H_2O_2 were used: 0 mM, 0.2 mM, 2 mM. Two pepper varieties were analysed: R3 (low retention; green), R8 (medium/high retention; orange). Student's T test was used to determine significant differences between the control condition (0 mM H_2O_2) and test concentrations ($p < 0.05$). At least 5 fruits were pooled per genotype, and three technical replicates were analysed ($n = 3$). Error bars = ± SE.

4.3. Discussion

An understanding of the changes in pepper colour during post-harvest storage is essential, as colour is one of the main consumer quality traits for pepper in determining fruit value. Carotenoids are the major pigment responsible for the observed red colour in pepper. Identification of lines which retain high levels of carotenoids throughout post-harvest storage is crucial in order to elucidate the mechanisms underlying this trait.

4.3.1. Analysing colour change by visual methods does not give an accurate representation of change in carotenoid content

Determining colour change of fruits has proven difficult as the use of different colour quantification methods results in variability and inconsistencies in colour change classification. Classification of fruit colour by eye is a very subjective method, as various factors, including fruit shape, size, amount of cuticle wax, and the light, may influence the colour observed. The diversity panel described here was previously analysed for colour retention phenotypes using various techniques. An image analysis method was used to determine change in colour in the diversity panel. In this experiment, whole fruits were stored over a nine month period. Using the image analysis method, colour was quantified using the RGB values of an image, which was captured under controlled experimental conditions. These values were then converted to CIELAB colour space (Commission Internationale de l'Eclairage) coordinates: *L**, *a**, and *b**, along with examining LCH (lightness, chroma, and hue angle) colour space coordinates (Berry, 2015). This allowed the colour retention phenotypes of the diversity panel to be characterised (Table 4-1). However, limitations exist with this method, as surface colour of the pepper fruit does not necessarily reflect the true colour phenotype of the pepper. It has been shown that image analysis values coordinated the occurrence of non-enzymatic browning in pepper (Lee et al., 1991). This is a process in which amino acids react with sugars within fruits, resulting in browning. If this colour interferes with image analysis results, the method of image analysis may be limited in measuring true red pigment of pepper fruits.

An alternative method has also been developed by the spice industry in order to allow standardised measuring of extractable colour from pepper fruits using an acetone extraction. The American Spice Trade Association (ASTA) developed a spectrophotometric method to analyse total pigment content of chilli powder, allowing comparisons to be made between countries (Wall and Bosland, 1998). The diversity panel used in this study was also previously subjected to ASTA colour analysis (Berry, 2015). However, ASTA measurements proved to be highly variable and were not

consistent with previously allocated retention phenotypes, suggesting this method was not optimal for accurate quantification of colour of pepper fruits. Interestingly, ASTA extractable red colour has previously been reported to be a better measure of pepper colour, when compared to image analysis measurements (Krajayklang et al., 2000). Furthermore, it was found that surface colour analysis was not sufficient for determining the colour of ground pepper, but the ASTA extractable method was more reliable (Vraþar et al., 2007). However, the use of HPLC provides the most accurate method for carotenoid quantification.

Carotenoid analysis using HPLC-PDA was also used previously to quantify the colour changes during storage of the diversity panel. As capsanthin content was found to be correlated with pepper fruit colour intensity, change in capsanthin content was used to determine changes in colour. However, the results from this experiment were highly variable and conclusions could not be drawn regarding the colour retention phenotypes of the diversity panel. It was concluded that a larger sample size would be required in order to accurately determine colour retention phenotypes (Berry, 2015).

Consequently, the storage experiment described here (Section 4.2.3) used a larger sample size, and colour retention phenotype was allocated based on change in total carotenoid content. Results were less variable when compared to the previous study, and therefore, carotenoid retention phenotypes could be allocated (Table 4-2).

Comparing the results of this experiment with the visual characterisation of carotenoid retention carried out previously, it was evident that there were some discrepancies in the allocation of colour retention phenotype. This may be due to subjectivity of the visual method. Consequently, colour retention phenotypes of the diversity panel were reallocated based on the carotenoid quantification experiment carried out in this study, although the visual colour retention classifications were not entirely disregarded. Lines in which differences in the allocation of colour retention phenotypes occurred, for example, R8, have been subsequently referred to using both allocated retention phenotypes. In the case of R8, this line has been referred to as medium/high retention.

4.3.2. High retention pepper varieties increase in carotenoid content during post-harvest storage

As was observed in Chapter 3, dried pepper fruits appear to increase in carotenoid content during post-harvest storage. This was particularly evident in lines identified as high retention, such as R5 and R6, which showed increases of 48% and 24% respectively, following oven drying. This increase in carotenoids during post-harvest storage has previously been observed in pepper varieties (Park and Lee, 1975,

Minguez-Mosquera and Hornero-Mendez, 1994a, Minguez-Mosquera et al., 2000). Understanding how these pepper varieties undergo carotenogenesis to such an extreme level, once they have been harvested from the fruit and left to dry, is fundamental to understanding the carotenoid retention trait. Line R6, here identified as high retention, was also studied in chapter 3 as part of the DH population. R6 is the high carotenoid retention parent for the DH population, and therefore, it is possible to compare the changes in carotenoid content during post-harvest storage in two independent experiments, in which growth and storage conditions were slightly different. The study performed in chapter 3 was using material grown, dried, and stored in India, in commercial conditions, whereas the study performed here used material grown in glasshouses in the UK, followed by oven drying and small-scale cold storage conditions. Whilst the conditions described in this chapter were chosen to reflect the study performed in chapter 3 as closely as possible, it was not possible to replicate these conditions entirely. Furthermore, the Indian study was carried out on material stored for seven months, however the study described here analysed material after three months, due to time constraints. The high retention parent line, R6 showed similar increases in carotenoid content in both Indian and UK conditions, with carotenoid increases of 32 % observed in the Indian study, and an increase of 24 % in the UK study. The slightly greater increase in the Indian study may have been due to the longer storage period.

The low retention line, R4, which is also the low retention parent of the DH population studied in chapter 3, was also analysed under both Indian and UK growth and storage conditions. However, this line showed drastically different changes in carotenoids dependent on the location of the study. Whilst in the study presented here, the low retention R4 line showed a decrease in carotenoid content of 21 %, in the Indian study, the same line showed an increase in carotenoid content of 43 %. This drastic difference in carotenoid change during post-harvest storage may reflect an array of differences in the studies, including the drying and storage conditions, sunlight, temperature, and humidity, of the two experiments. Whilst fruits were sun dried post-harvest in the experiment described in chapter 3, fruits were oven-dried under artificial light in the experiment described in this chapter. This difference in drying method may have caused the large discrepancy in carotenoid content. It has previously been reported that sun-dried peppers increased in carotenoid content by 4 %, whilst oven dried peppers decreased in carotenoid content by 30 % (Park and Lee, 1975). This difference reflects a change in carotenoid content on a similar scale to that observed in this study. The presence of sunlight during drying may have stimulated carotenogenesis as a defence mechanism against photooxidation of carotenoids,

resulting in carotenoid degradation. It is thought that photooxidation produces free radical species thought to be carotenoid radical cations (Mortensen et al., 1997, Boon et al., 2010). The presence of sunlight may have initiated the synthesis of increased carotenoids. It is known that the phytochrome interacting factor (PIF) family of transcription factors regulate the expression of the phytoene synthase gene, which is the first step in carotenoid biosynthesis. PIFs control this pathway by specifically repressing the *PSY1* gene which encodes the rate-limiting enzyme of the carotenoid biosynthesis pathway, phytoene synthase. Upon illumination, PIFs are degraded after interaction with photoactivated phytochromes, and the expression of *PSY1* is rapidly derepressed, resulting in carotenoid biosynthesis (Toledo-Ortiz et al., 2010). Consequently, line R4 may have undergone significant carotenogenesis upon drying under sunlight, which was not initiated when pepper fruits were oven dried, due to a lack of light intensity. This may explain the significant difference in change in carotenoid content of the low retention line R4 when stored in Indian and UK conditions. Interestingly, line R4 also had a cracked cuticle upon drying, and this may suggest that fruits of this variety were more susceptible to light-induced carotenogenesis, if light can more easily penetrate the fruit.

Interestingly, this difference in carotenogenesis observed in fruits dependent on storage location was only observed in the low retention line, R4. Line R6, the high retention parent line, was also subjected to the same conditions, however showed approximately the same changes in carotenoid content during post-harvest storage in both Indian and UK conditions. Whilst the low retention line underwent significantly greater carotenogenesis during sun-drying and post-harvest storage, the high retention line did not. This suggests that other factors influence carotenogenesis and carotenoid retention.

4.3.3. Pepper fruit cuticle plays a crucial role in controlling carotenoid retention

A major difference observed between low and medium/high carotenoid retention pepper fruits is the morphology of the cuticle upon fruit drying. Whilst high retention lines retain a smooth fruit cuticle upon drying, low retention lines have cuticles which are prone to wrinkling and cracking upon drying. As discussed in Section 4.3.2, a clear difference was observed between high and low carotenoid retention lines, R6 and R4, in the change in carotenoid content during post-harvest storage, dependent on drying and storage conditions. Whilst the low retention line displayed a vast difference in carotenoid retention dependent on post-harvest drying and storage location, R6 did not show such a difference. This suggests that other factors must influence the change in carotenoid content during post-harvest storage, as both lines were not affected in the

same way. A clear difference between lines R4 and R6 is the cuticle morphology upon drying. Whilst R6 retains a smooth cuticle upon fruit drying and storage, R4 rapidly displays a cuticle wrinkling and cracking phenotype upon drying. This may suggest that the cuticle structure influences the carotenoid retention phenotype. It may be hypothesised that the wrinkled and cracked cuticle observed in low carotenoid retention pepper lines results in the pepper fruit being more susceptible to carotenoid degradation. If this is the case, carotenogenesis in low retention lines may be initiated more so than in high retention lines in certain conditions in order to prevent excessive oxidative damage. Sun-drying, and other environmental factors, in combination with a cuticle prone to cracking, appears to initiate increased carotenogenesis, compared to oven drying.

Upon disruption of the pepper fruit cuticle by homogenisation, it was evident that high carotenoid retention lines did not show the same increase in carotenoid content as observed when fruits were stored as whole. Therefore, the cuticle structure clearly plays an important role in facilitating the biosynthesis and accumulation of carotenoids, along with the prevention of carotenoid degradation. It may be hypothesised that the smooth cuticle of high carotenoid retention pepper varieties allows the accumulation of increased carotenoids in these fruits. As the cuticle of low carotenoid retention peppers tends to be wrinkled and cracked, there appears to be a correlation between cuticle texture and carotenoid retention. Upon disruption of the fruit cuticle under controlled conditions, either in the cracking observed in low retention lines, or during homogenisation, carotenoids do not appear to accumulate in the same way as they do when the cuticle is intact. This may be due to the role that carotenoids play as antioxidants, in scavenging reactive oxygen species (ROS) and protecting against harmful singlet oxygen (Edge et al., 1997, Ramel et al., 2012, Edge and Truscott, 2018). Disrupted cuticles may be more permeable to reactive oxygen species or their precursors, and therefore carotenoids are required to scavenge these harmful species.

Analysis of the low retention line, R3, and the high retention line, R8, showed that fruits with a cracked cuticle at the point of treatment (R3, dried fruits) with hydrogen peroxide as an oxidative agent, displayed a greater degradation in carotenoid content, compared to smooth cuticle fruits (R8, fresh and dry). Capsorubin, capsanthin, and capsanthin diesters, the major red pigments in chilli peppers, are well characterised as having a high quenching capacity for singlet oxygen and hydroxyl free radicals (Nishino et al., 2016). Clearly, dried pepper fruits with a cracked cuticle, which showed a greater loss in carotenoid content than smooth cuticle fruits following treatment with hydrogen peroxide, had greater levels of reactive oxygen species to scavenge. This was

presumably due to the cracked cuticle being more permeable to hydrogen peroxide, therefore resulting in increased reactive oxygen species within the fruit.

4.4. Conclusion

It appears as though the carotenoid retention phenotype in some pepper varieties may be due to the post-harvest drying and storage conditions, as differences were observed in some varieties when treated under different experimental conditions. The low carotenoid retention variety, R4, increased in carotenoid content when dried under sunlight but decreased in carotenoid content when oven-dried. This may be due to abiotic factors, such as amount of sunlight or temperature, affecting the rate of carotenogenesis in some pepper varieties. Increased sunlight may initiate increased carotenogenesis in some lines in order to prevent photooxidative damage (Figure 4-8). As R4 also had a cracked cuticle, this also suggests that fruits with a compromised cuticle may be more susceptible to photo-induced carotenogenesis.

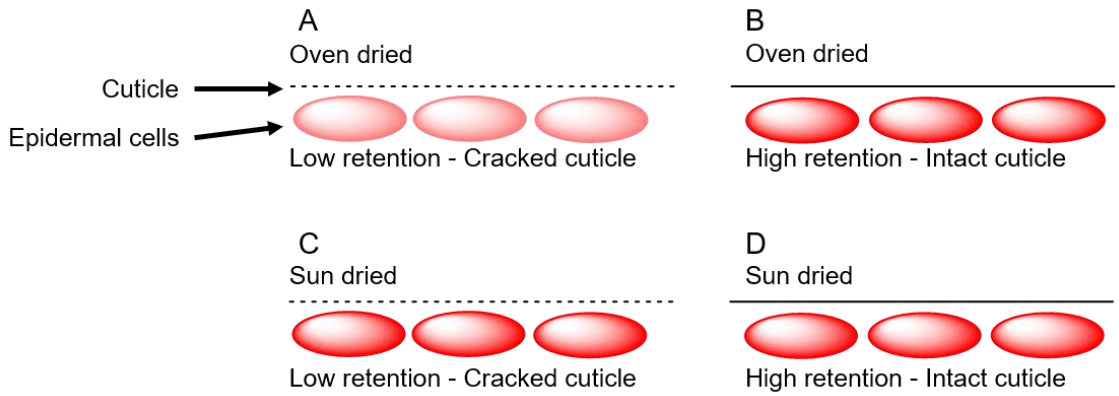


Figure 4-8 Low retention pepper varieties increase in carotenoid content when dried under sunlight.

Low carotenoid retention peppers, with cracked cuticles, dried in an oven showed a decrease in carotenoid content following storage (A), whilst peppers dried under sunlight showed an increase in carotenoid content (C). High retention peppers, with intact cuticles, showed little difference in carotenoid content between oven-dried (B) and sun-dried (D) conditions. This suggests drying conditions, along with cuticle morphology, may influence carotenoid retention.

Further to this, fruit cuticle morphology appears to play an important role in controlling pepper fruit carotenoid retention. It appears as though low carotenoid retention lines tend to have a cuticle prone to wrinkling and cracking following harvest, whereas medium and high carotenoid retention lines tend to have a smooth cuticle. Lines with a disrupted cuticle tended to show increased carotenoid degradation. Upon disruption of smooth cuticles following harvest using homogenisation, carotenoids degraded more rapidly, suggesting the cuticle plays a protective role. Treatment with hydrogen peroxide as an oxidative agent resulted in greater carotenoid loss in lines with a compromised cuticle following drying, as opposed to smooth cuticle varieties (Figure 4-9).

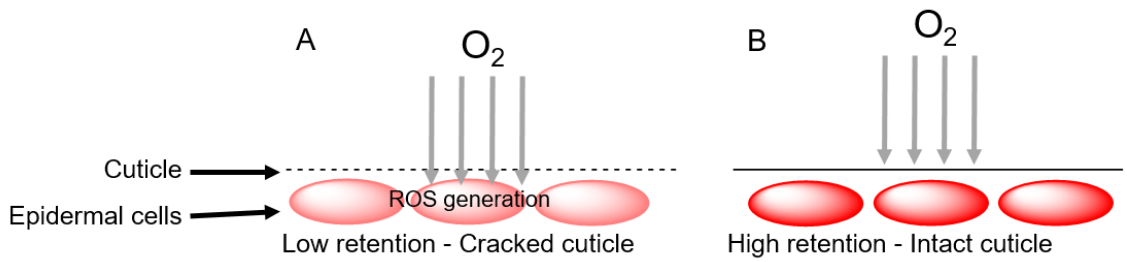


Figure 4-9 Cracked pepper fruit cuticles are more permeable to oxidative species, resulting in increased carotenoid degradation.

Pepper fruits with cracked cuticles showed greater significant decreases in carotenoid content following H_2O_2 treatment, as a source of ROS (A), whereas pepper fruits with intact cuticles did not show any difference in carotenoid content after treatment with ROS (B). This indicates that cuticle morphology and integrity is crucial in controlling pepper fruit carotenoid retention.

Clearly, the fruit cuticle plays an important role in the carotenoid retention trait, and therefore will be studied further.

5. The role of fruit surface structure in controlling carotenoid retention

5.1. Introduction

Waxy cuticles are often an essential element of a plant's physiology, playing crucial roles in leaves and in fruit. Cuticles play a fundamental role in the plant's interaction with the environment, and can protect the plant from environmental stressors including the negative effects of excessive water loss and pathogen attack, and in controlling organ expansion (Domínguez et al., 2011). Indeed, it has been suggested that the evolution of the cuticle was one of the critical factors in allowing the colonisation of land by the bryophytes, pteridophytes, and spermatophytes. By developing a hydrophobic interphase between their interior and surroundings, water loss could be limited (Kozioł and Whatley, 2016).

The cuticle is composed of two components: a cutin-rich section, known as the 'cuticular layer', and the waxes which lie on top of the cuticular layer, known as the 'cuticle proper'. Waxes embedded within the cutin matrix are referred to as intracuticular waxes, whilst waxes accumulating on the surface as crystals or films are referred to as epicuticular waxes (Yeats and Rose, 2013). Very long chain fatty acids (VLCFAs), with carbon chain length ranging from C_{20} to C_{34} are the precursors for cuticle wax synthesis, and cuticle waxes include a range of alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. Lipophilic secondary metabolites, such as triterpenoids or flavonoids may also be associated with cuticular waxes (Jetter et al., 2006). Cutin monomers are derived from fatty acids with chain lengths of C_{16} and C_{18}, and include oxygenated fatty acid-glycerol esters, which are known as monoacylglycerols (Graça et al., 2002).

Whilst the cuticle has long been known to play a crucial role in plant development and growth in modulating the interaction between plant and environment, it has also been suggested that the cuticle may play an important role in post-harvest quality of fruit. Once harvested, the cuticle of fruits acts as a barrier against drying, chemical attack, and mechanical damage, along with protecting against microbial infection (Lara et al., 2014). Clearly, the cuticle can play just as important a role in protecting the fruit during post-harvest storage, as it does during fruit growth and development.

The cuticle provides an effective barrier to control the entrance and exit of many elements, some of which include gases. Whilst the evolution of the fruit cuticle allowed the colonisation of land due to the minimisation of water loss, this also presented a challenge to these plants in the form of controlling gaseous exchange. Whilst many green plant tissues overcame this issue with the development of stomata to allow the entrance and exit of gases for essential processes such as photosynthesis and

respiration, fruit pericarp tends to be entirely covered in waxy cuticle. Therefore, it is suggested that fruit cuticles must be to some extent permeable to gases. Intact pepper fruit cuticle has been shown to be permeable to a small amount of carbon dioxide and oxygen, though this permeability increases significantly upon wounding of the fruit cuticle (Banks and Nicholson, 2000). Whilst gaseous exchange is essential for plant processes such as respiration and photosynthesis, negative effects may be observed as a result of this exchange. For example, the role of antioxidants is evident in protecting against the harmful effects of reactive oxygen species, which may be formed during the process of gaseous exchange.

Interestingly, the cuticle of *Capsicum annuum* has previously been reported to extend through the apoplast of multiple cell layers (Martin and Rose, 2014). There is some dispute over whether this 'cuticular' layer, which penetrates several cell layers deep into the fruit, can be termed the 'cuticle'. For this reason, the 'cuticular layer' spanning several cell layers, will here be termed the 'exocarp', which is defined as the outermost layer of the pericarp of an angiosperm fruit, external to the mesocarp (Botany, 2002). The term 'cuticle' will be used to define only the outermost wax layer, deposited above the epidermal cells.

It is therefore hypothesised that the antioxidant content, specifically carotenoid content in this case, of fruits is directly associated with cuticle, or exocarp, structure. As pepper fruits dry during post-harvest storage, cuticle structure may change or be wounded, resulting in fruit surface cracking. For this reason, exocarp structures of pepper lines with varying carotenoid retention phenotypes have been studied to determine whether there is a correlation between exocarp structure and carotenoid retention.

5.2. Results

5.2.1. Pepper diversity panel displays variation in exocarp thickness

Pepper fruit exocarps were observed using light microscopy in order to determine whether variation in exocarp structure is observed between different pepper varieties. The 12 line diversity panel supplied by Syngenta, and the line CM334 were used in this study. A staining method was developed in order to clearly distinguish fruit lipid exocarp from fruit pericarp. Nile Red to stain wax exocarp and Fast Green for pericarp was found to be the stain combination displaying the clearest differentiation between these two tissue types. This method showed that there was a clear difference in exocarp thickness between the 13 lines studied (Figure 5-1). Staining showed that epidermal cells were embedded within the wax exocarp layer, and in some cases, the wax exocarp layer penetrated several cell layers deep into the fruit. Whilst some lines had very thin exocarps, for example R3 and R7, which showed just one layer of cells

embedded within the wax exocarp layer, other lines, such as R5 and R6, showed up to four or five layers of cells embedded within the wax exocarp.

As clear differences were observed in the thickness of the wax exocarp layer between different lines within the pepper diversity panel, measurements were made using ImageJ software to accurately quantify exocarp thickness. As expected, significant differences were observed between lines (Figure 5-1, Figure 5-2). Whilst lines R1, R5, R6, and R8 all had exocarp thicknesses ranging from 90-120 µm, lines R3 and R7 both had thinner exocarps with thicknesses between 30-40 µm. Statistical testing using ANOVA demonstrated that these varieties were significantly different from one another.

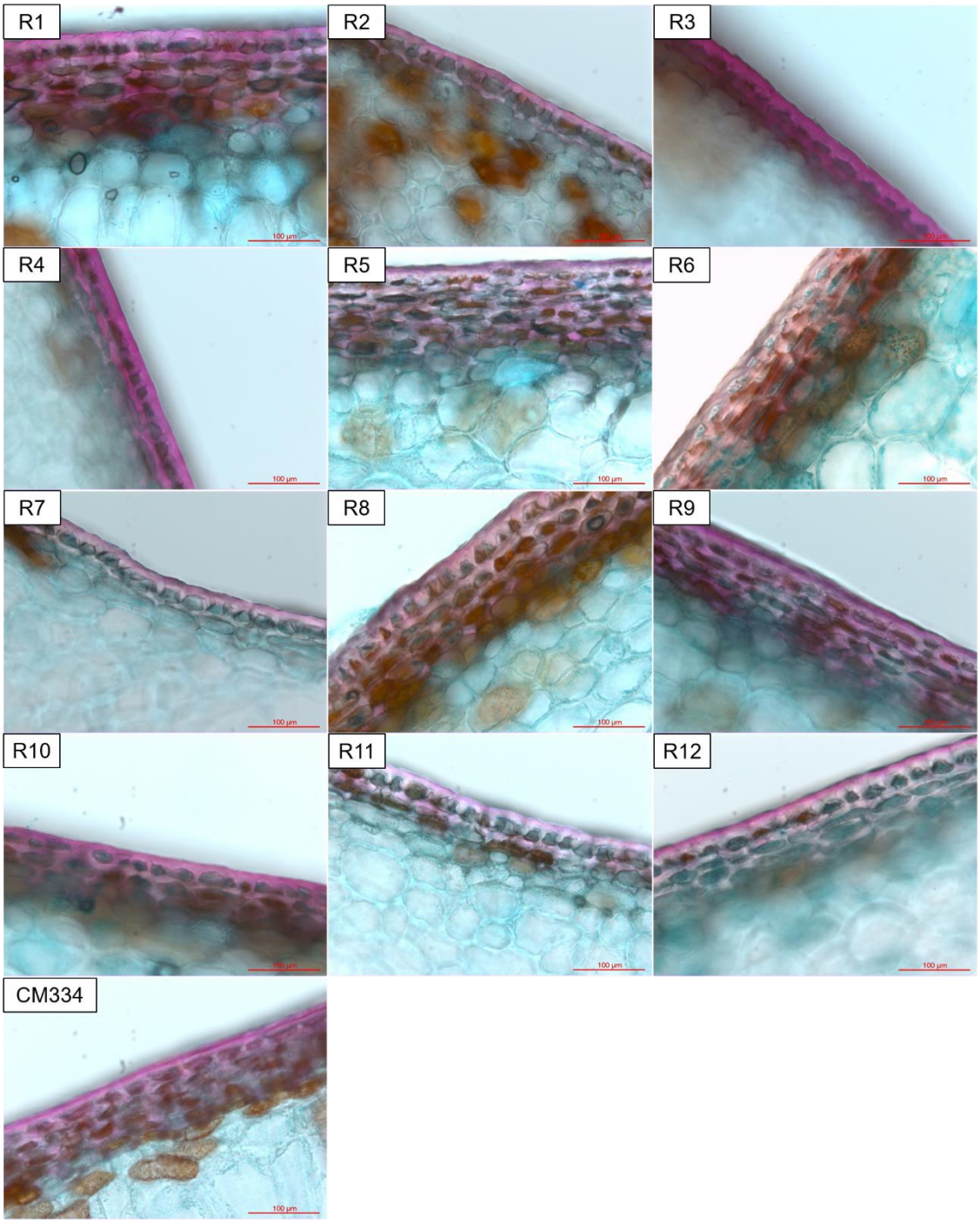


Figure 5-1 Variation in exocarp structure in pepper diversity panel.

Fine cross-sections of pepper fruit were cut and stained using Nile Red and Fast Green stains, before observing using light microscopy. Wax exocarp was stained pink. Variation in exocarp thickness of 12 line diversity panel, and line CM334 observed in this way. Three fruits per line were observed (n = 3).

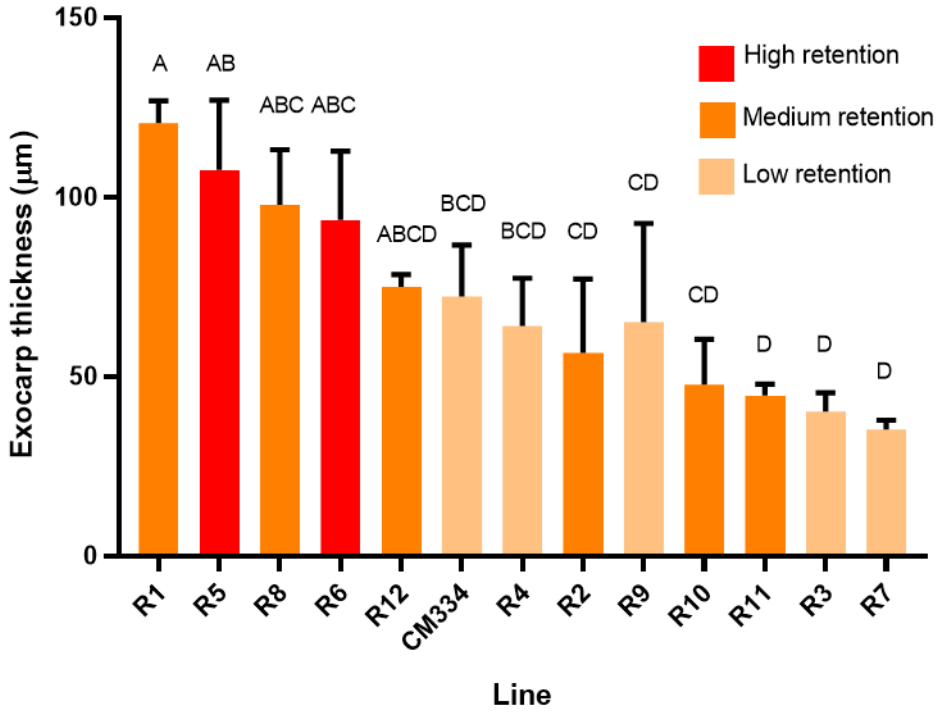


Figure 5-2 Pepper diversity panel exocarp thickness measurements.

Cross-sections of pepper fruit were isolated and exocarp thickness was measured based on extent of lipid staining by Nile Red stain. At least three fruits per line were measured, and six measurements per image were recorded per technical replicate (n = 3; standard error of the mean reported). ANOVA followed by Tukey post-hoc testing was used to determine statistical significance between lines ($p < 0.05$). Lines were coloured based on their carotenoid retention phenotype assigned using HPLC-PDA measurements, described in Chapter 4: high retention (red), medium retention (dark orange), low retention (pale orange).

5.2.2. Exocarp thickness is correlated with carotenoid retention

As the diversity panel had previously been characterised for carotenoid retention, it was possible to determine whether a correlation exists between post-harvest carotenoid retention and exocarp thickness. A general trend appeared to show that lines characterised using HPLC-PDA in Chapter 4 as high or medium carotenoid retention also had thicker exocarps (Figure 5-2).

5.2.3. Fruit surface texture is correlated with carotenoid retention

It was previously noted that fruits of some pepper varieties formed surface cracks upon post-harvest drying. This resulted in fruits of the 12 line diversity panel being classified as possessing either a ‘cracked’ cuticle, or a ‘smooth’ cuticle. A trend emerged, in which the cracked cuticle phenotype correlated with the low carotenoid retention phenotype. Consequently, scanning electron microscopy (SEM) was used to further characterise morphological differences in cuticle structure of post-harvest dried pepper fruit varieties. SEM clearly demonstrated the difference in cuticle structure between high and low carotenoid retention lines. As expected, low carotenoid retention lines, R3 and R4, both had a ‘wrinkled’ and ‘cracked’ cuticle structure (Figure 5-3A-D). In

contrast, high carotenoid retention lines, R6 and R8, both had smooth cuticle structures, with no cracks on the surface (Figure 5-3E-H). At 3000x magnification, the low carotenoid retention line R3 displayed clear cracks across the surface of the pepper, whereas high carotenoid retention line displayed an entirely in tact fruit surface.

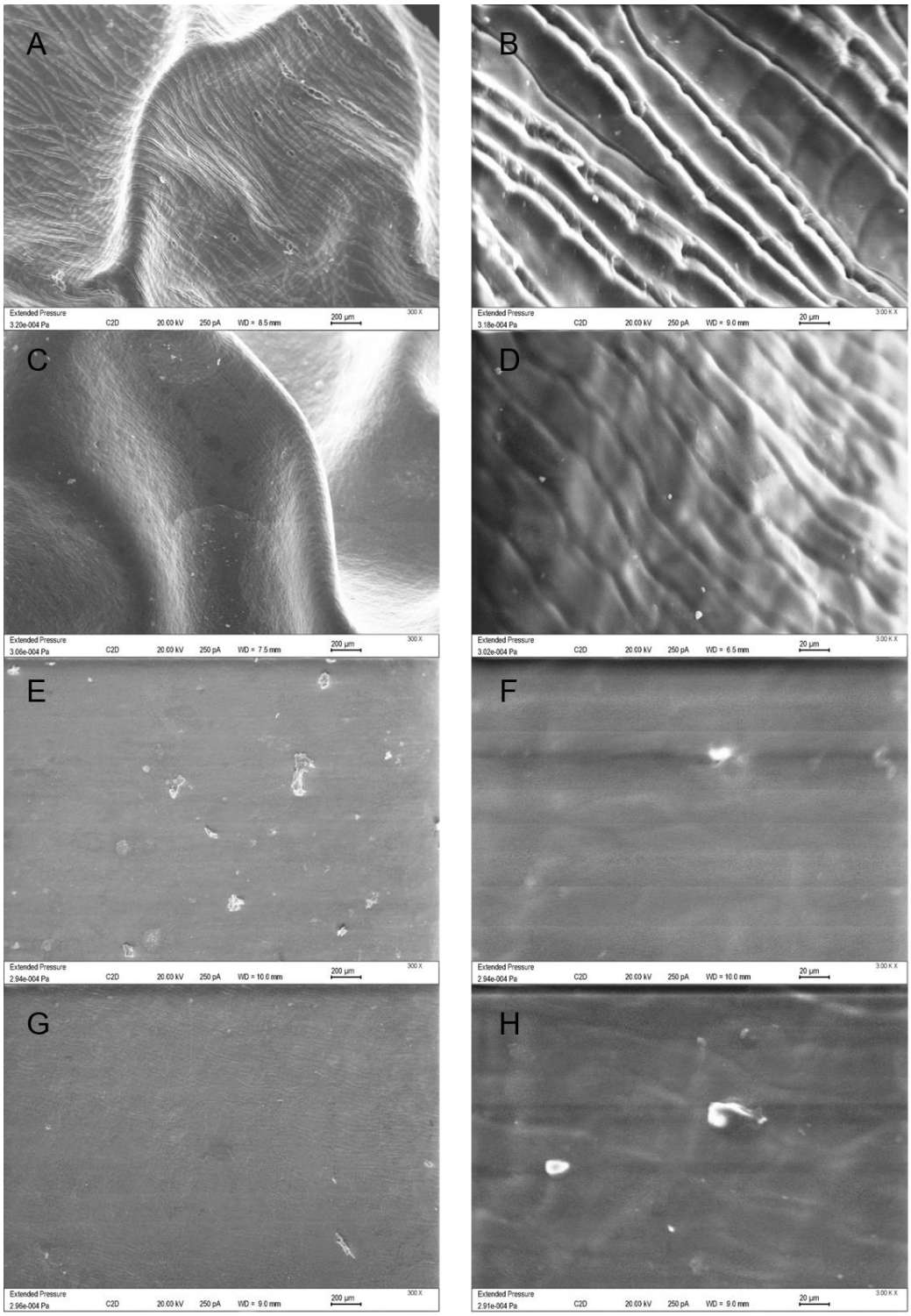


Figure 5-3 Scanning electron microscopy (SEM) images of pepper fruit cuticle surface.

Pepper fruits were harvested and oven dried for two weeks. Fruits were sectioned and the surface was analysed by a Zeiss EVO LS15 SEM. Four pepper varieties were analysed in this way: R3 – low carotenoid retention, cracked fruit surface (A: 300x magnification, B: 3000x magnification); R4 – low carotenoid retention, cracked fruit surface (C: 300x magnification, D: 3000x magnification); R6 – high carotenoid retention, smooth fruit surface (E: 300x magnification, F: 3000x magnification); R8 – medium/high carotenoid retention, smooth fruit surface (G: 300x magnification, H: 3000x magnification). Cracks are clearly visible in low retention lines, whilst high retention lines have a smooth fruit surface.

5.2.4. Cuticle content is correlated with carotenoid retention

Fruit cuticles are made up of several different components, including epicuticular waxes, intracuticular waxes, and cutin monomers. Whilst the 12 line pepper diversity panel displayed variation in exocarp thickness, biochemical profiling of the fruit cuticles was subsequently used to determine differences between lines in cuticle biochemical composition. Five pepper varieties were observed in the cuticle biochemical profiling study, being a high carotenoid retention line (R8), low carotenoid retention line (R3), high carotenoid retention parent line (R6), low carotenoid retention parent line (R4), and the wild variety (CM334).

5.2.4.1. Cutin monomer composition

Cutin monomer analysis was carried out to determine whether differences exist between thick and thin cuticle pepper lines, and whether cutin monomer composition correlated with the carotenoid retention phenotype. Cutin monomer composition was measured at four fruit developmental time points (anthesis + 20 days, anthesis + 30 days, anthesis + 45 days, red ripe), to determine the change in composition through fruit development. Total cutin monomer content increased throughout development in all 5 pepper varieties analysed as expected, as the cuticle is produced during fruit development. High retention lines R6 and R8, and CM334 line, had increased total cutin monomers at the ripe time point when compared to the low retention lines R3 and R4. Total cutin monomer amounts are displayed in Table 5-1.

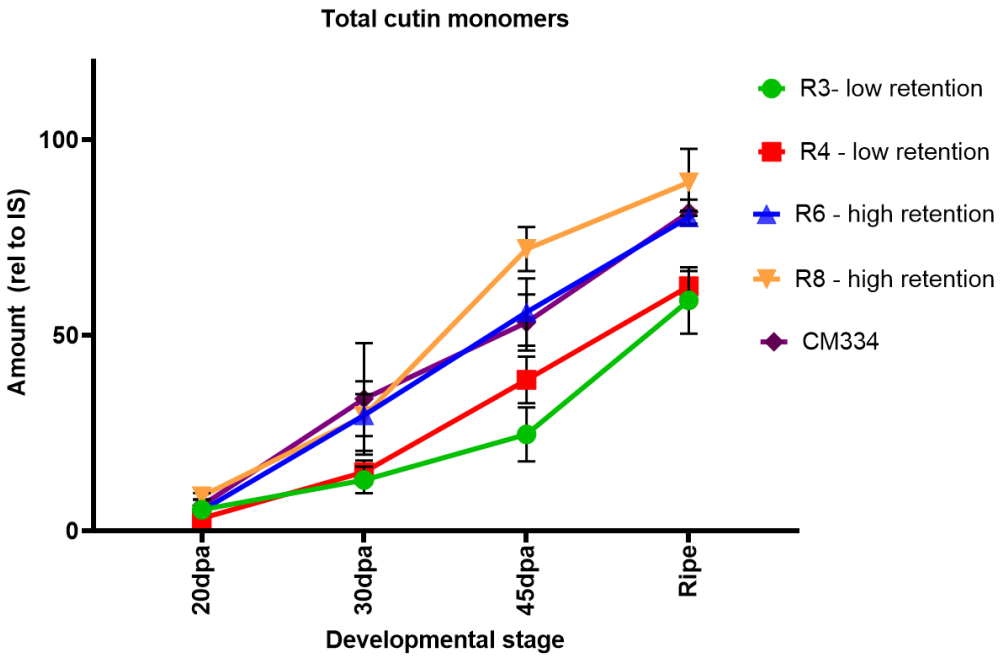


Figure 5-4 Total cutin monomer content of pepper cuticles.

Cutin monomers were isolated via a process of delipidation and depolymerisation. GCMS was used to identify and quantify cutin monomers. Quantification was determined relative to the internal standard (C_{32} alkane). Five pepper varieties were analysed: R3 (low carotenoid retention line; green), R4 (low carotenoid retention parent line; red), R6 (high carotenoid retention parent line; blue), R8 (high carotenoid retention line; yellow), CM334 (wild pepper line; purple). Seven cuticle discs per biological replicate were used, and three biological replicates were analysed (n = 3). Significance values displayed in Figure 5-6.

Clearly, the cuticle develops during fruit development, as an increase in cutin monomer content was observed as the development of the fruit progressed. 10,16-dihydroxyhexadecanoic acid was the compound responsible for the greatest proportion of cutin monomers in all 5 lines analysed, and levels increased significantly throughout fruit development in all lines studied (Figure 5-5K). Analysis of thick and thin exocarp pepper lines not only showed that there was a difference in total cutin monomer amounts, but that there were also differences in individual cutin monomer amounts. Whilst the levels of some cutin monomers, such as 9,10-epoxy-hydroxyoctadecanoic acid, 9,10-dihydroxyoctadecanedioic acid, and 9,10,18-trihydroxyoctadecanoic acid increased throughout development in all lines studied particularly towards the end of fruit development, other compounds detected did not reflect this same trend. Phenolic compounds including coumaric acid, ferulic acid, and caffeic acid showed relatively stable levels throughout fruit development, suggesting that these components of the cuticle were produced very early in fruit development, and did not change as the fruit continued to grow.

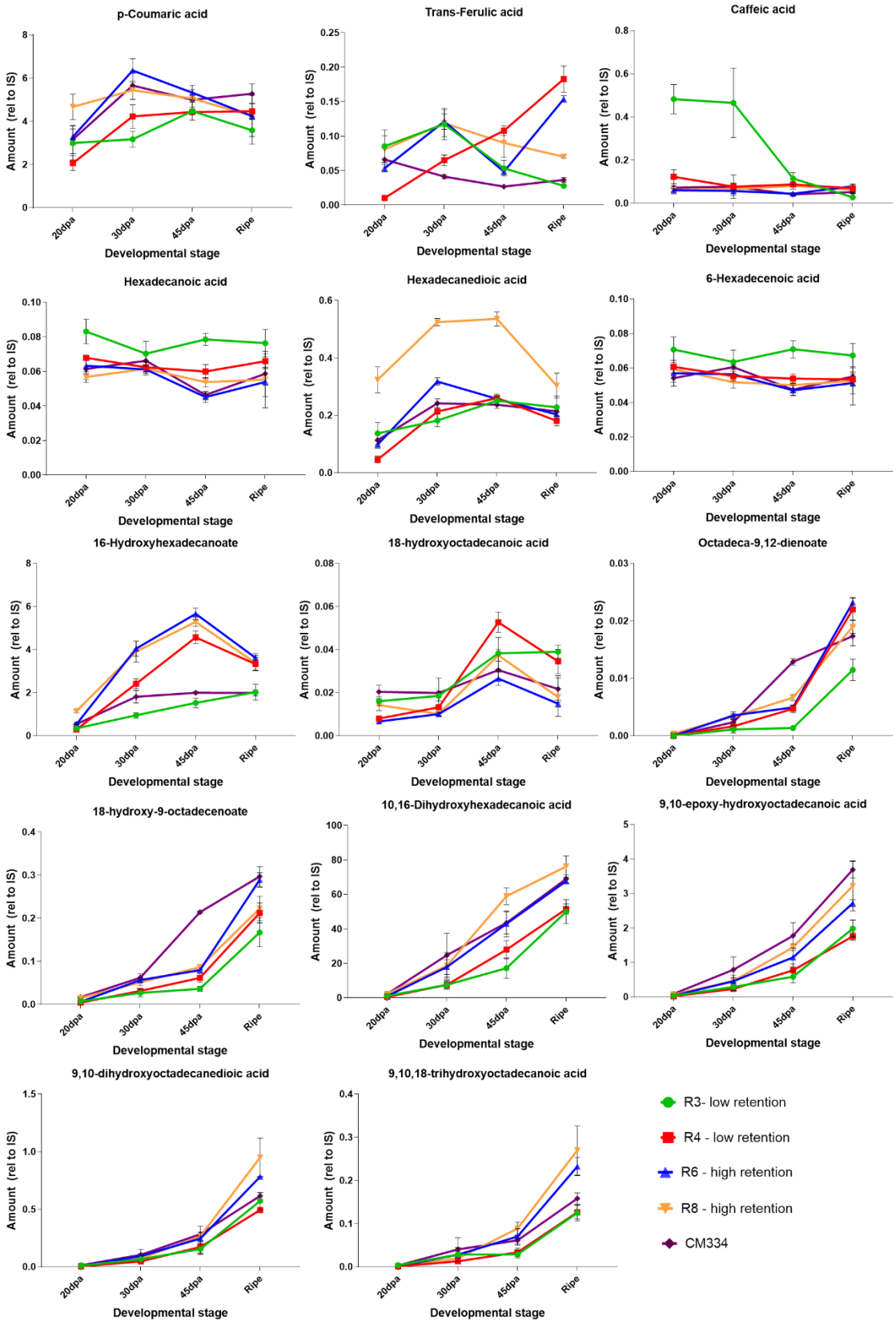


Figure 5-5 Changes in pepper cutin monomer content through fruit development.

Cutin monomers were isolated from pepper fruit cuticles and analysed by GC-MS. Cutin monomer compounds were identified based on comparison with published spectra, and quantified relative to an internal standard (C_{32} alkane). Five pepper varieties were analysed: R3 (green), R4 (red), R6 (blue), R8 (yellow), CM334 (purple). 13 cutin monomers were identified: p-Coumaric acid (A), trans-Ferulic acid (B), Caffeic acid (C), Hexadecanoic acid (D), Hexadecanedioic acid (E), 6-Hexadecenoic acid (F), 16-Hydroxyhexadecanoate (G), 18-Hydroxyoctadecanoic acid (H), Octadeca-9,12-dienoate (I), 18-Hydroxy-9-

0ctadecanoate (J), 10,16-Dihydroxyhexadecanoic acid (K), 9,10-epoxy-hydroxyoctadecanoic acid (L), 9,10-dihydroxyoctadecanedioic acid (M), 9,10,18-trihydroxyoctadecanoic acid (N). Seven cuticle discs per biological replicate were used; three biological replicates per line were analysed (n = 3). Significance values for ripe fruit displayed in Figure 5-6. Values displayed in Table 5-1.

Significant differences in compound quantities between lines was determined using ANOVA followed by Tukey post-hoc tests at the ripe fruit stage. The ripe fruit stage was considered to be the most biologically relevant fruit development stage for the hypothesis being tested. The carotenoid retention phenotype is only observed upon ripening of the pepper fruit, and therefore cuticle composition at this stage was determined to be the most relevant when considering whether cuticle composition affects carotenoid retention. At the ripe time point, R3 (low carotenoid retention) had significantly lower levels of ferulic acid (Figure 5-6B) and octadeca-9,12-dienoate (Figure 5-6I) compared to R4 (low retention parent), R6 (high retention parent), and R8 (high carotenoid retention line). Both low retention lines had significantly lower levels of 9,10,18-trihydroxyoctadecanoic acid (Figure 5-6N) and 10,16-dihydroxyhexadecanoic acid (Figure 5-6K) compared to the high retention line (R8), but not compared to the high retention parent (R6). 10,16-dihydroxyhexadecanoic acid was the most abundant cutin monomer in all lines analysed. A significant increase in this cutin monomer in high retention line R8 suggests its importance reflects the increase in total cutin monomers in this line, and suggests a significant contribution to exocarp thickness. Interestingly, none of the compounds analysed showed differences between the high and low carotenoid retention parent lines (R4 and R6). Whilst these lines showed differences in exocarp thickness (Figure 5-1), no significant difference was observed in cutin composition (Figure 5-6). The low extreme carotenoid retention line (R3) had significantly lower total cutin monomer content when compared to the high extreme carotenoid retention line (R8) (Figure 5-6O), but no significant difference was observed between other lines.

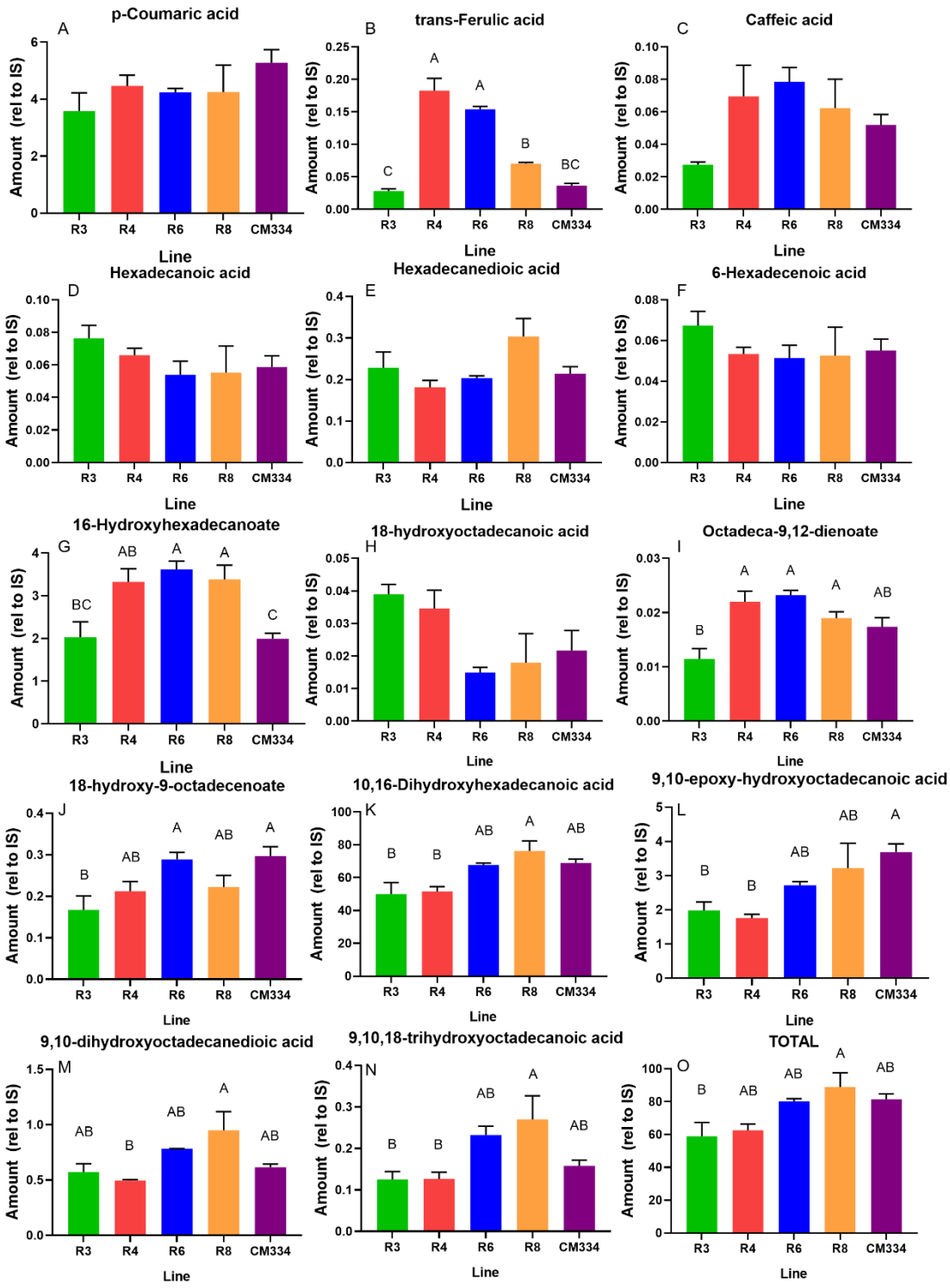


Figure 5-6 Differences in pepper fruit cutin monomer content at ripe time point.

Cutin monomers were isolated from pepper fruit and analysed by GC-MS. An internal standard (C_{32} alkane) was used for quantification of compounds. Five pepper varieties were analysed: R3 (green), R4 (red), R6 (blue), R8 (yellow), CM334 (purple). Seven cuticle discs per biological replicate were isolated, and three biological replicates per line were analysed (n = 3). ANOVA testing followed by a Tukey HSD post-hoc test was used to determine significance between lines at the ripe time point for each compound identified, and for total cutin monomers ($p < 0.05$).

Table 5-1 Pepper fruit cutin monomer values, measured throughout fruit development.

Cutin monomers were isolated from pepper fruit and analysed by GC-MS. An internal standard (C_{32} alkane) was used for relative quantification of compounds. Five pepper varieties were analysed: R3, R4, R6, R8, CM334. Seven cuticle discs per biological replicate were isolated, and three biological replicates per line were analysed (n = 3). Average values (± standard error) presented.

Developmental Stage	A + 20					A + 30					A + 45					Ripe				
Line	**R3**	**R4**	**R6**	**R8**	**CM334**	**R3**	**R4**	**R6**	**R8**	**CM334**	**R3**	**R4**	**R6**	**R8**	**CM334**	**R3**	**R4**	**R6**	**R8**	**CM334**
p-Coumaric acid	3.00	2.06	3.28	4.67	3.16	3.17	4.23	6.35	5.44	5.66	4.47	4.43	5.33	5.06	5.00	3.58	4.47	4.25	4.25	5.28
±SE	0.79	0.34	0.37	0.59	0.66	0.36	0.54	0.55	0.43	0.64	0.42	0.11	0.34	0.41	0.10	0.64	0.37	0.13	0.95	0.46
trans-Ferulic acid	0.09	0.01	0.05	0.08	0.07	0.12	0.07	0.12	0.12	0.04	0.05	0.11	0.05	0.09	0.03	0.03	0.18	0.15	0.07	0.04
±SE	0.02	0.00	0.01	0.02	0.02	0.02	0.01	0.01	0.02	0.00	0.01	0.01	0.00	0.02	0.00	0.00	0.02	0.00	0.00	0.00
Caffeic acid	0.48	0.12	0.06	0.06	0.07	0.47	0.08	0.06	0.07	0.08	0.11	0.09	0.04	0.08	0.04	0.03	0.07	0.08	0.06	0.05
±SE	0.07	0.03	0.01	0.02	0.00	0.16	0.01	0.01	0.03	0.05	0.03	0.01	0.00	0.02	0.01	0.00	0.02	0.01	0.02	0.01
Hexadecanoic acid	0.08	0.07	0.06	0.06	0.06	0.07	0.06	0.06	0.06	0.07	0.08	0.06	0.05	0.05	0.05	0.08	0.07	0.05	0.06	0.06
±SE	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.02	0.01
Hexadecanedioic acid	0.14	0.05	0.10	0.32	0.11	0.18	0.21	0.32	0.52	0.24	0.25	0.26	0.26	0.54	0.24	0.23	0.18	0.20	0.30	0.21
±SE	0.04	0.01	0.01	0.05	0.02	0.02	0.03	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.01	0.04	0.02	0.01	0.04	0.02
6-Hexadecenoic acid	0.07	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.05	0.06	0.07	0.05	0.05	0.05	0.05	0.07	0.05	0.05	0.05	0.06
±SE	0.01	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01
16-Hydroxyhexadecanoate	0.34	0.29	0.54	1.15	0.54	0.95	2.42	4.04	3.91	1.81	1.53	4.57	5.66	5.29	2.00	2.03	3.33	3.62	3.38	1.99
±SE	0.11	0.02	0.02	0.06	0.09	0.13	0.23	0.34	0.48	0.29	0.22	0.29	0.27	0.21	0.04	0.36	0.30	0.19	0.34	0.13
18-hydroxyoctadecanoic acid	0.02	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.02	0.04	0.05	0.03	0.04	0.03	0.04	0.03	0.01	0.02	0.02
±SE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01
Octadeca-9,12-dienoate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.02	0.02	0.02	0.02
±SE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18-hydroxy-9-octadecenoate	0.01	0.00	0.01	0.01	0.02	0.03	0.03	0.06	0.05	0.06	0.04	0.06	0.08	0.09	0.21	0.17	0.21	0.29	0.22	0.30
±SE	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.03	0.02	0.02	0.03	0.02
10,16-Dihydroxyhexadecanoic acid	1.11	0.50	0.96	2.34	2.28	7.55	7.59	17.92	18.56	24.76	17.23	27.94	42.94	58.97	43.51	49.97	51.55	67.70	76.21	68.97
±SE	0.37	0.09	0.19	0.05	0.57	2.56	2.07	4.27	7.68	12.79	5.95	5.28	7.60	4.85	6.53	7.02	2.95	1.14	6.19	2.31
9,10-epoxy-hydroxyoctadecanoic acid	0.04	0.02	0.04	0.07	0.09	0.29	0.23	0.46	0.46	0.79	0.59	0.77	1.15	1.44	1.78	1.98	1.76	2.72	3.22	3.69
±SE	0.01	0.00	0.01	0.01	0.05	0.11	0.05	0.11	0.17	0.37	0.18	0.18	0.28	0.10	0.38	0.25	0.11	0.10	0.73	0.24
9,10-dihydroxyoctadecanedioic acid	0.01	0.00	0.01	0.01	0.01	0.07	0.05	0.09	0.07	0.10	0.15	0.17	0.25	0.27	0.28	0.57	0.49	0.78	0.95	0.62
±SE	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.02	0.02	0.05	0.05	0.05	0.06	0.00	0.07	0.07	0.01	0.00	0.17	0.03
9,10,18-trihydroxyoctadecanoic acid	0.00	0.00	0.00	0.00	0.00	0.03	0.01	0.03	0.02	0.04	0.03	0.03	0.07	0.09	0.06	0.13	0.13	0.23	0.27	0.16
±SE	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.01	0.03	0.01	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.06	0.01
TOTAL	5.38	3.20	5.17	8.85	6.50	13.01	15.05	29.58	29.36	33.74	24.65	38.61	55.94	72.05	53.28	58.91	62.55	80.17	89.08	81.46
±SE	1.43	0.51	0.63	0.81	1.42	3.44	2.97	5.34	8.87	14.26	6.90	5.98	8.59	5.66	7.16	8.46	3.85	1.64	8.56	3.25

5.2.4.2. Cuticle wax composition

Subsequently, cuticle waxes were extracted and measured from pepper fruits as the second major biochemical component of the cuticle structure. Importantly, cuticle waxes were isolated using a chloroform dip, and therefore, all compounds soluble in chloroform were removed from the tissue. This included compounds not specific to the cuticle wax. Cuticle waxes are comprised of compounds with carbon chain length equal to, or greater than, C_{20}. Consequently, compounds with chain length smaller than C_{20} were still included in this analysis but would not contribute to the cuticle wax composition. Further to this, cuticle wax has been shown to include very long chain fatty acids, with carbon chain lengths up to C_{34}. The GC-MS system used to analyse cuticle wax composition in this study did not facilitate the analysis of very long chain fatty acids or alkanes with chain lengths as long as the maximum chain lengths observed in cuticle waxes, and therefore this analysis does not comprehensively determine differences in cuticle wax composition between high and low carotenoid retention lines.

Unlike in cutin composition, where an increase in cutin monomer content was observed as a general trend throughout fruit development, cuticle wax content largely remained stable throughout fruit development. Further to this, there was no clear difference between lines in cuticle wax composition. Whilst the content of some fatty acids, such as eicosanoic acid (Figure 5-7J) and hexacosanoic acid (Figure 5-7M), were greater in the low carotenoid retention line R3 throughout development, other fatty acids showed very little variation throughout fruit development when comparing between high and low carotenoid retention pepper varieties. Alkane composition also did not show a clear trend throughout fruit development in any pepper varieties, and there were no clear differences in the pepper lines in terms of alkane content (Figure 5-7N-T). There was no clear difference between lines in total cuticle wax component content, and levels remained stable throughout fruit development, suggesting that these components are synthesised early in fruit development and stay at this level as the fruit continues to grow.

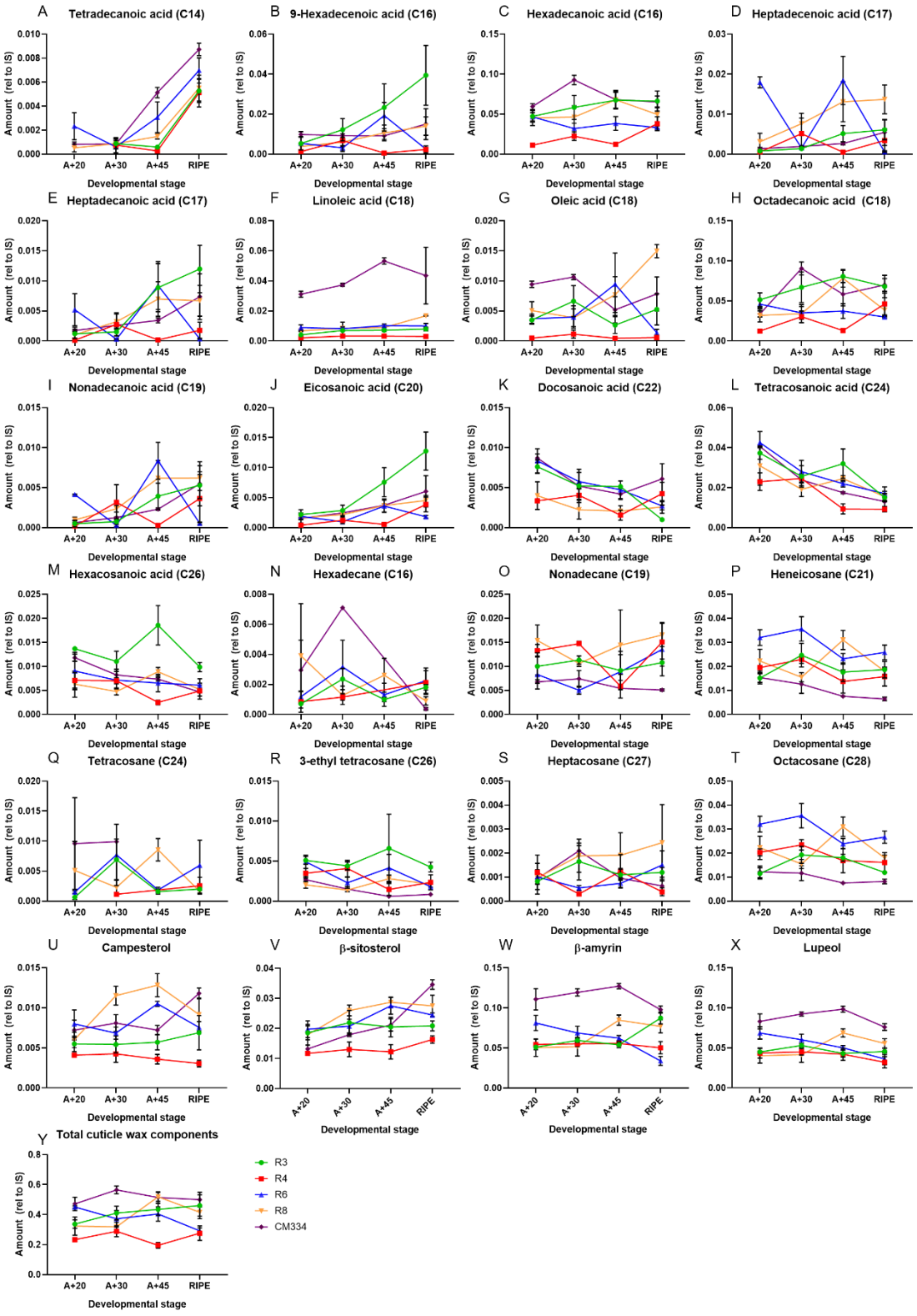


Figure 5-7 Changes in pepper fruit cuticle wax composition through development.

Cuticle waxes were extracted from 1cm diameter pepper fruit pericarp discs by washing the cuticle surface in chloroform for 10 seconds. Internal standard (D-27 Myristic acid; 10 µg) was added to allow relative quantification of compounds. Five pepper varieties were analysed: R3 (green), R4 (red), R6 (blue), R8 (yellow), CM334 (purple). 24 individual wax components were identified and quantified relative to the internal standard: Tetradecanoic acid (A), 9-Hexadecenoic acid (B), Hexadecanoic acid (C), Heptadecenoic acid (D), Heptadecanoic acid (E), Linoleic acid (F), Oleic acid (G), Octadecanoic acid (H), Nonadecanoic acid (I), Eicosanoic acid (J), Docosanoic acid (K), Tetracosanoic acid (L), Hexacosanoic acid (M), Hexadecane (N), Nonadecane (O), Heneicosane (P), Tetracosane (Q), 3-ethyl Tetracosane (R),

Heptacosane (S), Octacosane (T), Campesterol (U), β-sitosterol (V), β-amyrin (W), Lupeol (X), Total cuticle wax components (Y). 10 fruit discs per biological replicate were isolated, and three biological replicates per line were analysed (n = 3). Significance values for major wax component classes at ripe fruit stage displayed in Figure 5-8. Values for major wax cuticle component classes displayed in Table 5-2.

ANOVA testing followed by Tukey HSD post-hoc tests was used to determine significant differences in total cuticle wax components between pepper varieties at the ripe time point. As the carotenoid retention phenotype is observed at the ripe time point, this developmental stage was the most biologically relevant to determine significant differences. No significant differences were observed between the five pepper varieties in total fatty acid content (Figure 5-8A). As total fatty acids were measured when extracting from the pepper surface, some fatty acids identified were not constituents of the cuticle wax. This included all fatty acids with a carbon chain length smaller than C_{20}. Consequently, total fatty acid waxes, representing fatty acids with a chain length between C_{20} and C_{26}, were analysed alone as these waxes constitute the cuticle wax. No significant difference was observed between high and low carotenoid retention lines in total fatty acid wax content (Figure 5-8B).

Significant differences were not observed in total alkane content between the four lines sourced from Syngenta identified as either high or low carotenoid retention (R3, R4, R6, R8), however the two high retention lines (R6, R8) had significantly increased alkane content when compared to the wild line (CM334) (Figure 5-8C). Again, total alkane wax content was analysed, being alkane constituents with a chain length between C_{20} and C_{28}, and the high retention parent line, R6, was found to have significantly increased alkane wax components compared to the low retention lines R3 and R4. However, the high retention line, R8, did not have increased alkane wax content compared to the low retention lines (Figure 5-8D).

A significant difference was observed in total triterpenoid/sterol content (Figure 5-8E) between the two parent lines (R4: low, and R6: high retention) of the DH population and the extreme high carotenoid retention line (R8). However, this difference does not correlate to carotenoid retention phenotype, and therefore may be linked to other phenotypic differences between these lines. This does, however, display the similarity between the two DH population parent lines (R4, R6), which has been shown previously.

No significant difference was observed in total wax components between the four pepper varieties (R3, R4, R6, and R8) (Figure 5-8F). As there were no clear difference in cuticle wax components between high and low carotenoid retention pepper varieties, this suggests that cuticle wax composition does not influence the carotenoid retention phenotype. However, it should be noted that only waxes with chain lengths up to C_{26} in

the case of fatty acids, and C_{28} in the case of alkanes, were measured due to limitations of the column used in the GC-MS analysis system. Waxes may include fatty acids and alkanes with chain lengths up to and including C_{34}, and therefore it is evident that not all waxes were measured in this study. A further study needs to be carried out to determine longer chain wax content, using a more appropriate analysis system.

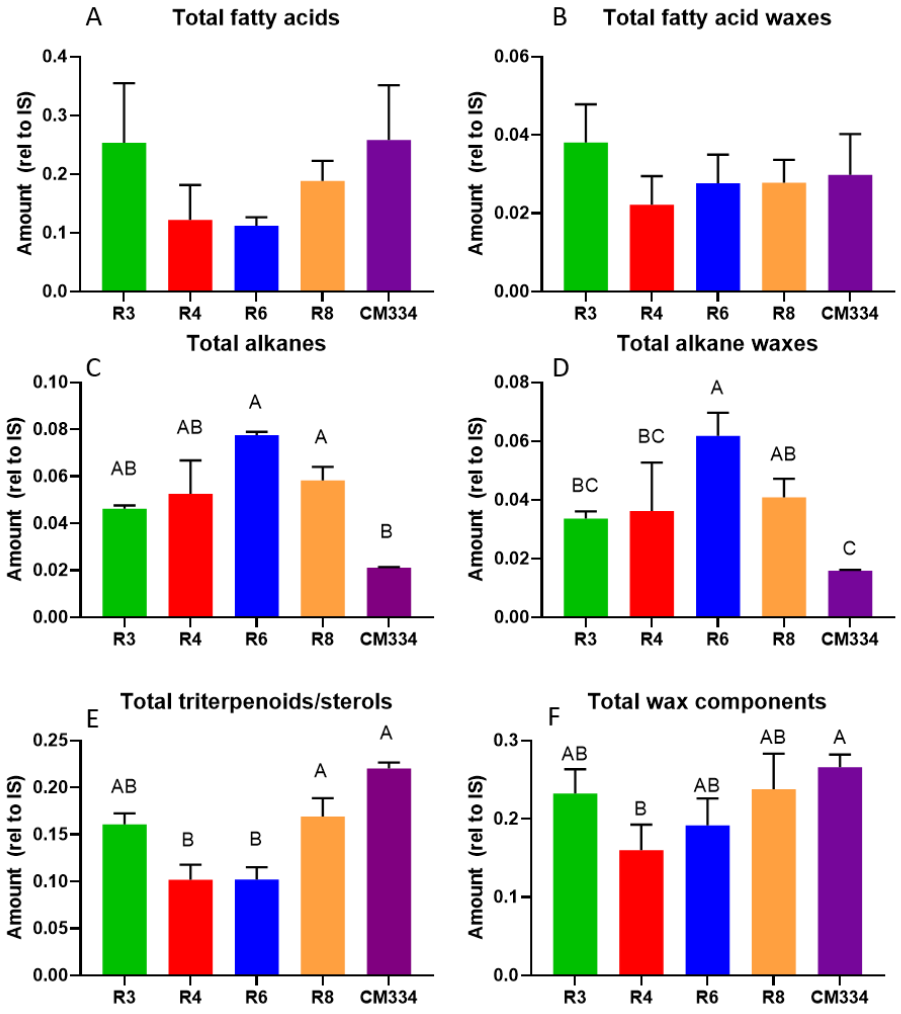


Figure 5-8 Differences in major wax component classes in pepper fruit cuticle throughout development.

Cuticle waxes were extracted from 1 cm diameter pepper fruit pericarp discs by washing the cuticle surface in chloroform for 10 seconds. Internal standard (D-27 Myristic acid; 10 µg) was added to allow quantification of individual components relative to the internal standard. Five pepper varieties were analysed: R3 (green), R4 (red), R6 (blue), R8 (yellow), CM334 (purple). 10 discs per biological replicate were isolated, and three biological replicates per line were analysed (n = 3). ANOVA testing, followed by Tukey HSD post-hoc test was used to determine significance between lines at the ripe time point, for the major classes of cuticle wax components ($p < 0.05$).

Table 5-2 Values for major cuticle wax component classes in pepper fruit, throughout fruit development.

Cuticle wax components were extracted from pepper fruit discs and analysed by GC-MS. An internal standard (D-27 Myristic acid; 10 µg) was used for relative quantification of compounds. Five pepper varieties were analysed: R3, R4, R6, R8, CM334. 10 discs per biological replicate were isolated, and three biological replicates per line were analysed (n = 3). Values (± standard error) for major cuticle wax component classes displayed.

Developmental stage	Line	Total fatty acids	Total wax fatty acids (C20-C34)	Total alkanes	Total wax alkanes (C20-C34)	Total triterpenoids/ sterols	Total wax components
	R3	0.17	0.06	0.04	0.03	0.12	0.21
	±SE	0.02	0.00	0.01	0.00	0.01	0.01
	R4	0.06	0.03	0.06	0.04	0.11	0.19
	±SE	0.01	0.01	0.00	0.00	0.01	0.01
	R6	0.19	0.06	0.08	0.07	0.18	0.31
	±SE	0.01	0.01	0.01	0.01	0.01	0.03
	R8	0.14	0.04	0.07	0.05	0.11	0.21
	±SE	0.03	0.01	0.02	0.01	0.02	0.04
	CM334	0.21	0.06	0.05	0.04	0.21	0.31
A+20	±SE	0.01	0.00	0.01	0.01	0.02	0.03
	R3	0.20	0.04	0.07	0.06	0.14	0.24
	±SE	0.04	0.01	0.01	0.01	0.01	0.01
	R4	0.10	0.03	0.07	0.05	0.12	0.20
	±SE	0.02	0.00	0.00	0.00	0.01	0.01
	R6	0.13	0.04	0.09	0.08	0.16	0.28
	±SE	0.02	0.01	0.02	0.02	0.02	0.04
	R8	0.14	0.03	0.05	0.04	0.13	0.19
	±SE	0.04	0.01	0.00	0.00	0.02	0.02
	CM334	0.29	0.04	0.04	0.03	0.24	0.31
A+30	±SE	0.01	0.00	0.02	0.01	0.01	0.01
	R3	0.26	0.06	0.05	0.04	0.12	0.23
	±SE	0.03	0.01	0.01	0.01	0.01	0.02
	R4	0.04	0.01	0.04	0.03	0.11	0.16
	±SE	0.00	0.00	0.01	0.01	0.01	0.02
	R6	0.19	0.04	0.06	0.05	0.15	0.24
	±SE	0.04	0.01	0.00	0.01	0.01	0.02
	R8	0.24	0.04	0.08	0.07	0.20	0.31
	±SE	0.01	0.00	0.02	0.01	0.01	0.02
	CM334	0.24	0.03	0.02	0.02	0.25	0.30
A+45	±SE	0.03	0.00	0.00	0.00	0.01	0.01
	R3	0.25	0.04	0.05	0.03	0.16	0.23
	±SE	0.06	0.01	0.00	0.00	0.01	0.02
	R4	0.12	0.02	0.05	0.04	0.10	0.16
	±SE	0.03	0.00	0.01	0.01	0.02	0.02
	R6	0.11	0.03	0.08	0.06	0.10	0.19
	±SE	0.01	0.00	0.00	0.00	0.01	0.02
	R8	0.19	0.03	0.06	0.04	0.17	0.24
	±SE	0.02	0.00	0.01	0.00	0.02	0.03
	CM334	0.26	0.03	0.02	0.02	0.22	0.27
RIPE	±SE	0.05	0.01	0.00	0.00	0.01	0.01

5.2.5. Carotenoid retention may be influenced by protective nature of fruit exocarp

As exocarp structure, thickness, and composition has been linked to the carotenoid retention phenotype of pepper fruits, understanding the causal mechanism behind this correlation is important. It has been found that carotenoids may be bound to, or even

embedded within the fruit exocarp layer, protecting them from degradation, and therefore causing the high retention phenotype.

5.2.5.1. Carotenoids may be associated with pepper fruit exocarp

Upon enzymatically isolating exocarp discs from pepper fruit for cutin monomer and cuticle wax analysis, it was unexpectedly noted that red pigment was retained within the wax exocarp tissue, despite the removal of all other pericarp tissue (Figure 5-9). Consequently, it was hypothesised that chromoplasts, containing carotenoids, may be located within the epidermal cells found in the isolated exocarp layer. This may affect the observed carotenoid retention phenotype, as a high concentration of pigment in the outer layers of the fruit may influence the perceived fruit colour.

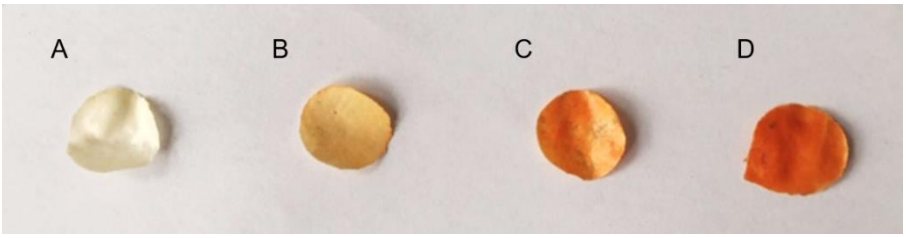


Figure 5-9 Isolated pepper fruit exocarp discs.

Discs (1 cm diameter) were cut from ripe pepper fruits. The waxy exocarp was enzymatically isolated using a solution containing pectinase and cellulase. Four pepper varieties were studied in this way: R3 (A), R4 (B), R6 (C), R8 (D).

Carotenoids were isolated from exocarp discs through a process of washing in chloroform and methanol over a period of three days. Carotenoids were analysed by HPLC, and the following compounds identified: violaxanthin, neoxanthin, antheraxanthin, antheraxanthin monoesters, capsanthin, capsanthin monoesters, capsanthin diesters, zeaxanthin, zeaxanthin diesters, β-cryptoxanthin, β-carotene, and capsorubin diesters. Carotenoid amounts extracted from exocarp discs were compared to carotenoid amounts extracted from whole fruit discs, and results were expressed as a ratio. A ratio was calculated in order that the quantity of exocarp-bound carotenoids could be compared relative to whole fruit carotenoid content across all lines analysed, regardless of the variation in total fruit carotenoid content between the lines. Compounds in which lines displayed significantly different ratio values are presented (Figure 5-10). Interestingly, three of the four compounds found to be significantly different between lines when looking at exocarp to whole fruit carotenoid composition ratio, were related to capsanthin. This suggests that capsanthin and esterified capsanthin may be responsible for causing the observed red colour of exocarp discs in some lines. A significantly greater exocarp to whole fruit ratio for capsanthin was observed in R8 (extreme high retention) when compared to R3

(extreme low retention) and R6 (high retention parent) (Figure 5-10B). In contrast, R8 had a significantly greater ratio of capsanthin monoesters only compared to R4 (low retention parent) (Figure 5-10C). This again suggests that R8 had a large amount of exocarp bound capsanthin compared to low retention lines. The starkest difference was observed in capsanthin diester exocarp to whole fruit ratio. R8 had a significantly greater ratio than all three other lines analysed (Figure 5-10D), and this was also reflected in total carotenoid ratio (Figure 5-10E). These findings support what was observed upon removing the exocarp from the fruit, as R8 exocarp discs retained the darkest pigment compared to the other three lines (Figure 5-9). As the R8 exocarp discs were both darker in colour, and were shown to have significantly greater exocarp bound carotenoid content compared to the other three lines, it was hypothesised that the ability to bind or trap carotenoids in the exocarp tissue may influence the observed colour retention phenotype. If carotenoids are exocarp bound, they may be less susceptible to degradation during post-harvest storage, and these peppers may therefore be deemed to be high carotenoid retaining.

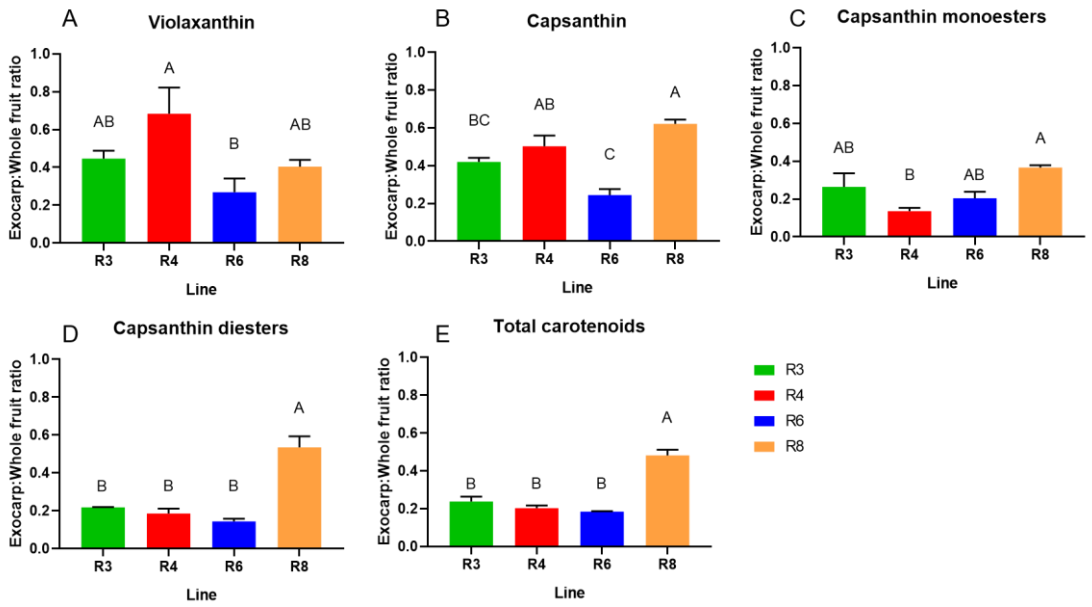


Figure 5-10 Exocarp to whole fruit carotenoid content ratio.

Discs (1 cm diameter) were cut from pepper fruit pericarp. The exocarp layer was removed from discs using cellulase and pectinase. Discs were washed in chloroform:methanol (10 mL) for 36 hours. Carotenoids were extracted from the wash solution, and analysed by HPLC. Exocarp disc carotenoid content was compared to whole fruit disc carotenoid content, analysed in the same way, and expressed as a ratio. Four pepper varieties were analysed in this way: R3 (green), R4 (red), R6 (blue), R8 (yellow). 10 discs per biological replicate were isolated, and three biological replicates per line were analysed (n = 3). All carotenoids as described in section 3.2.1 were measured. ANOVA testing followed by Tukey post-hoc tests were used to determine significant differences in carotenoid content ratio between whole fruit and exocarp disc in the four analysed lines ($p < 0.05$). Ratios are presented for carotenoids displaying significant differences between lines: Violaxanthin (A), Capsanthin (B), Capsanthin monoesters (C), Capsanthin diesters (D), total carotenoids (E). Standard error bars are shown.

5.2.5.2. Microscopy suggests increased number of chromoplasts in high retention line exocarp

Using light microscopy to examine the exocarp structure of the pepper fruit lines, it was noted that high carotenoid retention lines may have increased chromoplast numbers within their epidermal cells. Following wax exocarp staining, orange structures were observed in the epidermal cells of some fruits, and these structures were determined to be chromoplasts. Red arrows indicate high concentrations of epidermal cell chromoplasts in lines R6 and R8 (high retention lines) (Figure 5-11C, D). Whilst chromoplast structures were observed in low retention lines R3 and R4 (Figure 5-11A, B), the concentration of chromoplasts in these cells seemed to be lower than that observed in lines R6 and R8. Whilst counting of these chromoplast structures within these cells was attempted, the resolution of these images did not allow the accurate counting of chromoplasts. Therefore, an alternative method of molecular quantification of chromoplasts was used.

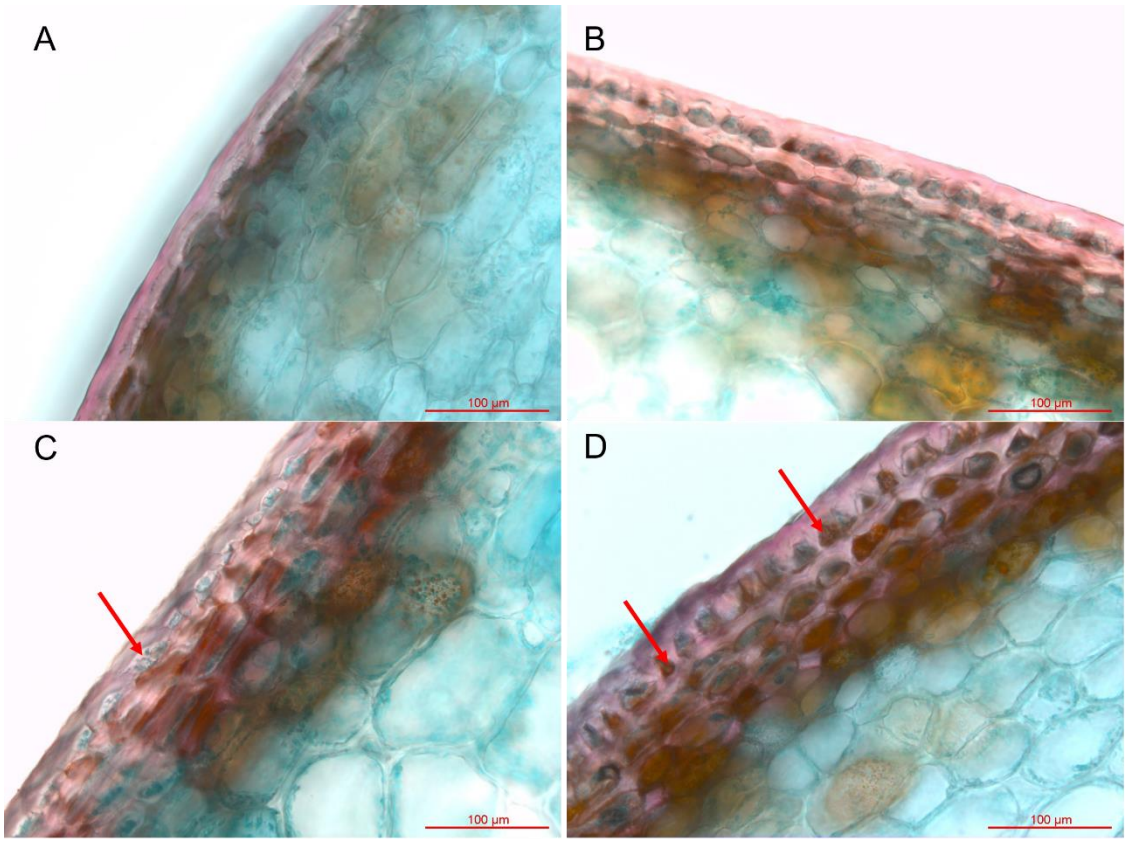


Figure 5-11 Identification of chromoplasts located within fruit epidermal cells.

Pepper fruits were hand sectioned and exocarp waxes were stained pink with Nile Red stain. Four lines were analysed: R3 (A), R4 (B), R6 (C), R8 (D). Red arrows indicate the presence of chromoplasts in epidermal cells.

5.2.5.3. Molecular quantification of chromoplasts within pepper fruit exocarp

As exocarp localised chromoplasts could not be counted accurately in the microscopy images, chromoplast number was molecularly quantified. Copy number of plastid localised *Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase* (*Rubisco*).was quantified relative to copy number of nuclear localised *Phytoene Desaturase* (*PDS*) and expressed as a ratio. Evidently, plastid DNA, and hence plastid number, in the exocarp of all four lines analysed was high relative to nuclear DNA. R4, which had the lowest plastid:genome ratio, had a ratio of over 300:1 (Figure 5-12). According to this method of molecularly quantifying plastid number, both R3 and R6 had significantly greater exocarp localised plastids compared to R4. R8 did not have significantly different plastid numbers compared to the other lines analysed. This result does not support the microscopy images, in which there appeared to be increased plastids in the epidermal cells of high retention lines, R6 and R8 (Figure 5-11). Furthermore, significantly higher carotenoid levels were observed in exocarp tissue in line R8 (Figure 5-10). The fact that plastid number in exocarp tissue in R8 was not significantly higher than lines R3 and R4 may suggest that carotenoid content was not linked to chromoplast number. Carotenoid content may be more concentrated within the chromoplasts of high retention lines R6 and R8. Whilst plastid number was not significantly higher in these lines, an increase in concentration of carotenoids within these plastids may explain the fact that greater levels of carotenoids were associated with the exocarp in these lines, as observed when carotenoids were extracted from the exocarp. Additionally, a higher concentration of carotenoids in exocarp localised chromoplasts in lines R6 and R8, may explain why plastids were more clearly visualised in microscopy images of these lines.

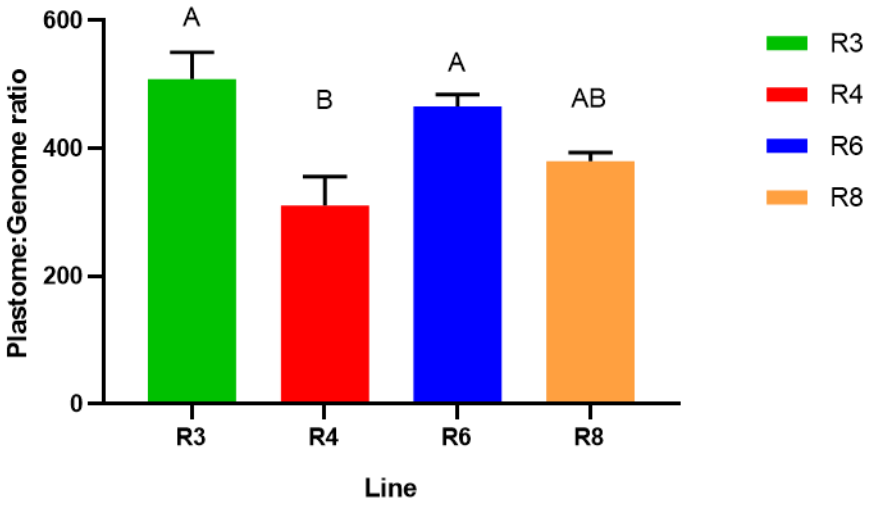


Figure 5-12 Chromoplast to genome ratio of pepper fruit exocarp tissue.

Genomic DNA was extracted from pepper fruit exocarp material, and the quantity of chromoplasts was calculated. Copy number of the plastid localised gene Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (Rubisco) was quantified relative to the nuclear localised Phytoene Desaturase (PDS). Genomic DNA was extracted from the exocarp of at least five fruits per biological replicate, and three biological replicates per pepper variety were analysed. Four pepper varieties were analysed: R3 (green), R4 (red), R6 (blue), R8 (yellow). Average values of three biological replicates are presented, with standard error bars. ANOVA testing followed by Tukey HSD post-hoc tests were used to determine significant differences between pepper varieties.

5.2.6. RNAseq reveals differentially expressed genes in fruit epidermal cells

As the exocarp appears to play an important role in controlling the carotenoid retention phenotype of pepper fruit, understanding gene expression is important to ascertain the molecular mechanisms controlling this trait. For this reason, RNAseq was used to determine gene expression patterns in pepper fruit epidermal cells during cuticle development. RNA was isolated from pepper fruit epidermal cells of lines R3 (low carotenoid retention; thin exocarp) and R8 (high carotenoid retention; thick exocarp) at the following development stages: anthesis + 20 days, anthesis + 30 days, anthesis + 45 days, and ripe (Figure 5-13). Epidermal cells were removed from the fruit using abrasion, and RNA was then extracted from these cells. Use of a Leica Laser Microdissection facility was attempted in order to isolate the cells of interest. However, the resources for this facility were limited, and the quality of the material dissected was poor due to the waxy nature of the tissue. The efficiency of cell dissection was also poor and thus, this method could not be carried out on the scale that was required for this study. The method of cell removal by abrasion yielded quality RNA, and was therefore deemed to be suitable. This method of isolating specific cell types provides spatiotemporal resolution to gene expression analyses. To date, this approach

represents one of the first examples of RNAseq studies performed in pepper that utilises spatiotemporal resolution.

Figure 5-13 Pepper fruits used for RNA extraction from epidermal cells.

Two pepper genotypes (R3; R8) were analysed by RNAseq for epidermal cell gene expression changes. RNA was extracted from epidermal cells removed from fruits using the abrasion method. Four development stages were analysed: Anthesis + 20 (A + 20), Anthesis + 30 (A + 30), Anthesis + 45 (A + 45), Ripe. A minimum of five fruits were used for epidermal cell isolation per biological replicate; three biological replicates per genotype were analysed at the three designated development stages.

5.2.6.1. Multivariate and differential expression analyses

Multivariate analysis and differential expression analysis were performed by Syngenta following read sequencing, adapter trimming and alignment to the CM334 pepper genome. Principal component analyses (PCA) showed that fruit development stage was responsible for the largest source of variance in this study, as shown by principal component 1 (Figure 5-14A). Ripe samples appeared to force this separation, as they displayed the greatest difference when compared to all other samples. This was not unexpected, as it is well known that a significant number of gene expression changes occur during the fruit ripening process.

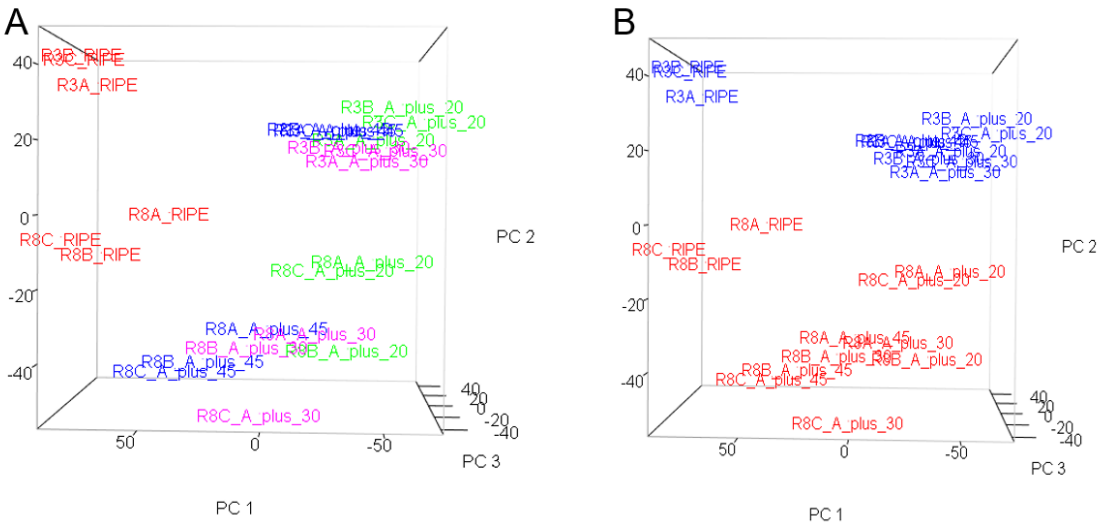


Figure 5-14 3D principal component analysis plots of cuticle RNAseq samples.

PCA plots of samples after normalisation, coloured by: developmental stage (A), genotype (B). Developmental stage responsible for greatest source of variation in this study.

Significant gene expression differences were identified through differential expression analyses. Thirteen comparisons, all of biological relevance, were tested and the number of statistically significant differentially expressed genes was determined for each comparison (FDR < 0.05; absolute log2 fold change > 1) (Table 5-3). The greatest number of differentially expressed genes were found in comparisons between anthesis + 20 days and ripe, for both genotypes studied. This reflects the significant changes that occur during development of fruit.

Table 5-3 Numbers of differentially expressed genes per comparison made.

Thirteen biologically relevant comparisons were made, between different development time points and genotypes. Number of up- and down-regulated genes presented (FDR < 0.05, absolute log2 fold change > 1), in A, when comparing A vs B.

Comparison (A vs B)	# Genes Up-regulated	# Genes Down-regulated
R3_A+20 vs. R8_A+20	376	385
R3_A+30 vs R8_A+30	689	899
R3_A+45 vs R8_A+45	1313	1439
R3_RIPE vs R8_RIPE	629	1059
R3_A+20 vs R3_A+30	79	26
R3_A+30 vs R3_A+45	164	77
R3_A+45 vs R3_RIPE	2702	2359
R8_A+20 vs R8_A+30	359	324
R8_A+30 vs R8_A+45	233	85
R8_A+45 vs R8_RIPE	1023	577
R3_A+20 vs R3_RIPE	3202	3127
R8_A+20 vs R8_RIPE	2425	2209
Genotype x DevStage Interaction	418	261

5.2.6.2. Identification of genes core to determining differences between genotypes

Genes were determined to be core to determining significant differences between the R3 and R8 genotypes if they were significantly differentially expressed in the same direction, when comparisons were made between the two genotypes throughout all four development stages studied. In this case, 114 genes were identified as following this trend. An example of these genes is CA10G18910, which encodes a cytochrome P450. This gene was significantly down-regulated in R3 compared to R8 at all four developmental stages. This cytochrome P450 has been predicted by the Plant Metabolic Network pathways database to be involved in cuticular wax biosynthesis. The fact that this gene was down-regulated in R3 compared to R8 correlates with previous findings that showed R3 to have a thinner cuticle than R8. The full list of significantly differentially expressed genes can be found in Supplementary Table 6. However, while this group of 114 genes may be fundamental in determining the differences between these two genotypes, there were many other genes found to be

significantly differentially expressed between the two genotypes, but not at all four developmental stages. Some traits may not be controlled by gene expression changes throughout development, but may rely on expression changes at a specific developmental time point. Therefore, whilst this group of 114 genes was found to be interesting in determining constitutive gene expression changes, it does not account for those gene expression changes which may occur at a single developmental time point.

5.2.6.3. Identification of cuticle genes potentially responsible for difference in carotenoid retention phenotype

A large number of genes were differentially expressed when comparing genotypes and developmental stages, thus simply mining these gene lists would be a challenging and superficial method for identifying cuticular genes responsible for controlling the carotenoid retention phenotype. Therefore, a statistical method of identifying gene sets, known as gene set enrichment analysis, was used in order to suggest biological explanations for the differences between the genotypes. In this study, ROAST (Wu et al. 2010) was used for gene set enrichment analysis. Gene sets were determined from CANNUUMCYC pathways (Plant Metabolic Network), Gene Ontology Biological Process, and Gene Ontology Molecular Function. ROAST considers the overall trend of genes in the gene set, whether they be up- or down-regulated, in order to identify gene sets which can be termed significant. This is regardless of whether any individual gene within the set is significantly differentially expressed, as all genes within the set are considered. Gene sets were deemed to show significant changes if the FDR from the enrichment was less than 0.05.

Gene sets related to carotenoid biosynthesis, specifically the capsanthin/capsorubin biosynthesis pathway, were not shown to be significantly enriched in the comparisons tested. This suggests that colour retention differences between R3 and R8 genotypes were unlikely to be due to differences in capsanthin biosynthesis. This supports previous findings: pepper colour intensity, and therefore biosynthesis, is a trait independent of the colour retention trait (Chapter 3). The hypothesis stating that the carotenoid retention trait is not controlled by biosynthesis, but is more likely influenced by physiological differences in the fruits, such as the cuticle structure, is therefore supported. Furthermore, R8 was shown to have significantly greater cuticle to whole fruit ratio of capsanthin diesters when compared to R3 (Section 5.2.5.1). This was not due to an increase in chromoplast number in epidermal cells (Section 5.2.5.3). This is supported by the fact that carotenoid biosynthesis genes were not up-regulated, as there were not more plastids in the high retention line (R8) acting as the site for carotenoid biosynthesis. Rather, these lines of evidence suggest several potential

hypotheses. Firstly, whilst carotenoid biosynthesis gene expression was not up-regulated in the high carotenoid retention line (R8), the carotenoid biosynthetic enzymes may be more active in the epidermal cells of the high retention lines. Secondly, as neither carotenoid biosynthesis gene expression is up-regulated, nor are there more plastids in the high retention line compared to the low retention line, carotenoids in the high retention line (R8) could be better protected from degradation compared to the low retention line (R3). This is likely to be due to the thick, waxy cuticle in the high retention line surrounding the epidermal cells, which could be less permeable to oxidative agents, capable of causing the degradation of carotenoids.

Whilst carotenoid biosynthesis gene sets were not enriched in the analysis, the cutin biosynthesis pathway, the xylogalacturonan biosynthesis pathway, and the suberin monomers biosynthesis pathway were all significantly down-regulated in R3 (low retention line) compared to R8 (high retention line). These three gene sets all play critical roles in the synthesis of the fruit cuticle. This down–regulation of these gene sets supports the biochemical differences observed in cuticle structure between lines R3 and R8, as described in section 5.2.2 and section 5.2.4, in which the low retention lines had a thinner cuticle, and decreased cutin monomers.

Genes involved in cuticle biosynthesis were identified in pepper, by identifying orthologs to genes in other species which have been reported to be involved in cuticle biosynthesis. Significantly differentially expressed genes between the high and low carotenoid retention lines are reported in Table 5-4. The pepper ortholog of the ABC transporter ABCG32, which functions in depositing cutin monomers at the cuticular surface in *Aradiposis thaliana* (Bessire et al. 2011), was identified as being significantly up-regulated in the high carotenoid retention line compared the low line. Lipid transfer proteins (LTPs) are considered to be involved in the transport of cutin monomers to the site of cuticle synthesis (Yeats and Rose, 2008). Several LTPs were identified as differentially expressed between the high and low carotenoid retention lines, however, some were up-regulated in the high retention line, whilst others were down-regulated. *Bodyguard* (*BDG*) has been identified as playing a role in cutin synthesis (Kurdyukov et al. 2006), and an ortholog of this gene was found to be up-regulated in the high carotenoid retention line compared to the low line. Interestingly, a MYB16 transcription factor was found to be down-regulated in the high carotenoid retention line early in fruit development. The ortholog of this gene has been shown to be involved in the regulation of cuticle development, along with *WIN1/SHN1* in *Arabidopsis thaliana* (Oshima et al., 2013), and so it may have been expected that this gene would be up-regulated in the high carotenoid retention line, as this line had a thicker fruit cuticle compared to the low carotenoid retention line. However, WIN1/SHN1 orthologs were

not found to be differentially expressed in pepper (Table 5-4). Two genes were annotated as *CER1* in the pepper Sol Genomics Network, and interestingly they displayed differences in expression between the high and low retention lines. CA09G18740 was shown to be up-regulated in the high retention line at anthesis + 45 days, whilst CA12G22670, also annotated as *CER1* was shown to be down-regulated in the high retention line in ripe fruit. *CER1* is involved in reduction and decarbonylation of very long chain fatty acids to cuticular alkanes (Bernard et al., 2012). The up-regulation of CA09G18740 therefore was consistent with the biochemical changes observed in Figure 5-8D, as an increase was observed in alkane wax components in high carotenoid retention lines. A cytochrome P450 enzyme MAH1, a midchain alkane hydroxylase, is involved in the oxidation of alkanes to secondary alcohols and ketones in *Arabidopsis* (Greer et al., 2007). *MAH1* genes were found to be up-regulated in the high carotenoid retention line compared to the low retention line throughout fruit development (Table 5-4).

Table 5-4 Cuticle biosynthesis-linked genes differentially expressed during fruit development.

RNAseq was performed on the epidermal tissue of the high retention line, R8, and low retention line, R3. Transcript number was assessed at four fruit development stages: anthesis +20, anthesis +30, anthesis +45, and ripe. Genes were identified based on literature searches, and using BLAST to identify gene orthologs. Sol Genomic Network gene descriptions are noted, if provided. Significant gene expression changes: R3 down-regulated = red, R3 up-regulated = blue. Genes were considered to be significantly differentially expressed if p < 0.05, FDR < 0.05. Three biological replicates per line were analysed.

Pepper gene	SGN annotation	Description	A+20	A+30	A+45	Ripe	Literature	Ortholog	Ortholog Function
CA06G14420	ABC transporter	Putative ABC transporter					Yeats and Rose 2013	At2G26910	ABCG32 full transporter
CA06G14430	Unknown function						Yeats and Rose 2013	At2G26910	ABCG32 full transporter
CA01G00270	Valacyclovir hydrolase	Putative					Yeats and Rose 2013	At4G24140	BDG3 BODYGUARD3
CA01G19070	CD2	Cutin deficient 2					Yeats and Rose 2013	Solyc01G091630	CD2 CUTIN DEFICIENT2
CA05G18530	Unknown function						Yeats and Rose 2013	At3G55360	CER10 ECERIFERUM10 Enoyl-CoA reductase
CA09G18740	CER3	Predicted protein ECERIFERUM 3-like					Yeats and Rose 2013	At5G7800	CER3 ECERIFERUM3 involved in alkane formation
CA11G06990	CER3	Predicted protein ECERIFERUM 3-like					Yeats and Rose 2013	At5G7800	CER3 ECERIFERUM3 involved in alkane formation
CA02G23370	3-ketoacyl-CoA synthase	3-ketoacyl-CoA synthase					Yeats and Rose 2013	At1G68530	CER6 ECERIFERUM6 b-Ketoacyl-CoA synthase
CA05G19790	Cytochrome P450	Cytochrome P450					Yeats and Rose 2013	At3G10570	CYP77A6 CYP77A subfamily
CA08G18140	Predicted long chain acyl-CoA synthetase 2-like	Predicted long chain acyl-CoA synthetase 2-like					Yeats and Rose 2013	At1G49430	Long chain acyl-CoA synthase
CA08G08360	Predicted long chain acyl-CoA synthetase 4-like	Predicted long chain acyl-CoA synthetase 4-like					Yeats and Rose 2013	At1G64400	Long chain acyl-CoA synthase
CA03G30090	Unknown function						Yeats and Rose 2013	At3G28910	MYB30 MYB transcription factor
CA02G28250	Transcription factor	Transcription factor					Yeats and Rose 2013	At3G28910	MYB30 MYB transcription factor

CA03G30090	Unknown function		Yeats and Rose 2013	At5G62470	MYB96 MYB transcription factor
CA02G28250	Transcription factor	Transcription factor	Yeats and Rose 2013	At5G62470	MYB96 MYB transcription factor
CA10G12030	LTP	Lipid transfer protein	Popovsky-Sarid et al. 2017		
CA10G12060	LTP	Lipid transfer protein	Popovsky-Sarid et al. 2017		
CA09G18740	CER1	Fatty acid hydroxylase	Popovsky-Sarid et al. 2017		
CA12G22670	CER1	Fatty acid hydroxylase	Popovsky-Sarid et al. 2017		
CA11G14620	MYB16	MYB-related transcription factor	Popovsky-Sarid et al. 2017		
CA02G20380	LCFL	Long chain fatty acid CoA ligase	Popovsky-Sarid et al. 2017		
CA03G16520	ERF	Ethylene responsive factor 2A	Popovsky-Sarid et al. 2017		
CA02G17970	PE	Pectinesterase	Popovsky-Sarid et al. 2017		
CA07G11250	ACC OXIDASE	Fruit ripening	Popovsky-Sarid et al. 2017		
CA10G16170	ACC OXIDASE	Fruit ripening	Popovsky-Sarid et al. 2017		
CA10G10710	LTP	Lipid transfer protein	Popovsky-Sarid et al. 2017		
CA10G10770	LTP	Lipid transfer protein	Popovsky-Sarid et al. 2017		
CA10G18900	CYP96A/MAH1	Cuticle development	Popovsky-Sarid et al. 2017		
CA10G18910	CYP96A/MAH1	Cuticle development	Popovsky-Sarid et al. 2017		
CA10G08490	LTP	Lipid transfer protein	Popovsky-Sarid et al. 2017		
CA10G18310	CYTB5	Cuticle development	Popovsky-Sarid et al. 2017		

5.3. Discussion

5.3.1. Pepper fruit cuticle structure characterisation

The cuticle of fruit species has been acknowledged as a modulator of post-harvest quality (Lara et al., 2014), and thus, it is important to understand the cuticle structure and how it may be influencing other post-harvest quality traits. Firstly, examining exocarp thickness indicated that carotenoid retention is correlated with exocarp thickness. Furthermore, when compared to the classification of lines as smooth or cracked cuticles (Chapter 4), smooth cuticle genotypes possessed a thicker cuticle. This trend has also been observed in cherry tomato, in which resistance to cuticle cracking correlates with a thicker fruit cuticle (Matas et al., 2004). Therefore, thicker exocarps are correlated with both high carotenoid retention and the smooth cuticle phenotype.

Previous studies examining pepper fruit cuticle structure have analysed this trait as a means of understanding fruit water loss mechanisms (Maalekuu et al., 2005, Parsons et al., 2012, Parsons et al., 2013). Studies assessing cuticle composition changes in relation to plant water loss have displayed vast variation in cuticular composition across different pepper genotypes. A study of 50 diverse pepper genotypes revealed a greater than 14-fold range for total wax amount, and a 16-fold range for cutin monomer amount, when comparing the most extreme genotypes (Parsons et al., 2013). Using this information, QTLs determining fruit post-harvest water loss in pepper have been revealed (Popovsky-Sarid et al., 2017). Whilst fruit water loss is an important post-harvest quality trait, the study presented here aims to understand further phenotypic changes upon fruit dehydration. Fruit water loss assays have not directly been performed in this study, however high carotenoid retention lines have been shown to be less prone to cuticle cracking. These lines appear to take longer to dry following harvest as the cuticle is not compromised, and could be said to have a low water loss rate. These high retention lines have been shown to have increased total cutin monomers compared to their low retention counterparts (Figure 5-6). Total cuticle wax amounts do not show any differences between high and low retention lines (Figure 5-8). It is important to note that analysis of cuticle wax composition was not comprehensive in this study, as wax components possessing a chain length up to C_{34} were not analysed. Therefore, cuticle waxes analysed in this study did not differ between high and low retention lines, however, longer chain wax components should be analysed to determine whether they contribute to carotenoid retention. It has previously been shown that in a diverse pepper collection, neither total cutin monomers nor total cuticle waxes correlated with water loss rates (Parsons et al., 2013). Therefore, whilst the lack of difference in total cuticle wax amounts between high and

low retention lines supports the previous findings, differences were observed between high and low retention lines in total cutin monomers, and this fails to support the previous study. Whilst these prior studies are useful for comparing cuticle composition and the resulting phenotypes, the nature and biological relevance of these studies is fundamentally different, and therefore it is unsurprising that differences in trends are observed.

5.3.2. Pepper exocarp provides a protective barrier to prevent carotenoid degradation during post-harvest storage

Carotenoid retention has been shown to be linked to fruit exocarp structure, as both thicker and smoother exocarps and greater total cutin monomers are associated with the high carotenoid retention phenotype. This correlation may be observed as the thicker waxy exocarp present in high carotenoid retention lines protects carotenoids within the fruit from being degraded during storage. High carotenoid retention lines have been shown to be less susceptible to fruit cuticle cracking, and thus, this may offer an explanation as to why carotenoids are more protected from degradation than in low retention lines. Charged molecules have been shown to permeate the cuticular membrane through aqueous pores in some leaves (Schönherr and Schreiber, 2004); and this shows that the cuticle is permeable to some extent by compounds in the environment. Oxygen, which may be converted to harmful oxidative agents, including singlet oxygen and hydroxyl radicals, may permeate into fruits. These harmful oxidative agents may result in the oxidative degradation of carotenoids, as carotenoids scavenge reactive oxygen species (ROS) (Stahl and Sies, 2003). Wounding of pepper fruit cuticle has previously been shown to dramatically increase the permeability of the fruit surface to oxygen (Banks and Nicholson, 2000). Permeability may increase in pepper lines with a cuticle more susceptible to cracking. This explains why low carotenoid retention lines, with thin cuticles more susceptible to cracking, may have a lower capacity to retain carotenoids during storage, as they are more readily degraded.

5.3.2.1. Cuticle biosynthesis

Numerous genes previously identified as playing a role in cuticle biosynthesis were demonstrated to differ in expression between the high and low carotenoid retention lines, during fruit development (Table 5-4). Genes identified previously as involved in pepper cuticle biosynthesis (Popovsky-Sarid et al., 2017) were amongst those identified as differentially expressed between the high and low carotenoid retention lines. It is important to note that these genes have not previously been functionally characterised in pepper cuticle biosynthesis. Several genes involved in the biosynthesis of *Arabidopsis thaliana* cuticle (Yeats and Rose, 2013) were found to have

orthologs in pepper, which were differentially expressed between the high carotenoid retention line, with a thick exocarp, and the low carotenoid retention line, with a thinner fruit exocarp. An ortholog of the *Bodyguard* gene (*BDG*), known in *Arabidopsis thaliana* to be involved in cutin biosynthesis (Kurdyukov et al., 2006a), is significantly up-regulated in the high retention line. This supports the finding that cutin monomer content is also increased in this high retention line (Figure 5-4). However, the *Cutin Deficient 2* (*CD2*) gene, which in tomato has been reported to regulate cutin monomer biosynthesis (Isaacson et al., 2009), is up-regulated in the low retention pepper line. This finding was unexpected, as the high carotenoid retention lines have increased cutin monomer levels. This may demonstrate an example in which the regulation of cuticle biosynthesis differs between species. Despite the fact that a *cd2* tomato mutant was found to have a decrease in cutin content of 98 %, suggesting the crucial role of *CD2* in regulating cutin biosynthesis in tomato fruit (Isaacson et al., 2009), the same influence of this gene may not be exerted in pepper.

Two genes annotated as *CER1*, the gene shown to be involved in the reduction and decarbonylation of very long chain fatty acids to cuticular alkanes in *Arabidopsis thaliana* (Bernard et al., 2012), are differentially expressed in high and low carotenoid retention pepper fruits. However, one candidate was up-regulated in the high carotenoid retention line at anthesis + 45 days, whilst the second candidate was down-regulated in the high carotenoid retention line in ripe fruit. *CER3* genes are also involved in the formation of cuticular alkanes from very long chain fatty acids (Bernard et al., 2012), and two orthologs of the *Arabidopsis* gene were identified as differentially expressed in the pepper lines. Again, one *CER3* candidate was up-regulated in the high retention line at anthesis + 45 days, whilst the other candidate was down-regulated in the high carotenoid retention line at time points anthesis + 20 days, anthesis + 30 days, and anthesis + 45 days. Further to this, two genes annotated as *MAH1* were shown to be differentially expressed, with one candidate significantly up-regulated in the high retention line at anthesis + 30 days, and anthesis + 45 days, whilst the second *MAH1* candidate was up-regulated in the high carotenoid retention line throughout fruit ripening. *MAH1* has previously been identified in pepper (Popovsky-Sarid et al., 2017) as involved in the modification of alkanes to form secondary alcohols and ketones. The cytochrome P450 enzyme MAH1 has been shown to be a midchain alkane hydroxylase in *Arabidopsis* (Greer et al., 2007). The significant differential expression of these genes involved in cuticular wax biosynthesis is interesting as no difference was observed in cuticular wax content between the high and low carotenoid retention lines (Figure 5-8). However, it is important to note that not all cuticular wax components were measured in this study, and therefore, the gene

expression changes may explain changes in cuticular wax composition that are not reported here. The fact that some genes involved in cuticular wax biosynthesis are up-regulated whilst others are down-regulated in the high carotenoid retention line suggests the complexity of this biosynthetic process, and the importance of regulation of gene expression.

Interestingly, overexpression of the tomato MIXTA-like MYB transcription factor (*SlMX1*) resulted in increased cuticle deposition in the peel, along with an increase in total fruit carotenoid content (Ewas et al., 2016). This not only confirms the regulatory role of *SlMX1* in cuticle formation, as has previously been discussed in Chapter 1, but also suggests cuticle-associated genes influencing fruit carotenoid content. Whilst homologs of this gene were not found to be differentially expressed between the high and low carotenoid retention pepper lines, it suggests the contribution of the cuticle to influencing carotenoid content.

5.3.3. Carotenoids are associated with fruit exocarp in pepper

5.3.3.1. Exocarp-associated carotenoids

Interestingly, carotenoids have been shown to be associated with the ripe pepper fruit exocarp (Figure 5-10, Figure 5-11). This phenomenon has not previously been observed in pepper, nor in other similar fleshy fruit species, such as tomato. Whilst carotenoids have not previously been associated with fruit exocarp, citrus fruits contain significant amounts of carotenoids within their peel (Oberholster et al., 2001). The fruit exocarp of pepper and fruit peel of citrus fruits are not morphologically similar, however this does provide an example in which carotenoids are stored within tissues directly in contact with the environment. Furthermore, the peel of citrus fruit also provides a protective barrier to the interior fruit, in a similar way to the exocarp protecting pepper fruits from the environment. Whilst it is not currently understood why carotenoids are associated with the epidermal layer in pepper, a detailed understanding of this mechanism may shed light on the carotenoid retention phenotype, along with other processes such as cuticle biosynthesis in pepper. It may be interesting to determine the role of the phytochrome-interacting factor (PIF) family. As this family of transcription factors down-regulates carotenoid accumulation by repressing phytoene synthase (*PSY*) until light-triggered degradation of PIFs results in rapid derepression of *PSY* expression, and a burst in carotenoid synthesis (Toledo-Ortiz et al., 2010), it would be interesting to determine whether these transcription factors affect the accumulation of carotenoids within the epidermal layers of these fruits.

5.3.3.2. Increased exocarp carotenoid content is not due to an increase in chromoplast number

An increase in the carotenoid content in the exocarp relative to the whole fruit is observed in high retention lines compared to low retention lines, however this is neither due to an increase in chromoplast number in epidermal cells, nor due to an increase in carotenoid biosynthesis gene expression (Section 5.2.6.3). Interestingly, ROS are known to be central players in cell signalling (Mittler et al., 2011). ROS have been shown to induce chromoplast specific carotenoid gene expression in the process of chromoplast differentiation (Bouvier et al., 1998). Major differences were not observed in plastid number between high and low retention pepper lines (Figure 5-12), and this suggests that chromoplast differentiation occurs to a similar extent in the lines studied. Therefore, endogenous ROS levels may be similar at the ripening time point and only initiate differences in carotenoid content following chromoplast differentiation. This further supports the hypothesis that ROS-mediated carotenoid degradation upon fruit drying and cuticle cracking initiates the differences observed in carotenoid retention phenotype. The predominant difference between the high and low retention lines is the amount of carotenoid stored in these chromoplasts in ripe fruit (Figure 5-10). It appears as though epidermal chromoplasts within high retention lines have a greater capacity for carotenoids compared to low retention lines. This may be linked to subchromoplast organelle structures, for example, fibrillar plastoglobuli, which are involved in the sequestration and storage of esterified carotenoids in pepper (Deruère et al., 1994). An increase in fibrillar structures would facilitate the accumulation of increased pigment levels within the chromoplasts of high carotenoid retention lines, as has previously been demonstrated (Berry et al., 2019).

5.3.3.3. Esterification of exocarp carotenoids is associated with high retention phenotype

The ratio of exocarp to whole fruit capsanthin diester content is significantly greater in high retention lines than low retention lines. This suggests that the waxy, lipophilic environment of the exocarp favours the storage of more non-polar carotenoids, specifically diesters. Esterified carotenoids have been shown to be more stable than their non-esterified counterparts (Schweiggert et al., 2007). This could explain the increase in total carotenoid content of epidermal cells in high retention lines, as esterified carotenoids increase the stability of these compounds, and make them less susceptible to oxidative degradation.

Increased carotenoid content within high retention pepper fruit epidermal cells was not due to an increase in carotenoid biosynthesis gene expression, but may be due to an increase in carotenoid biosynthetic enzyme activity. This would allow carotenoid biosynthesis to continue, despite no increase in gene expression. Gene expression does not necessarily reflect enzyme activity, due to an array of post-translational modifications that may occur. In order to test this hypothesis, enzyme activity studies would need to be carried out, however enzyme activity measurements specifically of epidermal cells would be logistically challenging.

5.3.3.4. Carotenoid localisation within fruit may affect retention classification

Localisation of carotenoids within epidermal cells of pepper fruits could also directly influence the carotenoid retention phenotype, as increased carotenoids towards the surface of the fruit could affect the retention classification. If more carotenoids are located in the epidermal cells of some pepper fruits, they may be classified as high retention, as these fruits appear more intensely pigmented than those fruits which cannot store as many carotenoids in these epidermal cells. If these carotenoids are also less susceptible to degradation due to the level of esterification, these pepper varieties may appear to have a high capacity for retaining carotenoids.

5.4. Conclusion

Fruit surface structure is clearly closely associated with the carotenoid retention phenotype. Two fruit surface structure-associated mechanisms appear to play fundamental roles in controlling the carotenoid retention phenotype. Firstly, thicker fruit exocarps protect fruits from harmful reactive oxygen species (ROS), free radicals, and their precursors, by offering a thick barrier to oxygen, as the precursor to ROS. This is supported in the increase in cuticle biosynthetic gene expression observed in high carotenoid retention pepper fruits, along with an increase in cutin monomer content. Therefore, carotenoids in fruits with thick exocarps are less susceptible to oxidative degradation, compared to their thin exocarp fruit counterparts.

Secondly, fruits with thicker exocarps offer a more favourable environment for carotenoid storage and accumulation. Whilst plastid number within epidermal cells remains constant between high and low retention lines, and carotenoid biosynthesis gene expression is not affected, thick exocarp lines accumulate more carotenoids in their epidermal cells than thin exocarp lines at the ripe time point. The waxy exocarp layer offers a protective environment, and this may facilitate greater carotenoid biosynthetic enzyme activity. Greater levels of carotenoids are stored within epidermal

cells of high retention lines, and this may be due to the elevated activity of biosynthetic enzymes. This supports the findings of Chapter 3, in which carotenoid content increases during post-harvest storage, presumably due to increased enzyme activity as opposed to gene expression. A thicker exocarp protects the fruit through storage, and allows for ongoing carotenoid biosynthesis, due to increased biosynthetic enzyme activity. The lipophilic environment of the exocarp may also allow increased storage of esterified carotenoids, within fibrillar structures.

Together, this suggests that high carotenoid retention lines may have two fruit surface-associated mechanisms to facilitate the retention of carotenoids. In high retention lines, the cuticle protects carotenoids from being degraded, which is supported by an increase in total cuticle components and cuticle biosynthesis gene expression. Secondly, the waxy exocarp provides a protective environment in which carotenoid biosynthetic enzymes may continue to be active during post-harvest storage, and esterified carotenoids can be stored more efficiently.

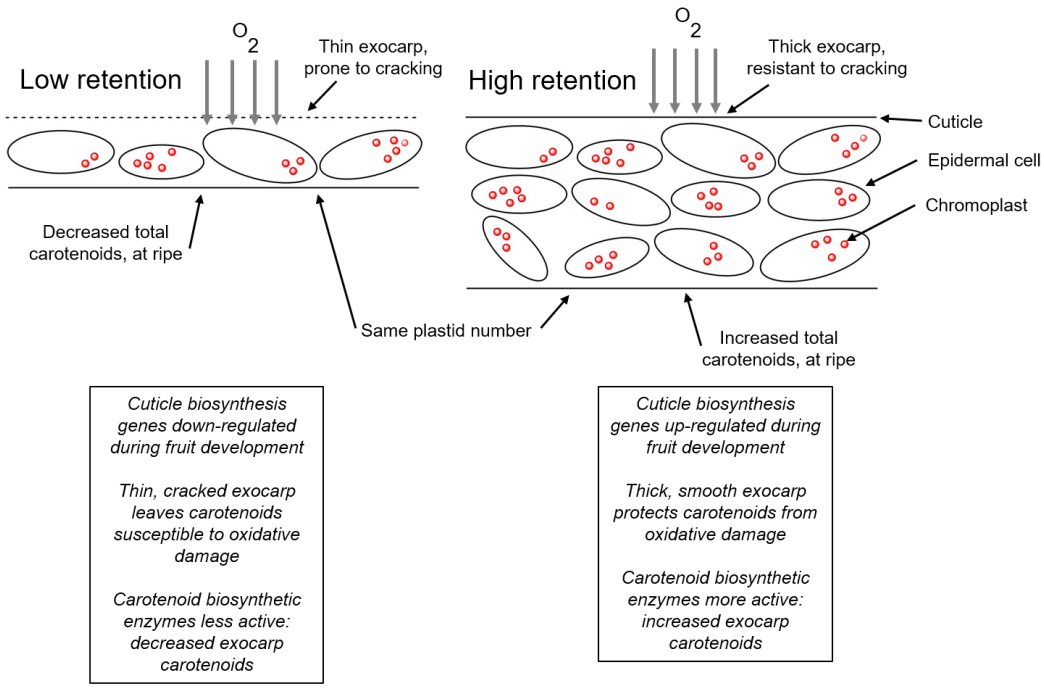


Figure 5-15 Proposed mechanisms by which fruit surface structure influences carotenoid retention phenotype.

6. Identification of genes involved in carotenoid retention and methods for gene functional characterisation

6.1. Introduction

Following the biochemical and physiological analysis of pepper varieties displaying variation in the colour retention phenotype, described in chapters 3, 4, and 5, the molecular basis for this trait was subsequently investigated.

Identification of candidate genes involved in the trait being investigated may be isolated using several methods. RNAseq studies determine the difference in gene expression between samples and experimental conditions, whilst QTL analysis is a method for identifying genomic regions associated with the trait of interest. Combined, these methods can be very powerful in narrowing down potential candidate genes.

Potential gene candidates which may be involved in controlling carotenoid retention include those involved in carotenoid biosynthesis, carotenoid sequestration and storage, along with the cuticle biosynthesis genes previously discussed in chapter 5.

Functional characterisation of genes is essential to prove their function in an organism. The ability to stably transform an organism is useful in being able to silence or overexpress genes, in order to prove their function. For example, in tomato, many studies have silenced or overexpressed genes using stable transformation. The *de-etiolated1* (*det1*) gene was suppressed in tomato to prove its function (Davuluri et al., 2005), and both *hmgr-1* and *dxs* were overexpressed in tomato to manipulate both the mevalonic acid (MVA) and the methylerythritol-4-phosphate (MEP) pathways resulting in increased levels of isoprenoid precursors (Enfissi et al., 2005). More recently, the development of sequence specific nucleases, such as CRISPR-Cas9 technology, has proven an excellent method for assessing the roles of genes within organisms. The simultaneous editing of six agronomically important genes by CRISPR-Cas9 in wild tomato plants, resulted in progeny which produced fruit similar to those observed in domesticated plants (Zsögön et al., 2018). Evidently, these methods of stable transformation are highly useful, not only for gene functional characterisation, but further for the genetic manipulation of plants, in order to produce varieties with improved metabolic and physiological traits.

However, whilst these methods are clearly of great use, not all plants are amenable to stable transformation, including chilli pepper. Therefore, alternative transient transformation must be used in these species, in order to functionally characterise genes of interest. This is an important tool in order to prove gene function, however its usefulness is limited, as stable lines can not be generated to produce varieties with improved traits. Virus Induced Gene Silencing (VIGS) is a method of transiently transforming plants, in order to functionally characterise genes.

6.2. Results

6.2.1. Transcriptomic analysis of extreme high and low carotenoid retention lines

The difference in expression of genes between the low carotenoid retention (R3) line, and the medium/high carotenoid retention (R8) line, was analysed in order to identify genes which may be linked to the carotenoid retention phenotype. Genes identified as significantly differentially expressed may be good candidates for genes controlling the retention trait, as their expression profile is different within these two differing pepper lines. Three time points in ripening were analysed in both lines, being at breaker, breaker + 3 days, and ripe (Figure 6-1). Different ripening stages were selected, as carotenoid biosynthesis and accumulation occurs during the ripening process. Therefore, it was considered that genes involved in the carotenoid retention trait were more likely to be expressed during ripening compared to earlier stages in development. Other factors may influence fruit phenotypes earlier in development, as seen in the cuticle in chapter 5. An independent gene expression study throughout development was carried out. Therefore, this study aims to identify ripening-associated carotenoid retention candidate genes.

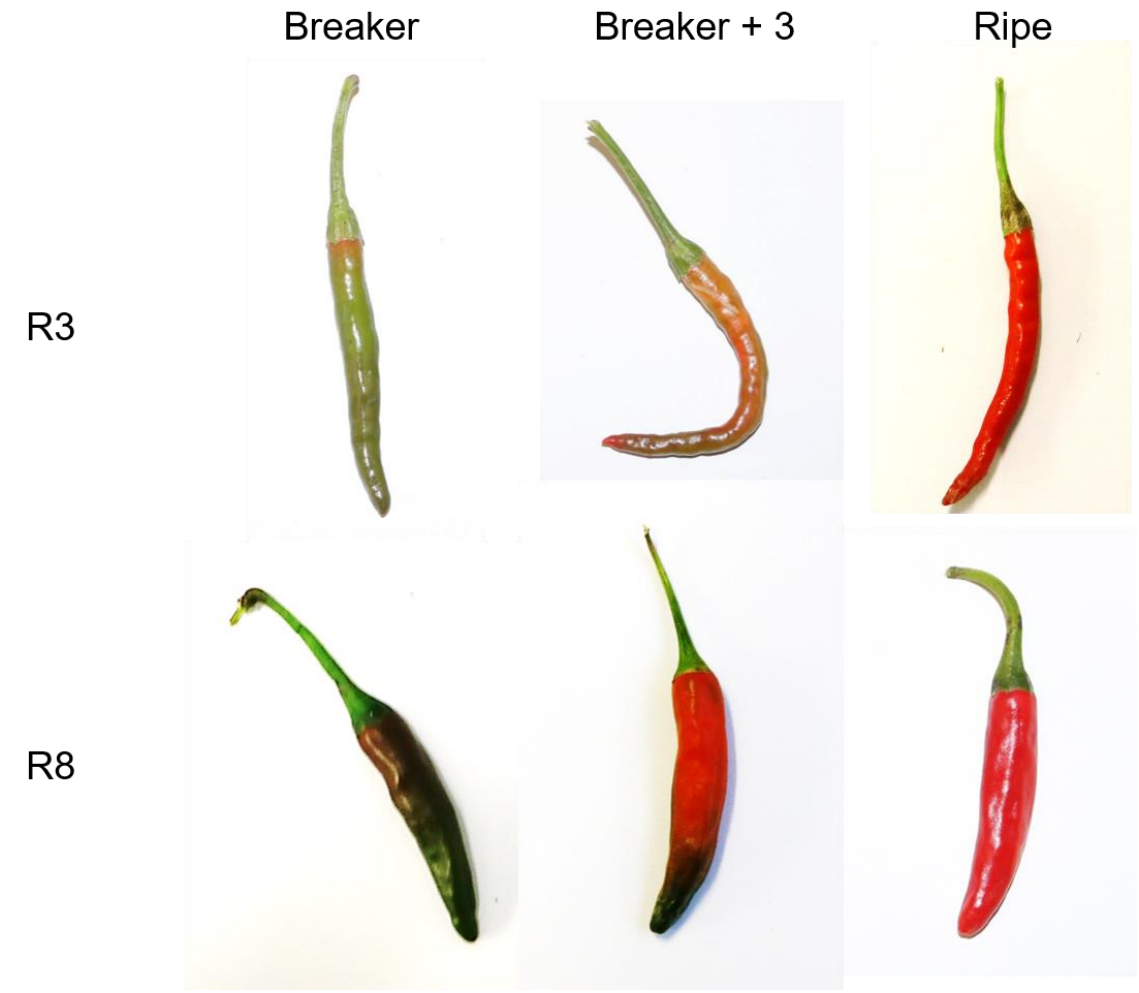


Figure 6-1 Pepper fruits analysed by RNAseq to determine difference in gene expression.

A low carotenoid retention and a high carotenoid retention pepper variety were analysed by RNAseq to determine differences in gene expression between these two varieties. Low retention line, R3, and medium/high carotenoid retention line, R8, were analysed. Each line was analysed at three independent time points during ripening: breaker, breaker + 3 days, ripe. At least three fruits per plant were pooled per time point, and three biological replicates were analysed (n = 3).

6.2.2. Global transcriptomic statistics

The Cufflinks software package was used to process data, which generated the differentially expressed transcripts (Trapnell et al., 2012). Comparisons were made between lines and time points, to determine the number of differentially expressed genes. Volcano plots show the relationship between fold change of gene expression, and the corresponding significance level (Figure 6-2). Significance level was defined on the y-axis as $-\log_{10}$(p value), whilst gene expression fold change was plotted on the x-axis. Genes with a significant difference in expression were plotted in red. Evidently, the level of significance of a differentially expressed gene is unaffected by the fold change in expression. The majority of points lie between log fold change values of -10 and 10, however significant values are unaffected by the fold change value. Many transcripts with fold change values of below -10 or greater than 10 were not found to be significant, whereas other transcripts with fold change values of close to one were

found to be significant. Consequently, identification of transcripts as significant is clearly sensitive. Some transcript fold changes were defined as infinite; this meant that the transcript was not detected in one of the samples, meaning that gene expression was infinitely increased in the sample in which it was detected.

6.2.2.1. Comparison of high and low carotenoid retention varieties

A comparison of differentially expressed genes was made between the low carotenoid retention line, R3, and the medium/high carotenoid retention line, R8, at three time points during the ripening process: breaker, breaker + 3 days, and ripe. Genes identified as differentially expressed between these two lines during ripening may be involved in the carotenoid retention trait. Significantly differentially expressed genes were identified, and volcano plots were created to display these genes and their corresponding fold change between two conditions (Figure 6-2). This showed that there were many more significantly differentially expressed genes between the two varieties at the breaker time point compared to the ripe time point.

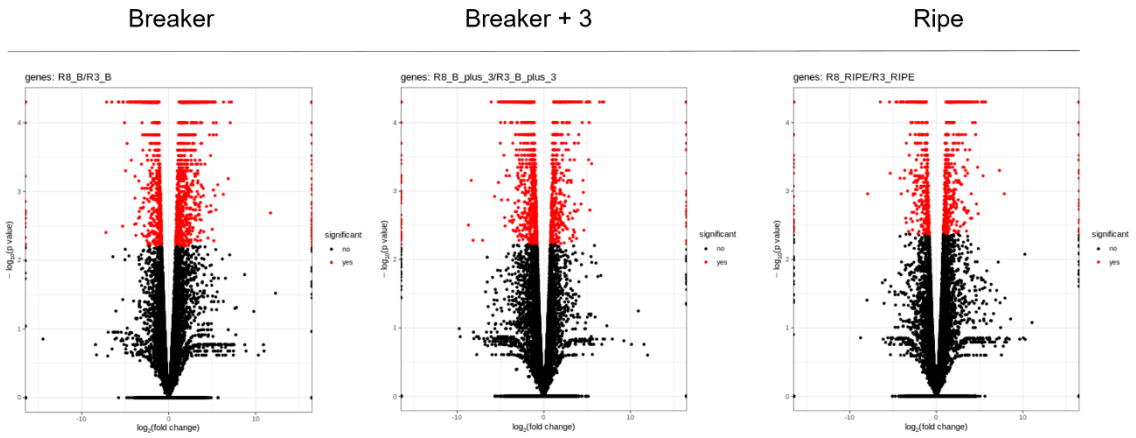


Figure 6-2 Volcano plots displaying the relationship between gene expression fold change and significance between two pepper varieties, through ripening.

Gene expression fold changes and significance were plotted for each comparison made: R3 Breaker vs R8 Breaker, R3 Breaker +3 vs R8 Breaker +3, R3 Ripe vs R8 Ripe. CummeRbund software package in R was used to generate plots. Significantly differentially expressed genes (red), non-significantly differentially expressed genes (black). Three biological replicates per line were analysed (n = 3).

As can be observed from the volcano plots, more genes were differentially expressed between lines R3 and R8 at the breaker time point, with 1869 genes differentially expressed between the two conditions, compared to the ripe time point, with 1131 genes differentially expressed (Table 6-1). This is unsurprising, as many biological changes occur at the start of fruit ripening.

Table 6-1 Number of differentially expressed genes when comparing pepper lines R3 and R8, at different ripening time points.

Gene expression comparisons were made between two pepper varieties: R3 and R8, at three ripening time points: breaker, breaker + 3 days, ripe. CummeRbund software package in R was used to calculate number of significantly differentially expressed genes. Three biological replicates per time point were analysed (n = 3).

Comparison	R8 down-regulated	R8 up-regulated	R8 infinitely down regulated	R8 infinitely up-regulated	Total
Breaker	770	863	28	198	1869
Breaker +3	929	520	116	113	1678
Ripe	433	515	56	127	1131

6.2.2.2. Comparison of ripening time points

6.2.2.2.1. Low carotenoid retention line

The number of significantly differentially expressed was calculated between ripening time points of the low carotenoid retention line, R3. The comparisons made were: breaker vs breaker + 3 days, breaker vs ripe, breaker + 3 days vs ripe. Volcano plots display the relationship between transcript fold change and significance (Figure 6-3), in which the greatest number of significantly differentially expressed genes were between breaker and ripe time points.

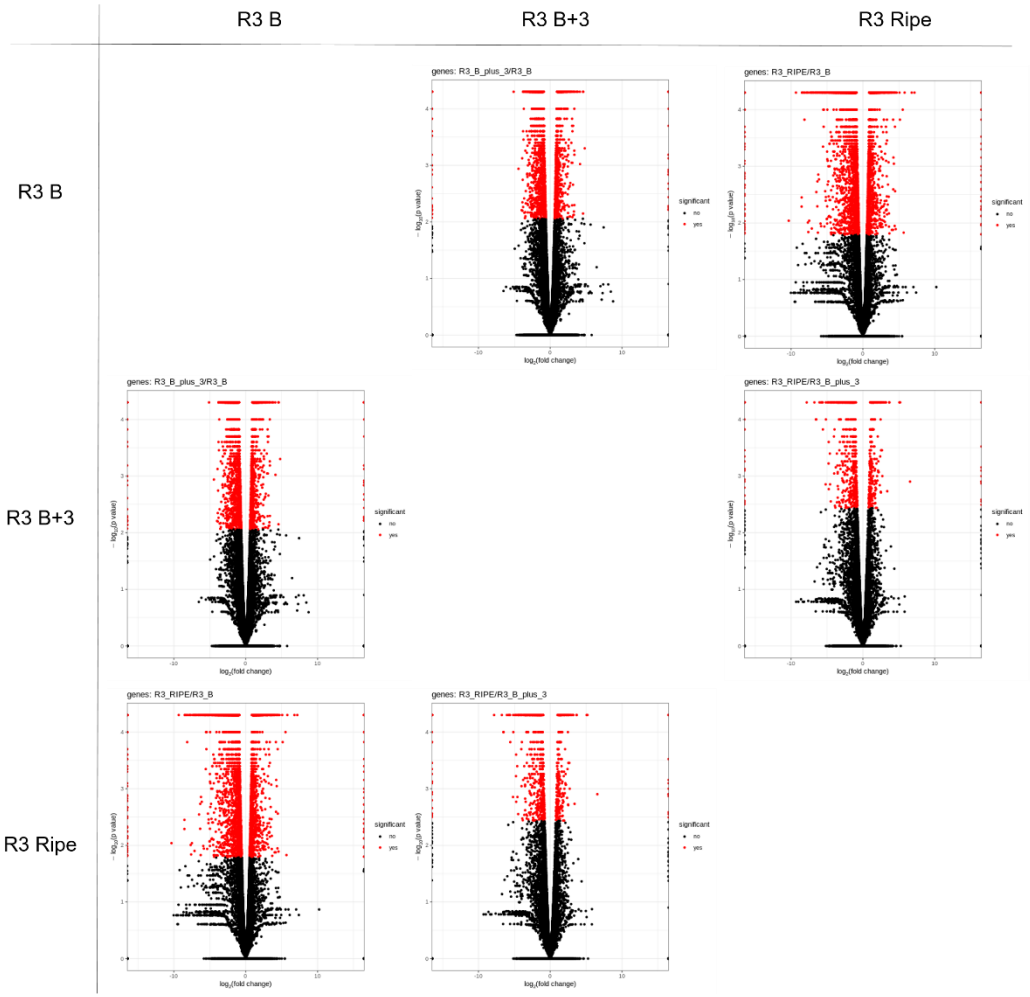


Figure 6-3 Volcano plots displaying relationship between gene expression fold change and statistical significance for line R3 throughout fruit ripening.

Gene expression fold changes and significance were plotted for each comparison made: R3 Breaker vs R3 Breaker +3 days, R3 Breaker vs R3 Ripe, R3 Breaker +3 days vs R3 Ripe. CummeRbund software package in R was used to generate plots. Three biological replicates per time point were analysed (n = 3).

It was evident that the greatest change in gene expression occurred at the breaker stage. The largest change in gene expression occurred between the breaker and ripe stages, with 4509 genes differentially expressed. Only 987 genes were differentially expressed between the breaker + 3 days and ripe stages (Table 6-2).

Table 6-2 Number of differentially expressed genes between ripening time points of low carotenoid retention line, R3.

Gene expression comparisons were made between three ripening time points: breaker, breaker + 3 days, and ripe, for the low carotenoid retention line: R3. CummeRbund software package in R was used to calculate number of significantly differentially expressed genes. Three biological replicates per time point were analysed (n = 3).

Comparison A vs B	B down-regulated	B up-regulated	B infinitely down regulated	B infinitely up-regulated	Total
Breaker vs Breaker +3	1469	960	41	38	2429
Breaker vs Ripe	2571	1636	253	49	4509
Breaker +3 vs Ripe	631	277	64	15	987

6.2.2.2.2. High carotenoid retention line

The number of significantly differentially expressed genes was also calculated between ripening stages for the medium/high carotenoid retention line, R8. Again, volcano plots suggest that the greatest change in gene expression occurred between the breaker and ripe stages, and few changes occurring between breaker + 3 days and ripe stages (Figure 6-4).

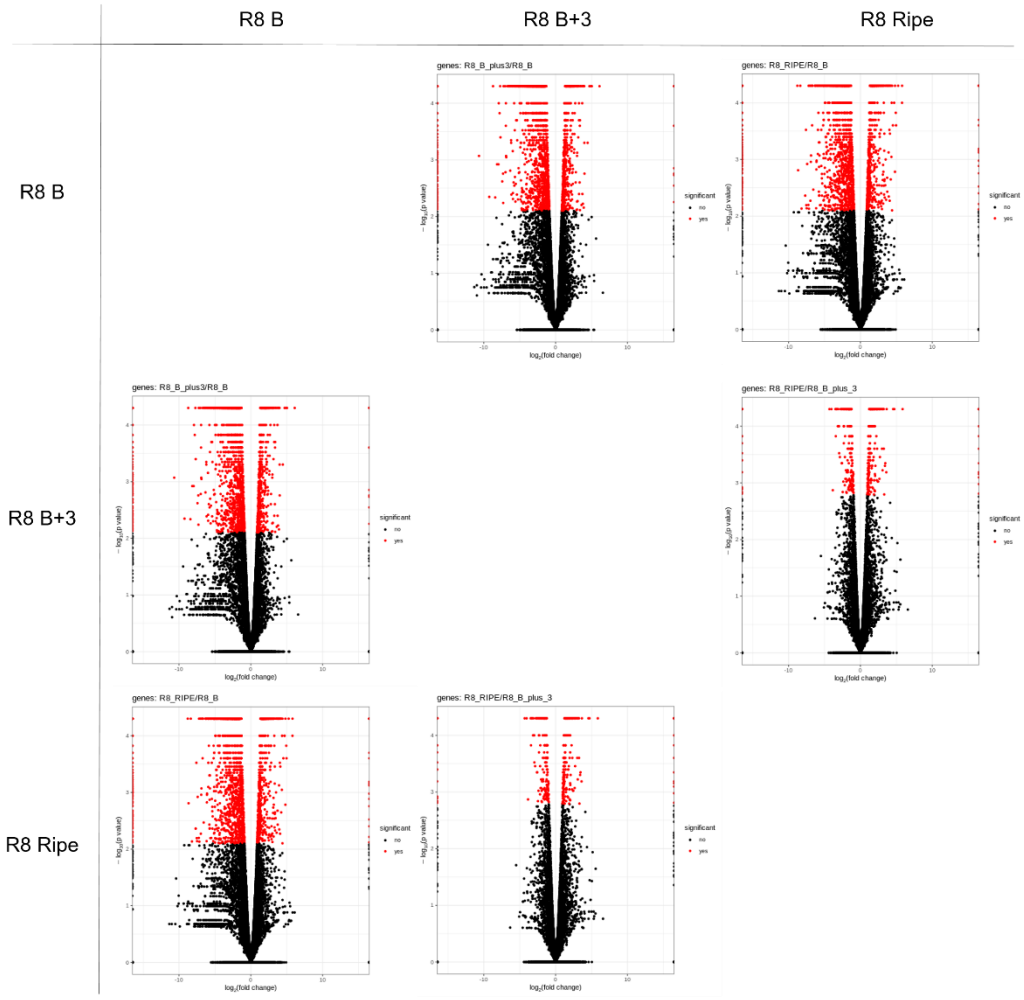


Figure 6-4 Volcano plots displaying relationship between gene expression fold change and statistical significance for line R8 throughout fruit ripening.

Gene expression fold changes and significance were plotted for each comparison made: R8 Breaker vs R8 Breaker +3 days, R8 Breaker vs R8 Ripe, R8 Breaker +3 days vs R8 Ripe. CummeRbund software package in R was used to generate plots. Three biological replicates per time point were analysed (n = 3).

Comparatively fewer genes were differentially expressed at the ripening stages in the high carotenoid retention line, R8, compared to R3. Again, the greatest change in gene expression occurred at the breaker stage, with 2372 genes differentially expressed between the breaker and ripe stages. This was in contrast to the 4509 genes differentially expressed between breaker and ripe in line R3, suggesting that greater transcriptomic changes occur in R3 during ripening compared to R8.

Table 6-3 Number of differentially expressed genes between ripening time points of high carotenoid retention line, R8.

Gene expression comparisons were made between three ripening time points: breaker, breaker + 3 days, and ripe, for the medium/high carotenoid retention line: R8. CummeRbund software package in R was used to calculate number of significantly differentially expressed genes. Three biological replicates per time point were analysed (n = 3).

Comparison A vs B	B down-regulated	B up-regulated	B infinitely down regulated	B infinitely up-regulated	Total
Breaker vs Breaker +3	1464	569	326	11	2370
Breaker vs Ripe	1373	628	348	23	2372
Breaker +3 vs Ripe	177	211	24	25	437

Despite the carotenoid retention trait being a post-harvest trait, this may be influenced by changes in transcript level during ripening, therefore it is important to evaluate changes in gene expression level throughout ripening.

6.2.3. Identification of candidate genes

In order to determine potential candidate genes controlling the carotenoid retention trait, it was important to understand the change in gene expression profile between the two pepper genotypes, throughout ripening. Whilst it was evident that many more genes were differentially expressed between the two pepper genotypes at the breaker time point compared to the ripe time point, some genes were continuously differentially expressed throughout ripening. Only 334 genes were continuously differentially expressed throughout ripening out of a total of 3298 differentially expressed genes at all time points, and therefore, this provided a core set of genes which could be deemed to be constitutively differentially expressed between the two genotypes during ripening (Figure 6-5). This core set of constitutively differentially expressed genes provided a workable sample set of genes, which could be mined to identify candidate genes.

However, carotenoid retention is a post-harvest trait, and therefore it is difficult to determine at which ripening time point gene expression changes may occur to influence the carotenoid retention trait. Consequently, analysing only genes constitutively differentially expressed through ripening may result in genes which are involved in the trait being missed, particularly if these genes are only differentially expressed at one time point during ripening. Therefore, genes differentially expressed at just one or two time points have also been studied to determine whether these may

play a role in carotenoid retention. However, this proved a much larger data set, containing 2964 genes (Figure 6-5).

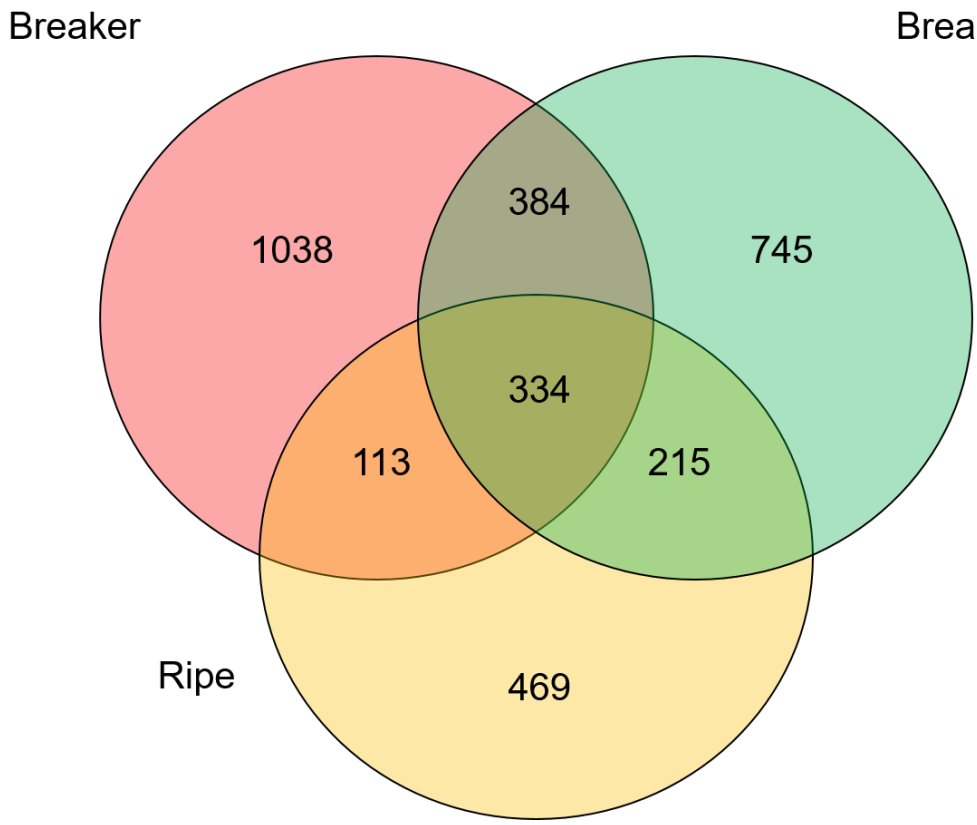


Figure 6-5 Venn diagram displaying number of differentially expressed genes between two pepper varieties, at three ripening time points.

6.2.3.1. Genes constitutively expressed throughout ripening

The 334 genes within the ripening-constitutively expressed core set were initially studied. Using the Plant Reg Map software (Jin et al., 2016), gene ontologies were identified for 259 of the 334 genes within this set. From this, 22 gene ontology terms were enriched, suggesting genes involved with these processes were differentially expressed between the high and low carotenoid retention lines, R8 and R3. For example, one of the gene ontology terms enriched was that for oxidoreductase activity: GO:0016491. 36 genes with oxidoreductase activity were identified by this ontology term. Interestingly, no carotenoid biosynthesis or degradation genes were identified within this ripening constitutive gene set. This is unsurprising, as carotenoid biosynthesis is often linked to specific time points within ripening. Furthermore, it has previously been shown that the carotenoid retention trait is not linked to rate of carotenoid biosynthesis.

6.2.3.1.1. Cu/Zn superoxide dismutase (Cu/Zn SOD)

The 36 genes within the gene ontology enriched term, GO:0016491 were further analysed, and gene CA01G25550 was identified as a potential candidate. This gene

was identified as Cu/Zn superoxide dismutase, which has previously been discussed as playing a potential role in the carotenoid retention trait (Berry, 2015). Superoxide radicals are converted to hydrogen peroxide by action of a group of enzymes known as superoxide dismutases. Within this family is the Cu/Zn SOD enzyme (Figure 6-6). In the comparison of the low carotenoid retention line, R3, and high carotenoid retention line, R8, this gene was consistently up-regulated in the high carotenoid retention line compared to the low retention line, at all three ripening time points studied.

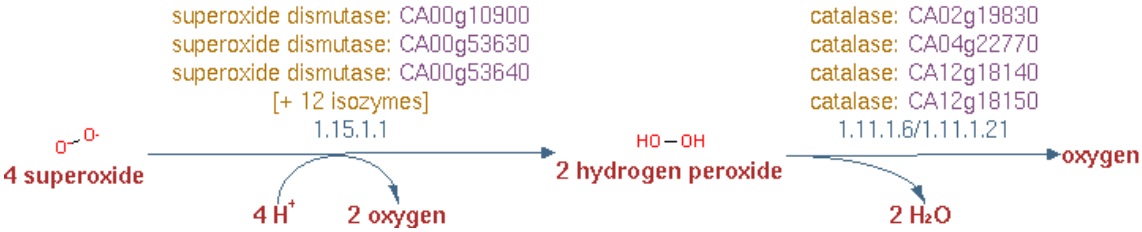


Figure 6-6 Superoxide radical degradation.

Superoxide radicals are converted to hydrogen peroxide by the action of a group of enzymes, known as superoxide dismutases. Within this group of enzymes, the gene Ca01g25550 is present, which encodes the Cu/Zn SOD enzyme. This gene was differentially expressed between high and low carotenoid retention lines throughout fruit ripening. The second step of superoxide radical detoxification converts hydrogen peroxide generated by superoxide dismutase to water and oxygen, by action of the catalase enzyme. Genes identified by Plant Metabolic Network software. Figure adapted from Plant Metabolic Network.

Catalase enzymes are responsible for converting hydrogen peroxide generated by superoxide dismutases to oxygen and water. Four genes were identified as encoding catalase enzymes by the Plant Metabolic Network software, including CA02G19830, CA04G22770, CA12G18140, and CA12G18150. As both superoxide dismutase and catalase enzymes are required for superoxide radical detoxification, these genes encoding catalase enzymes were mined for in the differentially expressed gene sets. None of these genes were found to be constitutively differentially expressed throughout ripening as the *Cu/Zn SOD* gene was. However, CA02G19830 was significantly up-regulated in the high retention line at the breaker time point, whilst CA12G18140 was significantly down-regulated in the high retention line at the breaker + 3 days time point. Whilst the up-regulation of CA02G19830 in the high retention line was unsurprising as both genes involved in the superoxide radical detoxification process were increased in the same direction, the fact that CA12G18140 was down-regulated in the high retention line was surprising, as this would appear that these two genes are expressed in different directions. This would suggest that the pathway does not work as efficiently as possible, as whilst the gene encoding the first step in the pathway is up-regulated, the gene encoding the second step in the pathway is down-regulated. Whilst gene expression does not necessarily reflect protein activity, it was surprising that this difference in expression was observed. The difference in expression of these

two related genes may also suggests that this gene is not linked to the carotenoid retention phenotype.

6.2.3.2. Differentially expressed genes in fruit at breaker stage

Of 1869 genes differentially expressed between the low and high carotenoid retention lines at breaker, 1468 genes were identified with gene ontology terms using Plant Reg Map software. 182 gene ontology terms were enriched. Within these 182 enriched terms, some key terms were noted. Three lipid associated gene ontology terms were enriched, including cellular lipid metabolic process (GO:0044255), lipid metabolic processes (GO:0006629), and lipid biosynthetic processes (GO:0008610). Four fatty acid associated terms were enriched: fatty acid biosynthesis (GO:0006633), fatty acid metabolic processes (GO:0006631), very long chain fatty acid-coA ligase activity (GO:0031957), and fatty acid synthase activity (GO:0004312). Four gene ontology terms for oxidoreductase activity were also enriched (GO:0016491, GO:0016903, GO:0016620, and GO:0016717). Whilst this large set of differentially expressed genes was difficult to mine for candidate genes, due to the number of genes present, understanding the gene ontology enrichment is a useful way to understand the processes in which these gene expression changes occurred. Significant changes were observed in lipid and fatty acid metabolism, along with oxidoreductase activity, and this supports findings presented in this chapter and chapter 5.

Pepper orthologs of genes known to be involved in fatty acid biosynthesis were identified, and significantly differentially expressed genes are presented in Table 6-4. The majority of fatty acid biosynthesis genes identified as differentially expressed displayed differential expression between the high and low carotenoid retention lines when fruit were at the breaker stage. 3-keto-acyl-CoA synthase homologs, responsible for the condensation of a long chain acyl-CoA with a malonyl-CoA, were generally found to be up-regulated in the low carotenoid retention line, suggesting increased fatty acid biosynthesis in the low retention line at this stage. Homologs of this gene were also found to be up-regulated in the low retention line at later stages of fruit ripening. However, one homolog: CA10G05460, was found to be up-regulated in the high retention line when fruit were at the breaker stage, as was the 3-keto-acyl- (acyl carrier protein)- synthase homolog: CA01G28560.

Table 6-4 Differential expression of fatty acid biosynthesis genes, throughout fruit ripening.

Pepper orthologs of genes known to be involved in fatty acid biosynthesis were identified, and differentially expressed genes between the high carotenoid retention line (R8) and low carotenoid retention line (R3) are presented. Significant gene expression changes: R3 down-regulated = red, R3 up-regulated = blue. Genes were considered to be significantly differentially expressed if p < 0.05, FDR < 0.05. Three biological replicates per line were analysed.

SGN Gene description	Gene	Process	Expression		
			Breaker	Breaker + 3	Ripe
3-ketoacyl- (acyl carrier protein) - synthase	CA01G28560	Fatty acid biosynthesis			
3-keto-acyl-CoA synthase	CA02G23370				
	CA03G06410				
	CA06G14290				
	CA10G05460				
2,3-enoyl-CoA reductase	CA05G18530				

Interestingly, the only carotenoid biosynthesis associated gene differentially expressed at this time point was *Phytoene synthase 1* (*PSY1*: CA04G04080). This gene was significantly up-regulated in the low carotenoid retention line. This is interesting, as previous data showed that R8 had higher carotenoid levels compared to R3 (Chapter 4). As *PSY1* is the first committed gene in the carotenoid biosynthetic pathway, it may have been expected that *PSY1* expression would be increased in the line containing increased carotenoids. However, as breaker is very early in the ripening process, this difference in expression may be explained if the low carotenoid retention line undergoes a rapid increase in carotenoid biosynthesis early in ripening, whereas the high retention line does not, but accumulates carotenoids over a longer period of time. Furthermore, it is important to note that gene expression does not always reflect enzyme activity, due to post-transcriptional changes, and consequently, an increase in *PSY1* expression in the low carotenoid retention line may not necessarily result in an increase in carotenoid content.

6.2.3.3. Differentially expressed genes in fruit at breaker + 3 days

1678 genes were differentially expressed between the high and low carotenoid retention lines at the breaker + 3 days stage. Of these genes, 1316 genes could be identified with gene ontology terms, and 183 gene ontology terms were enriched. Several of the enriched gene ontology terms related to photosynthesis-associated genes, including genes encoding photosystem subunits (CA02G00810, CA06G26270), chloroplast pigment binding proteins (CA08G15590, CA09G10320), and chlorophyll a/b binding proteins (CA10G02050). Interestingly, these photosynthesis-associated genes were all up-regulated in the low carotenoid retention line, R3. This line has previously been shown to contain lower levels of carotenoids compared to the high carotenoid

retention line, R8. Photosynthetic activity is often associated with increased carotenoid levels due to the essential role that carotenoids play in photosynthesis. Therefore, this increased gene expression in the low carotenoid retention line was surprising. However, as breaker + 3 days is during the middle of the ripening process, it may be that these two lines ripened at different rates. If fruits were not as ripe at breaker + 3 days in the low retention line compared to the high retention line, this would explain why increased photosynthesis was occurring in this line. For this reason, breaker + 3 days may not be a useful time point for elucidating candidate genes, as the ripening process may be slightly different in these two lines, resulting in less accurate comparisons.

6.2.3.4. Differentially expressed genes in ripe fruit

Differential expression was observed in 1131 genes when comparing the low and high carotenoid retention lines, and of these, 879 genes were identified with gene ontology terms. 148 gene ontology terms were enriched. Several carotenoid biosynthesis genes were significantly up-regulated in the high carotenoid retention line, R8, including zeaxanthin epoxidase (*ZEP*) and violaxanthin de-epoxidase (*VDE*). *DXS* (deoxyxylulose 5-phosphate synthase) which is required for synthesis of the precursor of carotenoid biosynthesis, IPP, was also significantly up-regulated in the high carotenoid retention line, R8.

Interestingly, many chloroplast-linked genes, including those involved in photosynthesis, light-harvesting, and thylakoid structure, were also up-regulated in the high carotenoid retention line, R8, at the ripe time point. Several gene ontology terms were enriched for these processes and structures, including photosynthesis and light harvesting (GO:0009765, GO:0009768), thylakoid membrane (GO:0042651, GO:0009535, GO:0055035, GO:0009579), and the chloroplast (GO:0044434, GO:0009507). This suggested that genes involved in photosynthesis and chloroplast structure and function were more highly expressed in the high carotenoid retention line than the low carotenoid retention line at the ripe time point. As the chloroplast is converted into a chromoplast during ripening, the fact that the high retention line had greater chloroplast-linked gene expression at the ripe time point suggests a slower chloroplast to chromoplast transition when compared to the low retention line. Whilst this line had greater expression of chloroplast-linked genes at the ripe time point compared to the low retention line, comparison of chloroplast-associated gene expression in the high retention line at breaker and ripe suggests that many of these genes were significantly down-regulated at ripe compared to breaker. This was expected, as at breaker, fruits were still largely green, and therefore photosynthesising, whereas at ripe, the fruits were red, and no trace of green colour was observed on the

fruit. This may suggest that the process of converting chloroplasts to chromoplasts was slower in the high retention line compared to the low retention line, and explains why greater transcript levels were present for chloroplast-associated genes in the high retention line at ripe.

6.2.3.5. Genes differentially expressed within putative carotenoid retention QTLs

Quantitative Trait Locus (QTL) mapping was previously performed by Syngenta for the colour retention trait in chilli peppers using image analysis data from the DH population, described in Chapter 3. This provided some information regarding potential genetic loci underlying the colour retention trait in pepper. Five QTL regions were identified as loci potentially underlying the trait, with QTL located on chromosomes 3, 7 (two regions located), 10, and 11. With this information, the differentially expressed transcripts identified in this transcriptomic study were combined with the QTL data, in order to further narrow down the list of potential candidate genes involved in pepper colour retention. Only differentially expressed genes between the high and low carotenoid retention lines located within these five genomic regions were analysed. 327 genes were found to be differentially expressed between the two genotypes at at least one ripening time point. Of these, 39 genes were differentially expressed throughout ripening, whilst 99 genes were only differentially expressed at breaker, 59 genes were only differentially expressed at breaker + 3 days, and 42 genes were only differentially expressed in ripe fruit (Figure 6-7).

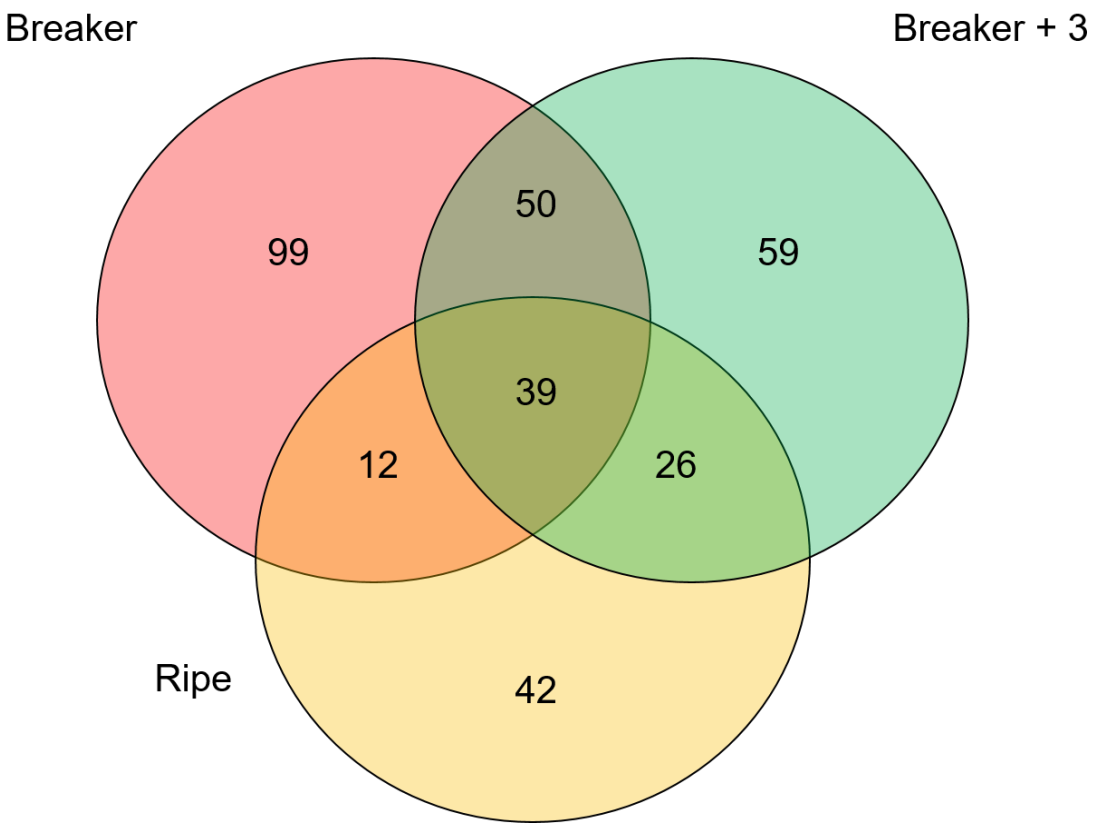


Figure 6-7 Venn diagram displaying number of differentially expressed genes located within carotenoid retention QTL regions.

Differential expression analysis was carried out to determine number of significantly differentially expressed genes between the high carotenoid retention line, R8, and low carotenoid retention line, R3. Three time points were analysed: breaker, breaker + 3 days, ripe. Only genes located within previously identified carotenoid retention QTLs were analysed.

Gene ontology enrichment analysis was carried out on the 39 core genes which were found to be differentially expressed throughout ripening using PlantRegMap software, however, no ontologies were found to be enriched within this gene set. Consequently, gene sets at individual time points were subjected to gene ontology enrichment. 200 genes were differentially expressed at breaker, and 25 gene ontology terms were enriched. 174 genes were differentially expressed at breaker + 3 days, and 35 gene ontology terms were enriched. 119 genes were differentially expressed in ripe fruits, and only two gene ontology terms were enriched.

The most notable enriched gene ontology terms were associated with the differentially expressed genes at breaker. Gene ontology terms were enriched for both lipid transport and lipid localisation, and this reflected the differential expression of four lipid transfer proteins. Three out of these four lipid transfer proteins were significantly down-regulated in the high retention line, R8, whilst one (CA10G14360) was up-regulated in R8. Fatty acid metabolism was also enriched, and differentially expressed genes were up-regulated in the high retention line, R8. Gene ontology terms for wax biosynthesis

and wax metabolism were also enriched, reflecting increased expression in the high retention line relative to the low retention line (Table 6-5).

Table 6-5 Gene ontology enrichment between high and low carotenoid retention lines, of genes located within carotenoid retention QTLs, at breaker stage.

Differentially expressed genes at breaker stage between the high carotenoid retention (R8) and low carotenoid retention (R3) lines were identified. Genes within the previously identified carotenoid retention QTLs were selected. Gene ontology enrichment analysis was performed. Gene ontology terms were selected if potentially linked to carotenoid retention trait. Gene codes and gene descriptions, based on Sol Genomic Network CM334 annotation, provided. Gene expression fold change expressed as log_2(fold change) for R3 transcript number / R8 transcript number.

GO.ID	Term	Gene	SGN description	log2(fold_change)
GO:0006869	Lipid transport	CA10G10710	Non-specific lipid-transfer protein 2-like	-3.46
GO:0010876	Lipid localization	CA10G10750	Non-specific lipid-transfer protein 2-like	-1.90
		CA10G10770	Non-specific lipid-transfer protein 2-like	-3.18
		CA10G14360	Non-specific lipid-transfer protein 2-like	inf
GO:0006631	Fatty acid metabolic process	CA03G33050	Alpha-dioxygenase 2	2.10
		CA10G05460	3-ketoacyl-CoA synthase	1.54
		CA10G11100	Acyl-CoA oxidase 4	1.47
		CA10G12310	12-oxophytodienoate reductase 1-like	2.47
		CA11G06990	Protein ECERIFERUM 3-like	-2.31
GO:0010025	Wax biosynthetic process	CA10G05460	3-ketoacyl-CoA synthase	1.54
GO:0010166	Wax metabolic process	CA11G04880	RNA-directed RNA polymerase	1.70

6.2.4. Virus Induced Silencing as a method for gene functional characterisation

In order to determine the function of candidate genes within pepper fruit, a method of functional characterisation had to be developed. As stated previously, stable transformation has not proved possible to date in pepper, and therefore, a transient method was used. Virus Induced Gene Silencing (VIGS) utilises viral vectors which carry a fragment of a gene of interest. Double stranded RNA is generated, and this initiates the silencing of the target gene within the plant. This technique has been used in several previous pepper studies. In this study, silencing of pepper carotenoid genes was carried out as a proof of concept that this method would work for further gene functional characterisation studies.

6.2.4.1. Construction of vectors for gene silencing

Silencing of pepper genes *Phytoene synthase 1* (*PSY1*), *Phytoene desaturase* (*PDS*), and *Capsanthin-capsorubin synthase* (*CCS*) was attempted. The SOL genomics VIGS silencing tool was used to design 300 base pair fragments of each gene, and these were cloned into the pTRV2 vector (Figure 6-8). Each construct was transformed into *Agrobacterium tumefaciens* and infiltrated into both tomato plants and pepper plants, in combination with the pTRV1 vector.

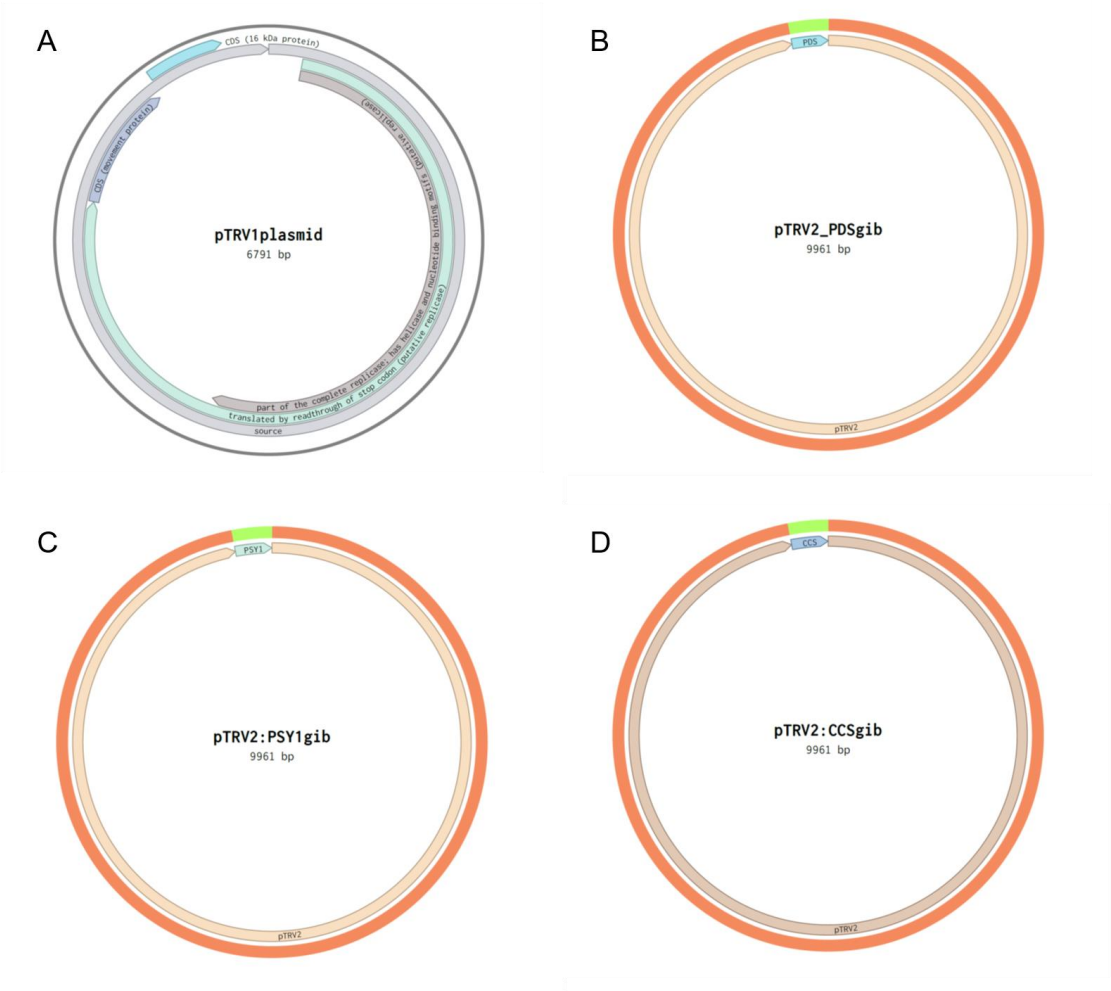


Figure 6-8 Vectors designed for VIGS of carotenoid genes in pepper and tomato.

300 base pair fragments of the carotenoid genes: PDS (B), PSY1 (C), and CCS (D), were cloned into the pTRV2 vector. pTRV2 vectors were infiltrated into tomato and pepper plants in combination with the pTRV1 vector (A).

Both pepper and tomato plants were transformed with the VIGS vectors. Tomato was used as an alternative system, to determine whether the same silencing phenotypes were observed in this organism as expected in pepper. Whilst pepper is known to be a difficult organism to work with for transformation experiments, tomato has been well studied in this way, and therefore was considered to be a possible alternative if no silencing was observed in pepper plants.

6.2.4.2. Ca*PDS* silencing in tomato leaf

The pepper *PDS* gene was silenced in tomato plants by means of VIGS. Two weeks following *Agrobacterium* transformation of the tomato leaves, phytoene accumulation was evident, as leaves started to display a bleached phenotype (Figure 6-9B). This was the first indication that the VIGS method was successful. Tomato leaves in which *PDS* was silenced displayed vastly increased phytoene levels, with 1 mg/g dry weight phytoene observed, compared to approximately 10 µg/g dry weight in empty vector

control leaves (Figure 6-9D). This represents a 100-fold increase in phytoene accumulation. Gene expression analysis demonstrated that the tomato *PDS* ortholog was indeed down regulated following silencing with the pepper gene construct, as gene expression of the tomato *PDS* was half of that observed in the empty vector control plants (Figure 6-9E).

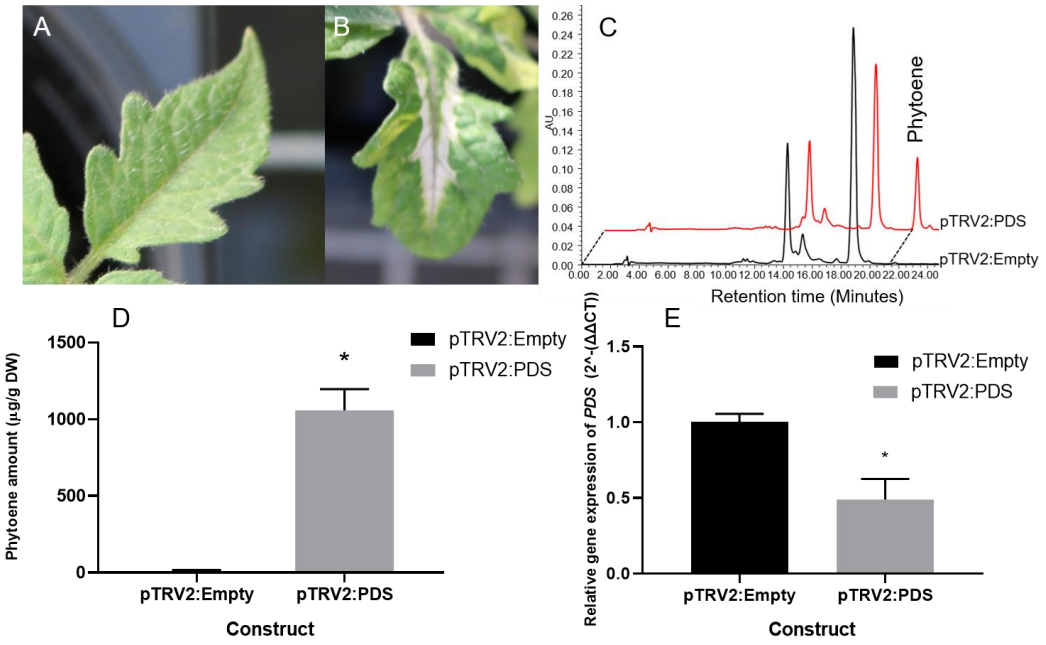


Figure 6-9 Silencing PDS gene in tomato leaf.

A pTRV2 construct was made containing a 300 bp fragment of the pepper PDS ortholog. This was agro-infiltrated into tomato plants. The tomato PDS gene was silenced, and analysed by several means. A: empty vector control leaf; B: PDS-silenced leaf, showing phytoene accumulation; C: HPLC chromatograms (286 nm), with a phytoene peak visible in the pTRV2:PDS samples, which was not observable in the empty vector control; D: Phytoene levels (mg/g dry weight) in PDS-silenced vs empty vector control plants; E: Tomato PDS gene expression in PDS-silenced vs empty vector control plants. Three biological replicates per sample were analysed (n = 3; error bars ± SE). Statistical significance determined using Student's T-test (p < 0.05).

6.2.4.3. Ca*PDS* silencing in tomato fruit

Four months after *Agrobacterium* infiltration of tomato seedlings with the pTRV2:*CaPDS* construct, phytoene accumulation was observed in tomato fruits. Tomato fruits showed a patchy yellow phenotype (Figure 6-10B). Following harvest, the yellow sections were dissected from fruits and the carotenoid profile was analysed. Indeed, the yellow colour was shown to be caused by an accumulation of phytoene, where a 2.4 fold increase was observed in *CaPDS*-silenced plants compared to controls, and an 8 fold decrease in the red pigment lycopene was observed (Figure 6-10D). A small significant decrease in β-carotene was observed in *CaPDS*-silenced fruits. A decrease in phytofluene content was observed, although this was not found to be significant at the $p < 0.05$ significance level. Changes in coloured carotenoids were presumably due to the fact that the biosynthetic pathway was blocked at *PDS*. As the

phytoene desaturase enzyme is required for converting phytoene to phytofluene and ζ-carotene (Fraser et al., 1993), which are precursors for lycopene and β-carotene biosynthesis, silencing of the gene encoding PDS prevented in the biosynthesis of these precursors, resulting in decreased accumulation of the coloured carotenoids.

Gene expression analysis showed that the tomato *PDS* ortholog was down-regulated following silencing with the pepper gene construct. A five-fold decrease in expression of *PDS* was observed in *PDS*-silenced fruits compared to control fruits (Figure 6-10E).

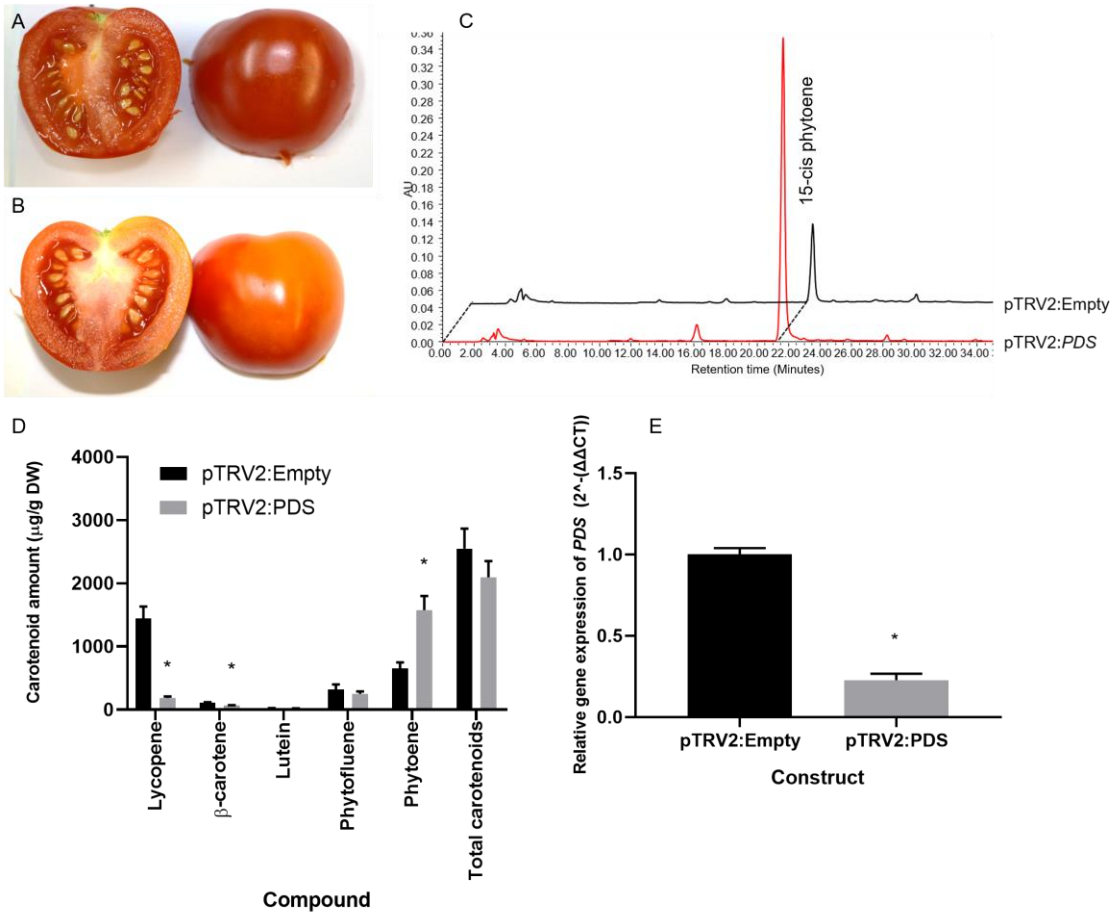


Figure 6-10 Silencing phytoene desaturase (PDS) gene in tomato fruit.

A pTRV2 construct was made containing a 300 bp fragment of the pepper PDS ortholog. This was agro-infiltrated into tomato plants. The tomato PDS gene was silenced in fruits, and analysed by several means. A: empty vector control fruit; B: PDS-silenced fruit, showing phytoene accumulation in yellow patches; C: HPLC chromatograms (286 nm), with a larger phytoene peak visible in the PDS-silenced samples; D: Carotenoid levels (mg/g dry weight) in PDS-silenced vs empty vector control fruits. The following carotenoids were measured: Lycopene, β-carotene, Lutein, Phytofluene, Phytoene, total carotenoids. E: Tomato PDS gene expression in PDS-silenced vs empty vector control plants. Three biological replicates per sample were analysed (n = 3; error bars ± SE). Statistical significance was determined using Student's T-test ($p < 0.05$).

6.2.4.4. Ca*PSY1* silencing in tomato fruit

A decrease in phytoene content was observed in tomato fruits from plants which had been infiltrated with the pTRV2:*CaPSY1* construct. A patchy yellow phenotype was

observed in fruits in which *PSY1* was silenced (Figure 6-11B) compared to the red colour observed in control fruits (Figure 6-11A). In this case, the patchy yellow phenotype was caused by a lack of carotenoids. As flavonoids are present in the tomato skin, the yellow colour observed here was most likely due to these yellow compounds being visible in the absence of the red coloured carotenoids. A ten-fold decrease in phytoene was observed in *PSY1*-silenced fruits compared to control fruits (Figure 6-11D), and significant decreases in lycopene, β-carotene, phytofluene, and total carotenoids were also observed. Decreases in these coloured carotenoids were observed as the phytoene synthase enzyme is responsible for synthesising phytoene: the precursor for all carotenoids. Consequently, a decrease in the precursor for carotenoid biosynthesis meant that the substrate for subsequent carotenoid biosynthesis was decreased, and therefore a six-fold decrease in total carotenoid content was observed in *PSY1*-silenced fruits.

Gene expression analysis showed that the tomato *PSY1* ortholog was down-regulated following virus-induced gene silencing with the pepper *PSY1* construct. *PSY1* expression was halved in *PSY1*-silenced fruits compared to control fruits (Figure 6-11E).

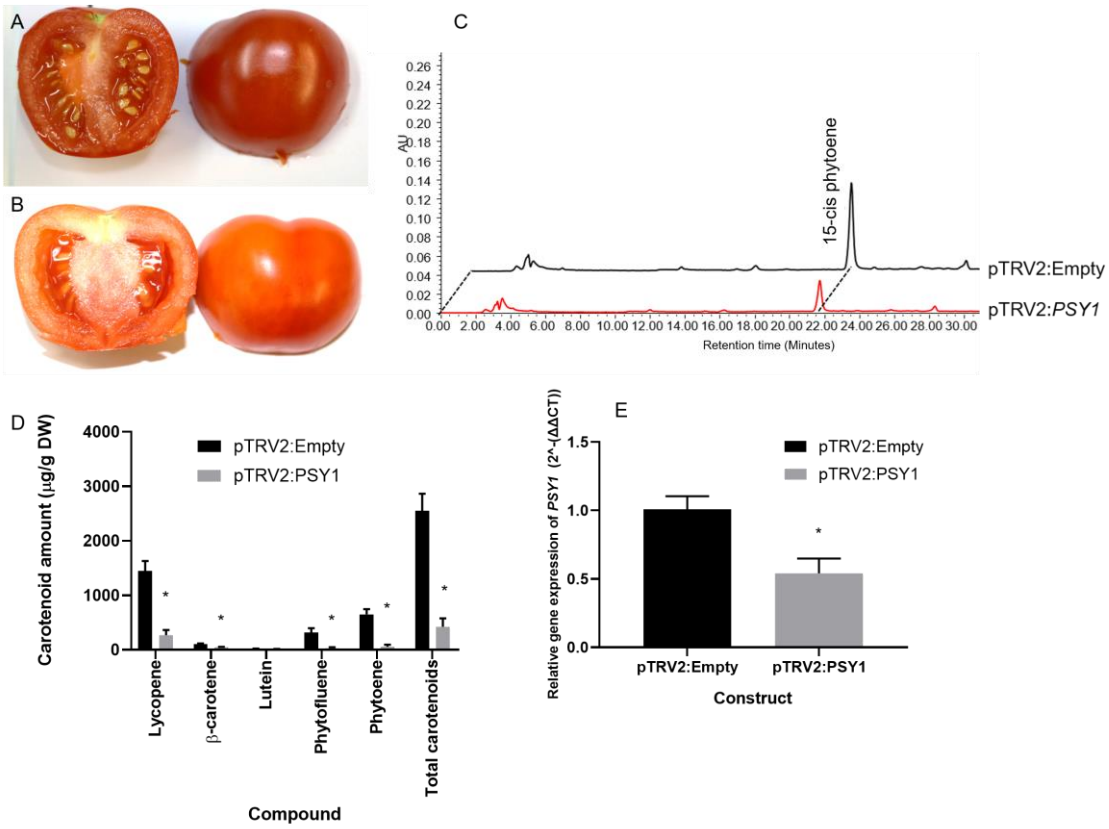


Figure 6-11 Silencing phytoene synthase 1 (PSY1) gene in tomato fruit.

A pTRV2 construct was made containing a 300 bp fragment of the pepper PSY1 ortholog. This was agro-infiltrated into tomato plants. The tomato PSY1 gene was silenced in fruits, and analysed by several means. A: empty vector control fruit; B: PSY1-silenced fruit, showing lack of phytoene in yellow patches; C: HPLC chromatograms (286 nm), with a smaller phytoene peak visible in the PDS-silenced samples; D: Carotenoid levels (mg/g dry weight) in PSY1-silenced vs empty vector control fruits. The following carotenoids were measured: Lycopene, ϐ-carotene, Lutein, Phytofluene, Phytoene, total carotenoids. E: Tomato PSY1 gene expression in PSY1-silenced vs empty vector control plants. Three biological replicates per sample were analysed (n = 3; error bars ± SE). Statistical significance was determined using Student's T-test ($p < 0.05$).

6.2.4.5. Ca*CCS* silencing in pepper fruit

Nine months following *Agrobacterium* infiltration of pepper seedlings with the pTRV2:*CCS* construct, ripe pepper fruits displayed a patchy yellow phenotype (Figure 6-12B), compared to the uniform red colour observed in control fruits (Figure 6-12A). The patchy yellow phenotype was a result of an accumulation of yellow carotenoids, including violaxanthin and zeaxanthin, and a decrease in the red carotenoid: capsanthin. A ten-fold decrease in total capsanthin content was observed in *CCS*-silenced pepper fruits compared to control fruits (Figure 6-12D), whilst a 12-fold increase in violaxanthin, and a three-fold increase in zeaxanthin were observed (Figure 6-12D). Luteoxanthin was also detected in *CCS*-silenced fruits, where it had not previously been detected in control fruits. Luteoxanthin is derived from violaxanthin, and therefore, its detection may be explained by the significant increase in violaxanthin in these fruits. As the substrate from which luteoxanthin is derived, violaxanthin, was increased, luteoxanthin levels were also increased. The enzyme responsible for

synthesis of the red pepper carotenoid, capsanthin, is capsanthin-capsorubin synthase, and therefore, silencing of the gene encoding this enzyme resulted in significantly decreased biosynthesis of capsanthin. Antheraxanthin is the substrate for capsanthin biosynthesis by CCS. No significant difference in antheraxanthin was observed between *CCS*-silenced fruits and control fruits, however, both violaxanthin and zeaxanthin content were increased in *CCS*-silenced fruits.

Gene expression analysis showed that the pepper *CCS* gene was down-regulated following virus-induced gene silencing with the pTRV2:*CCS* construct. An almost 20-fold decrease in *CCS* expression was observed in *CCS*-silenced fruits compared to control fruits (Figure 6-12E).

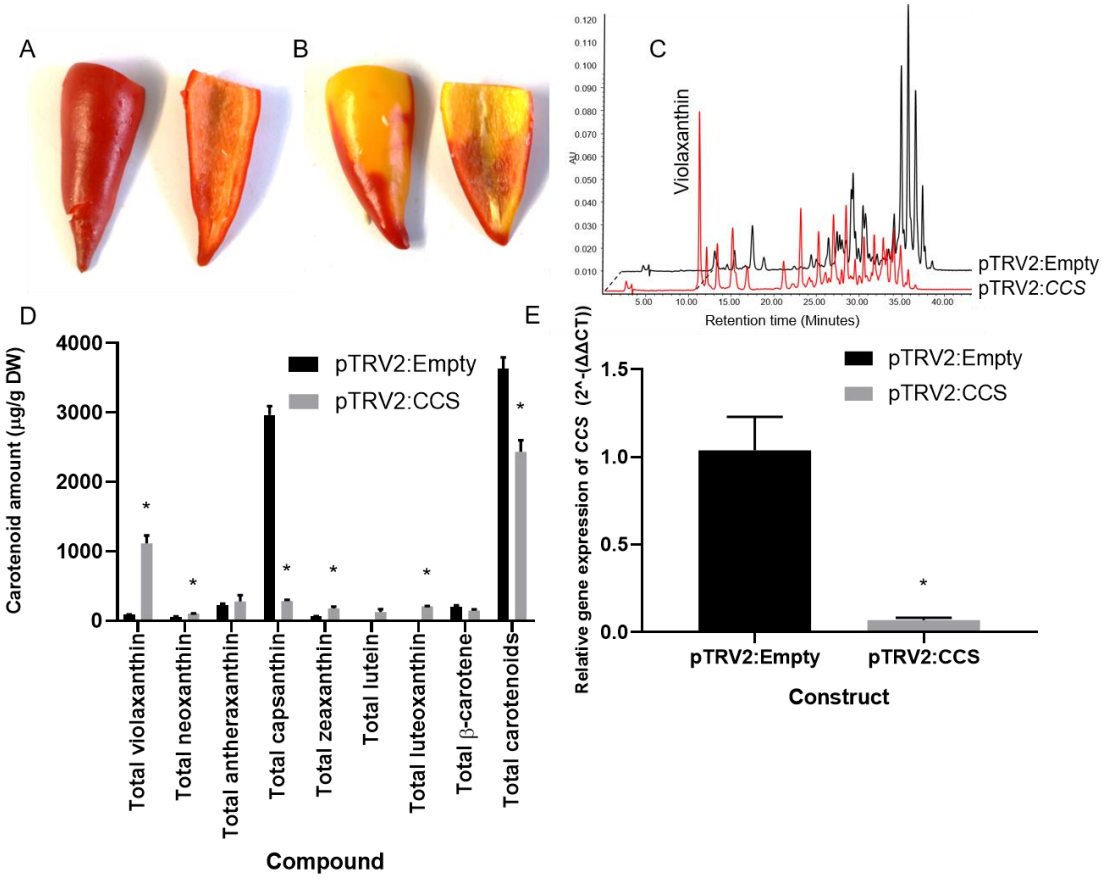


Figure 6-12 Silencing of capsanthin-capsorubin synthase (CCS) gene in pepper fruit.

A pTRV2 construct was made containing a 300 bp fragment of the pepper CCS ortholog. This was agro-infiltrated into pepper plants. The pepper CCS gene was silenced in fruits, and analysed by several means. A: empty vector control fruit; B: CCS-silenced fruit, showing lack of capsanthin in yellow patches; C: HPLC chromatograms (450 nm); D: Carotenoid levels (mg/g dry weight) in CCS-silenced vs empty vector control fruits. The following carotenoids were measured: Violaxanthin, Neoxanthin, Antheraxanthin, Capsanthin, Zeaxanthin, Lutein, Luteoxanthin, ϐ-carotene, total carotenoids. Free carotenoids and carotenoid esters were measured, and amounts were expressed for each carotenoid, with free- and esterified carotenoids summed together. E: Pepper CCS gene expression in CCS-silenced vs empty vector control plants. Three biological replicates per sample were analysed (n = 3; error bars ± SE). Statistical significance was determined using Student's T-test ($p < 0.05$).

6.2.4.6. Ca*PSY1* silencing in pepper fruits

An orange phenotype was observed in ripe fruits of plants infiltrated with the pTRV2:*PSY1* construct when compared to the control (Figure 6-13). However, this phenotype was observed on limited fruit numbers, and therefore, whilst it appeared as though silencing of *PSY1* in pepper fruits could be achieved using VIGS, efficiency was very low. This resulted in the harvesting of insufficient fruit numbers to allow robust analysis.

Figure 6-13 Silencing of phytoene synthase 1 (PSY1) gene in pepper fruit.

A pTRV2 construct was made containing a 300 bp fragment of the pepper PSY1 ortholog. This was agro-infiltrated into pepper plants. A visible phenotype was observed when comparing empty vector control fruits (A), and PSY1-silenced fruits (B), as PSY1-silenced fruits displayed a patchy orange phenotype. This difference was only observed in a very small number of fruits, due to low silencing efficiency, and insufficient fruits were harvested for carotenoid and gene expression analyses.

6.3. Discussion

6.3.1. *Capsicum* transcriptome

Whilst pepper is a widely cultivated crop, and is well-studied, genomic resources have only become available within recent years. The pepper genome is relatively complex compared to many plant species, comprising one of the largest genome sizes within the Solanaceae family at approximately 3.5 Gb, and contains many repetitive elements (Qin et al., 2014, Kim et al., 2014, Hulse-Kemp et al., 2018). The first pepper genome sequence to be assembled was for the cultivated *C. annuum Zunla-1* (Qin et al., 2014). This was rapidly followed by the genome sequence for the Mexican landrace *C. annuum* cv. CM334 (Criollo de Morelos 334) (Kim et al., 2014), and subsequently, a more recent CM334 genome using Linked-Read sequencing technology (Hulse-Kemp et al., 2018). These resources have facilitated transcriptomic studies of pepper to be performed, in order to investigate gene expression patterns. Transcriptome analysis of pepper has identified the role of brassinosteroids at the level of transcriptional control in inducing tolerance to chilling stress (Li et al., 2016), and RNAseq analysis assisted in the identification of a MYB transcription factor in controlling pungency in pepper (Han et al., 2019). Evidently, transcriptomics, and specifically here, RNAseq is a useful tool in elucidating candidate genes underlying traits of interest.

6.3.2. The use of transcriptomics to understand the carotenoid retention trait

RNAseq was utilised in this study to identify potential candidate genes involved in the carotenoid retention trait, and therefore, gene expression of a high carotenoid retention line was compared against a low carotenoid retention line. Three time points were analysed, and these were selected as ripening time points. The carotenoid retention trait is observed post-harvest, and as fruit carotenoid biosynthesis predominantly occurs during ripening, the following time points were deemed to be the most biologically relevant: breaker, breaker + 3 days, and ripe.

6.3.3. Identification of candidate genes for carotenoid retention trait

Unsurprisingly, the greatest number of gene expression changes were observed between the two genotypes when the fruit were at breaker stage, compared to other ripening stages. Fruits undergo significant changes at the onset of ripening, both molecularly and biochemically. Starches are converted to sugars, pigments are synthesised, aroma volatiles are produced, and softening occurs. Fruit ripening changes are controlled by a sophisticated interaction of hormones and transcription factors (Giovannoni et al., 2017), and therefore, significant changes in transcripts are expected at the onset of ripening.

6.3.3.1. Cu/ZnSOD

Several genes were constitutively differentially expressed throughout fruit ripening between the two genotypes, and several of these genes were annotated as possessing oxidoreductase activity. *Cu/ZnSOD* (*Superoxide dismutase*) was identified as a potential candidate gene involved in the carotenoid retention trait. This gene was found to be consistently up-regulated in the high carotenoid retention line throughout ripening. The *Superoxide dismutase* family of genes is considered to be the first line of defence in scavenging reactive oxygen species, and converting superoxide to hydrogen peroxide (Apel and Hirt, 2004). Consequently, increased reactive oxygen species may be scavenged by the SOD enzyme in high carotenoid retention lines, and result in decreased carotenoid degradation as a result of lipid peroxidation. This does, however, contradict previous observations, in which Cu/Zn SOD activity levels were shown to be increased in the low carotenoid retention line during post-harvest storage (Berry, 2015). However, these two observations were made at different time points. Despite *Cu/ZnSOD* gene expression being increased during fruit ripening in the high carotenoid retention line, the protein may degrade more rapidly, or be non-functional during post-harvest storage, due to an array of other protective mechanisms against lipid peroxidation and oxidative damage.

ROS is also known to initiate carotenogenesis (Bouvier et al., 1998). Expression of this superoxide dismutase enzyme may be increased in the high carotenoid retention line, if increased ROS are present, which has resulted in increased carotenogenesis.

Catalase acts immediately following SOD in order to convert hydrogen peroxide into oxygen and water (Apel and Hirt, 2004), and therefore, genes encoding this enzyme were mined for in differential expression data sets. Expression of one gene encoding catalase was found to be increased in the high retention line: CA02G19830, and this reflects the fact that both SOD and catalase are required for ROS detoxification.

6.3.3.2. Carotenoid-related genes

Both zeaxanthin epoxidase (*ZEP*) and violaxanthin de-epoxidase (*VDE*) were up-regulated in the high carotenoid retention line at the ripe time point. These genes are constitutents of the zeaxanthin epoxidase-violaxanthin de-epoxidase (ZEP/VDE) cycle, in which zeaxanthin is epoxidated to antheraxanthin, and subsequently to violaxanthin, whilst violaxanthin is de-epoxidated to antheraxanthin and subsequently to zeaxanthin (Yamamoto et al., 1962) (Figure 6-14).

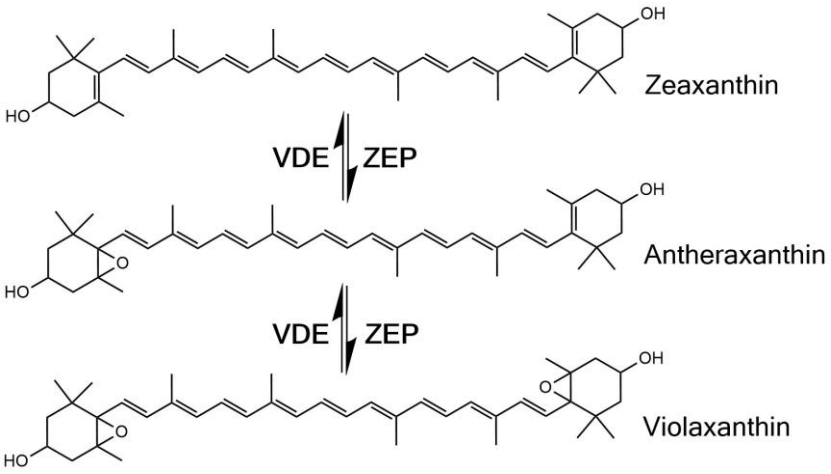


Figure 6-14 The xanthophyll cycle: conversion of zeaxanthin to violaxanthin.

As expression of both of these genes was increased in the high carotenoid retention line ripe fruit, this would suggest that flux through this pathway is increased. This demonstrates that carotenoid biosynthesis was pushed down the β- branch of the carotenoid biosynthesis pathway. Capsanthin, the pepper specific carotenoid, is synthesised from the precursor, antheraxanthin, by the *Capsanthin-capsorubin synthase* (*CCS*) gene (Bouvier et al., 1994). Whilst *CCS* was not shown to be differentially expressed between the genotypes, an increase in synthesis of the precursor may facilitate increased synthesis of the major red pigment. Whilst carotenoid retention has been shown to be independent of total carotenoid content, the high retention line studied here has been shown to have increased carotenoids relative to the low retention line. This explains the difference in xanthophyll cycle gene expression. Whilst carotenoid retention and carotenoid intensity are independent of one another, increased gene expression of genes involved in the xanthophyll cycle may facilitate ongoing carotenoid biosynthesis. The carotenoid retention trait may be the product of the relationship between synthesis and degradation, as carotenoid content was previously shown to increase in high carotenoid retention lines during post-harvest storage (Chapter 4). Therefore, increased gene expression of these xanthophyll genes may facilitate increased carotenoid biosynthesis during post-harvest storage.

6.3.3.3. Photosynthesis-related genes

Gene ontology terms relating to photosynthesis processes and structures were enriched when comparing gene expression levels between the high and low carotenoid retention lines. Upon fruit ripening, chloroplasts transition to form chromoplasts (Spurr and Harris, 1968, Bathgate et al., 1985), during which process, the grana and thylakoids of the chloroplast begin to lyse and disassemble (Spurr and Harris, 1968). Carotenoid crystals begin to form simultaneously (Egea et al., 2010). However, it has

been shown that the presence of fibrillar structures, or 'fibrils', which are important structures within pepper chromoplasts for storing carotenoids, may delay the disassembly of thylakoid structures. Fibrils are well characterised structures within pepper chromoplasts (Spurr and Harris, 1968), and have been shown to be particularly efficient at storing esterified carotenoids (Deruère et al., 1994), which are abundant in pepper fruits. Expression of the pepper fibrillin gene in tomato gave rise to such fibrillar structures, which are not usually present in tomato. Unexpectedly, expression of the fibrillin gene also resulted in a slower disassembly of thylakoids within chloroplasts, resulting in a slower chloroplast to chromoplast transition (Simkin et al., 2007). As the high carotenoid retention line was shown to have increased xanthophyll cycle gene expression and increased carotenoids in ripe fruit, the delay in the chloroplast to chromoplast transition in these fruits may be due to increased fibrillin within these plastids, resulting in slower thylakoid disassembly. This explains the increased expression of photosynthetic and thylakoid-related genes in the high carotenoid retention line, at the ripe time point.

6.3.3.4. Fatty acid biosynthesis genes

Several genes involved in the process of fatty acid biosynthesis were found to be differentially expressed between the low carotenoid retention line and high carotenoid retention line. Interestingly, several of these genes displayed increases in expression in the low retention line compared to the high retention line, particularly at the breaker stage. The 3-keto-acyl-CoA synthase gene: CA02G23370, was shown to be up-regulated in the low retention line at the breaker stage, as it was throughout fruit development. These genes are essential for cuticle biosynthesis (Lee et al., 2009). As the low retention line has been shown to have a significantly thinner cuticle layer compared to the high retention line (Chapter 5), it is interesting that expression of this gene was found to be up-regulated in the low retention line during fruit development and fruit ripening. As a thicker cuticle is present in fruits of the high retention line, it was anticipated that this gene would be up-regulated in the high retention line. However, it is possible that this homolog of the 3-keto-acyl-CoA synthase gene was not directly involved in fatty acid biosynthesis for cuticle waxes. As very long chain fatty acids are also required for sphingolipid biosynthesis, triacylglycerols, and phospholipids, it is possible that an increase in expression of this gene was to increase the fatty acid content for other purposes (Bach and Faure, 2010). An increase in fatty acid biosynthesis genes during fruit ripening may not be directly linked to fruit cuticle development. Alternatively, a 3-keto-acyl-CoA synthase was located within the carotenoid retention QTLs and did display an increase in expression in the high retention line compared to the low retention line. Therefore, it is more likely that this

gene is directly linked to carotenoid retention, particularly as it was found to be associated with the carotenoid retention QTL region, possibly in its role in cuticle biosynthesis (Section 6.3.4).

6.3.4. Identification of candidate genes within carotenoid retention QTLs

QTL regions which had previously been identified for the carotenoid retention trait were mined for differentially expressed genes, as these genes may be directly linked to the carotenoid retention trait. Several gene ontology terms were enriched in the breaker gene set, which were associated with lipid and fatty acid metabolism. Several lipid-transfer proteins (*LTPs*) were identified, which have previously been identified as involved in pepper fruit water loss. It was shown that increased expression of several lipid transfer proteins was associated with high plant water loss (Popovsky-Sarid et al., 2017). Pepper fruit water loss is dependent on the fruit cuticle structure, and cuticle biosynthesis has been shown to directly influence water loss (Popovsky-Sarid et al., 2017). Therefore, this suggests that the lipid transfer proteins described here are associated with cuticle structure. Increased expression of these *LTPs* was observed in the low carotenoid retention line, which has also been shown to have a thinner, cracked cuticle, which may be presumed to be more susceptible to water loss. As these *LTPs* are significantly differentially expressed between the high and low carotenoid retention lines, this provides further evidence that the carotenoid retention phenotype is associated with the fruit cuticle structure.

Fatty acid metabolism genes within the carotenoid retention QTL regions were more expressed in the high carotenoid retention line relative to the low retention line, as were other cuticle wax biosynthesis genes, such as CA10G05460 (3-ketoacyl-coA synthase) (Table 6-5). This again suggests that carotenoid retention is linked to cuticle structure. Furthermore, increased fatty acid metabolism may be linked to an increase in fatty acid content for esterification of carotenoids. Carotenoids in pepper are often esterified in order to increase their stability, and this is normally with fatty acids of chain length C_{12}, C_{14}, and C_{16} (Biacs et al., 1989, Pérez-Gálvez et al., 1999). Consequently, pepper fruits with increased carotenoids, such as the high carotenoid retention line studied here, will require increased fatty acids in order to esterify pigments. An increase in fatty acid esterification of carotenoids will also limit degradation during post-harvest storage, due to increased carotenoid stability. Therefore, genes encoding fatty acid metabolism may directly influence the carotenoid retention phenotype.

Whilst combining the QTL data from a previous study with the RNAseq data presented here is a useful tool for narrowing the search for potential candidate genes, it is important that genes located outside of these regions are also considered. The QTL

mapping described here was performed using data collected from an image analysis study. The limitations of this method have previously been discussed in Chapter 4, and therefore, these QTLs may not accurately reflect genomic regions underlying the carotenoid retention trait. The image analysis method may not fully explain the variation in carotenoid content, which was subsequently observed using chromatographic methods. QTL mapping based on the accurate carotenoid quantities presented in this study would be more robust for determining regions within which carotenoid retention genes may lie. Furthermore, the QTL regions previously determined provided very large genomic regions within which candidate genes may be located. Consequently, not all genes identified may be linked to carotenoid retention. Future QTL mapping should aim to specifically target the carotenoid retention trait, and to narrow the loci to smaller genomic regions.

6.3.5. The use of Virus Induced Gene Silencing as a tool for gene functional characterisation

Genetic transformation of pepper has proven to be challenging, and seemingly successful attempts have been unrepeatable (Chung et al., 2004). Consequently, Virus Induced Gene Silencing (VIGS) has been used to study gene function in pepper (Chung et al., 2004, Zhang et al., 2015, Kim et al., 2017). Although this has provided a more reliable method for understanding gene function in pepper, gene silencing in pepper fruits using this method is very time consuming and inefficient. VIGS was attempted in this study, in order to establish a tool for characterising candidate gene function. In order to establish the method, carotenoid genes were selected as the first candidates for silencing, as these genes would give a distinct, visible colour change upon gene silencing.

In this study, pTRV2 constructs containing pepper genes of interest were infiltrated into tomato. Tomato homologs were therefore targeted using the pepper construct. VIGS in tomato has long since been established as a method for gene functional characterisation (Orzaez et al., 2009, Ballester et al., 2010, Fantini et al., 2013), and tomato proved to be more amenable to *Agrobacterium* transformation compared to pepper.

Both the pepper *PDS* and *PSY1* orthologs were capable of silencing their respective tomato endogenous genes within the fruit, therefore suggesting that these genes may have the same function in both pepper and tomato. Whilst this method does not confirm that the function of these genes in pepper, as they have not been silenced in pepper, it does suggest the high homology between the pepper and tomato genes. Consequently, this suggests that the function of the genes may be conserved between

the two species. *PDS* was shown to only be partially silenced in tomato leaves, despite a 100-fold increase in phytoene accumulation. This was likely to be due to the fact that patchy silencing was observed throughout the leaf material, and therefore, the gene was not entirely silenced throughout the leaf. Furthermore, *PDS* is an essential plant carotenoid biosynthetic enzyme, key to the success of the plant, and consequently is the target of certain inhibitors, such as norflurazon (Koschmieder et al., 2017). Therefore, complete silencing of the *PDS* gene would likely have significantly affected the growth of the plant.

The pepper *CCS* gene was silenced in pepper fruits using VIGS. As a *CCS* ortholog does not exist in tomato, the function of this gene had to be determined in pepper. This represents a limitation with using the tomato as a model for pepper when determining gene function, as only genes with orthologs and high sequence similarity may be characterised in this way between the two species. Gene silencing within the pepper fruit was first observed nine months after *Agrobacterium* transformation of plants with VIGS constructs. This highlights the time consuming nature of this technique. As *CCS* is the gene responsible for synthesising capsanthin, the major red pigment, in pepper fruit, a visible phenotype was observed when silencing of this gene had been effective. Fruits exhibited yellow patches, owing to the lack of capsanthin, in pericarp regions in which gene silencing had been efficient.

A significant decrease in capsanthin and an increase in precursors was observed. Antheraxanthin is the intermediate in the zeaxanthin epoxidase/violaxanthin de-epoxidase (ZEP/VDE) cycle (Yamamoto et al., 1962). The accumulation of violaxanthin observed in *CCS*-silenced fruits suggests that the antheraxanthin, which would normally have been utilised in capsanthin synthesis, instead was converted to violaxanthin by the zeaxanthin epoxidase (ZEP) enzyme, rather than accumulated. Increased zeaxanthin was also observed in *CCS*-silenced fruits. Antheraxanthin may also be converted back to zeaxanthin in the ZEP/VDE cycle, and therefore the antheraxanthin which would previously have been directed to capsanthin biosynthesis may have been converted back to zeaxanthin upon blocking of the *CCS* gene. This suggests that blocking of the CCS pathway results in accumulation of the end products of the ZEP/VDE cycle, zeaxanthin and violaxanthin, as opposed to the intermediate and direct precursor of capsanthin: antheraxanthin.

6.3.6. Limitations of VIGS as a tool for gene functional characterisation

VIGS has proved a very useful tool for studying gene function in pepper, particularly as there are limited other methods to do this when compared to other similar species, such as the tomato.

Whilst studying pepper gene function in tomato may be possible for some genes with high homology between the two species, this method is limited. Further to this, this method does not directly characterise the pepper gene function, as it has not been studied endogenously within the pepper. It does however, suggest that the gene function in both the tomato and pepper may be similar. This provides a more efficient method for studying pepper gene functions, as opposed to the time consuming process of studying gene function directly in pepper fruit.

The study presented here silenced carotenoid biosynthetic genes within pepper fruit, in order to establish the VIGS method. It was hoped that this method would be used for functional characterisation of candidate genes identified using RNAseq. However, it is clear that this method is not particularly efficient. Gene silencing is only observed in parts of the fruit, resulting in a 'patchy' phenotype. Furthermore, many fruits on plants do not show silencing to any extent. This patchy distribution of phenotype is a result of partial and highly variable silencing between fruits (Orzaez et al., 2009, Fernandez-Moreno et al., 2013). Consequently, visual reporter systems have been developed in order to allow silencing of non-visual phenotypes to be traced within fruit. In tomato, a transgenic tomato line was produced which expressed *Antirrhinum majus* transcription factors: Delila and Rosea1, under to the control of the fruit specific E8 promoter. This resulted in purple fruits, which were rich in anthocyanins. Tobacco rattle virus VIGS vectors were modified to incorporate *Rosea1* and *Delila* sequence fragments, which, when silenced in these fruits, restored the fruit to the red coloured phenotype (Orzaez et al., 2009). This was shown to be an efficient method of tracing gene silencing. A similar method was used in pepper, using a gene involved in anthocyanin biosynthesis (*An2*) as a visible reporter for VIGS on pepper fruits. Unlike in tomato, where a transgenic tomato line was developed with high anthocyanin content, this was not possible due to limitations around *Agrobacterium* transformation of pepper, and consequently a cultivar naturally containing visible levels of anthocyanin: *Capsicum annuum* cv. NuMex Halloween, was used. Cosilencing of the *An2* gene along with *capsaicin synthase*, responsible for controlling pungency in pepper fruits, resulted in lower expression of *Capsaicin synthase* in anthocyanin-depleted tissues, suggesting that the *An2* gene was an effective method for visibly tracing gene silencing (Kim et al., 2017). This tool will be useful for future gene function studies. Experiments must be carefully designed to ensure co-silencing of reporter genes does not influence the phenotype observed by silencing the gene of interest.

The study presented here has shown the role of the fruit cuticle in influencing carotenoid retention. Candidate genes for the carotenoid retention trait may include cuticle biosynthesis genes. However, the cuticle may be structurally different in purple-

pigmented fruits compared to red-pigmented fruits. Silencing of such genes in purple pigmented fruits may not reveal the role of these genes in carotenoid retention, as this trait is not observed in these fruits. Therefore, whilst a visible reporter system for VIGS is valuable, care must be taken to avoid interaction effects between phenotypes. Clearly, there are still limitations in place for gene functional characterisation in pepper.

6.4. Conclusions

A transcriptomic study has been performed to compare high and low carotenoid retention lines through fruit development, in order to identify potential candidate genes for this trait. The use of previously performed QTL mapping data has narrowed down the genomic regions within which candidate genes may be located, although it is still important to consider genes outside of these regions. Potential gene candidates have been identified, and integration of this data, with carotenoid retention QTL mapping to be performed in the near future, will identify further candidates.

A method for gene functional characterisation has been established in pepper, which will be useful for determining candidate gene function. Tomato has also been shown to be a model for pepper gene function, although the use of this method is limited.

This work has provided a molecular basis to explain the carotenoid retention trait in pepper, with potential genetic mechanisms identified underlying this trait. Further work should confirm these findings, and may identify further genes involved in the control of this complex trait.

7. General Discussion

7.1. General Conclusions

Despite the fact that the presence and profile of carotenoids in *Capsicum annuum* fruits is well characterised, the mechanisms underlying the retention of these carotenoids during post-harvest storage is not fully understood. The mechanisms underlying the interplay between carotenoid biosynthesis and degradation, along with physiological mechanisms influencing carotenoid storage, and the molecular mechanisms controlling expression of genes involved in carotenoid biosynthesis, remain largely understudied.

This project aimed to determine the mechanisms controlling carotenoid retention in chilli pepper (*Capsicum annuum*). Biochemical, physiological, and molecular mechanisms were assessed to determine the contributions of these mechanisms to the carotenoid retention trait.

7.1.1. Aims and Objectives

The aims of this project can be divided into three sections: i) characterising the biochemical changes associated with pepper fruit carotenoid retention, ii) determining the role of physiological structures, such as the fruit cuticle, in influencing carotenoid retention, and iii) using a transcriptomic approach to identify candidate genes associated with controlling the fruit carotenoid retention phenotype. In order to achieve these aims, experiments were designed to fulfil the following objectives.

Biochemical profiling of pepper varieties displaying variation in the carotenoid retention phenotype:

- The carotenoid profile of a DH population was characterised in fresh and stored fruit.
- The carotenoid retention value for each pepper line was calculated.
- Intermediary metabolism of the DH population was assessed and it was determined that intermediary metabolism does not influence the carotenoid retention phenotype.
- Changes to the volatile profile of high and low carotenoid retention pepper lines following post-harvest storage were analysed.
- Changes to fruit metabolic profile and fruit structure during post-harvest storage were assessed.

Understanding the link between fruit physiology and carotenoid retention phenotype

- Changes in fruit morphology during post-harvest storage, and how these changes link to carotenoid retention were determined.

- The chemical composition of the fruit cuticle, and how this links to carotenoid retention phenotype was analysed.

- Changes to gene expression in fruit surface tissue, and how this may affect carotenoid retention were assessed.

Identifying gene candidates underlying the carotenoid retention phenotype, and developing a method for functional characterisation of candidate genes

- A transcriptomic approach to determine genes associated with the carotenoid retention phenotype was used.

- A transcriptomic approach was coupled with existing QTL data to further narrow down candidate genes for the trait.

- VIGS was developed as a method for gene functional characterisation of candidate genes in pepper

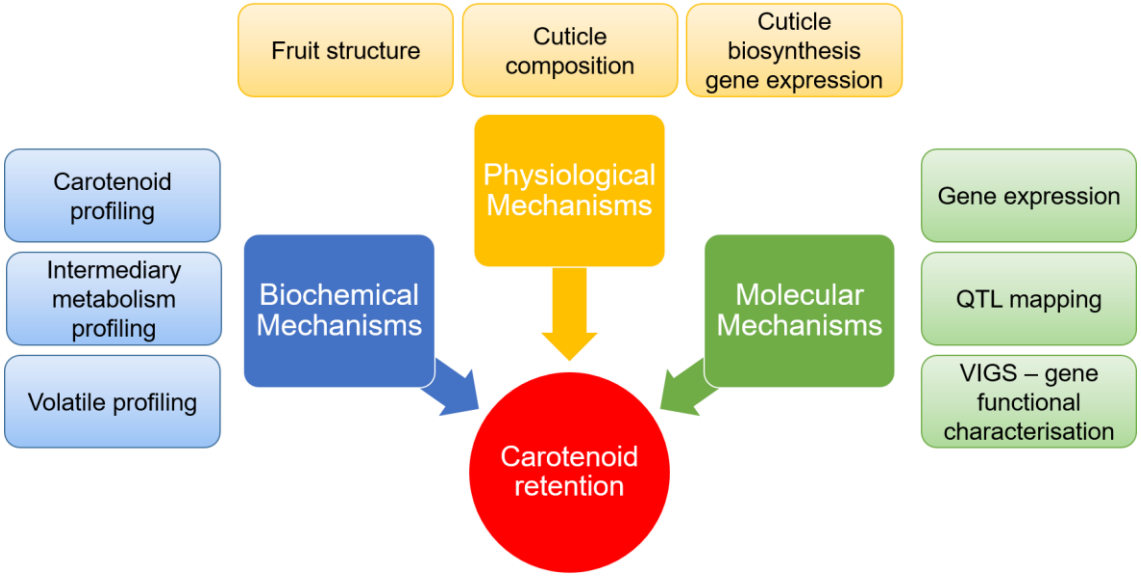


Figure 7-1 Outline of aims and experimental approaches used to determine mechanisms controlling carotenoid retention.

7.1.2. Summary of findings

Chapter 3 utilised metabolite profiling of a Doubled Haploid (DH) population, and revealed changes in the metabolite profile of chilli pepper during post-harvest storage. High Pressure Liquid Chromatography (HPLC) with photodiode array (PDA) detection, revealed that carotenoid amount may increase during post-harvest storage of chilli

peppers. Furthermore, it was demonstrated that carotenoid amount does not correlate with carotenoid retention phenotype, and the carotenoid retention trait is dependent on the entire carotenoid profile of the fruit, as opposed to one or two specific carotenoids. Intermediary metabolite profiling was carried out using Gas Chromatography Mass Spectrometry (GC-MS) of polar and non-polar extracts of fresh fruit, and the carotenoid retention trait was shown not to be associated with changes in fresh fruit metabolites. Changes in intermediary metabolites were observed during the storage of fruits from the sub-population, however, these changes were not linked to either the high or low carotenoid retention phenotypes. The volatile profile of pepper fruits also changed during post-harvest storage. High and low carotenoid retention lines showed increases in carotenoid derived volatiles during post-harvest storage, although greater increases were observed in high carotenoid retention lines. Lipid derived volatiles increased in stored high carotenoid retention lines, whilst they decreased in stored low carotenoid retention lines. This was considered to be due to high levels of lipid peroxidation in low carotenoid retention lines early in storage, resulting in a decrease in lipid-derived volatiles at the point of analysis. In high carotenoid retention lines, in which lipid peroxidation could be mediated by carotenoid antioxidant activity, lipid-derived volatiles were still being produced at the point of analysis.

Upon detailed analysis of the changes to carotenoid content during post-harvest storage, Chapter 4 demonstrated that quantification of carotenoids by HPLC was a more accurate method of determining the carotenoid retention phenotype of chilli pepper fruits, compared to the 'by eye' measurements previously used by breeders. Further to this, it was found that the drying and storage environment may have a significant effect on the rate to which carotenoid accumulation is observed, as a low retention line displayed drastically different carotenoid retention phenotypes when compared between storage in India and in replicated lab-based conditions. It was determined that the fruit surface texture, and presumably the fruit cuticle, plays a critical role in controlling carotenoid retention as high retention lines did not accumulate carotenoids to the same extent when stored as homogenised powder, as when stored as whole fruit. Initiation of lipid peroxidation through treatment with hydrogen peroxide demonstrated that pepper lines with a cracked surface were more susceptible to carotenoid degradation, than those which retained a smooth, intact surface.

Chapter 5 demonstrated that the carotenoid retention phenotype is correlated with fruit surface structure, including exocarp thickness and surface texture upon fruit drying. Further to this, the biochemical composition of the fruit cuticle was shown to be associated with the carotenoid retention phenotype, as cutin monomer composition was increased in high carotenoid retention lines. Carotenoids were shown to be

associated with the fruit exocarp, although the mechanisms and reason underlying this observation are uncertain, however it was suggested that an increase in esterified carotenoids in exocarp tissue may be linked to an increase in cuticle component precursors in the fruit epidermal cells. The localisation of carotenoids within the fruit exocarp may influence the carotenoid retention classification. The link between carotenoid retention phenotype and fruit surface structure was further supported by the increase in expression of cuticle biosynthesis genes in high carotenoid retention lines.

Analysis of some of the potential mechanisms underlying the carotenoid retention phenotype was carried out in Chapter 6. Gene expression analysis identified genes differentially expressed between high and low carotenoid retention lines, and these included genes involved in antioxidative processes, such as *Superoxide dismutase* (*SOD*). The increased expression in the high carotenoid retention line may mitigate against lipid peroxidation, and therefore result in fewer carotenoids being scavenged, and therefore degraded, to protect the fruit against excessive lipid peroxidation. An increase in expression of fatty acid and lipid metabolic processes suggests the changing composition of fatty acids and lipids through fruit ripening. As carotenoids are widely esterified by fatty acids in pepper fruits, an increase in fatty acid precursors is essential to allow for esterification to occur. Esterified carotenoids are more stable than free carotenoids, and therefore, an increase in fatty acid metabolic processes in high retention lines would explain the increased stability of carotenoids in these pepper fruits, and the fact that they are less susceptible to degradation. Virus Induced Gene Silencing has been demonstrated as a method for functional characterisation of genes in pepper, and will provide a useful tool in future functional characterisation of genes of interest.

7.2. Perspectives and Outlook

7.2.1. Biochemical mechanisms influencing carotenoid retention

The studies performed in this thesis demonstrated that the carotenoid profile, along with the profile of some other metabolites, including carotenoid-derived and lipid-derived volatiles, contribute to the carotenoid retention phenotype of chilli pepper fruit. The metabolic composition of chilli pepper fruits has been widely characterised (Deli et al., 1996, Howard et al., 2000, Berry et al., 2019), however, the direct influence this has on carotenoid retention has been understudied to date. This thesis has shown that the high carotenoid retention phenotype does not necessarily correlate with a high carotenoid content phenotype in fresh fruit. Therefore, these data suggest that the carotenoid retention phenotype is more complex than simply understanding the metabolic profile of these fruits. Carotenoid retention has been demonstrated to be the

result of the dynamic interplay between carotenoid biosynthesis and degradation, as was shown in Chapter 3, when comparing carotenoid profile with carotenoid-derived volatile profile.

Further to this, localisation of carotenoids within the fruit has been suggested as playing an essential role in determining fruit carotenoid retention phenotype. The sequestration of carotenoids into subcellular compartments has been shown to be essential to determining fruit carotenoid accumulation (Deruère et al., 1994, Nogueira et al., 2013, Berry et al., 2019), however, the work presented here suggests that spatial localisation of cells accumulating carotenoids may also influence the carotenoid retention phenotype. Chapter 5 identified the epidermal cells as a potential location for carotenoid accumulation in some pepper accessions. The preferential localisation of carotenoids in these cells may influence the carotenoid retention phenotype, as a concentration of carotenoids toward the outer cell layers of the fruit may influence the visual perception of the carotenoid retention phenotype. Carotenoid diesters were shown to preferentially accumulate in a high carotenoid retention line, and this may be due to the fact that cells embedded in the exocarp layer are within a very hydrophobic environment, therefore, favouring the accumulation of more hydrophobic compounds, including carotenoid diesters. Esterification of carotenoids has been shown to increase the stability of carotenoids (Biacs et al., 1989). In this case, the esterification of carotenoids may not only increase their stability, protecting them against oxidative damage, but further may facilitate their accumulation in specific fruit tissue layers. Whilst plastid number in epidermal cells was found to show no difference between high and low carotenoid retention lines, it would be interesting to determine whether a difference exists in sub-chromoplast organelles, such as the plastoglobuli. Fibrillar plastoglobuli preferentially sequester esterified carotenoids (Deruère et al., 1994), and therefore, it may be expected that an increase in fibrillar plastoglobuli would be observed in epidermal chromoplasts of high carotenoid retention varieties. Further, it has been shown that sub-chromoplast structures adapt to the metabolites located within the cell, as opposed to pre-determined structures accumulating the metabolites available, in transgenic potato (Mortimer et al., 2016). Therefore it may be hypothesised that an increase in carotenoid ester structures would result in an increase in fibrillar plastoglobuli within epidermal chromoplasts.

Whilst carotenoid retention is unlinked to the broader intermediary metabolic profile of pepper fruits, changes occur to the intermediary metabolic profile during post-harvest storage. Lipid-derived volatiles increased in high carotenoid retention lines, whilst they decreased in low carotenoid retention lines during post-harvest storage. This was suggested to be due to low carotenoid retention lines being more susceptible to lipid

peroxidation, resulting in the production of lipid-derived volatiles, early in storage. In contrast, high carotenoid retention lines have a greater capacity for preventing lipid peroxidation during post-harvest storage. Whilst carotenoids may mitigate the negative effects of excessive lipid-peroxidation, the production of Reactive Oxygen Species (ROS) is still essential as these compounds are vital for plant signalling (Mittler et al., 2004). Therefore, excessive lipid peroxidation is mitigated against in high carotenoid retention lines, resulting in a slower rate of lipid peroxidation. Consequently, following post-harvest storage, lipid-derived volatiles were still detected in these lines.

The fact that the carotenoid retention phenotype is unlinked to the broader intermediary metabolism profile in chilli pepper fruit may be of particular interest to plant breeders, as this suggests that breeding towards the advantageous high carotenoid retention phenotype will not influence other key metabolic traits, which ultimately influence flavour, aroma, or pungency profiles.

Taken together, the evidence presented in this thesis highlights the influence that the complex biochemical profile of the pepper fruit plays in contributing to carotenoid retention phenotype. Evidently, carotenoid content and carotenoid biosynthesis, cannot be studied or manipulated in isolation in order to influence the carotenoid retention phenotype of fruit. An example of broader metabolic phenotype being considered has been demonstrated in sorghum, in which biofortification for elevated β-carotene was engineered. Whilst engineering of increased β-carotene was successful, carotenoid instability resulted in the maximum benefit not being gained from this crop. However, engineering of increased vitamin E along with β-carotene resulted in improved β-carotene accumulation and stability (Che et al., 2016). Carotenoid content and retention should be considered simultaneously with other metabolic classes in order to gain the optimal storage and retention of carotenoids.

7.2.2. Physiological mechanisms influencing carotenoid retention

Whilst carotenoid retention is a largely metabolic phenotype, this thesis has presented a fruit structural element to controlling the retention phenotype. Observations of fruit surface structure upon fruit drying identified a stark difference between fruits, in which some fruits retained a smooth surface, whilst others displayed distinctive surface cracking. Microscopy confirmed the presence of these micro-cracks on the surface of some fruits. Identification of the cracked surface trait, along with these fruits possessing a thinner exocarp than their smooth surface counterparts, was subsequently linked to the carotenoid retention phenotype. This represents the first

example of fruit structure and texture being linked to carotenoid retention during post-harvest storage.

Whilst the fruit surface structure is evidently essential to plant success, in protecting against biotic and abiotic stresses, such as water loss and pathogen attack (Domínguez et al., 2011), the fruit surface structure can now be implicated in influencing the carotenoid retention phenotype. The importance of the fruit cuticle has previously been discussed as it plays a role in modulating post-harvest quality of fruits (Lara et al., 2014), however, this thesis represents the first example of the direct implication of the cuticle in the post-harvest carotenoid retention phenotype. Identification of the role of the fruit surface structure in controlling the carotenoid retention phenotype, may also suggest the role of the fruit surface in influencing other metabolic pathways.

Elevated carotenoid content has been engineered in a variety of crop plants, including rice (Beyer et al., 2002), maize (Zhu et al., 2008), and cassava (Welsch et al., 2010). Whilst these studies have been successful in engineering elevated carotenoid content, and consequently, increased provitamin A capacity, in these crops, significant problems have been encountered due to the fact that these crops tend not to retain these carotenoids during post-harvest storage (Hidalgo and Brandolini, 2008, Ortiz et al., 2016). Post-harvest storage is often unavoidable in the production of these crops in order to ensure that the consumer demand can be met. The lack of stability of these carotenoids is evidently detrimental to the economic value of these crops. Analysis of the surface structure of these crops may shed light on their inability to retain carotenoids during post-harvest storage. Previous studies engineering elevated carotenoid crops have focused solely on engineering the carotenoid pathway, whilst insufficient attention has been paid to carotenoid catabolism and changes during post-harvest storage. Consequently, greater consideration of these processes should be taken when engineering carotenoids in plants in order to minimise carotenoid degradation during post-harvest storage.

Clearly, engineering of elevated carotenoid levels in crop plants should not only consider modification of the carotenoid pathway, but must also consider the structural modifications necessary for the plant to be able to successfully retain these carotenoids.

7.2.3. Molecular mechanisms influencing carotenoid retention

Changes in gene expression have been demonstrated in this thesis to be involved in controlling the carotenoid retention phenotype. Genes differentially expressed between

the high and low carotenoid retention phenotype fruits included those involved in processes such as protecting against oxidative damage, some carotenoid biosynthesis genes, along with those involved in fatty acid metabolic processes. Interesting to note, was the fact that increasing the spatial resolution of the tissue used for gene expression analysis, by analysing transcripts from the fruit cuticle layer alone, revealed other genes which were also differentially expressed between the high and low carotenoid retention lines. Whilst some wax biosynthesis genes were found to be differentially expressed in the whole fruit analysis between high and low carotenoid retention lines, significantly more cuticle and wax biosynthesis genes were significantly differentially expressed in the tissue-specific study. This may be due to a dilution effect, in which some detail is lost when comparing transcript levels across the whole homogenised fruit tissue. This highlights the need for increased spatial resolution when analysing gene expression levels, particularly when transcript levels may be low, and when gene expression changes occur in a tissue-specific manner. The need for such spatial- and temporal-resolution has been noted in tomato, and the Tomato Expression Atlas resource has been developed, in order to facilitate analysis of gene expression at the cell and tissue level within an organ (Fernandez-Pozo et al., 2017). Clearly, this represents an invaluable tool in improving the specificity of studies. The study presented here represents one of the first transcriptomic studies in chilli pepper to account for tissue-specific gene expression changes.

A consideration of spatiotemporal changes to gene expression will also be critical in the functional characterisation of genes using VIGS. Whilst VIGS represents a valuable tool in being able to functionally characterise genes in chilli pepper, some limitations are presented, due to its inefficiency and 'patchy' silencing nature. An anthocyanin-reporter gene system has previously been used to identify fruit regions displaying gene silencing (Kim et al., 2017), however, it will also be essential to note that gene silencing may only occur in specific fruit tissues. In the case of the carotenoid retention phenotype, candidate gene silencing may only be expected in cuticle tissue, and therefore, the careful dissection of silenced material will be necessary to validate whether a gene of interest has been silenced, and the resulting phenotype. Consequently, further work may be required to establish robust gene functional characterisation systems in *Capsicum.*

Genes, including the *Or* gene have previously been shown to play an important role in carotenoid accumulation and storage (Lopez et al., 2008), and therefore it was considered that this gene may play a role in controlling carotenoid retention. However, the *Or* gene was not significantly differentially expressed between the high and low carotenoid retention lines. Despite the lack of differential expression, this does not

mean that this gene should be discounted, as a range of post-transcriptional mechanisms may mean that genes such as this are still involved in controlling the phenotype. Enzyme activity does not always reflect gene expression level, and therefore, differences may be observed in activity, despite a lack of difference in gene expression. *PSY1* was found to be significantly up-regulated in the low carotenoid retention line fruit at the breaker stage, which was unexpected, as this suggests an increase in carotenoid biosynthesis. However, it is important again to note that gene expression level does not necessarily reflect enzyme activity, and therefore, an increase in gene expression does not necessarily result in an increase in carotenoid biosynthesis. Whilst gene expression analyses have been carried out in this study, enzyme activity has not been determined, and therefore, changes in gene expression should not be concluded to be definitive changes in their respective pathways.

7.3. Future Directions

Mechanisms underlying the carotenoid retention phenotype have been studied in this thesis, however, further work may be carried out to confirm the specific role of these mechanisms, and to shed further light on the roles of identified candidate genes on this trait.

Employing QTL mapping, using the carotenoid retention values calculated for the DH pepper population may identify further genomic loci linked to the carotenoid retention trait. Whilst an initial QTL mapping study has been performed to identify significant genomic loci, performing mapping with this dataset should identify other loci, or narrow down the loci currently identified. Combining this with the RNAseq studies performed in this thesis will allow for a more focused analysis of genes potentially involved in controlling carotenoid retention.

Further QTL mapping, utilising intermediary metabolism profiling data, generated by GC-MS, will facilitate the identification of metabolite QTL (mQTL) regions to be identified, which underlie the genomic regions encoding for other metabolic traits of interest. This will allow breeders to select combinatorially for both the carotenoid retention trait, and other key metabolic quality traits.

Upon identification of further candidate genes of interest underlying the carotenoid retention phenotype, further gene functional characterisation may be carried out, using the VIGS method described in this thesis. Developing the use of an anthocyanin marker gene as a reporter gene, as has previously been used in other pepper fruit gene functional characterisation studies, may be required in order to determine the fruit phenotype as a result of gene silencing.

7.4. Project Outcomes

This thesis has produced several key findings regarding the carotenoid retention trait of pepper fruits. These have been summarised.

7.4.1. Carotenoid retention is a metabolic trait independent of intermediary metabolism

The carotenoid retention trait has been demonstrated to be independent of intermediary metabolism, and does not appear to be influenced by the composition of other metabolites in fresh fruit. This discovery may be beneficial to breeders, as this suggests that breeding towards the carotenoid retention trait will not have detrimental effects on other metabolites, which may play roles in other key quality traits, including flavour, aroma, and pungency.

7.4.2. Fruit surface structure plays a critical role in controlling carotenoid retention

Identification of the fruit surface structure as providing a protective mechanism against carotenoid degradation suggests a previously undescribed role for the fruit surface. This also suggests a novel mechanism by which carotenoid content may be controlled in fruit crops. Studies aiming to engineer elevated carotenoid levels should consider the fruit surface structure as a mechanism contributing to carotenoid accumulation and retention.

Further to this, the fruit surface structure may provide an efficient screening method for growers to select lines, which may have a high carotenoid retention phenotype. Upon fruit drying, a screen may be performed to select for ‘smooth’ surface pepper fruits. According to this thesis, these pepper varieties will be less susceptible to cuticle ‘cracking’, and therefore, will have greater carotenoid retention properties. This provides an efficient, and low-cost method for rapid identification of varieties interesting in breeding this trait. Upon crude identification of these lines, detailed molecular mapping may be performed, analysing molecular markers associated with cuticle biosynthesis. This would provide strong evidence to support such lines as high carotenoid retention candidates. These analyses could be carried out in a matter of weeks, which would save the costly long-term storage of several months for many pepper varieties to determine which lines are high carotenoid retention candidates.

7.4.3. Development of a functional characterisation tool to assess genes putatively identified as underlying the carotenoid retention trait

Candidate genes for the trait of interest have been identified using transcriptomics and QTL mapping, and further candidate genes may be identified upon further QTL mapping, however, without a method for functionally characterising the roles of these

genes of interest, they would remain as putatively characterised. Therefore, a method for characterising the function of genes was essential. Performing VIGS in chilli pepper fruits has been shown to be an effective method for determining the function of genes. Consequently, genes identified to be of interest in the carotenoid retention trait may now be characterised using this method. Whilst some further development may be needed to ensure an efficient method for identifying regions of silencing, the work carried out here provides foundations on which this further development may be carried out.

7.5. Overview

Table 7-1 Summary of experimental objectives of this thesis, and their outcomes.

Objective	Outcome
Determine carotenoid retention values for DH population	Data is discussed in Chapter 3. DH population displayed variation in carotenoid retention trait. The population showed heterosis relative to parents. Carotenoid content was shown to increase during post-harvest storage. Data to be used for QTL mapping of the trait.
Profile intermediary metabolites in DH population	Data is discussed in Chapter 3. Intermediary metabolism is independent of carotenoid retention trait. Data to be used for mQTL mapping.
Create carotenoid retention sub-population and validate metabolic profiling	Data is discussed in Chapter 3. Sub-population reflected results observed in initial screen. Volatile analysis was carried out to determine degradation products formed during post-harvest storage.
Determine whether a link between fruit morphology and carotenoid retention exists	Data is discussed in Chapter 4 and 5. Wrinkled dry fruit is correlated with low carotenoid retention pepper varieties, whilst smooth dry fruit peppers are associated with high carotenoid retention.
Determine the role that the fruit surface structure may play in controlling carotenoid retention	Data is discussed in Chapter 5. Thicker exocarp is associated with high carotenoid retention. Increased cutin monomer components associated with high carotenoid retention. Increased cuticle biosynthetic gene expression associated with high carotenoid retention.

Use RNA-seq to identify candidate genes for the carotenoid retention trait	Data is discussed in Chapter 6. Candidate genes identified by comparing transcript number between high and low carotenoid retention varieties. RNAseq data combined with QTL data to identify genes differentially expressed found within carotenoid retention loci.
Develop a method for functional characterisation of genes of interest in pepper	Data is discussed in Chapter 6. VIGS used to silence carotenoid biosynthetic genes in tomato and pepper fruit. This could be used as a method for functional characterisation of candidate genes for carotenoid retention trait.

7.6. Concluding remarks

The carotenoid retention phenotype is an important quality trait to breeders and growers of chilli pepper, however, it is also evident that this trait is highly complex, and controlled by multiple layers of regulation. Breeding for this trait will be achieved upon consideration of the various levels of control discussed in this thesis, including the biochemical, physiological, and molecular forms of regulation of carotenoid retention. A holistic approach must be taken in order to achieve pepper varieties retaining carotenoids to a high level.

The carotenoid retention trait should be considered as a priority when performing carotenoid biofortification studies in diverse crop plants, as the ability to retain carotenoids is essential in order for biofortified crops to be nutritionally beneficial to consumers. Further to this, the work performed here to determine mechanisms controlling carotenoid retention should be translated to other crops in order to optimise the retention trait, and to gain the maximum benefit from engineering carotenoids in plants.

8. References

AGUILAR-MELÉNDEZ, A., MORRELL, P. L., ROOSE, M. L. & KIM, S.-C. 2009. Genetic diversity and structure in semiwild and domesticated chiles (*Capsicum annuum*; Solanaceae) from Mexico. *American Journal of Botany,* 96**,** 1190-1202.

AHARONI, A., DIXIT, S., JETTER, R., THOENES, E., VAN ARKEL, G. & PEREIRA, A. 2004. The SHINE Clade of AP2 Domain Transcription Factors Activates Wax Biosynthesis, Alters Cuticle Properties, and Confers Drought Tolerance when Overexpressed in Arabidopsis. *The Plant Cell,* 16**,** 2463.

AJIKUMAR, P. K., TYO, K., CARLSEN, S., MUCHA, O., PHON, T. H. & STEPHANOPOULOS, G. 2008. Terpenoids: Opportunities for Biosynthesis of Natural Product Drugs Using Engineered Microorganisms. *Molecular Pharmaceutics,* 5**,** 167-190.

ALBRECHT, M., KLEIN, A., HUGUENEY, P., SANDMANN, G. & KUNTZ, M. 1995. Molecular cloning and functional expression in *E. coli* of a novel plant enzyme mediating ξ-carotene desaturation. *FEBS Letters,* 372**,** 199-202.

ALPHA-TOCOPHROL, B. C. C. P. S. G. 1994. The Effect of Vitamin E and Beta Carotene on the Incidence of Lung Cancer and Other Cancers in Male Smokers. *New England Journal of Medicine,* 330**,** 1029-1035.

ALSCHER, R. G., ERTURK, N. & HEATH, L. S. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany,* 53**,** 1331-1341.

ALSEEKH, S., TOHGE, T., WENDENBERG, R., SCOSSA, F., OMRANIAN, N., LI, J., KLEESSEN, S., GIAVALISCO, P., PLEBAN, T., MUELLER-ROEBER, B., ZAMIR, D., NIKOLOSKI, Z. & FERNIE, A. R. 2015. Identification and Mode of Inheritance of Quantitative Trait Loci for Secondary Metabolite Abundance in Tomato. *The Plant Cell,* 27**,** 485.

ANDREWS, J. 1993. Diffusion of Mesoamerican Food Complex to Southeastern Europe. *Geographical Review,* 83**,** 194-204.

APEL, K. & HIRT, H. 2004. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. *Annual Review of Plant Biology,* 55**,** 373-399.

AULDRIDGE, M. E., BLOCK, A., VOGEL, J. T., DABNEY-SMITH, C., MILA, I., BOUZAYEN, M., MAGALLANES-LUNDBACK, M., DELLAPENNA, D., MCCARTY, D. R. & KLEE, H. J. 2006. Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. *The Plant Journal,* 45**,** 982-993.

AUSTIN, J. R., FROST, E., VIDI, P.-A., KESSLER, F. & STAEHELIN, L. A. 2006. Plastoglobules Are Lipoprotein Subcompartments of the Chloroplast That Are Permanently Coupled to Thylakoid Membranes and Contain Biosynthetic Enzymes. *The Plant Cell,* 18**,** 1693.

AZA-GONZÁLEZ, C., NÚÑEZ-PALENIUS, H. G. & OCHOA-ALEJO, N. 2011. Molecular biology of capsaicinoid biosynthesis in chili pepper (Capsicum spp.). *Plant Cell Reports,* 30**,** 695-706.

BACH, L. & FAURE, J.-D. 2010. Role of very-long-chain fatty acids in plant development, when chain length does matter. *Comptes Rendus Biologies,* 333**,** 361-370.

BACH, L., MICHAELSON, L. V., HASLAM, R., BELLEC, Y., GISSOT, L., MARION, J., DA COSTA, M., BOUTIN, J.-P., MIQUEL, M., TELLIER, F., DOMERGUE, F., MARKHAM, J. E., BEAUDOIN, F., NAPIER, J. A. & FAURE, J.-D. 2008. The very-long-chain hydroxy fatty acyl-CoA dehydratase PASTICCINO2 is essential and limiting for plant development. *Proceedings of the National Academy of Sciences,* 105**,** 14727.

BALLESTER, A.-R., MOLTHOFF, J., DE VOS, R., HEKKERT, B. T. L., ORZAEZ, D., FERNÁNDEZ-MORENO, J.-P., TRIPODI, P., GRANDILLO, S., MARTIN, C.,

HELDENS, J., YKEMA, M., GRANELL, A. & BOVY, A. 2010. Biochemical and Molecular Analysis of Pink Tomatoes: Deregulated Expression of the Gene Encoding Transcription Factor SlMYB12 Leads to Pink Tomato Fruit Color. *Plant Physiology,* 152**,** 71.

BANERJEE, A. & SHARKEY, T. D. 2014. Methylerythritol 4-phosphate (MEP) pathway metabolic regulation. *Natural Product Reports,* 31**,** 1043-1055.

BANKS, N. H. & NICHOLSON, S. E. 2000. Internal atmosphere composition and skin permeance to gases of pepper fruit. *Postharvest Biology and Technology,* 18**,** 33-41.

BATHGATE, B., PURTON, M. E., GRIERSON, D. & GOODENOUGH, P. W. 1985. Plastid changes during the conversion of chloroplasts to chromoplasts in ripening tomatoes. *Planta,* 165**,** 197-204.

BEAUDOIN, F., WU, X., LI, F., HASLAM, R. P., MARKHAM, J. E., ZHENG, H., NAPIER, J. A. & KUNST, L. 2009. Functional Characterization of the Arabidopsis β-Ketoacyl-Coenzyme A Reductase Candidates of the Fatty Acid Elongase. *Plant Physiology,* 150**,** 1174.

BECHOFF, A., TOMLINS, K., DHUIQUE-MAYER, C., DOVE, R. & WESTBY, A. 2011. On-farm evaluation of the impact of drying and storage on the carotenoid content of orange-fleshed sweet potato (*Ipomea batata* Lam.). *International Journal of Food Science & Technology,* 46**,** 52-60.

BEMER, M., KARLOVA, R., BALLESTER, A. R., TIKUNOV, Y. M., BOVY, A. G., WOLTERS-ARTS, M., ROSSETTO, P. D. B., ANGENENT, G. C. & DE MAAGD, R. A. 2012. The Tomato FRUITFULL Homologs TDR4/FUL1 and MBP7/FUL2 Regulate Ethylene-Independent Aspects of Fruit Ripening. *The Plant Cell,* 24**,** 4437.

BENNETT, D. J. & KIRBY, G. W. 1968. Constitution and biosynthesis of capsaicin. *Journal of the Chemical Society C: Organic***,** 442-446.

BERNARD, A., DOMERGUE, F., PASCAL, S., JETTER, R., RENNE, C., FAURE, J.-D., HASLAM, R. P., NAPIER, J. A., LESSIRE, R. & JOUBÈS, J. 2012. Reconstitution of Plant Alkane Biosynthesis in Yeast Demonstrates That Arabidopsis ECERIFERUM1 and ECERIFERUM3 Are Core Components of a Very-Long-Chain Alkane Synthesis Complex. *The Plant Cell,* 24**,** 3106.

BERRY, H. 2015. *Elucidation of the molecular and biochemical mechanisms associated with colour intensity and colour retention in fresh and dry chilli peppers (Capsicum annuum).* PhD, Royal Holloway, University of London.

BERRY, H. M., RICKETT, D. V., BAXTER, C. J., ENFISSI, E. M. A. & FRASER, P. D. 2019. Carotenoid biosynthesis and sequestration in red chilli pepper fruit and its impact on colour intensity traits. *Journal of Experimental Botany,* 70**,** 2637-2650.

BESSIRE, M., BOREL, S., FABRE, G., CARRAÇA, L., EFREMOVA, N., YEPHREMOV, A., CAO, Y., JETTER, R., JACQUAT, A.-C., MÉTRAUX, J.-P. & NAWRATH, C. 2011. A Member of the PLEIOTROPIC DRUG RESISTANCE Family of ATP Binding Cassette Transporters Is Required for the Formation of a Functional Cuticle in Arabidopsis. *The Plant Cell,* 23**,** 1958.

BEYER, P., AL-BABILI, S., YE, X., LUCCA, P., SCHAUB, P., WELSCH, R. & POTRYKUS, I. 2002. Golden Rice: Introducing the β-Carotene Biosynthesis Pathway into Rice Endosperm by Genetic Engineering to Defeat Vitamin A Deficiency. *The Journal of Nutrition,* 132**,** 506S-510S.

BIACS, P. A., DAOOD, H. G., PAVISA, A. & HAJDU, F. 1989. Studies on the carotenoid pigments of paprika (*Capsicum annuum* L. var Sz-20). *Journal of agricultural and food chemistry,* 37**,** 350-353.

BIRCHLER, J. A., YAO, H., CHUDALAYANDI, S., VAIMAN, D. & VEITIA, R. A. 2010. Heterosis. *The Plant Cell,* 22**,** 2105.

BIRD, D., BEISSON, F., BRIGHAM, A., SHIN, J., GREER, S., JETTER, R., KUNST, L., WU, X., YEPHREMOV, A. & SAMUELS, L. 2007. Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion†. *The Plant Journal,* 52**,** 485-498.

BONE, R. A., LANDRUM, J. T., FRIEDES, L. M., GOMEZ, C. M., KILBURN, M. D., MENENDEZ, E., VIDAL, I. & WANG, W. 1997. Distribution of Lutein and Zeaxanthin Stereoisomers in the Human Retina. *Experimental Eye Research,* 64**,** 211-218.

BOON, C. S., MCCLEMENTS, D. J., WEISS, J. & DECKER, E. A. 2010. Factors Influencing the Chemical Stability of Carotenoids in Foods. *Critical Reviews in Food Science and Nutrition,* 50**,** 515-532.

BORLAUG, N. E. 1983. Contributions of Conventional Plant Breeding to Food Production. *Science,* 219**,** 689.

BOSLAND, P. & VOTAVA, E. 2000. Peppers: vegetable and spices Capsicums. Crop roduction science in horticultura. *Peppers: vegetable and spice Capsicums***,** 204.

BOTANY, D. O. 2002. *Dictionary of Botany* [Online]. Available: http://botanydictionary.org/exocarp.html [Accessed].

BOTTÉ, C. Y. & MARÉCHAL, E. 2014. Plastids with or without galactoglycerolipids. *Trends in Plant Science,* 19**,** 71-78.

BOUVIER, F., D'HARLINGUE, A., HUGUENEY, P., MARIN, E., MARION-POLL, A. & CAMARA, B. 1996. Xanthophyll Biosynthesis: CLONING, EXPRESSION, FUNCTIONAL RECONSTITUTION, AND REGULATION OF β-CYCLOHEXENYL CAROTENOID EPOXIDASE FROM PEPPER (*CAPSICUM ANNUUM*). *Journal of Biological Chemistry,* 271**,** 28861-28867.

BOUVIER, F., HUGUENEY, P., D'HARLINGUE, A., KUNTZ, M. & CAMARA, B. 1994. Xanthophyll biosynthesis in chromoplasts: isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid. *The Plant Journal,* 6**,** 45-54.

BOUVIER, F., KELLER, Y., D'HARLINGUE, A. & CAMARA, B. 1998. Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (*Capsicum annuum* L.). *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 1391**,** 320-328.

BRAMLEY, P. M. 2002. Regulation of carotenoid formation during tomato fruit ripening and development. *Journal of Experimental Botany,* 53**,** 2107-2113.

BRITTON, G. 1995. Structure and properties of carotenoids in relation to function. *The FASEB Journal,* 9**,** 1551-1558.

BRUNO, M., BEYER, P. & AL-BABILI, S. 2015. The potato carotenoid cleavage dioxygenase 4 catalyzes a single cleavage of β-ionone ring-containing carotenes and non-epoxidated xanthophylls. *Archives of Biochemistry and Biophysics,* 572**,** 126-133.

BURT, A. J., GRAINGER, C. M., YOUNG, J. C., SHELP, B. J. & LEE, E. A. 2010. Impact of Postharvest Handling on Carotenoid Concentration and Composition in High-Carotenoid Maize (*Zea mays* L.) Kernels. *Journal of Agricultural and Food Chemistry,* 58**,** 8286-8292.

CAMARA, B. & MONÉGER, R. 1978. Free and esterified carotenoids in green and red fruits of *Capsicum annuum*. *Phytochemistry,* 17**,** 91-93.

CARMONA, L., ZACARÍAS, L. & RODRIGO, M. J. 2012. Stimulation of coloration and carotenoid biosynthesis during postharvest storage of 'Navelina' orange fruit at 12°C. *Postharvest Biology and Technology,* 74**,** 108-117.

CATERINA, M. J., SCHUMACHER, M. A., TOMINAGA, M., ROSEN, T. A., LEVINE, J. D. & JULIUS, D. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* 389**,** 816-824.

CAZZONELLI, C. I. & POGSON, B. J. 2010. Source to sink: regulation of carotenoid biosynthesis in plants. *Trends in Plant Science,* 15**,** 266-274.

CHARLESWORTH, D. & WILLIS, J. H. 2009. The genetics of inbreeding depression. *Nature Reviews Genetics,* 10**,** 783.

CHAVEZ, A. L., BEDOYA, J. M., SÁNCHEZ, T., IGLESIAS, C., CEBALLOS, H. & ROCA, W. 2000. Iron, Carotene, and Ascorbic Acid in Cassava Roots and Leaves. *Food and Nutrition Bulletin,* 21**,** 410-413.

CHE, P., ZHAO, Z.-Y., GLASSMAN, K., DOLDE, D., HU, T. X., JONES, T. J., GRUIS, D. F., OBUKOSIA, S., WAMBUGU, F. & ALBERTSEN, M. C. 2016. Elevated vitamin E content improves all-trans β-carotene accumulation and stability in biofortified sorghum. *Proceedings of the National Academy of Sciences,* 113**,** 11040.

CHUNG, E., SEONG, E., KIM, Y. C., CHUNG, E. J., OH, S. K., LEE, S., PARK, J. M., JOUNG, Y. H. & CHOI, D. 2004. A method of high frequency virus induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). *Mol Cells,* 17**,** 377-80.

CONGDON, N. G. & WEST, K. P. 1999. Nutrition and the eye. *Current Opinion in Ophthalmology,* 10.

CROMBIE, W. M. 1958. Fatty Acids in Chloroplasts and Leaves. *Journal of Experimental Botany,* 9**,** 254-261.

CROSBY, K. M. 2008. Pepper. *In:* PROHENS, J. & NUEZ, F. (eds.) *Vegetables II: Fabaceae, Liliaceae, Solanaceae, and Umbelliferae.* New York, NY: Springer New York.

CUNNINGHAM, F. X. & GANTT, E. 2001. One ring or two? Determination of ring number in carotenoids by lycopene ε-cyclases. *Proceedings of the National Academy of Sciences,* 98**,** 2905.

CURL, A. L. 1962. Red pepper carotenoids, the carotenoids of red bell peppers. *Journal of Agricultural and Food Chemistry,* 10**,** 504-509.

CURRY, J., ALURU, M., MENDOZA, M., NEVAREZ, J., MELENDREZ, M. & O'CONNELL, M. A. 1999. Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent Capsicum spp. *Plant Science,* 148**,** 47-57.

CVETKOVIC, D. & NIKOLIC, G. 2017. *Carotenoids*, IntechOpen.

DAVISON, P. A., HUNTER, C. N. & HORTON, P. 2002. Overexpression of β-carotene hydroxylase enhances stress tolerance in Arabidopsis. *Nature,* 418**,** 203-206.

DAVULURI, G. R., VAN TUINEN, A., FRASER, P. D., MANFREDONIA, A., NEWMAN, R., BURGESS, D., BRUMMELL, D. A., KING, S. R., PALYS, J., UHLIG, J., BRAMLEY, P. M., PENNINGS, H. M. J. & BOWLER, C. 2005. Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nature Biotechnology,* 23**,** 890-895.

DE MOURA, F. F., MILOFF, A. & BOY, E. 2015. Retention of Provitamin A Carotenoids in Staple Crops Targeted for Biofortification in Africa: Cassava, Maize and Sweet Potato. *Critical Reviews in Food Science and Nutrition,* 55**,** 1246-1269.

DEBONO, A., YEATS, T. H., ROSE, J. K. C., BIRD, D., JETTER, R., KUNST, L. & SAMUELS, L. 2009. Arabidopsis LTPG Is a Glycosylphosphatidylinositol-Anchored Lipid Transfer Protein Required for Export of Lipids to the Plant Surface. *The Plant Cell,* 21**,** 1230.

DELI, J., MATUS, Z. & TÓTH, G. 1996. Carotenoid Composition in the Fruits of *Capsicum annuum* Cv. Szentesi Kosszarvú during Ripening. *Journal of Agricultural and Food Chemistry,* 44**,** 711-716.

DERUÈRE, J., RÖMER, S., HARLINGUE, A., BACKHAUS, R. A., KUNTZ, M. & CAMARA, B. 1994. Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. *The Plant Cell,* 6**,** 119.

DICKINSON, A. J., LEHNER, K., MI, J., JIA, K.-P., MIJAR, M., DINNENY, J., AL-BABILI, S. & BENFEY, P. N. 2019. β-Cyclocitral is a conserved root growth regulator. *Proceedings of the National Academy of Sciences,* 116**,** 10563.

DIETRICH, C. R., PERERA, M. A. D. N., D. YANDEAU-NELSON, M., MEELEY, R. B., NIKOLAU, B. J. & SCHNABLE, P. S. 2005. Characterization of two GL8 paralogs reveals that the 3-ketoacyl reductase component of fatty acid elongase is essential for maize (*Zea mays* L.) development. *The Plant Journal,* 42**,** 844-861.

DOGBO, O. & CAMARA, B. 1987. Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from Capsicum chromoplasts by

affinity chromatography. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 920**,** 140-148.
DOMÍNGUEZ, E., CUARTERO, J. & HEREDIA, A. 2011. An overview on plant cuticle biomechanics. *Plant Science,* 181**,** 77-84.
EDGE, R., MCGARVEY, D. J. & TRUSCOTT, T. G. 1997. The carotenoids as anti-oxidants — a review. *Journal of Photochemistry and Photobiology B: Biology,* 41**,** 189-200.
EDGE, R. & TRUSCOTT, G. T. 2018. Singlet Oxygen and Free Radical Reactions of Retinoids and Carotenoids—A Review. *Antioxidants,* 7.
EDWARDS, D. 1993. Cells and tissues in the vegetative sporophytes of early land plants. *New Phytologist,* 125**,** 225-247.
EGEA, I., BARSAN, C., BIAN, W., PURGATTO, E., LATCHÉ, A., CHERVIN, C., BOUZAYEN, M. & PECH, J.-C. 2010. Chromoplast Differentiation: Current Status and Perspectives. *Plant and Cell Physiology,* 51**,** 1601-1611.
EISENREICH, W., ROHDICH, F. & BACHER, A. 2001. Deoxyxylulose phosphate pathway to terpenoids. *Trends in Plant Science,* 6**,** 78-84.
ENFISSI, E. M. A., FRASER, P. D., LOIS, L.-M., BORONAT, A., SCHUCH, W. & BRAMLEY, P. M. 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnology Journal,* 3**,** 17-27.
ENFISSI, E. M. A., NOGUEIRA, M., BRAMLEY, P. M. & FRASER, P. D. 2017. The regulation of carotenoid formation in tomato fruit. *The Plant Journal,* 89**,** 774-788.
ENFISSI, E. M. A., NOGUEIRA, M., D'AMBROSIO, C., STIGLIANI, A. L., GIORIO, G., MISAWA, N. & FRASER, P. D. 2019. The road to astaxanthin production in tomato fruit reveals plastid and metabolic adaptation resulting in an unintended high lycopene genotype with delayed over-ripening properties. *Plant Biotechnology Journal,* 17**,** 1501-1513.
ESHBAUGH, W. H. 1975. Genetic and Biochemical Systematic Studies of Chili Peppers (Capsicum- Solanaceae). *Bulletin of the Torrey Botanical Club,* 102**,** 396-403.
ESTÉVEZ, J. M., CANTERO, A., REINDL, A., REICHLER, S. & LEÓN, P. 2001. 1-Deoxy-d-xylulose-5-phosphate Synthase, a Limiting Enzyme for Plastidic Isoprenoid Biosynthesis in Plants. *Journal of Biological Chemistry,* 276**,** 22901-22909.
EWAS, M., GAO, Y., WANG, S., LIU, X., ZHANG, H., NISHAWY, E. M. E., ALI, F., SHAHZAD, R., ZIAF, K., SUBTHAIN, H., MARTIN, C. & LUO, J. 2016. Manipulation of SlMXl for enhanced carotenoids accumulation and drought resistance in tomato. *Science Bulletin,* 61**,** 1413-1418.
FANTINI, E., FALCONE, G., FRUSCIANTE, S., GILIBERTO, L. & GIULIANO, G. 2013. Dissection of Tomato Lycopene Biosynthesis through Virus-Induced Gene Silencing. *Plant Physiology,* 163**,** 986.
FAOSTAT. 2017. Available: www.fao.org/faostat/en/#data/QC [Accessed].
FERNANDEZ-MORENO, J.-P., ORZAEZ, D. & GRANELL, A. 2013. VIGS: A Tool to Study Fruit Development in Solanum lycopersicum. *In:* BECKER, A. (ed.) *Virus-Induced Gene Silencing: Methods and Protocols.* Totowa, NJ: Humana Press.
FERNANDEZ-POZO, N., ROSLI, HERNAN G., MARTIN, GREGORY B. & MUELLER, LUKAS A. 2015. The SGN VIGS Tool: User-Friendly Software to Design Virus-Induced Gene Silencing (VIGS) Constructs for Functional Genomics. *Molecular Plant,* 8**,** 486-488.
FERNANDEZ-POZO, N., ZHENG, Y., SNYDER, S. I., NICOLAS, P., SHINOZAKI, Y., FEI, Z., CATALA, C., GIOVANNONI, J. J., ROSE, J. K. C. & MUELLER, L. A. 2017. The Tomato Expression Atlas. *Bioinformatics,* 33**,** 2397-2398.
FICH, E. A., SEGERSON, N. A. & ROSE, J. K. C. 2016. The Plant Polyester Cutin: Biosynthesis, Structure, and Biological Roles. *Annual Review of Plant Biology,* 67**,** 207-233.

FOREMAN, J., DEMIDCHIK, V., BOTHWELL, J. H. F., MYLONA, P., MIEDEMA, H., TORRES, M. A., LINSTEAD, P., COSTA, S., BROWNLEE, C., JONES, J. D. G., DAVIES, J. M. & DOLAN, L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature,* 422**,** 442-446.

FRANK, H. A., CUA, A., CHYNWAT, V., YOUNG, A., GOSZTOLA, D. & WASIELEWSKI, M. R. 1994. Photophysics of the carotenoids associated with the xanthophyll cycle in photosynthesis. *Photosynthesis Research,* 41**,** 389-395.

FRANKEL, E. N. 1983. Volatile lipid oxidation products. *Progress in lipid research,* 22**,** 1-33.

FRASER, P. D. & BRAMLEY, P. M. 2004. The biosynthesis and nutritional uses of carotenoids. *Progress in Lipid Research,* 43**,** 228-265.

FRASER, P. D., LINDEN, H. & SANDMANN, G. 1993. Purification and reactivation of recombinant Synechococcus phytoene desaturase from an overexpressing strain of Escherichia coli. *Biochemical Journal,* 291**,** 687.

FRASER, P. D., PINTO, M. E. S., HOLLOWAY, D. E. & BRAMLEY, P. M. 2000. Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *The Plant Journal,* 24**,** 551-558.

FRIDOVICH, I. 1989. Superoxide dismutases. An adaptation to a paramagnetic gas. *Journal of Biological Chemistry,* 264**,** 7761-7764.

FUJIWAKE, H., SUZUKI, T. & IWAI, K. 1982. Intracellular Distributions of Enzymes and Intermediates Involved in Biosynthesis of Capsaicin and Its Analogues in Capsicum Fruits. *Agricultural and Biological Chemistry,* 46**,** 2685-2689.

GAUR, R., SHARMA, V., CHHAPEKAR, S. S., DAS, J., KUMAR, A., YADAVA, S. K., NITIN, M., BRAHMA, V., ABRAHAM, S. K. & RAMCHIARY, N. 2016. Comparative Analysis of Fruit Metabolites and Pungency Candidate Genes Expression between Bhut Jolokia and Other Capsicum Species. *PLOS ONE,* 11**,** e0167791.

GAYEN, D., ALI, N., SARKAR, S. N., DATTA, S. K. & DATTA, K. 2015. Down-regulation of lipoxygenase gene reduces degradation of carotenoids of golden rice during storage. *Planta,* 242**,** 353-363.

GIOVANNONI, J., NGUYEN, C., AMPOFO, B., ZHONG, S. & FEI, Z. 2017. The Epigenome and Transcriptional Dynamics of Fruit Ripening. *Annual Review of Plant Biology,* 68**,** 61-84.

GIRARD, A.-L., MOUNET, F., LEMAIRE-CHAMLEY, M., GAILLARD, C., ELMORJANI, K., VIVANCOS, J., RUNAVOT, J.-L., QUEMENER, B., PETIT, J., GERMAIN, V., ROTHAN, C., MARION, D. & BAKAN, B. 2012. Tomato GDSL1 Is Required for Cutin Deposition in the Fruit Cuticle. *The Plant Cell,* 24**,** 3119.

GIULIANO, G. & DIRETTO, G. 2007. Of chromoplasts and chaperones. *Trends in Plant Science,* 12**,** 529-531.

GONZALEZ-JORGE, S., HA, S.-H., MAGALLANES-LUNDBACK, M., GILLILAND, L. U., ZHOU, A., LIPKA, A. E., NGUYEN, Y.-N., ANGELOVICI, R., LIN, H., CEPELA, J., LITTLE, H., BUELL, C. R., GORE, M. A. & DELLAPENNA, D. 2013. CAROTENOID CLEAVAGE DIOXYGENASE4 Is a Negative Regulator of β-Carotene Content in Arabidopsis Seeds. *The Plant Cell,* 25**,** 4812.

GRAÇA, J., SCHREIBER, L., RODRIGUES, J. & PEREIRA, H. 2002. Glycerol and glyceryl esters of ω-hydroxyacids in cutins. *Phytochemistry,* 61**,** 205-215.

GREER, S., WEN, M., BIRD, D., WU, X., SAMUELS, L., KUNST, L. & JETTER, R. 2007. The Cytochrome P450 Enzyme CYP96A15 Is the Midchain Alkane Hydroxylase Responsible for Formation of Secondary Alcohols and Ketones in Stem Cuticular Wax of Arabidopsis. *Plant Physiology,* 145**,** 653.

GUTENSOHN, M. & DUDAREVA, N. 2016. Chapter Thirteen - Tomato Fruits—A Platform for Metabolic Engineering of Terpenes. *In:* O'CONNOR, S. E. (ed.) *Methods in Enzymology.* Academic Press.

GUZMAN, I., BOSLAND, P. W. & O'CONNELL, M. A. 2011. Heat, Color, and Flavor Compounds in Capsicum Fruit. *In:* GANG, D. R. (ed.) *The Biological Activity of Phytochemicals.* New York, NY: Springer New York.

GUZMAN, I., HAMBY, S., ROMERO, J., BOSLAND, P. W. & O'CONNELL, M. A. 2010. Variability of carotenoid biosynthesis in orange colored Capsicum spp. *Plant Science,* 179**,** 49-59.

HA, S.-H., KIM, J.-B., PARK, J.-S., LEE, S.-W. & CHO, K.-J. 2007. A comparison of the carotenoid accumulation in Capsicum varieties that show different ripening colours: deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. *Journal of Experimental Botany,* 58**,** 3135-3144.

HALIKOWSKI SMITH, S. 2015. In the shadow of a pepper-centric historiography: Understanding the global diffusion of capsicums in the sixteenth and seventeenth centuries. *Journal of Ethnopharmacology,* 167**,** 64-77.

HALL, R. D. 2006. Plant metabolomics: from holistic hope, to hype, to hot topic. *New Phytologist,* 169**,** 453-468.

HALLIWELL, B. & CHIRICO, S. 1993. Lipid peroxidation: its mechanism, measurement, and significance. *The American Journal of Clinical Nutrition,* 57**,** 715S-725S.

HAN, K., JANG, S., LEE, J.-H., LEE, D.-G., KWON, J.-K. & KANG, B.-C. 2019. A MYB transcription factor is a candidate to control pungency in *Capsicum annuum*. *Theoretical and Applied Genetics,* 132**,** 1235-1246.

HASLAM, T. M., MAÑAS-FERNÁNDEZ, A., ZHAO, L. & KUNST, L. 2012. Arabidopsis ECERIFERUM2 Is a Component of the Fatty Acid Elongation Machinery Required for Fatty Acid Extension to Exceptional Lengths. *Plant Physiology,* 160**,** 1164.

HAVAUX, M. 2014. Carotenoid oxidation products as stress signals in plants. *The Plant Journal,* 79**,** 597-606.

HEISER, C. B. & PICKERSGILL, B. 1969. Names for the Cultivated Capsicum Species (Solanaceae). *Taxon,* 18**,** 277-283.

HEMMERLIN, A., HOEFFLER, J.-F., MEYER, O., TRITSCH, D., KAGAN, I. A., GROSDEMANGE-BILLIARD, C., ROHMER, M. & BACH, T. J. 2003. Cross-talk between the Cytosolic Mevalonate and the Plastidial Methylerythritol Phosphate Pathways in Tobacco Bright Yellow-2 Cells. *Journal of Biological Chemistry,* 278**,** 26666-26676.

HEN-AVIVI, S., LASHBROOKE, J., COSTA, F. & AHARONI, A. 2014. Scratching the surface: genetic regulation of cuticle assembly in fleshy fruit. *Journal of Experimental Botany,* 65**,** 4653-4664.

HIDALGO, A. & BRANDOLINI, A. 2008. Kinetics of Carotenoids Degradation during the Storage of Einkorn (*Triticum monococcum* L. ssp. monococcum) and Bread Wheat (*Triticum aestivum* L. ssp. aestivum) Flours. *Journal of Agricultural and Food Chemistry,* 56**,** 11300-11305.

HILL, C. B., TAYLOR, J. D., EDWARDS, J., MATHER, D., LANGRIDGE, P., BACIC, A. & ROESSNER, U. 2015. Detection of QTL for metabolic and agronomic traits in wheat with adjustments for variation at genetic loci that affect plant phenology. *Plant Science,* 233**,** 143-154.

HORNERO-MÉNDEZ, D., GÓMEZ-LADRÓN DE GUEVARA, R. & MÍNGUEZ-MOSQUERA, M. I. 2000. Carotenoid Biosynthesis Changes in Five Red Pepper (*Capsicum annuum* L.) Cultivars during Ripening. Cultivar Selection for Breeding. *Journal of Agricultural and Food Chemistry,* 48**,** 3857-3864.

HORTON, P., RUBAN, A. V. & WALTERS, R. G. 1996. REGULATION OF LIGHT HARVESTING IN GREEN PLANTS. *Annual Review of Plant Physiology and Plant Molecular Biology,* 47**,** 655-684.

HOU, X., RIVERS, J., LEÓN, P., MCQUINN, R. P. & POGSON, B. J. 2016. Synthesis and Function of Apocarotenoid Signals in Plants. *Trends in Plant Science,* 21**,** 792-803.

HOWARD, L. R., TALCOTT, S. T., BRENES, C. H. & VILLALON, B. 2000. Changes in Phytochemical and Antioxidant Activity of Selected Pepper Cultivars (Capsicum Species) As Influenced by Maturity. *Journal of Agricultural and Food Chemistry,* 48**,** 1713-1720.

HUANG, F.-C., MOLNÁR, P. & SCHWAB, W. 2009. Cloning and functional characterization of carotenoid cleavage dioxygenase 4 genes. *Journal of Experimental Botany,* 60**,** 3011-3022.

HUGUENEY, P., BADILLO, A., CHEN, H.-C., KLEIN, A., HIRSCHBERG, J., CAMARA, B. & KUNTZ, M. 1995. Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum* chromoplasts. *The Plant Journal,* 8**,** 417-424.

HUGUENEY, P., RÖMER, S., KUNTZ, M. & CAMARA, B. 1992. Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and ζ-carotene in Capsicum chromoplasts. *European Journal of Biochemistry,* 209**,** 399-407.

HUH, J. H., KANG, B. C., NAHM, S. H., KIM, S., HA, K. S., LEE, M. H. & KIM, B. D. 2001. A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (Capsicum spp.). *Theoretical and Applied Genetics,* 102**,** 524-530.

HULSE-KEMP, A. M., MAHESHWARI, S., STOFFEL, K., HILL, T. A., JAFFE, D., WILLIAMS, S. R., WEISENFELD, N., RAMAKRISHNAN, S., KUMAR, V., SHAH, P., SCHATZ, M. C., CHURCH, D. M. & VAN DEYNZE, A. 2018. Reference quality assembly of the 3.5-Gb genome of *Capsicum annuum* from a single linked-read library. *Horticulture Research,* 5**,** 4.

HURTADO-HERNANDEZ, H. & SMITH, P. G. 1985. Inheritance of mature fruit color in *Capsicum annuum* L. *Journal of Heredity,* 76**,** 211-213.

HUSSAIN, B., KHAN, M. A., ALI, Q. & SHAUKAT, S. 2012. Double haploid production is the best method for genetic improvement and genetic studies of wheat. *Int J Agro Vet Med Sci,* 6**,** 216-228.

IBDAH, M., AZULAY, Y., PORTNOY, V., WASSERMAN, B., BAR, E., MEIR, A., BURGER, Y., HIRSCHBERG, J., SCHAFFER, A. A., KATZIR, N., TADMOR, Y. & LEWINSOHN, E. 2006. Functional characterization of CmCCD1, a carotenoid cleavage dioxygenase from melon. *Phytochemistry,* 67**,** 1579-1589.

ISAACSON, T., KOSMA, D. K., MATAS, A. J., BUDA, G. J., HE, Y., YU, B., PRAVITASARI, A., BATTEAS, J. D., STARK, R. E., JENKS, M. A. & ROSE, J. K. C. 2009. Cutin deficiency in the tomato fruit cuticle consistently affects resistance to microbial infection and biomechanical properties, but not transpirational water loss. *The Plant Journal,* 60**,** 363-377.

ISAACSON, T., RONEN, G., ZAMIR, D. & HIRSCHBERG, J. 2002. Cloning of tangerine from Tomato Reveals a Carotenoid Isomerase Essential for the Production of β-Carotene and Xanthophylls in Plants. *The Plant Cell,* 14**,** 333.

JASWIR, I., SHAHIDAN, N., OTHMAN, R., HAS-YUN HASHIM, Y. Z., OCTAVIANTI, F. & SALLEH, M. N. B. 2014. Effects of Season and Storage Period on Accumulation of Individual Carotenoids in Pumpkin Flesh (*Cucurbita moschata*). *Journal of Oleo Science,* 63**,** 761-767.

JEONG, H.-B., KANG, M.-Y., JUNG, A., HAN, K., LEE, J.-H., JO, J., LEE, H.-Y., AN, J.-W., KIM, S. & KANG, B.-C. 2019. Single-molecule real-time sequencing reveals diverse allelic variations in carotenoid biosynthetic genes in pepper (Capsicum spp.). *Plant Biotechnology Journal,* 17**,** 1081-1093.

JETTER, R., KUNST, L. & SAMUELS, A. L. 2006. Biology of the plant cuticle. *Composition of plant cuticular waxes,* 144**,** 181.

JIN, J., TIAN, F., YANG, D.-C., MENG, Y.-Q., KONG, L., LUO, J. & GAO, G. 2016. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Research,* 45**,** D1040-D1045.

KANNANGARA, R., BRANIGAN, C., LIU, Y., PENFIELD, T., RAO, V., MOUILLE, G., HÖFTE, H., PAULY, M., RIECHMANN, J. L. & BROUN, P. 2007. The Transcription Factor WIN1/SHN1 Regulates Cutin Biosynthesis in Arabidopsis thaliana. *The Plant Cell,* 19**,** 1278.

KASHA, K. J. 2005. Chromosome Doubling and Recovery of Doubled Haploid Plants. *In:* DON PALMER, C. E., KELLER, W. A. & KASHA, K. J. (eds.) *Haploids in Crop Improvement II.* Berlin, Heidelberg: Springer Berlin Heidelberg.

KENDE, A., LIM, P. P., LAI, F., JESSOP, M., SWINDALE, L., OLIVER, M., HURR, B., RICKETT, D. & BAXTER, C. 2019. High throughput quantitative volatile profiling of melons with silicone rod extraction – thermal desorption – GC–MS for plant breeding line selection. *Food Chemistry,* 270**,** 368-374.

KIM, D., PERTEA, G., TRAPNELL, C., PIMENTEL, H., KELLEY, R. & SALZBERG, S. L. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology,* 14**,** R36.

KIM, H., LEE, S. B., KIM, H. J., MIN, M. K., HWANG, I. & SUH, M. C. 2012. Characterization of Glycosylphosphatidylinositol-Anchored Lipid Transfer Protein 2 (LTPG2) and Overlapping Function between LTPG/LTPG1 and LTPG2 in Cuticular Wax Export or Accumulation in *Arabidopsis thaliana*. *Plant and Cell Physiology,* 53**,** 1391-1403.

KIM, J., PARK, M., JEONG, E. S., LEE, J. M. & CHOI, D. 2017. Harnessing anthocyanin-rich fruit: a visible reporter for tracing virus-induced gene silencing in pepper fruit. *Plant Methods,* 13**,** 3.

KIM, O. R., CHO, M.-C., KIM, B.-D. & HUH, J. H. 2010. A splicing mutation in the gene encoding phytoene synthase causes orange coloration in Habanero pepper fruits. *Molecules and Cells,* 30**,** 569-574.

KIM, S., PARK, M., YEOM, S.-I., KIM, Y.-M., LEE, J. M., LEE, H.-A., SEO, E., CHOI, J., CHEONG, K., KIM, K.-T., JUNG, K., LEE, G.-W., OH, S.-K., BAE, C., KIM, S.-B., LEE, H.-Y., KIM, S.-Y., KIM, M.-S., KANG, B.-C., JO, Y. D., YANG, H.-B., JEONG, H.-J., KANG, W.-H., KWON, J.-K., SHIN, C., LIM, J. Y., PARK, J. H., HUH, J. H., KIM, J.-S., KIM, B.-D., COHEN, O., PARAN, I., SUH, M. C., LEE, S. B., KIM, Y.-K., SHIN, Y., NOH, S.-J., PARK, J., SEO, Y. S., KWON, S.-Y., KIM, H. A., PARK, J. M., KIM, H.-J., CHOI, S.-B., BOSLAND, P. W., REEVES, G., JO, S.-H., LEE, B.-W., CHO, H.-T., CHOI, H.-S., LEE, M.-S., YU, Y., DO CHOI, Y., PARK, B.-S., VAN DEYNZE, A., ASHRAFI, H., HILL, T., KIM, W. T., PAI, H.-S., AHN, H. K., YEAM, I., GIOVANNONI, J. J., ROSE, J. K. C., SØRENSEN, I., LEE, S.-J., KIM, R. W., CHOI, I.-Y., CHOI, B.-S., LIM, J.-S., LEE, Y.-H. & CHOI, D. 2014. Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species. *Nature Genetics,* 46**,** 270.

KNAPP, S., BOHS, L., NEE, M. & SPOONER, D. M. 2004. Solanaceae 2014;A Model for Linking Genomics with Biodiversity. *Comparative and Functional Genomics,* 5.

KOSCHMIEDER, J., FEHLING-KASCHEK, M., SCHAUB, P., GHISLA, S., BRAUSEMANN, A., TIMMER, J. & BEYER, P. 2017. Plant-type phytoene desaturase: Functional evaluation of structural implications. *PLOS ONE,* 12**,** e0187628.

KOSMA, D. K., BOURDENX, B., BERNARD, A., PARSONS, E. P., LÜ, S., JOUBÈS, J. & JENKS, M. A. 2009. The Impact of Water Deficiency on Leaf Cuticle Lipids of Arabidopsis. *Plant Physiology,* 151**,** 1918.

KOZIOŁ, M. J. & WHATLEY, F. R. 2016. *Gaseous Air Pollutants and Plant Metabolism*, Elsevier Science.

KOZUKUE, N., HAN, J.-S., KOZUKUE, E., LEE, S.-J., KIM, J.-A., LEE, K.-R., LEVIN, C. E. & FRIEDMAN, M. 2005. Analysis of Eight Capsaicinoids in Peppers and Pepper-Containing Foods by High-Performance Liquid Chromatography and Liquid Chromatography−Mass Spectrometry. *Journal of Agricultural and Food Chemistry,* 53**,** 9172-9181.

KRAFT, K. H., BROWN, C. H., NABHAN, G. P., LUEDELING, E., LUNA RUIZ, J. D. J., COPPENS D'EECKENBRUGGE, G., HIJMANS, R. J. & GEPTS, P. 2014. Multiple lines of evidence for the origin of domesticated chili pepper, *Capsicum annuum*, in Mexico. *Proceedings of the National Academy of Sciences,* 111**,** 6165.

KRAJAYKLANG, M., KLIEBER, A. & DRY, P. R. 2000. Colour at harvest and post-harvest behaviour influence paprika and chilli spice quality. *Postharvest Biology and Technology,* 20**,** 269-278.

KUMAR, D. & KALITA, P. 2017. Reducing Postharvest Losses during Storage of Grain Crops to Strengthen Food Security in Developing Countries. *Foods,* 6.

KUNST, L., TAYLOR, D. C. & UNDERHILL, E. W. 1992. Fatty acid elongation in developing seeds of *Arabidopsis thaliana*. *Plant Physiol Biochem,* 30**,** 425-434.

KURDYUKOV, S., FAUST, A., NAWRATH, C., BÄR, S., VOISIN, D., EFREMOVA, N., FRANKE, R., SCHREIBER, L., SAEDLER, H., MÉTRAUX, J.-P. & YEPHREMOV, A. 2006a. The Epidermis-Specific Extracellular BODYGUARD Controls Cuticle Development and Morphogenesis in Arabidopsis. *The Plant Cell,* 18**,** 321.

KURDYUKOV, S., FAUST, A., TRENKAMP, S., BÄR, S., FRANKE, R., EFREMOVA, N., TIETJEN, K., SCHREIBER, L., SAEDLER, H. & YEPHREMOV, A. 2006b. Genetic and biochemical evidence for involvement of HOTHEAD in the biosynthesis of long-chain α-,ω-dicarboxylic fatty acids and formation of extracellular matrix. *Planta,* 224**,** 315-329.

KWAK, J. M., MORI, I. C., PEI, Z.-M., LEONHARDT, N., TORRES, M. A., DANGL, J. L., BLOOM, R. E., BODDE, S., JONES, J. D. G. & SCHROEDER, J. I. 2003. NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *The EMBO Journal,* 22**,** 2623-2633.

LALOI, C., APEL, K. & DANON, A. 2004. Reactive oxygen signalling: the latest news. *Current Opinion in Plant Biology,* 7**,** 323-328.

LANGE, M., YELLINA, A. L., ORASHAKOVA, S. & BECKER, A. 2013. Virus-Induced Gene Silencing (VIGS) in Plants: An Overview of Target Species and the Virus-Derived Vector Systems. *In:* BECKER, A. (ed.) *Virus-Induced Gene Silencing: Methods and Protocols.* Totowa, NJ: Humana Press.

LANGMEAD, B. 2010. Aligning Short Sequencing Reads with Bowtie. *Current Protocols in Bioinformatics,* 32**,** 11.7.1-11.7.14.

LARA, I., BELGE, B. & GOULAO, L. F. 2014. The fruit cuticle as a modulator of postharvest quality. *Postharvest Biology and Technology,* 87**,** 103-112.

LASHBROOKE, J., ADATO, A., LOTAN, O., ALKAN, N., TSIMBALIST, T., RECHAV, K., FERNANDEZ-MORENO, J.-P., WIDEMANN, E., GRAUSEM, B., PINOT, F., GRANELL, A., COSTA, F. & AHARONI, A. 2015. The Tomato MIXTA-Like Transcription Factor Coordinates Fruit Epidermis Conical Cell Development and Cuticular Lipid Biosynthesis and Assembly. *Plant Physiology,* 169**,** 2553.

LEE, D. S., CHUNG, S. K., KIM, H. K. & YAM, K. L. 1991. NONENZYMATIC BROWNING IN DRIED RED PEPPER PRODUCTS. *Journal of Food Quality,* 14**,** 153-163.

LEE, S.-B., JUNG, S.-J., GO, Y.-S., KIM, H.-U., KIM, J.-K., CHO, H.-J., PARK, O. K. & SUH, M.-C. 2009. Two Arabidopsis 3-ketoacyl CoA synthase genes, KCS20 and KCS2/DAISY, are functionally redundant in cuticular wax and root suberin biosynthesis, but differentially controlled by osmotic stress. *The Plant Journal,* 60**,** 462-475.

LEFEBVRE, V., KUNTZ, M., CAMARA, B. & PALLOIX, A. 1998. The capsanthin-capsorubin synthase gene: a candidate gene for the y locus controlling the red fruit colour in pepper. *Plant Molecular Biology,* 36**,** 785-789.

LI-BEISSON, Y., POLLARD, M., SAUVEPLANE, V., PINOT, F., OHLROGGE, J. & BEISSON, F. 2009. Nanoridges that characterize the surface morphology of flowers require the synthesis of cutin polyester. *Proceedings of the National Academy of Sciences,* 106**,** 22008.

LI, F., WU, X., LAM, P., BIRD, D., ZHENG, H., SAMUELS, L., JETTER, R. & KUNST, L. 2008. Identification of the Wax Ester Synthase/Acyl-Coenzyme A:Diacylglycerol Acyltransferase WSD1 Required for Stem Wax Ester Biosynthesis in Arabidopsis. *Plant Physiology,* 148**,** 97.

LI, J., YANG, P., KANG, J., GAN, Y., YU, J., CALDERÓN-URREA, A., LYU, J., ZHANG, G., FENG, Z. & XIE, J. 2016. Transcriptome Analysis of Pepper (*Capsicum annuum*) Revealed a Role of 24-Epibrassinolide in Response to Chilling. *Frontiers in Plant Science,* 7**,** 1281.

LI, L., YANG, Y., XU, Q., OWSIANY, K., WELSCH, R., CHITCHUMROONCHOKCHAI, C., LU, S., VAN ECK, J., DENG, X.-X., FAILLA, M. & THANNHAUSER, T. W. 2012. The Or Gene Enhances Carotenoid Accumulation and Stability During Post-Harvest Storage of Potato Tubers. *Molecular Plant,* 5**,** 339-352.

LI, Y., BEISSON, F., KOO, A. J. K., MOLINA, I., POLLARD, M. & OHLROGGE, J. 2007. Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. *Proceedings of the National Academy of Sciences,* 104**,** 18339.

LINDQVIST, A. & ANDERSSON, S. 2002. Biochemical Properties of Purified Recombinant Human β-Carotene 15,15′-Monooxygenase. *Journal of Biological Chemistry,* 277**,** 23942-23948.

LIU, Z., YAN, H., WANG, K., KUANG, T., ZHANG, J., GUI, L., AN, X. & CHANG, W. 2004. Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature,* 428**,** 287-292.

LIVINGSTONE, K. D., LACKNEY, V. K., BLAUTH, J. R., VAN WIJK, R. & JAHN, M. K. 1999. Genome Mapping in Capsicum and the Evolution of Genome Structure in the Solanaceae. *Genetics,* 152**,** 1183.

LOIS, L. M., RODRÍGUEZ-CONCEPCIÓN, M., GALLEGO, F., CAMPOS, N. & BORONAT, A. 2000. Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *The Plant Journal,* 22**,** 503-513.

LOIZZO, M. R., PUGLIESE, A., BONESI, M., MENICHINI, F. & TUNDIS, R. 2015. Evaluation of chemical profile and antioxidant activity of twenty cultivars from *Capsicum annuum*, *Capsicum baccatum*, *Capsicum chacoense* and *Capsicum chinense*: A comparison between fresh and processed peppers. *LWT - Food Science and Technology,* 64**,** 623-631.

LOPEZ, A. B., VAN ECK, J., CONLIN, B. J., PAOLILLO, D. J., O'NEILL, J. & LI, L. 2008. Effect of the cauliflower Or transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *Journal of Experimental Botany,* 59**,** 213-223.

LÜ, S., SONG, T., KOSMA, D. K., PARSONS, E. P., ROWLAND, O. & JENKS, M. A. 2009. Arabidopsis CER8 encodes LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. *The Plant Journal,* 59**,** 553-564.

LU, S., VAN ECK, J., ZHOU, X., LOPEZ, A. B., HALLORAN, D. M., COSMAN, K. M., CONLIN, B. J., PAOLILLO, D. J., GARVIN, D. F., VREBALOV, J., KOCHIAN, L. V., KÜPPER, H., EARLE, E. D., CAO, J. & LI, L. 2006. The Cauliflower Or Gene Encodes a DnaJ Cysteine-Rich Domain-Containing Protein That Mediates High Levels of β-Carotene Accumulation. *The Plant Cell,* 18**,** 3594.

LUNING, P. A., DE RIJK, T., WICHERS, H. J. & ROOZEN, J. P. 1994. Gas chromatography, mass spectrometry, and sniffing port analyses of volatile compounds of fresh bell peppers (*Capsicum annuum*) at different ripening stages. *Journal of agricultural and food chemistry,* 42**,** 977-983.

MA, Y.-Z., HOLT, N. E., LI, X.-P., NIYOGI, K. K. & FLEMING, G. R. 2003. Evidence for direct carotenoid involvement in the regulation of photosynthetic light harvesting. *Proceedings of the National Academy of Sciences,* 100**,** 4377.

MAALEKUU, K., SHARON, T.-A., YAVIN, S., ELAZAR, F., YONATAN, E., MATTHEW, A. J. & MARK, S. G. 2005. Characterization of Physiological and Biochemical Factors Associated with Postharvest Water Loss in Ripe Pepper Fruit during Storage. *Journal of the American Society for Horticultural Science jashs,* 130**,** 735-741.

MAHJOUB, A., HERNOULD, M., JOUBÈS, J., DECENDIT, A., MARS, M., BARRIEU, F., HAMDI, S. & DELROT, S. 2009. Overexpression of a grapevine R2R3-MYB factor in tomato affects vegetative development, flower morphology and flavonoid and terpenoid metabolism. *Plant Physiology and Biochemistry,* 47**,** 551-561.

MALLICK, N. & MOHN, F. H. 2000. Reactive oxygen species: response of algal cells. *Journal of Plant Physiology,* 157**,** 183-193.

MAO, G. D., THOMAS, P. D., LOPASCHUK, G. D. & POZNANSKY, M. J. 1993. Superoxide dismutase (SOD)-catalase conjugates. Role of hydrogen peroxide and the Fenton reaction in SOD toxicity. *Journal of Biological Chemistry,* 268**,** 416-420.

MAOKA, T. 2009. Recent progress in structural studies of carotenoids in animals and plants. *Archives of Biochemistry and Biophysics,* 483**,** 191-195.

MARTIN, L. B. B. & ROSE, J. K. C. 2014. There's more than one way to skin a fruit: formation and functions of fruit cuticles. *Journal of Experimental Botany,* 65**,** 4639-4651.

MARTIN, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200*.

MATAS, A. J., EWARD, D. C., DOMINICK, J. P. & KARL, J. N. 2004. Crack Resistance in Cherry Tomato Fruit Correlates with Cuticular Membrane Thickness. *HortScience HortSci,* 39**,** 1354-1358.

MAZIDA, M. M., SALLEH, M. M. & OSMAN, H. 2005. Analysis of volatile aroma compounds of fresh chilli (*Capsicum annuum*) during stages of maturity using solid phase microextraction (SPME). *Journal of Food Composition and Analysis,* 18**,** 427-437.

MAZOUREK, M., PUJAR, A., BOROVSKY, Y., PARAN, I., MUELLER, L. & JAHN, M. M. 2009. A Dynamic Interface for Capsaicinoid Systems Biology. *Plant Physiology,* 150**,** 1806.

MCLEOD, M. J., GUTTMAN, S. I. & ESHBAUGH, W. H. 1982. Early Evolution of Chili Peppers (Capsicum). *Economic Botany,* 36**,** 361-368.

MILLAR, A. A., CLEMENS, S., ZACHGO, S., GIBLIN, E. M., TAYLOR, D. C. & KUNST, L. 1999. CUT1, an Arabidopsis Gene Required for Cuticular Wax Biosynthesis and Pollen Fertility, Encodes a Very-Long-Chain Fatty Acid Condensing Enzyme. *The Plant Cell,* 11**,** 825.

MINGUEZ-MOSQUERA, M. I. & HORNERO-MENDEZ, D. 1994a. Comparative study of the effect of paprika processing on the carotenoids in peppers (*Capsicum annuum*) of the Bola and Agridulce varieties. *Journal of Agricultural and Food Chemistry,* 42**,** 1555-1560.

MINGUEZ-MOSQUERA, M. I. & HORNERO-MENDEZ, D. 1994b. Formation and transformation of pigments during the fruit ripening of *Capsicum annuum* cv. Bola and Agridulce. *Journal of Agricultural and Food Chemistry,* 42**,** 38-44.

MINGUEZ-MOSQUERA, M. I., PÉREZ-GÁLVEZ, A. & GARRIDO-FERNÁNDEZ, J. 2000. Carotenoid Content of the Varieties Jaranda and Jariza (*Capsicum a nnuum* L.) and Response during the Industrial Slow Drying and Grinding Steps in Paprika Processing. *Journal of agricultural and food chemistry,* 48**,** 2972-2976.

MISHRA, S., JHA, A. B. & DUBEY, R. S. 2011. Arsenite treatment induces oxidative stress, upregulates antioxidant system, and causes phytochelatin synthesis in rice seedlings. *Protoplasma,* 248**,** 565-577.

MITTLER, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science,* 7**,** 405-410.

MITTLER, R., VANDERAUWERA, S., GOLLERY, M. & VAN BREUSEGEM, F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science,* 9**,** 490-498.

MITTLER, R., VANDERAUWERA, S., SUZUKI, N., MILLER, G., TOGNETTI, V. B., VANDEPOELE, K., GOLLERY, M., SHULAEV, V. & VAN BREUSEGEM, F. 2011. ROS signaling: the new wave? *Trends in Plant Science,* 16**,** 300-309.

MOOSE, S. P. & MUMM, R. H. 2008. Molecular Plant Breeding as the Foundation for 21st Century Crop Improvement. *Plant Physiology,* 147**,** 969.

MORTENSEN, A., SKIBSTED, L. H., SAMPSON, J., RICE-EVANS, C. & EVERETT, S. A. 1997. Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. *FEBS Letters,* 418**,** 91-97.

MORTIMER, C. L., MISAWA, N., DUCREUX, L., CAMPBELL, R., BRAMLEY, P. M., TAYLOR, M. & FRASER, P. D. 2016. Product stability and sequestration mechanisms in *Solanum tuberosum* engineered to biosynthesize high value ketocarotenoids. *Plant Biotechnology Journal,* 14**,** 140-152.

NAMBARA, E. & MARION-POLL, A. 2005. ABSCISIC ACID BIOSYNTHESIS AND CATABOLISM. *Annual Review of Plant Biology,* 56**,** 165-185.

NASCIMENTO, P., FERNANDES, N. S., MAURO, M. A. & KIMURA, M. BETA-CAROTENE STABILITY DURING DRYING AND STORAGE OF CASSAVA AND SWEET POTATO. 2009. International Society for Horticultural Science (ISHS), Leuven, Belgium, 363-366.

NATIONAL INSTITUTES OF HEALTH, N. 2019. *https://ods.od.nih.gov/factsheets/VitaminA-HealthProfessional/#en14* [Online]. Available: https://ods.od.nih.gov/factsheets/VitaminA-HealthProfessional/#en14 [Accessed].

NELSON, E. & DAWSON, L. 1923. The constitution of capsaicin, the pungent principle of Capsicum. III. *Journal of the American Chemical Society,* 45**,** 2179-2181.

NISHINO, A., YASUI, H. & MAOKA, T. 2016. Reaction of Paprika Carotenoids, Capsanthin and Capsorubin, with Reactive Oxygen Species. *Journal of Agricultural and Food Chemistry,* 64**,** 4786-4792.

NISHINO, H., MURAKOSHI, M., TOKUDA, H. & SATOMI, Y. 2009. Cancer prevention by carotenoids. *Archives of Biochemistry and Biophysics,* 483**,** 165-168.

NODA, K.-I., GLOVER, B. J., LINSTEAD, P. & MARTIN, C. 1994. Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. *Nature,* 369**,** 661-664.

NOGUEIRA, M., ENFISSI, E. M. A., ALMEIDA, J. & FRASER, P. D. 2018. Creating plant molecular factories for industrial and nutritional isoprenoid production. *Current Opinion in Biotechnology,* 49**,** 80-87.

NOGUEIRA, M., ENFISSI, E. M. A., MARTÍNEZ VALENZUELA, M. E., MENARD, G. N., DRILLER, R. L., EASTMOND, P. J., SCHUCH, W., SANDMANN, G. & FRASER, P. D. 2017. Engineering of tomato for the sustainable production of ketocarotenoids and its evaluation in aquaculture feed. *Proceedings of the National Academy of Sciences,* 114**,** 10876.

NOGUEIRA, M., MORA, L., ENFISSI, E. M. A., BRAMLEY, P. M. & FRASER, P. D. 2013. Subchromoplast sequestration of carotenoids affects regulatory mechanisms in tomato lines expressing different carotenoid gene combinations. *The Plant Cell,* 25**,** 4560-4579.

NUNES-NESI, A., ALSEEKH, S., DE OLIVEIRA SILVA, F. M., OMRANIAN, N., LICHTENSTEIN, G., MIRNEZHAD, M., GONZÁLEZ, R. R. R., SABIO Y GARCIA, J., CONTE, M., LEISS, K. A., KLINKHAMER, P. G. L., NIKOLOSKI, Z., CARRARI, F. & FERNIE, A. R. 2019. Identification and characterization of metabolite quantitative trait loci in tomato leaves and comparison with those reported for fruits and seeds. *Metabolomics,* 15**,** 46.

NUNN, N. & QIAN, N. 2010. The Columbian Exchange: A History of Disease, Food, and Ideas. *Journal of Economic Perspectives,* 24**,** 163-88.

OBERHOLSTER, R., COWAN, A. K., MOLNÁR, P. & TÓTH, G. 2001. Biochemical Basis of Color as an Aesthetic Quality in Citrus sinensis. *Journal of Agricultural and Food Chemistry,* 49**,** 303-307.

OKAZAKI, Y. & SAITO, K. 2012. Recent advances of metabolomics in plant biotechnology. *Plant Biotechnology Reports,* 6**,** 1-15.

OLSON, J. A. 1964. The biosynthesis and metabolism of carotenoids and retinol (vitamin A). *Journal of Lipid Research,* 5**,** 281-299.

OMENN, G. S., GOODMAN, G. E., THORNQUIST, M. D., BALMES, J., CULLEN, M. R., GLASS, A., KEOGH, J. P., MEYSKENS, F. L., VALANIS, B., WILLIAMS, J. H., BARNHART, S. & HAMMAR, S. 1996. Effects of a Combination of Beta Carotene and Vitamin A on Lung Cancer and Cardiovascular Disease. *New England Journal of Medicine,* 334**,** 1150-1155.

OMS-OLIU, G., HERTOG, M. L. A. T. M., VAN DE POEL, B., AMPOFO-ASIAMA, J., GEERAERD, A. H. & NICOLAÏ, B. M. 2011. Metabolic characterization of tomato fruit during preharvest development, ripening, and postharvest shelf-life. *Postharvest Biology and Technology,* 62**,** 7-16.

OP DEN CAMP, R. G. L., PRZYBYLA, D., OCHSENBEIN, C., LALOI, C., KIM, C., DANON, A., WAGNER, D., HIDEG, É., GÖBEL, C., FEUSSNER, I., NATER, M. & APEL, K. 2003. Rapid Induction of Distinct Stress Responses after the Release of Singlet Oxygen in Arabidopsis. *The Plant Cell,* 15**,** 2320.

ORTIZ, D., ROCHEFORD, T. & FERRUZZI, M. G. 2016. Influence of Temperature and Humidity on the Stability of Carotenoids in Biofortified Maize (*Zea mays* L.) Genotypes during Controlled Postharvest Storage. *Journal of Agricultural and Food Chemistry,* 64**,** 2727-2736.

ORZAEZ, D., MEDINA, A., TORRE, S., FERNÁNDEZ-MORENO, J. P., RAMBLA, J. L., FERNÁNDEZ-DEL-CARMEN, A., BUTELLI, E., MARTIN, C. & GRANELL, A. 2009. A Visual Reporter System for Virus-Induced Gene Silencing in Tomato Fruit Based on Anthocyanin Accumulation. *Plant Physiology,* 150**,** 1122.

OSHIMA, Y., SHIKATA, M., KOYAMA, T., OHTSUBO, N., MITSUDA, N. & OHME-TAKAGI, M. 2013. MIXTA-Like Transcription Factors and WAX INDUCER1/SHINE1 Coordinately Regulate Cuticle Development in Arabidopsis and *Torenia fournieri*. *The Plant Cell,* 25**,** 1609.

PANIKASHVILI, D., SHI, J. X., SCHREIBER, L. & AHARONI, A. 2011. The Arabidopsis ABCG13 transporter is required for flower cuticle secretion and patterning of the petal epidermis. *New Phytologist,* 190**,** 113-124.

PARAN, I. & VAN DER KNAAP, E. 2007. Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. *Journal of Experimental Botany,* 58**,** 3841-3852.

PARK, C.-R. & LEE, K.-J. 1975. A Study on the influence of drying methods upon the chemical changes in red pepper-2. Changes of free amino acid, free sugar. *Journal of Nutrition and Health,* 8**,** 33-37.

PARK, H., KREUNEN, S. S., CUTTRISS, A. J., DELLAPENNA, D. & POGSON, B. J. 2002. Identification of the Carotenoid Isomerase Provides Insight into Carotenoid Biosynthesis, Prolamellar Body Formation, and Photomorphogenesis. *The Plant Cell,* 14**,** 321.

PARSONS, E. P., POPOPVSKY, S., LOHREY, G. T., ALKALAI-TUVIA, S., PERZELAN, Y., BOSLAND, P., BEBELI, P. J., PARAN, I., FALLIK, E. & JENKS, M. A. 2013. Fruit cuticle lipid composition and water loss in a diverse collection of pepper (Capsicum). *Physiologia Plantarum,* 149**,** 160-174.

PARSONS, E. P., POPOPVSKY, S., LOHREY, G. T., LÜ, S., ALKALAI-TUVIA, S., PERZELAN, Y., PARAN, I., FALLIK, E. & JENKS, M. A. 2012. Fruit cuticle lipid composition and fruit post-harvest water loss in an advanced backcross generation of pepper (Capsicum sp.). *Physiologia Plantarum,* 146**,** 15-25.

PASCAL, A. A., LIU, Z., BROESS, K., VAN OORT, B., VAN AMERONGEN, H., WANG, C., HORTON, P., ROBERT, B., CHANG, W. & RUBAN, A. 2005. Molecular basis of photoprotection and control of photosynthetic light-harvesting. *Nature,* 436**,** 134-137.

PASCAL, S., BERNARD, A., SOREL, M., PERVENT, M., VILE, D., HASLAM, R. P., NAPIER, J. A., LESSIRE, R., DOMERGUE, F. & JOUBÈS, J. 2013. The Arabidopsis cer26 mutant, like the cer2 mutant, is specifically affected in the very long chain fatty acid elongation process. *The Plant Journal,* 73**,** 733-746.

PECKER, I., GABBAY, R., CUNNINGHAM, F. X. & HIRSCHBERG, J. 1996. Cloning and characterization of the cDNA for lycopene β-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Molecular Biology,* 30**,** 807-819.

PÉNICAUD, C., ACHIR, N., DHUIQUE-MAYER, C., DORNIER, M. & BOHUON, P. 2011. Degradation of β-carotene during fruit and vegetable processing or storage: reaction mechanisms and kinetic aspects: a review. *Fruits,* 66**,** 417-440.

PÉREZ-GÁLVEZ, A., GARRIDO-FERNÁNDEZ, J., MÍNGUEZ-MOSQUERA, M. I., LOZANO-RUIZ, M. & MONTERO-DE-ESPINOSA, V. 1999. Fatty acid composition of two new pepper varieties (*Capsicum annuum* L. cv. Jaranda and Jariza). Effect of drying process and nutritional aspects. *Journal of the American Oil Chemists' Society,* 76**,** 205-208.

PÉREZ-GÁLVEZ, A., HORNERO-MÉNDEZ, D. & MÍNGUEZ-MOSQUERA, M. I. 2009. Stability of Paprika without Supplementary Antioxidants during Storage under Industrial Controlled Conditions. *Journal of Agricultural and Food Chemistry,* 57**,** 4718-4723.

PÉREZ-GÁLVEZ, A. & MÍNGUEZ-MOSQUERA, M. I. 2001. Structure−Reactivity Relationship in the Oxidation of Carotenoid Pigments of the Pepper (*Capsicum annuum* L.). *Journal of Agricultural and Food Chemistry,* 49**,** 4864-4869.

PERRY, L., DICKAU, R., ZARRILLO, S., HOLST, I., PEARSALL, D. M., PIPERNO, D. R., BERMAN, M. J., COOKE, R. G., RADEMAKER, K., RANERE, A. J., RAYMOND, J. S., SANDWEISS, D. H., SCARAMELLI, F., TARBLE, K. & ZEIDLER, J. A. 2007. Starch Fossils and the Domestication and Dispersal of Chili Peppers (Capsicum spp. L.) in the Americas. *Science,* 315**,** 986.

PICKERSGILL, B. 1971. Relationships Between Weedy and Cultivated Forms in Some Species of Chili Peppers (Genus capsicum). *Evolution,* 25**,** 683-691.

PICKERSGILL, B. 1988. The genus Capsicum: a multidisciplinay approach to the taxonomy of cultivated and wild plants. v.107:381-389.

PICKERSGILL, B. 2007. Domestication of Plants in the Americas: Insights from Mendelian and Molecular Genetics. *Annals of Botany,* 100**,** 925-940.

PIGHIN, J. A., ZHENG, H., BALAKSHIN, L. J., GOODMAN, I. P., WESTERN, T. L., JETTER, R., KUNST, L. & SAMUELS, A. L. 2004. Plant Cuticular Lipid Export Requires an ABC Transporter. *Science,* 306**,** 702.

POPOVSKY-SARID, S., BOROVSKY, Y., FAIGENBOIM, A., PARSONS, E. P., LOHREY, G. T., ALKALAI-TUVIA, S., FALLIK, E., JENKS, M. A. & PARAN, I. 2017. Genetic and biochemical analysis reveals linked QTLs determining natural variation for fruit post-harvest water loss in pepper (Capsicum). *Theoretical and Applied Genetics,* 130**,** 445-459.

POPOVSKY, S. & PARAN, I. 2000. Molecular genetics of the y locus in pepper: its relation to capsanthin-capsorubin synthase and to fruit color. *Theoretical and Applied Genetics,* 101**,** 86-89.

PRICE, E. 2016. *Metabolomics of Dioscorea spp. (Yam): Biochemical diversity of an understudied and underutilised crop.* PhD, Royal Holloway, University of London.

PRUSKY, D. 2011. Reduction of the incidence of postharvest quality losses, and future prospects. *Food Security,* 3**,** 463-474.

PYKE, K. A. 1999. Plastid Division and Development. *The Plant Cell,* 11**,** 549.

QIN, C., YU, C., SHEN, Y., FANG, X., CHEN, L., MIN, J., CHENG, J., ZHAO, S., XU, M., LUO, Y., YANG, Y., WU, Z., MAO, L., WU, H., LING-HU, C., ZHOU, H., LIN, H., GONZÁLEZ-MORALES, S., TREJO-SAAVEDRA, D. L., TIAN, H., TANG, X., ZHAO, M., HUANG, Z., ZHOU, A., YAO, X., CUI, J., LI, W., CHEN, Z., FENG, Y., NIU, Y., BI, S., YANG, X., LI, W., CAI, H., LUO, X., MONTES-HERNÁNDEZ, S., LEYVA-GONZÁLEZ, M. A., XIONG, Z., HE, X., BAI, L., TAN, S., TANG, X., LIU, D., LIU, J., ZHANG, S., CHEN, M., ZHANG, L., ZHANG, L., ZHANG, Y., LIAO, W., ZHANG, Y., WANG, M., LV, X., WEN, B., LIU, H., LUAN, H., ZHANG, Y., YANG, S., WANG, X., XU, J., LI, X., LI, S., WANG, J., PALLOIX, A., BOSLAND, P. W., LI, Y., KROGH, A., RIVERA-BUSTAMANTE, R. F., HERRERA-ESTRELLA, L., YIN, Y., YU, J., HU, K. & ZHANG, Z. 2014. Whole-genome sequencing of cultivated and wild peppers provides insights into Capsicum domestication and specialization. *Proceedings of the National Academy of Sciences,* 111**,** 5135.

RAMEL, F., BIRTIC, S., GINIES, C., SOUBIGOU-TACONNAT, L., TRIANTAPHYLIDÈS, C. & HAVAUX, M. 2012. Carotenoid oxidation products

are stress signals that mediate gene responses to singlet oxygen in plants. *Proceedings of the National Academy of Sciences,* 109**,** 5535.
RAO, A. V. & RAO, L. G. 2007. Carotenoids and human health. *Pharmacological Research,* 55**,** 207-216.
REDMOND, T. M., GENTLEMAN, S., DUNCAN, T., YU, S., WIGGERT, B., GANTT, E. & CUNNINGHAM, F. X. 2001. Identification, Expression, and Substrate Specificity of a Mammalian β-Carotene 15,15′-Dioxygenase. *Journal of Biological Chemistry,* 276**,** 6560-6565.
REILLY, K., GÓMEZ-VÁSQUEZ, R., BUSCHMANN, H., TOHME, J. & BEECHING, J. R. 2003. Oxidative stress responses during cassava post-harvest physiological deterioration. *Plant Molecular Biology,* 53**,** 669-685.
RHINN, M. & DOLLÉ, P. 2012. Retinoic acid signalling during development. *Development,* 139**,** 843.
RHOADS, A. & AU, K. F. 2015. PacBio Sequencing and Its Applications. *Genomics, Proteomics & Bioinformatics,* 13**,** 278-289.
RIEDERER, M. & MULLER, C. 2008. *Annual Plant Reviews, Biology of the Plant Cuticle*, Wiley.
RINALDI, R., VAN DEYNZE, A., PORTIS, E., ROTINO, G. L., TOPPINO, L., HILL, T., ASHRAFI, H., BARCHI, L. & LANTERI, S. 2016. New Insights on Eggplant/Tomato/Pepper Synteny and Identification of Eggplant and Pepper Orthologous QTL. *Frontiers in Plant Science,* 7**,** 1031.
RISSO, D. 2013. EDASeq: Exploratory Data Analysis and Normalization for RNA-Seq.
ROCKHOLM, D. C. & YAMAMOTO, H. Y. 1996. Violaxanthin De-Epoxidase (Purification of a 43-Kilodalton Lumenal Protein from Lettuce by Lipid-Affinity Precipitation with Monogalactosyldiacylglyceride). *Plant Physiology,* 110**,** 697.
RODRÍGUEZ-SÁIZ, M., DE LA FUENTE, J. L. & BARREDO, J. L. 2010. *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin. *Applied Microbiology and Biotechnology,* 88**,** 645-658.
RODRÍGUEZ-VILLALÓN, A., GAS, E. & RODRÍGUEZ-CONCEPCIÓN, M. 2009. Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown Arabidopsis seedlings. *The Plant Journal,* 60**,** 424-435.
ROHMER, M. 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Natural product reports,* 16**,** 565-574.
ROHMER, M., SEEMANN, M., HORBACH, S., BRINGER-MEYER, S. & SAHM, H. 1996. Glyceraldehyde 3-Phosphate and Pyruvate as Precursors of Isoprenic Units in an Alternative Non-mevalonate Pathway for Terpenoid Biosynthesis. *Journal of the American Chemical Society,* 118**,** 2564-2566.
ROMER, S., HUGUENEY, P., BOUVIER, F., CAMARA, B. & KUNTZ, M. 1993. Expression of the Genes Encoding the Early Carotenoid Biosynthetic-Enzymes in *Capsicum annuum*. *Biochemical and Biophysical Research Communications,* 196**,** 1414-1421.
ROTTET, S., DEVILLERS, J., GLAUSER, G., DOUET, V., BESAGNI, C. & KESSLER, F. 2016. Identification of Plastoglobules as a Site of Carotenoid Cleavage. *Frontiers in Plant Science,* 7**,** 1855.
ROWLAND, O., ZHENG, H., HEPWORTH, S. R., LAM, P., JETTER, R. & KUNST, L. 2006. CER4 Encodes an Alcohol-Forming Fatty Acyl-Coenzyme A Reductase Involved in Cuticular Wax Production in Arabidopsis. *Plant Physiology,* 142**,** 866.
RUYTER-SPIRA, C., AL-BABILI, S., VAN DER KROL, S. & BOUWMEESTER, H. 2013. The biology of strigolactones. *Trends in Plant Science,* 18**,** 72-83.
SÄRKINEN, T., BOHS, L., OLMSTEAD, R. G. & KNAPP, S. 2013. A phylogenetic framework for evolutionary study of the nightshades (Solanaceae): a dated 1000-tip tree. *BMC Evolutionary Biology,* 13**,** 214.
SCANDALIOS, J. G. 1993. Oxygen Stress and Superoxide Dismutases. *Plant physiology,* 101**,** 7-12.

SCHÖNHERR, J. & SCHREIBER, L. 2004. Size selectivity of aqueous pores in astomatous cuticular membranes isolated from *Populus canescens* (Aiton) Sm. leaves. *Planta,* 219**,** 405-411.

SCHWARTZ, S. H., QIN, X. & ZEEVAART, J. A. D. 2001. Characterization of a Novel Carotenoid Cleavage Dioxygenase from Plants. *Journal of Biological Chemistry,* 276**,** 25208-25211.

SCHWEIGGERT, U., KAMMERER, D. R., CARLE, R. & SCHIEBER, A. 2005. Characterization of carotenoids and carotenoid esters in red pepper pods (*Capsicum annuum* L.) by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid Communications in Mass Spectrometry,* 19**,** 2617-2628.

SCHWEIGGERT, U., KURZ, C., SCHIEBER, A. & CARLE, R. 2007. Effects of processing and storage on the stability of free and esterified carotenoids of red peppers (*Capsicum annuum* L.) and hot chilli peppers (*Capsicum frutescens* L.). *European Food Research and Technology,* 225**,** 261-270.

SEDDON, J. M., AJANI, U. A., SPERDUTO, R. D., HILLER, R., BLAIR, N., BURTON, T. C., FARBER, M. D., GRAGOUDAS, E. S., HALLER, J., MILLER, D. T., YANNUZZI, L. A. & WILLETT, W. 1994. Dietary Carotenoids, Vitamins A, C, and E, and Advanced Age-Related Macular Degeneration. *JAMA,* 272**,** 1413-1420.

SHARMA, P. & DUBEY, R. S. 2005. Drought Induces Oxidative Stress and Enhances the Activities of Antioxidant Enzymes in Growing Rice Seedlings. *Plant Growth Regulation,* 46**,** 209-221.

SHARMA, P., JHA, A. B., DUBEY, R. S. & PESSARAKLI, M. 2012. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany,* 2012**,** 26.

SHI, J. X., ADATO, A., ALKAN, N., HE, Y., LASHBROOKE, J., MATAS, A. J., MEIR, S., MALITSKY, S., ISAACSON, T., PRUSKY, D., LESHKOWITZ, D., SCHREIBER, L., GRANELL, A. R., WIDEMANN, E., GRAUSEM, B., PINOT, F., ROSE, J. K. C., ROGACHEV, I., ROTHAN, C. & AHARONI, A. 2013. The tomato SlSHINE3 transcription factor regulates fruit cuticle formation and epidermal patterning. *New Phytologist,* 197**,** 468-480.

SHI, J. X., MALITSKY, S., DE OLIVEIRA, S., BRANIGAN, C., FRANKE, R. B., SCHREIBER, L. & AHARONI, A. 2011. SHINE Transcription Factors Act Redundantly to Pattern the Archetypal Surface of Arabidopsis Flower Organs. *PLOS Genetics,* 7**,** e1001388.

SIMKIN, A. J., GAFFÉ, J., ALCARAZ, J.-P., CARDE, J.-P., BRAMLEY, P. M., FRASER, P. D. & KUNTZ, M. 2007. Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Phytochemistry,* 68**,** 1545-1556.

SIMKIN, A. J., SCHWARTZ, S. H., AULDRIDGE, M., TAYLOR, M. G. & KLEE, H. J. 2004. The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles β-ionone, pseudoionone, and geranylacetone. *The Plant Journal,* 40**,** 882-892.

SMITH, A. M., COUPLAND, G., DOLAN, L., HARBERD, N., JONES, J., MARTIN, C., SABLOWSKI, R. & AMEY, A. 2009. *Plant Biology*, Taylor & Francis Group.

SNAPE, J. W. The use of doubled haploids in plant breeding. 1981 1981. 52-58.

SORA, G. T. D. S., SOUZA, A. H. P., ZIELINSKI, A. A. F., HAMINIUK, C. W. I., MATSUSHITA, M. & PERALTA, R. M. 2015. FATTY ACID COMPOSITION OF Capsicum GENUS PEPPERS. *Ciência e Agrotecnologia,* 39**,** 372-380.

SPRENGER, G. A., SCHÖRKEN, U., WIEGERT, T., GROLLE, S., DE GRAAF, A. A., TAYLOR, S. V., BEGLEY, T. P., BRINGER-MEYER, S. & SAHM, H. 1997. Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. *Proceedings of the National Academy of Sciences,* 94**,** 12857.

SPURR, A. R. & HARRIS, W. M. 1968. ULTRASTRUCTURE OF CHLOROPLASTS AND CHROMOPLASTS IN *CAPSICUM ANNUUM* I. THYLAKOID MEMBRANE

CHANGES DURING FRUIT RIPENING. *American Journal of Botany,* 55**,** 1210-1224.
STAHL, W. & SIES, H. 2003. Antioxidant activity of carotenoids. *Molecular Aspects of Medicine,* 24**,** 345-351.
STEINMÜLLER, D. & TEVINI, M. 1985. Composition and function of plastoglobuli. *Planta,* 163**,** 201-207.
STEWART JR, C., KANG, B.-C., LIU, K., MAZOUREK, M., MOORE, S. L., YOO, E. Y., KIM, B.-D., PARAN, I. & JAHN, M. M. 2005. The Pun1 gene for pungency in pepper encodes a putative acyltransferase. *The Plant Journal,* 42**,** 675-688.
SUKRASNO, N. & YEOMAN, M. 1993. Phenylpropanoid metabolism during growth and development of *Capsicum frutescens* fruits. *Phytochemistry,* 32**,** 839-844.
SUZUKI, T., FUJIWAKE, H. & IWAI, K. 1980. Intracellular localization of capsaicin and its analogues, capsaicinoid, in Capsicum fruit 1. Microscopic investigation of the structure of the placenta of *Capsicum annuum* var. annuum cv. Karayatsubusa1. *Plant and Cell Physiology,* 21**,** 839-853.
TALEON, V., MUGODE, L., CABRERA-SOTO, L. & PALACIOS-ROJAS, N. 2017. Carotenoid retention in biofortified maize using different post-harvest storage and packaging methods. *Food Chemistry,* 232**,** 60-66.
TAN, B.-C., JOSEPH, L. M., DENG, W.-T., LIU, L., LI, Q.-B., CLINE, K. & MCCARTY, D. R. 2003. Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. *The Plant Journal,* 35**,** 44-56.
TAYLOR, A., JACQUES, P. F. & DOREY, C. K. 1993. Oxidation and Aging: Impact on Vision. *Toxicology and Industrial Health,* 9**,** 349-371.
TEFERA, T. 2012. Post-harvest losses in African maize in the face of increasing food shortage. *Food Security,* 4**,** 267-277.
TEWKSBURY, J. J. & NABHAN, G. P. 2001. Directed deterrence by capsaicin in chillies. *Nature,* 412**,** 403-404.
THONUSIN, C., IGLAYREGER, H. B., SONI, T., ROTHBERG, A. E., BURANT, C. F. & EVANS, C. R. 2017. Evaluation of intensity drift correction strategies using MetaboDrift, a normalization tool for multi-batch metabolomics data. *Journal of Chromatography A,* 1523**,** 265-274.
TOLEDO-ORTIZ, G., HUQ, E. & RODRÍGUEZ-CONCEPCIÓN, M. 2010. Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. *Proceedings of the National Academy of Sciences,* 107**,** 11626.
TOPUZ, A. & OZDEMIR, F. 2004. Influences of gamma irradiation and storage on the capsaicinoids of sun-dried and dehydrated paprika. *Food Chemistry,* 86**,** 509-515.
TRAPNELL, C., ROBERTS, A., GOFF, L., PERTEA, G., KIM, D., KELLEY, D. R., PIMENTEL, H., SALZBERG, S. L., RINN, J. L. & PACHTER, L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols,* 7**,** 562.
VAN BREUSEGEM, F. & DAT, J. F. 2006. Reactive Oxygen Species in Plant Cell Death. *Plant Physiology,* 141**,** 384.
VAN RUYSKENSVELDE, V., VAN BREUSEGEM, F. & VAN DER KELEN, K. 2018. Post-transcriptional regulation of the oxidative stress response in plants. *Free Radical Biology and Medicine,* 122**,** 181-192.
VON LINTIG, J., WELSCH, R., BONK, M., GIULIANO, G., BATSCHAUER, A. & KLEINIG, H. 1997. Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *The Plant Journal,* 12**,** 625-634.
VRAÞAR, L. O., TEPIÜ, A. N., VUJIÞIÜ, B. L. & ŠOLAJA, S. 2007. Influence of the heat treatment on the colour of ground pepper (*Capsicum annuum*).
WAHYUNI, Y., BALLESTER, A.-R., SUDARMONOWATI, E., BINO, R. J. & BOVY, A. G. 2011. Metabolite biodiversity in pepper (Capsicum) fruits of thirty-two diverse

accessions: Variation in health-related compounds and implications for breeding. *Phytochemistry,* 72**,** 1358-1370.
WAHYUNI, Y., BALLESTER, A.-R., TIKUNOV, Y., DE VOS, R. C. H., PELGROM, K. T. B., MAHARIJAYA, A., SUDARMONOWATI, E., BINO, R. J. & BOVY, A. G. 2013. Metabolomics and molecular marker analysis to explore pepper (Capsicum sp.) biodiversity. *Metabolomics,* 9**,** 130-144.
WALL, M. M. & BOSLAND, P. W. 1998. Analytical methods for color and pungency of chiles (capsicums). *In:* WETZEL, D. L. B. & CHARALAMBOUS, G. (eds.) *Developments in Food Science.* Elsevier.
WALTER, M. H. & STRACK, D. 2011. Carotenoids and their cleavage products: biosynthesis and functions. *Natural product reports,* 28**,** 663-692.
WANG, J. E., LI, D. W., GONG, Z. H. & ZHANG, Y. L. 2013. Optimization of virus-induced gene silencing in pepper (*Capsicum annuum* L.). *Genet Mol Res,* 12**,** 2492-2506.
WELSCH, R., ARANGO, J., BÄR, C., SALAZAR, B., AL-BABILI, S., BELTRÁN, J., CHAVARRIAGA, P., CEBALLOS, H., TOHME, J. & BEYER, P. 2010. Provitamin A Accumulation in Cassava (*Manihot esculenta*) Roots Driven by a Single Nucleotide Polymorphism in a Phytoene Synthase Gene. *The Plant Cell,* 22**,** 3348.
WELSCH, R., BEYER, P., HUGUENEY, P., KLEINIG, H. & VON LINTIG, J. 2000. Regulation and activation of phytoene synthase, a key enzyme in carotenoid biosynthesis, during photomorphogenesis. *Planta,* 211**,** 846-854.
WORLD HEALTH ORGANIZATION, W. 2019. *https://www.who.int/nutrition/topics/vad/en/* [Online]. Available: https://www.who.int/nutrition/topics/vad/en/ [Accessed].
WU, D., LIM, E., VAILLANT, F., ASSELIN-LABAT, M.-L., VISVADER, J. E. & SMYTH, G. K. 2010. ROAST: rotation gene set tests for complex microarray experiments. *Bioinformatics (Oxford, England),* 26**,** 2176-2182.
XU, J., DUAN, X., YANG, J., BEECHING, J. R. & ZHANG, P. 2013. Enhanced Reactive Oxygen Species Scavenging by Overproduction of Superoxide Dismutase and Catalase Delays Postharvest Physiological Deterioration of Cassava Storage Roots. *Plant Physiology,* 161**,** 1517.
YAMAMOTO, H. Y., KAMITE, L. & WANG, Y.-Y. 1972. An Ascorbate-induced Absorbance Change in Chloroplasts from Violaxanthin De-epoxidation. *Plant Physiology,* 49**,** 224.
YAMAMOTO, H. Y., NAKAYAMA, T. O. M. & CHICHESTER, C. O. 1962. Studies on the light and dark interconversions of leaf xanthophylls. *Archives of Biochemistry and Biophysics,* 97**,** 168-173.
YANG, W., POLLARD, M., LI-BEISSON, Y., BEISSON, F., FEIG, M. & OHLROGGE, J. 2010. A distinct type of glycerol-3-phosphate acyltransferase with sn-2 preference and phosphatase activity producing 2-monoacylglycerol. *Proceedings of the National Academy of Sciences,* 107**,** 12040.
YEATS, T. H., MARTIN, L. B. B., VIART, H. M. F., ISAACSON, T., HE, Y., ZHAO, L., MATAS, A. J., BUDA, G. J., DOMOZYCH, D. S., CLAUSEN, M. H. & ROSE, J. K. C. 2012. The identification of cutin synthase: formation of the plant polyester cutin. *Nature Chemical Biology,* 8**,** 609.
YEATS, T. H. & ROSE, J. K. C. 2008. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Science,* 17**,** 191-198.
YEATS, T. H. & ROSE, J. K. C. 2013. The Formation and Function of Plant Cuticles. *Plant Physiology,* 163**,** 5.
YOUNG, N. D. 1996. QTL MAPPING AND QUANTITATIVE DISEASE RESISTANCE IN PLANTS. *Annual Review of Phytopathology,* 34**,** 479-501.
YTTERBERG, A. J., PELTIER, J.-B. & VAN WIJK, K. J. 2006. Protein Profiling of Plastoglobules in Chloroplasts and Chromoplasts. A Surprising Site for Differential Accumulation of Metabolic Enzymes. *Plant Physiology,* 140**,** 984.
ZHANG, X., LIU, H., GUO, Q., ZHENG, C., LI, C., XIANG, X., ZHAO, D., LIU, J., LUO, J. & ZHAO, D. 2016. Genome-wide identification, phylogenetic relationships,

and expression analysis of the carotenoid cleavage oxygenase gene family in pepper. *Genetics and molecular research: GMR,* 15.

ZHANG, Z., LI, D.-W., JIN, J.-H., YIN, Y.-X., ZHANG, H.-X., CHAI, W.-G. & GONG, Z.-H. 2015. VIGS approach reveals the modulation of anthocyanin biosynthetic genes by CaMYB in chili pepper leaves. *Frontiers in Plant Science,* 6**,** 500.

ZHAO, L., CHANG, W.-C., XIAO, Y., LIU, H.-W. & LIU, P. 2013. Methylerythritol Phosphate Pathway of Isoprenoid Biosynthesis. *Annual Review of Biochemistry,* 82**,** 497-530.

ZHENG, H., ROWLAND, O. & KUNST, L. 2005. Disruptions of the Arabidopsis Enoyl-CoA Reductase Gene Reveal an Essential Role for Very-Long-Chain Fatty Acid Synthesis in Cell Expansion during Plant Morphogenesis. *The Plant Cell,* 17**,** 1467.

ZHOU, X., WELSCH, R., YANG, Y., ÁLVAREZ, D., RIEDIGER, M., YUAN, H., FISH, T., LIU, J., THANNHAUSER, T. W. & LI, L. 2015. Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis. *Proceedings of the National Academy of Sciences,* 112**,** 3558.

ZHU, C., NAQVI, S., BREITENBACH, J., SANDMANN, G., CHRISTOU, P. & CAPELL, T. 2008. Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proceedings of the National Academy of Sciences,* 105**,** 18232.

ZSÖGÖN, A., ČERMÁK, T., NAVES, E. R., NOTINI, M. M., EDEL, K. H., WEINL, S., FRESCHI, L., VOYTAS, D. F., KUDLA, J. & PERES, L. E. P. 2018. De novo domestication of wild tomato using genome editing. *Nature biotechnology*.

9. Appendices

9.1. DH population retention classification

Table 9-1 DH population retention classification.

DH population lines were assigned a carotenoid retention classification following carotenoid analysis of fresh and stored fruits. Carotenoid retention values were scaled from 0-100. Lines with retention values below the values of the population parents were defined as low retention. Lines with retention values above the values of the population parents were defined as high retention. Lines with retention values between the values of the population parents were defined as medium retention. Change in total carotenoid amount were expressed as a percentage. Lines were assigned a cluster number, dependent on the cluster they were grouped into using agglomerative hierarchical clustering (Figure 3-7).

DH Line	Scaled Total Carotenoid Retention Value	Retention Phenotype	Total Carotenoid Change (%)	Dendrogram Cluster Number
11-2091	0.00	Low	-53.71	1
11-2075	3.67	Low	-25.34	1
11-1967	4.63	Low	-17.94	1
11-1764	5.01	Low	-14.96	2
11-1937	5.17	Low	-13.75	1
12-1844	6.03	Low	-7.11	2
11-1773	6.07	Low	-6.82	1
11-2031	6.80	Low	-1.19	1
11-1973	7.87	Low	7.07	2
11-1949	8.01	Low	8.17	2
11-2094	8.18	Low	9.49	1
12-1803	8.21	Low	9.75	2
11-1792	8.29	Low	10.35	2
12-1791	8.43	Low	11.40	3
11-1976	8.43	Low	11.47	2
11-1896	8.62	Low	12.90	1
11-2081	8.63	Low	12.95	1
11-1999	8.69	Low	13.46	3
12-1796	9.00	Low	15.87	1
11-2052	9.02	Low	16.00	1
11-1881	9.67	Low	21.02	3
11-2104	9.94	Low	23.11	1
11-1926	10.30	Low	25.89	3
11-1877	10.55	Low	27.78	1
11-1854	10.67	Low	28.73	1
11-1904	10.70	Low	28.98	1
11-2105	10.91	Low	30.59	1
11-1808	10.97	Low	31.04	2
12-1799	11.06	Low	31.77	3
11-1900	11.10	Low	32.10	1
11-1905	11.22	Low	32.97	1
11-1953	11.36	Low	34.08	1
11-1929	11.37	Low	34.16	3
11-1852	11.65	Low	36.35	3
11-2009	11.69	Low	36.59	3
11-1987	11.86	Low	37.90	3
11-1888	11.94	Low	38.59	3
11-1840	12.01	Low	39.12	1
11-1925	12.19	Low	40.51	1
12-1787	12.24	Low	40.86	2
11-2086	12.29	Low	41.25	3
11-1765	12.33	Low	41.61	2
11-1856	12.35	Low	41.70	3
12-1857	12.38	Low	41.98	3
11-1784	12.54	Low	43.17	2
11-1818	12.55	Low	43.25	2
11-1975	12.59	Low	43.59	1
11-1907	12.61	Low	43.71	1
11-2098	12.64	Low	43.93	3

12-1802	12.67	Low	44.23	3
11-1970	12.75	Low	44.85	2
11-1972	12.85	Low	45.62	1
11-1981	12.91	Low	46.09	3
11-1927	13.06	Low	47.21	3
11-2100	13.14	Low	47.85	1
11-2113	13.15	Low	47.94	3
12-1860	13.23	Low	48.53	2
11-1868	13.26	Low	48.75	1
11-1989	13.28	Low	48.91	2
12-1815	13.29	Low	48.97	3
11-1793	13.33	Low	49.29	3
11-2007	13.47	Low	50.35	1
11-1857	13.63	Low	51.65	1
11-1759	14.24	Low	56.36	1
11-1778	14.25	Low	56.41	1
11-1928	14.26	Low	56.50	2
11-1853	14.30	Low	56.82	1
11-1757	14.36	Low	57.27	1
11-1883	14.37	Low	57.30	1
11-1850	14.37	Low	57.32	1
11-2076	14.37	Low	57.35	1
11-1805	14.39	Low	57.49	3
11-2044	14.43	Low	57.82	1
11-1887	14.44	Low	57.91	3
11-1889	14.46	Low	57.99	3
11-1966	14.46	Low	58.07	1
11-2145	14.50	Low	58.36	3
12-1797	14.57	Low	58.85	3
11-2078	14.64	Low	59.43	3
11-1898	14.79	Low	60.62	2
11-2089	14.82	Low	60.84	2
11-1938	14.89	Low	61.35	2
12-1813	14.96	Low	61.91	3
11-1952	14.99	Low	62.11	1
11-1836	15.00	Low	62.24	3
11-1873	15.04	Low	62.55	1
11-2109	15.21	Low	63.82	1
11-1791	15.23	Low	64.01	1
11-2039	15.28	Low	64.40	3
12-1783	15.31	Low	64.60	3
11-1797	15.41	Low	65.39	3
11-1806	15.42	Low	65.48	2
12-1845	15.53	Low	66.30	3
11-1763	15.59	Low	66.78	1
11-1990	15.64	Low	67.16	1
12-1814	15.80	Low	68.36	2
11-2134	16.09	Low	70.64	1
11-1830	16.10	Low	70.74	1
11-1964	16.11	Low	70.77	1
11-1843	16.13	Low	70.96	1
11-2025	16.15	Low	71.12	1
11-1882	16.21	Low	71.52	1
11-1770	16.28	Low	72.09	2
11-2099	16.31	Low	72.32	3
11-1846	16.42	Low	73.17	3
11-2010	16.43	Low	73.26	3
11-1977	16.56	Low	74.28	1
12-1817	16.68	Low	75.17	3
11-1962	16.70	Low	75.31	3
11-1787	16.74	Low	75.68	1
11-1827	16.78	Low	75.99	1
12-1765	16.89	Low	76.78	1

1175	16.90	Low	76.92	3
11-2126	16.91	Medium	76.97	2
11-1832	16.92	Medium	77.07	1
11-2005	16.96	Medium	77.35	1
11-1956	16.99	Medium	77.59	1
11-1870	17.07	Medium	78.22	1
11-1802	17.11	Medium	78.53	1
12-1805	17.14	Medium	78.73	3
11-2027	17.23	Medium	79.41	1
11-2122	17.31	Medium	80.05	1
11-1847	17.31	Medium	80.07	3
11-1867	17.37	Medium	80.55	1
12-1793	17.45	Medium	81.17	1
12-1754	17.46	Medium	81.22	3
11-2061	17.48	Medium	81.38	3
12-1818	17.50	Medium	81.49	3
11-1901	17.54	Medium	81.85	1
11-2072	17.58	Medium	82.13	1
1179	17.59	High	82.21	3
11-1908	17.65	High	82.67	1
12-1771	17.67	High	82.85	3
12-1762	17.71	High	83.14	3
11-2035	17.74	High	83.40	1
12-1871	17.75	High	83.47	3
11-2068	17.81	High	83.95	1
11-1941	17.83	High	84.07	1
12-1812	17.83	High	84.10	3
11-1943	17.91	High	84.71	2
11-1946	18.12	High	86.29	1
11-1803	18.36	High	88.15	1
11-1948	18.38	High	88.34	2
11-2146	18.38	High	88.36	3
11-2112	18.44	High	88.76	3
12-1855	18.57	High	89.80	3
12-1775	18.63	High	90.27	1
12-1779	18.65	High	90.39	3
12-1759	18.69	High	90.68	3
11-2135	18.70	High	90.77	3
11-1831	18.75	High	91.21	1
11-1879	18.98	High	92.94	1
11-1909	19.02	High	93.26	1
11-2032	19.21	High	94.70	3
12-1772	19.21	High	94.75	3
11-1845	19.46	High	96.66	1
11-2093	19.50	High	96.96	3
12-1792	19.52	High	97.10	3
11-2103	19.70	High	98.53	2
11-2014	19.74	High	98.82	3
11-1838	19.81	High	99.40	2
11-1825	19.89	High	100.01	1
11-1796	20.04	High	101.14	2
11-2142	20.14	High	101.93	1
11-1906	20.16	High	102.07	1
11-2083	20.17	High	102.14	3
11-2059	20.21	High	102.49	2
11-1783	20.25	High	102.75	3
11-1820	20.31	High	103.26	3
11-1821	20.43	High	104.20	1
11-2021	20.57	High	105.28	3
11-1917	20.73	High	106.49	2
11-1940	20.80	High	107.03	1
11-1969	20.89	High	107.69	1
11-2071	20.94	High	108.07	3

11-2048	21.01	High	108.67	1
11-1920	21.09	High	109.26	2
11-1771	21.17	High	109.85	2
11-2066	21.24	High	110.46	2
11-2108	21.25	High	110.48	1
12-1833	21.26	High	110.54	1
11-2120	21.51	High	112.54	3
12-1798	21.56	High	112.87	3
11-1916	21.73	High	114.21	3
11-1923	21.89	High	115.47	1
12-1780	21.91	High	115.58	1
12-1829	21.97	High	116.05	2
11-1849	21.99	High	116.25	1
11-2138	22.05	High	116.65	1
11-1885	22.10	High	117.10	1
12-1774	22.13	High	117.34	3
11-1924	22.19	High	117.75	2
12-1785	22.26	High	118.33	3
11-2004	22.62	High	121.08	2
11-1755	22.72	High	121.88	1
11-1931	22.76	High	122.17	1
11-2022	22.85	High	122.84	1
11-1933	22.91	High	123.30	2
11-2115	22.94	High	123.58	3
11-1809	22.97	High	123.77	1
12-1854	22.99	High	123.98	1
11-1959	23.20	High	125.55	2
11-1798	23.21	High	125.64	1
11-1789	23.43	High	127.39	1
11-2060	23.47	High	127.64	1
12-1769	23.58	High	128.51	3
12-1788	23.87	High	130.73	3
11-1807	24.26	High	133.80	3
11-1876	24.29	High	133.99	2
11-1794	24.30	High	134.05	2
11-2015	24.42	High	135.03	3
12-1801	24.60	High	136.39	3
11-1950	24.67	High	136.95	1
11-1934	24.72	High	137.35	3
11-1884	24.74	High	137.45	3
11-2069	24.83	High	138.15	1
11-1815	24.95	High	139.10	1
11-1919	25.03	High	139.71	1
11-1869	25.05	High	139.89	1
11-2088	25.07	High	139.99	1
11-1747	25.07	High	140.02	3
11-1968	25.21	High	141.07	1
11-1824	25.21	High	141.08	1
11-1947	25.23	High	141.26	3
11-2124	25.25	High	141.39	1
12-1841	25.44	High	142.86	1
12-1773	25.45	High	142.95	3
12-1816	25.48	High	143.16	1
11-2001	25.57	High	143.91	1
12-1764	25.59	High	144.03	1
11-1951	25.68	High	144.74	3
11-1954	25.68	High	144.75	1
11-1930	25.85	High	146.05	1
12-1811	25.90	High	146.44	3
12-1863	26.10	High	148.00	2
11-1834	26.29	High	149.49	1
12-1761	26.35	High	149.90	3
11-1775	26.38	High	150.13	1

11-1875	26.46	High	150.78	2
12-1781	26.62	High	152.01	3
11-1761	26.66	High	152.34	1
12-1807	26.73	High	152.85	2
11-1871	26.79	High	153.32	2
12-1776	26.93	High	154.36	3
11-1822	27.04	High	155.27	1
11-1760	27.43	High	158.29	2
11-2106	27.50	High	158.80	1
12-1820	27.69	High	160.30	3
11-2140	27.79	High	161.03	3
11-1786	27.84	High	161.44	2
12-1838	27.98	High	162.51	3
11-2116	28.02	High	162.79	3
11-2064	28.06	High	163.09	3
11-1903	28.13	High	163.66	1
12-1760	28.28	High	164.81	3
11-1921	28.33	High	165.19	2
12-1810	28.60	High	167.28	1
11-1958	28.73	High	168.33	1
11-1994	28.88	High	169.48	1
11-1891	28.99	High	170.29	2
11-1858	29.16	High	171.65	3
11-1817	29.20	High	171.94	1
11-1750	29.24	High	172.28	1
11-1782	29.57	High	174.82	2
11-1897	29.81	High	176.64	1
11-1878	30.08	High	178.78	1
11-2034	30.10	High	178.86	1
11-2023	30.26	High	180.09	1
12-1768	30.64	High	183.04	3
11-2030	30.64	High	183.04	1
11-1790	30.90	High	185.10	1
12-1824	31.22	High	187.57	1
11-2143	31.56	High	190.21	1
11-1823	31.69	High	191.22	3
11-1902	32.00	High	193.60	1
12-1840	32.32	High	196.04	3
11-1939	32.43	High	196.93	2
11-1914	32.93	High	200.73	1
11-2013	33.09	High	202.03	3
12-1830	33.42	High	204.55	3
12-1842	33.51	High	205.24	3
11-2000	33.61	High	205.98	1
11-1984	33.62	High	206.07	3
12-1755	33.89	High	208.20	3
11-2074	34.05	High	209.41	3
11-1993	34.24	High	210.87	1
11-1844	34.41	High	212.16	1
12-1846	34.55	High	213.31	2
12-1851	34.57	High	213.47	2
12-1767	34.73	High	214.65	3
11-2092	34.80	High	215.25	3
11-1872	34.85	High	215.57	1
12-1868	35.10	High	217.50	3
12-1847	35.32	High	219.25	1
11-1799	35.33	High	219.29	1
11-1842	35.42	High	220.02	1
11-1911	35.59	High	221.28	1
11-1749	35.79	High	222.90	1
11-1960	35.99	High	224.38	3
12-1794	36.13	High	225.47	3
11-1785	36.15	High	225.61	3

12-1859	36.22	High	226.19	1
11-2024	36.64	High	229.43	1
11-1776	36.83	High	230.93	1
11-1774	36.87	High	231.20	1
12-1789	37.24	High	234.04	3
12-1828	37.27	High	234.29	2
11-1811	37.34	High	234.83	3
12-1869	37.44	High	235.65	3
11-2137	37.54	High	236.38	1
12-1827	37.54	High	236.39	3
11-1862	37.59	High	236.79	2
11-1915	37.63	High	237.07	2
12-1877	38.71	High	245.41	3
12-1804	38.82	High	246.27	1
12-1861	39.29	High	249.93	1
11-2006	40.28	High	257.60	3
11-1894	40.83	High	261.84	3
12-1873	40.95	High	262.74	3
11-1995	41.61	High	267.84	1
12-1825	41.90	High	270.05	2
11-2125	42.45	High	274.34	3
12-1848	42.82	High	277.18	1
11-2003	42.94	High	278.12	3
12-1790	43.00	High	278.56	1
11-1986	43.10	High	279.37	2
11-2141	43.67	High	283.74	2
11-1795	43.77	High	284.50	3
11-1865	44.00	High	286.30	1
11-2079	44.08	High	286.90	1
11-1860	44.33	High	288.89	2
12-1837	44.41	High	289.46	1
12-1819	44.65	High	291.31	3
11-1890	44.89	High	293.17	2
11-1942	44.94	High	293.54	1
11-2144	45.41	High	297.21	1
11-2049	45.46	High	297.60	3
12-1835	45.87	High	300.80	3
11-1997	46.42	High	305.03	3
12-1864	46.61	High	306.49	2
12-1853	47.22	High	311.20	2
12-1822	47.46	High	313.04	1
12-1834	47.69	High	314.86	2
12-1800	47.73	High	315.11	1
12-1876	48.67	High	322.40	3
11-2102	48.81	High	323.46	2
11-1863	49.20	High	326.52	1
12-1786	49.26	High	326.96	1
11-2002	49.57	High	329.34	3
11-2051	50.34	High	335.31	2
12-1839	50.56	High	337.02	1
11-2033	51.41	High	343.56	3
12-1858	51.71	High	345.89	3
12-1879	52.27	High	350.22	3
11-1864	52.96	High	355.52	2
11-1748	53.18	High	357.26	1
11-1835	54.63	High	368.46	1
11-1756	54.74	High	369.31	3
12-1832	55.83	High	377.76	2
12-1763	55.89	High	378.19	3
12-1843	58.49	High	398.30	1
11-2040	58.66	High	399.64	3
11-2020	59.08	High	402.81	2
11-2087	59.76	High	408.14	1

12-1878	60.44	High	413.32	3
11-2136	60.55	High	414.24	2
12-1777	62.09	High	426.11	1
12-1866	64.90	High	447.82	2
12-1867	69.84	High	485.99	1
11-2012	69.88	High	486.30	2
12-1850	70.30	High	489.58	1
12-1809	72.45	High	506.16	2
12-1821	73.13	High	511.44	2
12-1852	73.29	High	512.64	3
11-2041	73.94	High	517.70	1
11-1945	99.58	High	715.82	1
12-1865	100.00	High	719.07	2

9.2. Metabolites identified in polar and non-polar extracts of chilli pepper fruit following GC-MS analysis

Table 9-2 Metabolites identified in chilli pepper fruit extracts following GC-MS analysis.

Metabolite	Retention index	Retention time	Reference Ion Peak	Identification	Phase
Lactic acid	1044.2	8.7	117	Standard	NP
1-Dodecanol	1133.6	11.6	281	NIST Database	P
Malonic acid	1197.3	13.7	147	NIST Database	NP
Benzoic acid	1197.6	13.7	179	NIST Database	P
Valine (2TMS)	1213.2	12.7	144	Standard	P
Ethanolamine	1223.1	14.5	174	NIST Database	P
Phosphate (3TMS)	1230.7	14.5	299	Standard	P
Phosphoric acid	1257.4	15.7	299	NIST Database	P
Glycerol	1257.4	15.7	205	Standard	NP
Proline (2TMS)	1276.2	16.3	142	Standard	P
Glycine	1284	16.6	174	Standard	P
Butanedioic acid, methylene-	1296.3	17	147	NIST Database	P
Unknown (P) - 17.3	1299.2	17.3	188	Unknown	P
Dopamine (3TMS)	1331	18.2	174	NIST Database	P
Threonine	1341.7	18.5	218	Standard	P
L-Serine (3TMS)	1354.5	17.7	204	Standard	P
Pentanoic acid-like	1355	18.9	201	NIST Database	NP
Malic acid	1443.1	21.7	233	Standard	P
5-oxo-Proline	1470.3	22.6	156	NIST Database	P
Aspartic acid	1475.3	22.7	232	Standard	P
GABA	1502.1	23.5	174	Standard	P
Erythronic acid	1515.4	23.7	292	Standard	P
Phenylalanine	1560	25.2	218	Standard	P
Gluconic acid, 2-methoxime	1595.5	26.3	204	Standard	P
Lyxose	1603.5	26.5	217	Standard	P
Asparagine	1612.3	26.8	231	NIST Database	P
Dodecanoic acid	1623.5	27.1	257	Standard	NP
Unknown1	1630.4	27.3	315	Unknown	P
Xylitol (5TMS)	1666.7	28.3	217	Standard	P
Propanoic acid	1692	29	292	NIST Database	P
1-Propene-1,2,3-tricarboxylic acid (3TMS)	1693.7	29.1	375	NIST Database	P
Glycerol-3-Phosphate (4TMS)	1706.2	29.4	299	Standard	P
Arabinofuranose (4TMS)	1710	29.5	217	NIST Database	P
1-Nonene	1715.3	29.7	111	NIST Database	NP
D-Ribo-Hexitol (5TMS)	1733.3	30.2	231	NIST Database	P
Fructose (5TMS) isomer 1	1743.5	30.4	437	Standard	P
Pentaric Acid	1764.4	31	273	NIST Database	P
2-Ketoglutaric acid	1780.9	31.2	316	NIST Database	P
Citric acid	1789.5	31.6	363	NIST Database	P
Tetradecanoic acid	1815.2	32.3	285	Standard	NP
D-Fructose	1822	32.3	217	Standard	P
D-Galactose	1837.3	32.9	319	Standard	P

Pentadecanoic acid	1847.5	33.2	299	NIST Database	NP
D-Glucose	1851.2	33.03	319	Standard	P
Ascorbic acid	1882	34.1	332	Standard	P
Mannose (4TMS)	1923.4	35.1	204	NIST Database	P
Galacturonic acid	1948.6	35.7	292	NIST Database	NP
Unknown2	1951.1	35.8	319	Unknown	NP
Unknown (P) - 35.8	1952	35.8	319	Unknown	P
Hexadecanoic acid	2009.2	37.2	313	Standard	NP
Myo-Inositol	2042.7	38	305	Standard	P
Sedoheptulose	2067.2	38.5	319	NIST Database	P
Heptadecanoic acid (1TMS)	2104.5	39.4	327	Standard	NP
Linoleic acid	2127.5	39.9	337	Standard	NP
Oleic acid	2140.4	40.2	399	Standard	NP
Linolenic acid	2144.2	40.3	335	Standard	NP
Octadecanoic acid	2203.5	41.6	341	Standard	NP
D-Glucuronic acid	2308.2	43.9	375	NIST Database	P
Myo-inositol phosphate	2333.8	44.4	318	NIST Database	P
Myristic acid	2357.7	44.9	343	NIST Database	NP
Eicosanoic acid	2397.7	45.7	369	Standard	NP
Hexanedioic acid-like	2418.5	46.2	446	NIST Database	NP
Unknown (P) - 46.5	2433.2	46.5	217	Unknown	P
10'-Apo-β-carotenoic acid	2493	47.6	512	NIST Database	NP
2-Monopalmitin	2514.1	48.05	313	NIST Database	NP
Sucrose	2554.3	48.8	361	Standard	P
1-Monopalmitin (2 TMS)	2582.6	47.6	371	NIST Database	NP
Dihydrocapsaicin	2585.4	49.4	377	NIST Database	NP
Docosanoic acid	2592.3	49.5	397	Standard	NP
Maltose (8TMS)	2600.3	49.7	361	NIST Database	P
2-Monostearin derivative	2667.5	50.9	408	NIST Database	NP
2-Monostearin	2684.4	51.2	399	NIST Database	NP
Melibiose	2691.1	51.4	204	NIST Database	P
Tetracosanoic acid	2786.4	52.2	425	Standard	NP
Unknown C (similar to Tricosanoic acid)	2883.5	54.8	411	NIST Database	NP
γ-tocopherol derivative	3016.8	57	502	NIST Database	NP
a-Tocopherol	3075.6	57.9	502	Standard	NP
Stigmasterol	3147	59.1	394	Standard	NP
Campesterol (1TMS)	3180	59.6	343	Standard	NP
β-Sitosterol	3266.8	60.9	396	Standard	NP
β-amyrin	3284.5	61.2	498	Standard	NP
Unknown (NP) - 66.36	3624	66.4	407	Unknown	NP

9.3. Carotenoid retention diversity panel storage experiment carotenoid amounts

Table 9-3 Carotenoid retention diversity panel storage experiment carotenoid amounts

Pepper fruits of 13 varieties were harvested at the ripe fruit stage, dried in an oven for two weeks (30-40 °C), and stored in refrigerated, dark conditions (4 °C) for up to 12 weeks. Fruits were removed from storage at four week intervals for analysis by HPLC-PDA. At least three fruits per plant were analysed at each time point, and three biological replicates were analysed (n = 3).

R1	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE
Violaxanthin	133.62	5.60	102.27	3.63	87.59	5.65	94.71	5.77	98.68	10.23
Neoxanthin	78.95	1.70	74.79	1.76	68.00	2.14	70.09	3.01	71.85	2.01
Antheraxanthin	51.43	4.22	41.15	2.42	31.89	2.47	38.88	3.28	35.20	3.84
Antheraxanthin (monoester)	179.54	9.36	206.15	17.93	169.52	26.41	196.27	21.35	160.87	36.59
Capsanthin	558.06	32.92	392.48	4.87	359.49	14.36	380.75	14.63	374.79	18.79
Capsanthin (monoester)	1059.93	89.57	1218.41	154.24	1578.98	504.33	1404.84	122.88	1198.25	104.13
Capsanthin (diester)	1921.74	102.52	2371.96	141.13	1685.76	206.91	2399.49	169.93	2113.95	122.39
β-carotene	182.07	41.58	150.40	25.83	76.90	6.45	102.96	11.69	121.64	31.68
β-cryptoxanthin	53.37	13.32	60.92	7.98	42.13	4.45	45.47	3.71	44.59	5.15
Zeaxanthin	28.73	1.73	15.36	0.95	13.57	0.60	14.88	0.95	14.55	0.51
Zeaxanthin (diester)	124.88	11.28	97.15	31.73	87.64	27.92	82.11	30.11	86.66	22.18
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	4327.78	241.79	4710.73	303.69	4201.46	398.33	4807.08	289.60	4288.14	169.20

R2	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE
Violaxanthin	272.76	15.85	157.17	9.03	166.99	12.88	153.69	7.54	167.43	11.55
Neoxanthin	138.23	9.99	86.42	0.73	90.82	5.08	89.79	3.34	95.91	6.12
Antheraxanthin	89.63	3.59	74.79	3.64	103.55	16.36	85.94	9.00	86.19	6.54
Antheraxanthin (monoester)	792.21	24.07	789.91	43.76	758.91	133.27	725.04	10.43	800.38	76.32
Capsanthin	956.66	50.12	497.74	29.86	541.22	43.49	560.08	57.71	567.19	36.76
Capsanthin (monoester)	2737.32	144.95	2919.39	121.53	3276.51	106.10	3354.88	256.82	3166.21	397.30
Capsanthin (diester)	6189.50	719.13	6652.47	414.44	6906.83	497.12	6596.64	84.75	6858.15	534.94
β-carotene	977.65	18.26	597.51	39.37	794.92	97.40	673.69	13.57	575.55	98.38
β-cryptoxanthin	207.26	71.45	156.29	20.63	217.99	60.35	175.89	23.89	187.25	33.77
Zeaxanthin	47.01	1.35	29.38	2.96	33.34	2.67	33.01	3.78	31.25	2.62
Zeaxanthin (diester)	305.49	50.60	233.87	8.10	334.95	13.35	375.63	76.60	342.51	136.81
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	12713.72	895.11	12194.95	489.80	13226.02	888.23	12824.26	358.10	12878.01	1244.31

R3	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE	Average (µg/g DW)	± SE
Violaxanthin	54.58	0.98	55.45	0.32	54.73	1.36	58.46	0.04	55.45	1.21
Neoxanthin	0.00	0.00	0.00	0.00	0.00	0.00	52.06	0.43	0.00	0.00
Antheraxanthin	12.83	0.75	12.79	0.74	10.54	0.14	13.00	0.78	10.92	1.10
Antheraxanthin (monoester)	103.23	19.01	94.71	4.72	90.23	15.07	78.98	7.81	77.21	14.40
Capsanthin	273.02	2.56	258.93	5.28	253.25	2.71	258.25	0.16	250.17	6.45
Capsanthin (monoester)	361.72	14.19	399.76	34.62	403.86	75.73	373.35	3.07	358.99	41.01
Capsanthin (diester)	1276.21	43.94	1248.55	25.78	1104.41	88.27	1123.46	2.33	1034.16	186.13
β-carotene	173.27	25.78	156.47	8.58	130.76	30.38	98.94	15.69	128.70	26.59
β-cryptoxanthin	31.37	3.36	23.22	0.32	25.55	3.23	24.22	0.68	26.75	0.00
Zeaxanthin	24.87	1.63	16.53	0.96	13.72	0.67	15.32	0.83	14.33	1.81
Zeaxanthin (diester)	179.46	51.30	133.79	10.49	129.32	22.43	166.66	51.82	150.05	31.62
Capsorubin (diester)	252.77	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	2574.82	77.98	2392.46	12.06	2189.62	232.94	2262.70	42.26	2088.89	313.35

R4	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	178.41	16.20	156.78	12.04	151.13	1.00	135.91	6.32	126.75	25.10
Neoxanthin	80.03	7.17	75.46	4.89	77.26	1.24	74.47	4.16	68.00	5.39
Antheraxanthin	77.18	9.04	71.35	7.07	72.98	2.59	65.65	6.05	58.77	19.91
Antheraxanthin (monoester)	500.44	71.94	290.65	19.73	318.55	37.17	305.89	36.93	220.06	8.40
Capsanthin	431.64	34.62	346.20	22.31	352.50	15.29	349.95	19.07	323.00	39.49
Capsanthin (monoester)	1966.04	454.75	2415.61	230.73	2253.13	240.08	2210.96	198.25	1758.08	456.16
Capsanthin (diester)	4103.99	543.71	4505.22	280.24	4608.65	439.72	3662.00	144.78	3310.66	533.99
β-carotene	243.99	63.53	144.37	13.58	271.61	42.48	93.75	22.32	144.46	16.98
β-cryptoxanthin	86.48	4.59	117.54	8.26	102.20	22.55	71.35	24.74	95.57	17.29
Zeaxanthin	39.59	4.18	24.64	1.82	24.09	1.40	23.36	1.53	19.81	4.36
Zeaxanthin (diester)	194.57	32.56	73.34	24.65	137.63	57.30	76.95	23.60	75.69	21.69
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	317.65	22.55	0.00	0.00
TOTAL	7902.35	964.11	8221.15	567.16	8343.97	537.49	7282.00	167.49	6200.86	1120.50

R5	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	94.42	8.73	96.61	4.77	97.60	1.84	92.88	3.62	96.01	6.23
Neoxanthin	61.04	1.19	68.78	1.13	71.95	6.63	69.77	5.13	65.50	2.39
Antheraxanthin	27.14	3.54	44.77	5.24	51.27	13.66	56.90	15.35	37.56	3.09
Antheraxanthin (monoester)	203.66	15.87	287.01	40.95	192.20	41.19	190.36	27.97	266.56	24.17
Capsanthin	365.90	28.46	363.27	24.13	365.67	37.73	386.70	44.22	369.39	18.62
Capsanthin (monoester)	586.31	41.96	1596.09	56.45	1139.92	42.29	1186.38	164.02	1198.06	183.07
Capsanthin (diester)	2203.65	107.82	3305.36	145.06	2657.02	550.72	2355.14	348.71	3155.47	284.90
β-carotene	197.55	15.91	91.58	3.45	218.16	81.31	161.84	33.70	223.07	62.32
β-cryptoxanthin	33.05	0.00	28.83	0.83	41.51	10.18	47.97	4.30	50.77	0.67
Zeaxanthin	19.94	2.71	14.57	0.98	13.14	0.19	13.67	1.39	14.31	1.17
Zeaxanthin (diester)	73.07	7.42	93.34	17.85	68.15	18.60	69.40	27.16	140.85	34.99
Capsorubin (diester)	0.00	0.00	0.00	0.00	238.86	0.00	255.38	0.00	0.00	0.00
TOTAL	3812.21	223.04	5935.08	196.74	4963.68	624.80	4685.17	272.96	5585.53	494.29

R6	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	91.82	7.96	94.02	7.03	87.90	2.35	111.96	5.86	106.20	11.12
Neoxanthin	60.96	1.96	68.42	2.79	64.35	2.41	70.15	0.46	70.17	2.93
Antheraxanthin	24.23	0.98	25.56	1.89	23.62	1.26	29.31	4.34	26.14	1.85
Antheraxanthin (monoester)	342.61	11.46	418.60	45.70	421.02	16.17	465.86	66.00	366.60	55.48
Capsanthin	348.80	6.77	305.31	13.14	303.61	9.66	316.70	5.94	317.74	6.61
Capsanthin (monoester)	748.13	40.29	1208.91	155.00	1183.41	148.28	1258.67	28.90	1240.50	106.03
Capsanthin (diester)	3525.77	84.91	4867.55	257.18	4389.55	213.54	4903.55	546.65	4359.45	493.68
β-carotene	284.69	20.91	288.33	33.17	300.75	59.02	391.35	56.26	326.07	64.29
β-cryptoxanthin	62.66	20.54	87.44	10.71	51.18	7.89	111.58	15.15	110.81	17.16
Zeaxanthin	22.78	1.53	15.85	0.77	17.20	3.38	16.71	1.46	16.29	1.01
Zeaxanthin (diester)	217.25	16.25	217.78	28.31	202.20	63.94	232.41	30.53	160.23	32.35
Capsorubin (diester)	0.00	0.00	0.00	0.00	262.10	0.00	0.00	0.00	0.00	0.00
TOTAL	5699.11	98.50	7543.61	370.74	7081.41	354.89	7870.92	737.82	7064.79	706.14

R7	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	361.54	29.77	180.33	34.82	181.22	9.42	261.74	27.97	202.30	44.52
Neoxanthin	134.71	10.04	94.31	8.82	94.92	2.46	108.43	13.38	97.04	7.19
Antheraxanthin	125.03	7.35	87.74	9.31	91.02	1.99	110.57	16.78	93.53	6.62
Antheraxanthin (monoester)	1077.27	105.06	642.30	131.90	888.64	62.55	1105.17	89.53	783.69	184.55
Capsanthin	722.36	23.81	510.53	41.43	537.08	11.91	567.88	37.60	495.22	15.83
Capsanthin (monoester)	4147.52	195.76	3542.08	265.51	3265.66	202.81	3993.00	581.26	3586.68	202.16
Capsanthin (diester)	9028.95	346.52	6433.29	951.51	6829.21	445.33	8333.79	1209.85	6855.18	1100.40
β-carotene	1690.45	74.28	860.49	55.68	838.74	126.29	908.75	111.99	722.22	108.63
β-cryptoxanthin	425.33	67.68	186.05	57.10	110.19	27.89	187.99	28.22	176.04	9.45
Zeaxanthin	81.89	13.41	43.11	2.41	37.08	1.00	39.12	0.93	33.94	3.79
Zeaxanthin (diester)	448.69	23.23	431.04	42.05	400.74	110.65	446.42	46.55	249.41	50.75
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	289.91	0.00
TOTAL	18123.22	693.16	12951.16	1507.75	13214.09	915.17	15975.60	2170.30	13324.45	1525.93

R8	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	82.93	6.84	78.45	2.42	75.90	1.32	74.24	7.36	79.64	5.70
Neoxanthin	57.05	2.59	62.01	1.90	59.93	1.70	59.08	2.28	59.81	1.73
Antheraxanthin	16.74	1.37	40.29	15.49	29.34	6.31	34.34	6.65	34.52	8.59
Antheraxanthin (monoester)	173.99	17.37	203.38	8.56	144.18	12.51	137.85	13.10	166.24	17.59
Capsanthin	273.52	6.31	324.93	49.15	296.28	33.39	301.55	35.26	292.07	21.07
Capsanthin (monoester)	517.97	92.09	897.49	198.70	849.66	141.08	864.04	182.11	701.04	96.86
Capsanthin (diester)	2572.98	348.42	2776.54	15.39	2646.71	164.73	2065.59	116.89	2254.77	174.15
β-carotene	198.93	30.96	185.21	39.06	192.07	28.89	145.82	47.51	169.95	19.93
β-cryptoxanthin	37.37	8.88	65.73	11.20	41.86	6.61	61.48	27.78	54.38	12.87
Zeaxanthin	13.24	1.00	13.51	2.16	12.72	0.06	11.74	1.60	11.33	1.06
Zeaxanthin (diester)	144.13	21.53	100.80	27.14	86.19	28.75	82.89	15.00	117.20	33.24
Capsorubin (diester)	0.00	0.00	0.00	0.00	270.72	0.00	275.34	11.51	290.02	0.00
TOTAL	4042.19	465.70	4701.52	215.46	4475.56	358.72	3997.42	299.41	3991.15	348.58

R9	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	175.52	18.33	113.66	10.76	111.07	2.93	105.43	0.22	110.25	10.71
Neoxanthin	88.34	4.54	71.02	0.34	68.72	1.72	68.07	1.27	66.46	0.66
Antheraxanthin	53.11	4.63	36.12	1.36	31.74	2.51	29.69	1.35	32.38	4.44
Antheraxanthin (monoester)	373.62	39.22	358.54	30.24	291.74	4.74	260.10	11.92	299.11	73.54
Capsanthin	501.24	59.84	302.36	2.73	296.18	12.07	297.46	6.43	302.75	9.69
Capsanthin (monoester)	1975.01	215.12	1569.54	117.61	1601.07	44.85	1281.08	74.20	1591.28	173.15
Capsanthin (diester)	4043.99	490.34	3916.26	310.13	3739.25	381.06	3007.86	76.61	3652.01	344.14
β-carotene	112.73	16.05	116.72	18.40	68.77	12.40	35.47	5.23	46.83	12.06
β-cryptoxanthin	102.16	20.52	76.22	7.29	100.79	15.57	58.45	0.88	66.20	3.78
Zeaxanthin	17.64	2.10	12.00	0.41	9.88	0.26	15.10	0.00	15.78	0.72
Zeaxanthin (diester)	132.47	44.22	45.75	27.08	150.35	16.90	81.89	23.12	86.54	32.08
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	7517.33	719.98	6552.65	421.38	6429.26	419.21	5195.41	196.45	6205.42	593.81

R10	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	77.87	17.71	80.86	5.87	72.36	0.60	70.54	5.05	71.08	6.23
Neoxanthin	58.47	2.44	0.00	0.00	57.06	0.00	0.00	0.00	0.00	0.00
Antheraxanthin	23.29	3.61	27.59	1.53	22.81	0.76	24.16	2.63	21.43	1.84
Antheraxanthin (monoester)	178.47	26.11	292.80	26.04	208.76	27.62	241.83	51.40	199.30	16.92
Capsanthin	317.94	15.71	267.50	4.12	259.40	3.80	257.91	4.78	252.69	4.61
Capsanthin (monoester)	557.55	81.32	958.11	73.80	778.71	38.27	866.05	99.61	755.70	76.24
Capsanthin (diester)	2173.13	438.99	2821.40	173.72	2414.16	98.23	2359.44	536.22	2330.60	60.71
β-carotene	156.31	21.17	218.23	112.18	113.96	16.51	79.06	31.59	90.61	10.85
β-cryptoxanthin	51.53	15.89	37.13	3.94	83.64	12.82	34.89	2.94	56.33	4.59
Zeaxanthin	22.87	0.74	14.61	0.36	13.03	0.74	14.08	1.31	13.29	0.75
Zeaxanthin (diester)	250.92	84.92	119.88	33.03	131.70	18.39	110.22	31.57	191.13	73.99
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	601.46	12.76	0.00	0.00
TOTAL	3842.39	610.55	4722.00	296.06	4093.44	135.36	4435.65	420.36	3958.48	21.47

R11	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	109.52	12.06	104.19	7.71	93.77	8.09	90.10	0.00	113.97	5.40
Neoxanthin	67.46	4.12	0.00	0.00	61.62	1.38	59.11	2.33	0.00	0.00
Antheraxanthin	38.39	2.02	39.79	5.17	35.57	6.62	36.11	0.84	39.56	3.31
Antheraxanthin (monoester)	360.43	20.12	214.62	29.23	223.03	17.57	170.32	74.52	172.37	29.90
Capsanthin	322.46	12.32	308.31	12.98	294.60	5.47	297.86	9.28	307.62	5.01
Capsanthin (monoester)	920.09	104.46	1383.50	122.61	1156.58	96.76	924.61	80.15	1347.54	185.98
Capsanthin (diester)	3103.48	240.56	3094.65	227.62	2641.32	312.95	2207.83	290.45	3345.66	231.87
β-carotene	232.02	14.50	59.99	11.09	101.89	22.66	130.20	18.61	124.38	30.01
β-cryptoxanthin	60.02	18.30	49.55	5.29	37.02	8.22	42.38	3.98	71.55	12.40
Zeaxanthin	43.11	3.98	26.52	0.17	21.11	2.21	22.91	1.98	24.52	2.13
Zeaxanthin (diester)	235.94	50.22	87.92	12.46	78.20	11.92	74.70	12.50	87.77	8.72
Capsorubin (diester)	0.00	0.00	239.99	0.00	0.00	0.00	252.45	8.39	261.39	0.00
TOTAL	5436.39	378.47	5414.32	333.91	4713.44	428.32	4263.54	399.34	5684.06	372.93

R12	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	84.63	2.96	67.64	3.44	73.73	0.37	71.68	0.00	72.35	0.00
Neoxanthin	60.56	1.65	0.00	0.00	0.00	0.00	54.70	0.86	0.00	0.00
Antheraxanthin	48.79	8.52	32.17	6.42	29.49	3.48	27.65	1.00	29.74	2.02
Antheraxanthin (monoester)	157.08	20.11	101.68	14.64	123.29	16.03	104.42	18.73	126.93	3.84
Capsanthin	498.56	55.27	304.86	17.62	293.66	9.33	290.26	2.89	306.45	4.23
Capsanthin (monoester)	919.52	11.41	863.21	17.25	782.92	23.46	761.55	123.54	1084.78	80.92
Capsanthin (diester)	1622.87	76.81	1220.91	53.05	1622.27	119.61	1405.11	253.40	1955.26	20.84
β-carotene	120.87	10.09	54.69	6.61	127.22	26.95	99.69	15.97	74.78	10.22
β-cryptoxanthin	31.86	3.63	25.44	0.00	41.53	9.95	62.62	7.77	65.18	0.00
Zeaxanthin	45.01	5.01	17.31	1.29	17.53	1.83	15.01	0.28	18.56	0.44
Zeaxanthin (diester)	195.77	71.24	151.05	61.62	72.88	3.99	54.08	16.72	45.21	16.52
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	3757.30	71.02	2799.45	66.95	3159.94	197.46	2910.94	444.51	3710.48	3.41

CM334	Fresh		Dry		Dry + 4 weeks		Dry + 8 weeks		Dry + 12 weeks	
Compound	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**	**Average (µg/g DW)**	**± SE**
Violaxanthin	85.69	10.77	85.55	3.16	87.11	5.28	94.01	4.07	87.09	9.38
Neoxanthin	62.54	5.80	61.34	0.68	59.53	2.14	62.27	1.19	60.68	2.16
Antheraxanthin	41.46	1.52	29.78	2.21	33.95	6.51	31.13	1.73	46.11	8.94
Antheraxanthin (monoester)	374.87	74.55	140.07	15.17	136.86	5.77	194.80	29.14	135.42	22.72
Capsanthin	408.76	13.68	327.06	4.34	305.24	15.21	328.17	4.44	335.17	28.94
Capsanthin (monoester)	1289.40	160.75	1319.31	131.29	1194.33	132.12	1412.55	97.41	1277.67	155.91
Capsanthin (diester)	3100.26	424.85	2681.21	228.27	2496.36	166.10	2739.53	230.89	2483.25	163.94
β-carotene	267.04	116.33	59.66	6.85	64.05	8.31	44.44	3.26	83.82	12.33
β-cryptoxanthin	32.75	0.00	123.72	46.81	106.25	28.22	91.51	21.94	82.72	7.34
Zeaxanthin	43.14	9.18	12.19	0.72	11.94	0.88	11.88	0.25	13.10	1.39
Zeaxanthin (diester)	192.31	46.38	57.68	5.37	69.10	24.58	108.46	66.86	80.43	11.80
Capsorubin (diester)	0.00	0.00	0.00	0.00	0.00	0.00	312.41	26.84	0.00	0.00
TOTAL	5826.99	665.82	4864.99	391.56	4535.69	353.01	5395.86	354.40	4656.41	281.09

9.4. Carotenoid candidate genes for VIGS sequence alignment

Tomato and pepper homologs of genes silenced in fruits using VIGS have been aligned using the CLUSTALW algorithm. 300 base pair gene fragments were designed using Sol Genomics Network VIGS tool. VIGS fragments were designed using the pepper homolog of respective genes: PDS, PSY1, and CCS. These genes were silenced in both pepper and tomato fruit, hence it is important to understand the homology between pepper and tomato gene homologs. In this thesis, CCS was not silenced in tomato, however the alignment between the pepper CCS gene and tomato LCYb gene is presented here.

9.4.1. Ca*PDS*

```
SlPDS           TACAGAATTATACGCTTTTACTAGTTATAGCATTCGGTATCTTTTTCTGGGTAACTGCCA
CaPDS           ------------------------------------------------------------
CaPDS_VIGS      ------------------------------------------------------------


SlPDS           AACCACCACAAATTTCAAGTTTCCATTTAACTCTTCAACTTCAACCCAACCAAATTTATT
CaPDS           ------------------------------------------------------------
CaPDS_VIGS      ------------------------------------------------------------


SlPDS           TGCTTAATTGTGCAGAACCACTCCCTATATCTTCTAGGTGCTTTCATTCGTTCCGAGGTT
CaPDS           ------------------------------------------------------------
CaPDS_VIGS      ------------------------------------------------------------


SlPDS           TTACTGTTATTTTTCAGTAAAATGCCTCAAATTGGACTTGTTTCTGCTGTTAACTTGAGA
CaPDS           ----------------TCCAgATGCCCCAAATTGGACTTGTTTCTGCTGTCAACTTGAGA
CaPDS_VIGS      ----------------------ATGCCCCAAATTGGACTTGTTTCTGCTGTCAACTTGAGA
                                     ***** ********************** *********

SlPDS           GTCCAAGGTAGTTCAGCTTATCTTTGGAGCTCGAGGTCGTCTTCTTTGGGAACTGAAAGT
CaPDS           GTCCAAGGTAATTCAGCTTATCTTTGGAGCTCAAGGTC---TTCTTTGGGAACTGATAGT
CaPDS_VIGS      GTCCAAGGTAATTCAGCTTATCTTTGGAGCTCAAGGTC---TTCTTTGGGAACTGATAGT
                ********** ******************** *****   *************** ***

SlPDS           CGAGATGGTTGCTTGCAAAGGAATTCGTTATGTTTTGCTGGTAGCGAATCAATGGGTCAT
CaPDS           CAAGATGGTTGCTCGCAAAGGAATTCGTTATGTTTTGGTGGTAGTGACTCAATGAGTCAT
CaPDS_VIGS      CAAGATGGTTGCTCGCAAAGGAATTCGTTATGTTTTGGTGGTAGTGACTCAATGAGTCAT
                * *********** ********************** ****** ** ****** *****

SlPDS           AAGTTAAAGATTCGTACTCCCCATGCCACGACCAGAAGATTGGTTAAGGACTTGGGGCCT
CaPDS           AGGTTAAAGATTCGTAATCCCCATTCCATAACGAGAAGATTGGCTAAGGATTTCCGGCCT
CaPDS_VIGS      AGGTTAAAGATTCGTAATCCCCATTCCATAACGAGAAGATTGGCTAAGGATTTCCGGCCT
                * ************** ******* ***  ** ********** ****** **  *****

SlPDS           TTAAAGGTCGTATGCATTGATTATCCAAGACCAGAGCTGGACAATACAGTTAACTATTTG
CaPDS           TTAAAGGTTGTTTGCATTGATTATCCAAGGCCAGAGCTAGACAATACAGTTAACTATTTG
CaPDS_VIGS      TTAAAGGTTGTTTGCATTGATTATCCAAGGCCAGAGCTAGACAATACAGTTAACTATTTG
                ******** ** ***************** ******** **********************

SlPDS           GAGGCTGCATTTTTATCATCAACGTTCCGTGCTTCTCCGCGCCCAACTAAACCATTGGAG
CaPDS           GAGGCTGCATTCTTATCATCATCATTCCGATCTTCTCCGCGCCCAACCAAACCACTGGAG
CaPDS_VIGS      GAGGCTGCATTCTTATCATCATCA-----------------------------------
                *********** ********* *

SlPDS           ATTGTTATTGCTGGTGCAGGTTTGGGTGGTTTGTCTACAGCAAAATATTTGGCAGATGCT
CaPDS           ATTGTTATTGCTGGTGCAGGTTTGGGTGGTTTGTCTACAGCAAAATATTTGGCAGATGCT
CaPDS_VIGS      ------------------------------------------------------------


SlPDS           GGTCACAAACCGATACTGCTGGAGGCAAGGGATGTTCTAGGTGGAAAGGTAGCTGCATGG
CaPDS           GGTCACAAACCAATACTGCTGGAGGCAAGGGATGTTCTAGGTGGAAAGGTAGCTGCATGG
CaPDS_VIGS      ------------------------------------------------------------


SlPDS           AAAGATGATGATGGAGATTGGTACGAGACTGGTTTGCATATATTCTTTGGGGCTTACCCA
CaPDS           AAAGATGATGATGGAGATTGGTATGAGACTGGTTTGCACATATTCTTTGGGGCTTACCCA
CaPDS_VIGS      ------------------------------------------------------------
```

```
SlPDS           AATATTCAGAACCTGTTTGGAGAATTAGGGATTAACGATCGATTGCAATGGAAGGAACAT
CaPDS           AATATGCAGAACCTATTTGGAGAATTAGGGATAAATGATCGATTGCAATGGAAGGAACAT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           TCAATGATATTTGCAATGCCAAGCAAGCCAGGAGAATTCAGCCGCTTTGATTTCTCCGAA
CaPDS           TCGATGATATTTGCAATGCCAAACAAGCCAGGAGAATTCAGCCGCTTTGATTTCCCCGAA
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GCTTTACCCGCTCCTTTAAATGGAATTTTAGCCATCTTAAAGAATAACGAAATGCTTACA
CaPDS           GCTTTACCTGCTCCTTTAAATGGAATTTTGGCAATCCTAAAGAACAATGAAATGCTTACA
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           TGGCCAGAGAAAGTCAAATTTGCAATTGGACTCTTGCCAGCAATGCTTGGAGGGCAATCT
CaPDS           TGGCCAGAAAAATTCAAATTTGCAATTGGACTCTTGCCAGCAATGCTTGGTGGGCAATCT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           TATGTTGAAGCTCAAGATGGGATAAGTGTTAAGGACTGGATGAGAAAGCAAGGTGTGCCG
CaPDS           TATGTTGAAGCTCAAGACGGGATAAGTGTTAAGGACTGGATGAGAAAACAAGGTGTGCCG
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GACAGGGTGACAGATGAGGTGTTCATTGCTATGTCAAAGGCACTCAACTTTATAAACCCT
CaPDS           GATAGGGTGACGGATGAGGTGTTCATCGCCATGTCAAAGGCACTTAACTTCATAAATCCT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GACGAACTTTCAATGCAGTGCATTTTGATCGCATTGAACAGGTTTCTTCAGGAGAAACAT
CaPDS           GATGAGCTTTCGATGCAGTGCATCTTGATCGCGTTGAACAGATTTCTTCAGGAGAAACAT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GGTTCAAAAATGGCCTTTTTAGATGGTAATCCTCCTGAGAGACTTTGCATGCCGATTGTT
CaPDS           GGTTCAAAAATGGCCTTTTTAGATGGTAATCCTCCTGAGAGACTTTGCATGCCGATTGTT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GAACACATTGAGTCAAAAGGTGGCCAAGTCAGACTGAACTCACGAATAAAAAAGATTGAG
CaPDS           GAACATATCGAGTCAAAAGGTGGACAAGTCAGACTGAACTCACGAATAAAAAAGATTGAG
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           CTGAATGAGGATGGAAGTGTCAAGAGTTTTATACTGAGTGACGGTAGTGCAATCGAGGGA
CaPDS           CTGAATGAGGATGGAAGTGTCAAGTGTTTTATACTGAACGATGGTAGTACAATTGAGGGA
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GATGCTTTTGTGTTTGCCGCTCCAGTGGATATTTTCAAGCTTCTATTGCCTGAAGACTGG
CaPDS           GATGCTTTTGTGTTTGCGACTCCAGTGGATATTTTCAAGCTTCTTTTGCCTGAAGACTGG
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           AAAGAGATTCCATATTTCCAAAAGTTGGAGAAGTTAGTCGGAGTACCTGTGATAAATGTA
CaPDS           AAAGAGATTCCATATTTCCAAAAGTTGGAGAAGTTAGTCGGAGTACCTGTGATAAATGTC
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           CATATATGGTTTGACAGAAAACTGAAGAACACATATGATCATTTGCTCTTCAGCAGAAGC
CaPDS           CATATATGGTTTGACAGAAAACTGAAGAACACATCTGATAATTTGCTCTTCAGCAGAAGC
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           TCACTGCTCAGTGTGTATGCTGACATGTCTGTTACATGTAAGGAATATTACAACCCCAAT
CaPDS           CCACTGCTCAGTGTGTATGCTGACATGTCCGTCACATGTAAGGAATATTACGACCCCAAC
CaPDS_VIGS      ------------------------------------------------------------
```

```
SlPDS           CAGTCTATGTTGGAATTGGTTTTTGCACCTGCAGAAGAGTGGATATCTCGCAGCGACTCA
CaPDS           AAGTCCATGTTGGAATTGGTCTTTGCGCCTGCAGAAGAGTGGGTATCTCGCAGTGACTCT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GAAATTATTGATGCAACGATGAAGGAACTAGCAACGCTTTTTCCTGATGAAATTTCAGCA
CaPDS           GAAATTATTGATGCTACAATGAAGGAACTAGCAAAGCTATTTCCTGATGAAATTTCGGCG
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GATCAAAGCAAAGCAAAAATATTGAAGTACCATGTTGTCAAAACTCCGAGGTCTGTTTAT
CaPDS           GATCAGAGCAAAGCAAAAATATTGAAGTATCATGTTGTCAAAACTCCAAGGTCTGTATAT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           AAAACTGTGCCAGGTTGTGAACCCTGTCGGCCTTTACAAAGATCCCCAATAGAGGGGTTT
CaPDS           AAAACTGTGCCAGGTTGTGAACCCTGTCGGCCCTTGCAAAGATCCCCTGTAGAGGGGTTT
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           TATTTAGCCGGTGACTACACGAAACAGAAATACTTGGCTTCAATGGAAGGCGCTGTCTTA
CaPDS           TATTTAGCTGGTGACTACACGAAACAGAAATACTTGGCTTCAATGGAAGGTGCTGTCTTA
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           TCAGGAAAGCTTTGTGCTCAAGCTATTGTACAGGATTATGAGTTACTTGTTGGACGTAGC
CaPDS           TCAGGAAAGTTTTGTGCACAAGCTATTGTACAGGATTACGAGTTACTTGTTGGCCGGAGC
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           CAAAAGAAGTTGTCGGAAGCAAGCGTAGTTTAGCTTTGTGGTTATTATTTAGCTTCTGTA
CaPDS           CAGAGGAAGTTGGCAGAAACAAGTGTAGTTTAG---------------------------
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           CACTAAATTTATGATGCAAGAAGCGTTGTACACAACATATAGAAGAAGAGTGCGAGGTGA
CaPDS           ------------------------------------------------------------
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           AGCAAGTAGGAGAAATGTTAGGAAAGCTCCTATACAAAAGGATGGCATGTTGAAGATTAG
CaPDS           ------------------------------------------------------------
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           CATCTTTTTAATCCCAAGTTTAAATATAAAGCATATTTTATGTACCACTTTCTTTATCTG
CaPDS           ------------------------------------------------------------
CaPDS_VIGS      ------------------------------------------------------------

SlPDS           GGGTTTGTAATCCCTTTATATCTTTATGCAATCTTTACGTTAAAATT
CaPDS           -----------------------------------------------
CaPDS_VIGS      -----------------------------------------------
```

9.4.2. Ca*PSY1*

```
SlPSY1          GATTTCACTATATTGTAATATTAACTTGAGGTCACTATAGGAGCTCAAAAACTTCTAATT
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          TTGAATCAATGTCTGGTTATACTTTTTTTGTCATAACTGTATCTCAAATGTGGTGTTTGG
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          TTTATCTCATTTTGCAGAAGTCAAGAAACAGGTTACTCCTGTTTGAGTGAGGAAAAGTTG
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GTTTGCCTGTCTGTGGTCTTTTTATAATCTTTTTCTACAGAAGAGAAAGTGGGTAATTTT
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GTTTGAGAGTGGAAATATTCTCTAGTGGGAATCTACTAGGAGTAATTTATTTTCTATAAA
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          CTAAGTAAAGTTTGGAAGGTGACAAAAAGAAAGACAAAAATCTTGGAATTGTTTTAGACA
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          ACCAAGGTTTTCTTGCTCAGAATGTCTGTTGCCTTGTTATGGGTTGTTTCTCCTTGTGAC
CaPSY1          ----------------------ATGTCTGTTGCCTTGTTATGGGTTGTTTCTCCTTGTGAC
CaPSY1_VIGS     ----------------------ATGTCTGTTGCCTTGTTATGGGTTGTTTCTCCTTGTGAC
                                       ************************************

SlPSY1          GTCTCAAATGGGACAAGTTTCATGGAATCAGTCCGGGAGGGAAACCGTTTTTTTGATTCA
CaPSY1          GTCTCAAACGGGACAGGATTCTTGGTATCCGTTCGTGAGGGAAACCGGATTTTTGATTCG
CaPSY1_VIGS     GTCTCAAACGGGACAGGATTCTTGGTATCCGTTCGTGAGGGAAACCGGATTTTTGATTCG
                ******** ****** * *** *** *** ** ** ***********  **********

SlPSY1          TCGAGGCATAGGAATTTGGTGTCCAATGAGAGAATCAATAGAGGTGGTGGAAAG------
CaPSY1          TCGGGGCGTAGGAATTTGGCGTGCAATGAGAGAATCAAGAGAGGAGGTGGAAAACAAAGG
CaPSY1_VIGS     TCGGGGCGTAGGAATTTGGCGTGCAATGAGAGAATCAAGAGAGGAGGTGGAAAACAAAGG
                *** *** *********** ** *************** ***** ********

SlPSY1          ------------------------------CAAACTAATAATGGACGGAAATTTTCTGTA
CaPSY1          TGGAGTTTTGGTTCTTGCTTGGGAGGAGCACAAACTGGAAGTGGACGGAAATTTTCTGTA
CaPSY1_VIGS     TGGAGTTTTGGTTCTTGCTTGGGAGGAGCACAAACTGGAAGTGGACGGAAATTTTCTGTA
                                        ******   * ******************

SlPSY1          CGGTCTGCTATTTTGGCTACTCCATCTGGAGAACGGACGATGACATCGGAACAGATGGTC
CaPSY1          CGTTCTGCTATCGTGGCTACTCCGGCTGGAGAAATGACGATGTCATCAGAACGGATGGTA
CaPSY1_VIGS     CGTTCTGCTATCGTGGCTACTCCGGCTGGAGAAATGACGATGTCATCAGAACGGATGGTA
                ** ********  **********  *********  ******* **** **** ******

SlPSY1          TATGATGTGGTTTTGAGGCAGGCAGCCTTGGTGAAGAGGCAACTGAGATCTACCAATGAG
CaPSY1          TATGATGTGGTTTTGAGGCAGGCAGCCTTGGTGAAGAGACAGCTGAGATCGACCGATGAG
CaPSY1_VIGS     TATGATGTGGTTTTGAGGCAG----------------------------------------
                ********************

SlPSY1          TTAGAAGTGAAGCCGGATATACCTATTCCGGGGAATTTGGGCTTGTTGAGTGAAGCATAT
CaPSY1          TTAGATGTGAAGAAGGATATACCTATTCCGGGGACTTTGGGCTTGTTGAGTGAAGCATAT
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GATAGGTGTGGTGAAGTATGTGCAGAGTATGCAAAGACGTTTAACTTAGGAACTATGCTA
CaPSY1          GATAGGTGTAGTGAAGTATGTGCAGAGTACGCAAAGACGTTTTACTTAGGAACGATGCTA
CaPSY1_VIGS     ------------------------------------------------------------
```

```
SlPSY1          ATGACTCCCGAGAGAAGAAGGGCTATCTGGGCAATATATGTATGGTGCAGAAGAACAGAT
CaPSY1          ATGACTCCGGAGAGAAGAAAGGCTATCTGGGCAATATACGTATGGTGCAGGAGAACAGAC
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GAACTTGTTGATGGCCCAAACGCATCATATATTACCCCGGCAGCCTTAGATAGGTGGGAA
CaPSY1          GAACTTGTTGATGGTCCGAATGCATCACACATTACTCCGGCGGCCTTAGATAGGTGGGAA
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          AATAGGCTAGAAGATGTTTTCAATGGGCGGCCATTTGACATGCTCGATGGTGCTTTGTCC
CaPSY1          GACAGGCTAGAAGATGTTTTCAGTGGACGGCCATTTGACATGCTCGATGCTGCTTTGTCC
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GATACAGTTTCTAACTTTCCAGTTGATATTCAGCCATTCAGAGATATGATTGAAGGAATG
CaPSY1          GACACAGTTTCCAAATTTCCAGTTGATATTCAGCCATTCAGAGATATGATTGAAGGAATG
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          CGTATGGACTTGAGAAAATCGAGATACAAAAACTTCGACGAACTATACCTTTATTGTTAT
CaPSY1          CGTATGGACTTGAGGAAGTCAAGATACAGAAACTTTGACGAACTATACCTATATTGTTAT
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          TATGTTGCTGGTACGGTTGGGTTGATGAGTGTTCCAATTATGGGTATCGCCCCTGAATCA
CaPSY1          TACGTTGCTGGTACGGTTGGGTTGATGAGTGTTCCAATTATGGGCATCGCACCTGAATCA
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          AAGGCAACAACAGAGAGCGTATATAATGCTGCTTTGGCTCTGGGGATCGCAAATCAATTA
CaPSY1          AAGGCAACAACGGAGAGCGTATATAATGCTGCTTTGGCTTTGGGGATCGCAAATCAGCTG
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          ACTAACATACTCAGAGATGTTGGAGAAGATGCCAGAAGAGGAAGAGTCTACTTGCCTCAA
CaPSY1          ACCAACATACTTAGAGATGTTGGAGAAGATGCCAGAAGAGGAAGAGTCTATTTGCCTCAA
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GATGAATTAGCACAGGCAGGTCTATCCGATGAAGATATATTTGCTGGAAGGGTGACCGAT
CaPSY1          GATGAATTAGCACAGGCAGGTCTATCCGACGAAGACATATTTGCTGGAAGAGTGACCGAT
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          AAATGGAGAATCTTTATGAAGAAACAAATACATAGGGCAAGAAAGTTCTTTGATGAGGCA
CaPSY1          AAATGGAGAATCTTCATGAAGAAACAAATTCAGAGGGCAAGAAAGTTCTTTGACGAGGCA
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GAGAAAGGCGTGACAGAATTGAGCTCAGCTAGTAGATTCCCTGTATGGGCATCTTTGGTC
CaPSY1          GAGAAAGGAGTGACCGAATTGAGCGCAGCTAGTAGATGGCCTGTGTTGGCATCTCTGCTG
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          TTGTACCGCAAAATACTAGATGAGATTGAAGCCAATGACTACAACAACTTCACAAAGAGA
CaPSY1          TTGTACCGCAGGATACTGGACGAGATCGAAGCCAATGACTACAACAACTTCACAAAGAGA
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GCATATGTGAGCAAATCAAAGAAGTTGATTGCATTACCTATTGCATATGCAAAATCTCTT
CaPSY1          GCTTATGTGAGCAAACCAAAGAAGTTGATTGCATTACCTATTGCATATGCAAAATCTCTT
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GTGCCTCCTACAAAAACTGCCTCTCTTCAAAGATAAAGCATGAAATGAAGATATATATAT
CaPSY1          GTGCCTTCTACAAGAACATGA----------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------
```

```
SlPSY1          ATATATATATAGCAATATACATTAGAAGAAAAAAAGGAAGAAGAAATGTTGTTGTATTGA
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          TATAAATGTATATCATAAATATTAGGTTGTAGTAACATTCAATATAATTATCTCTTGTAG
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          TTGTTGTATCTTCACTTTATCTCAACTCCTTTGAGAGAACTTTCCGTAGTTATCTGCTTT
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GCACTTGGTTACTCAGAATTTTACTGTGGGCATGATAATTGATATACCAAATTCAGTTTT
CaPSY1          ------------------------------------------------------------
CaPSY1_VIGS     ------------------------------------------------------------

SlPSY1          GATTCTATCGAAAAATTTGTTATTACATTTTTTTGGGGGGAAAGGAA
CaPSY1          -----------------------------------------------
CaPSY1_VIGS     -----------------------------------------------
```

9.4.3. Ca*CCS*

```
SlLCYB          ATGGAAACTCTTCTCAAGCCTTTTCCATCTCTTTTACTTTCCTCTCCTACACCCTATAGG
CaCCS            ATGGAAACCCTTCTAAAGCCTTTTCCATCTCCTTTACTTTCCATTCCTACTCCTAACATG
CaCCS_VIGS       ATGGAAACCCTTCTAAAGCCTTTTCCATCTCCTTTACTTTCCATTCCTACTCCTAACATG
                 ******** ***** *************** **********  ****** **  * * *

SlLCYB          TCTATTGTCCAACAAAATCCTTCTTTTCTAAGTCCCACCACCAAAAAAAAATCAAGAAAA
CaCCS             TATAGTTTCAAACACAACTCCACTTTTCCAAATCCAACCAAACAAAAAGATTCAAGAAAG
CaCCS_VIGS       TATAGTTTCAAACACAACTCCACTTTTCCAAATCCAACCAAACAAAAAGATTCAAGAAAG
                 * ** * ** **** **  *  ****** ** *** ****   ***** * ********

SlLCYB          TGTCTTCTTAGAAACAAAAGTAGTAAACTTTTTTGTAGCTTTCTTGATTTAGCACCCACA
CaCCS             TTCCATTATAGAAACAAAAGCAGTACACATTTTTGTAGCTTTCTTGATTTAGCACCCACA
CaCCS_VIGS       TTCCATTATAGAAACAAAAGCAGTACACATTTTTGTAGCTTTCTTGATTTAGCACCCACA
                 *  * *  ************ **** ** ********************************

SlLCYB          TCAAAGCCAGAGTCTTTAGATGTTAACATCTCATGGGTTGATCCTAATTCGAATCGGGCT
CaCCS             TCAAAGCCAGAGTCTTTAGATGTTAACATCTCATGGGTTGATACTGATCTGGACCGGGCT
CaCCS_VIGS       TCAAAGCCAGAGTCTTTAGATGTTAACATCTCATGGGTTGATACTGATCTGGACCGGGCT
                 ****************************************** ** **  * * ******

SlLCYB          CAATTCGACGTGATCATTATCGGAGCTGGCCCTGCTGGGCTCAGGCTAGCTGAACAAGTT
CaCCS             GAATTCGACGTGATCATCATTGGAACTGGCCCTGCCGGGCTTCGGCTAGCTGAACAAGTT
CaCCS_VIGS       GAATTCGACGTGATCATCATTGGAACTGGCCCTGCCGGGCTTCGGCTAGCTGAACAAGTT
                  *************** ** *** ********** *****  *****************

SlLCYB          TCTAAATATGGTATTAAGGTATGTTGTGTTGACCCTTCACCACTCTCCATGTGGCCAAAT
CaCCS             TCTAAATATGGTATTAAGGTATGTTGCGTTGACCCTTCACCACTTTCCATGTGGCCAAAT
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          AATTATGGTGTTTGGGTTGATGAGTTTGAGAATTTAGGACTGGAAGATTGTTTAGATCAT
CaCCS             AATTATGGTGTTTGGGTTGATGAGTTTGAAAAGTTGGGATTAGAAGATTGTCTAGATCAT
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          AAATGGCCTATGACTTGTGTGCATATAAATGATAACAAAACTAAGTATTTGGGAAGACCA
CaCCS             AAGTGGCCTGTGAGTTGTGTTCATATAAGTGATCACAAGACTAAGTATTTGGACAGACCA
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          TATGGTAGAGTTAGTAGAAAGAAGCTGAAGTTGAAATTGTTGAATAGTTGTGTTGAGAAC
CaCCS             TATGGTAGAGTAAGTAGAAAGAAGTTGAAGTTGAAATTGTTGAATAGTTGTGTTGAAAAT
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          AGAGTGAAGTTTTATAAAGCTAAGGTTTGGAAAGTGGAACATGAAGAATTTGAGTCTTCA
CaCCS             AGAGTGAAGTTTTATAAAGCCAAGGTTTTGAAAGTGAAGCATGAAGAATTTGAGTCTTCG
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          ATTGTTTGTGATGATGGTAAGAAGATAAGAGGTAGTTTGGTTGTGGATGCAAGTGGTTTT
CaCCS             ATTGTTTGTGATGATGGTAGGAAGATAAGTGGTAGCTTGATTGTTGATGCAAGTGGCTAT
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          GCTAGTGATTTTATAGAGTATGACAGGCCAAGAAACCATGGTTATCAAATTGCTCATGGG
CaCCS             GCTAGTGATTTTATAGAGTATGACAAGCCAAGAAACCATGGTTATCAAGTTGCTCATGGG
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          GTTTTAGTAGAAGTTGATAATCATCCATTTGATTTGGATAAAATGGTGCTTATGGATTGG
CaCCS             ATTTTAGCAGAAGTTGATAATCATCCATTTGATTTGGATAAAATGATGCTTATGGATTGG
CaCCS_VIGS       ------------------------------------------------------------


SlLCYB          AGGGATTCTCATTTGGGTAATGAGCCATATTTAAGGGTGAATAATGCTAAAGAACCAACA
CaCCS             AGGGATTCTCATTTAGGTAATGAGCCATATCTGAGGGTGAAGAATACTAAAGAACCAACA
CaCCS_VIGS       ------------------------------------------------------------
```

```
SlLCYB          TTCTTGTATGCAATGCCATTTGATAGAGATTTGGTTTTCTTGGAAGAGACTTCTTTGGTG
CaCCS           TTCTTGTATGCAATGCCATTTGATAGGAATTTGGTATTCTTGGAAGAGACTTCTTTAGTG
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          AGTCGTCCTGTTTTATCGTATATGGAAGTAAAAAGAAGGATGGTGGCAAGATTAAGGCAT
CaCCS           AGTCGGCCTATGTTATCGTATATGGAAGTGAAAAGAAGGATGGTAGCAAGATTAAGACAT
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          TTGGGGATCAAAGTGAAAAGTGTTATTGAGGAAGAGAAATGTGTGATCCCTATGGGAGGA
CaCCS           TTGGGGATCAAAGTGAGAAGTGTCCTTGAGGAAGAGAAGTGTGTGATCACTATGGGAGGA
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          CCACTTCCGCGGATTCCTCAAAATGTTATGGCTATTGGTGGGAATTCAGGGATAGTTCAT
CaCCS           CCACTTCCGCGGATTCCTCAAAATGTTATGGCTATTGGTGGGACTTCAGGGATAGTTCAT
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          CCATCAACAGGGTACATGGTGGCTAGGAGCATGGCTTTAGCACCAGTACTAGCTGAAGCC
CaCCS           CCATCGTCTGGGTACATGGTGGCTCGTAGCATGGCATTGGCACCAGTACTGGCTGAGGCC
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          ATCGTCGAGGGGCTTGGCTCAACAAGAATGATAAGAGGGTCTCAACTTTACCATAGAGTT
CaCCS           ATCGTCGAAAGCCTTGGCTCAACAAGAATGATAAGAGGGTCTCAACTTTACCATAGAGTT
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          TGGAATGGTTTGTGGCCTTTGGATAGAAGATGTGTTAGAGAATGTTATTCATTTGGGATG
CaCCS           TGGAATGGTTTGTGGCCTTCGGATAGAAGACGTGTTAGAGAATGTTATTGTTTCGGAATG
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          GAGACATTGTTGAAGCTTGATTTGAAAGGGACTAGGAGATTGTTTGACGCTTTCTTTGAT
CaCCS           GAGACTTTGTTGAAGCTTGATTTGGAAGGTACTAGGAGATTGTTTGATGCTTTCTTTGAT
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          CTTGATCCTAAATACTGGCAAGGGTTCCTTTCTTCAAGATTGTCTGTCAAAGAACTTGGT
CaCCS           GTTGATCCCAAGTACTGGCACGGGTTCCTTTCTTCAAGATTGTCTGTCAAAGAACTTGCT
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          TTACTCAGCTTGTGTCTTTTCGGACATGGCTCAAACATGACTAGGTTGGATATTGTTACA
CaCCS           GTACTCAGTTTGTACCTTTTTGGACATGCCTCTAATTTGGCTAGGTTGGATATTGTTACA
CaCCS_VIGS      ------------------------------------------------------------

SlLCYB          AAATGTCCTCTTCCTTTGGTTAGACTGATTGGCAATCTAGCAATAGAGAGCCTTTGA
CaCCS           AAGTGCACTGTCCCCTTGGTTAAACTGCTGGGCAATCTAGCAATAGAGAGCCTTTGA
CaCCS_VIGS      ------------------------------------------------------
```

9.5. Supplementary tables

9.5.1. Supplementary Table 1 – DH population fresh fruit carotenoid screen

See attached disc

9.5.2. Supplementary Table 2 – DH population stored fruit carotenoid screen

See attached disc

9.5.3. Supplementary Table 3 – DH population carotenoid retention screen

See attached disc

9.5.4. Supplementary Table 4 – DH population fresh fruit GC-MS screen

See attached disc

9.5.5. Supplementary Table 5 – DH sub-population GC-MS analysis

See attached disc

9.5.6. Supplementary Table 6 – Low vs High retention cuticle RNAseq

See attached disc

9.5.7. Supplementary Table 7 – Low vs High retention RNAseq

See attached disc