The Physiology and Transcriptomics Underlying Dahlia Flower Senescence

A thesis submitted to Royal Holloway, University of London for the degree of Doctor of Philosophy

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2018

Declaration of Authorship

I, Matthew Casey, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

Date:

Abstract

Dahlias are a popular commercial flower due to their variety in colour and morphology. Unfortunately, they are currently not commercially viable as cut flowers due to an unreliable vase-life. Analysis of the cut flower trade suggests that minimum longevity needs to be 10-14 days from harvest to provide time for transport to a pack house and on to the supermarket, three days in-store shelf life, and still allow a 5-day consumer guarantee. Dahlia inflorescences present unique challenges due to their complex composite floral structure: florets develop sequentially within the capitulum hence in each flower head flowers of different ages are represented. Another problem has been the limited data on dahlia and the lack of conclusive data regarding ethylene sensitivity. Therefore it was necessary to address some key questions on dahlia during this project. Firstly, dahlia flower senescence was characterised and cut flower senescence compared to senescence on the plant. The response of cut dahlia to traditional and novel postharvest treatments, including ethylene inhibitors, exogenous phytohormones and sugars, and application methods, including sprays, pulses, and holding solutions was also analysed. It was found that treatment with ethylene action inhibitors and exogenous cytokinins may significantly extend dahlia vase life. Finally, RNA-sequencing has been carried out on material from three different developmental stages of dahlia flowers to examine transcriptomic changes during the process and analyse the process at a molecular level. The differential expression analysis of this data found upregulation of putative genes including ethylene biosynthetic genes and downregulation of cytokinin biosynthetic genes, along with changes in expression in the signalling pathways of both cytokinins and ethylene. This work will provide a database for further research on dahlia flower senescence.

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List of Abbreviations

Abbreviation	**Full Name**
ABA	Abscisic Acid
AOA	Aminooxyacetic Acid
AVG	Aminoethoxyvinyl glycine
BA	6-Benzylaminopurine
CEPA	Chloroethylphosphonic acid
ERF	Ethylene Response Factor
GA	Gibberellic Acid
KEGG	Kyoto Encyclopaedia of Genes and Genomes
1-MCP	1-Methylcyclopropene
PCR	Polymerase Chain Reaction
RHS	Royal Horticultural Society
STS	Silver Thiosulphate

Dedication

This thesis is dedicated to the memory of my late grandfather Kenneth Turner who instilled in me a love of all plant life, and who loved to grow dahlias in his own garden, I think he would happily approve of the work performed in this project.

Acknowledgements

First and foremost, I would like to thank my supervisors, Tony Stead and Hilary Rogers for their guidance and advice throughout the PhD, it goes without saying that this project would not have happened without them. I would also like to thank Paul Devlin, Martin Squire at Chrysal and Paula Edgington at Waitrose for support during the project. I would also like to thank BBSRC for their generous support of this project.

I am particularly grateful for the baked goods, Christmas dinners, emotional support and life and lab advice provided by my fellow lab members, Lauren Edwards, Kate Orman, Bianca Lear and Clive Hill. And also, a big thank you to my fellow PhD students George Skinner, Stacey Vincent, Francesca Baylis, Sebastien Lambertucci and Shaoli Das Gupta for the help they gave me when I needed it. Special thanks for Bianca for keeping me on the straight and narrow in my final year and for always being a voice of reason, sobriety and moderation.

Thank you to Pauline Baker, Tracey Jeffries and Michelle Jux for support during the project and for performing invaluable work for the department.

And a final thanks to my whole family for emotional, and sometimes financial, support during this project even if you didn't always understand what I actually did.

1. Introduction

1.1. The cut flower industry

Cut flowers are a considerable industry, with the UK ornamental plant and flower industry worth £1.2 billion in 2016, a rise of 4.7% on the previous year (DEFRA, 2017). It is also an increasingly globalised market, with developing countries generally supplying developed countries. However, the cut flower market is the domain of relatively few plants such as roses, lilies, and chrysanthemums. Many other species popular as garden plants, such as the dahlia, are excluded from the cut flower market due to their limited or unreliable vase life. The cut flower industry involves a supply chain that encompasses growing, harvesting, transportation and storage, retail, and finally a consumer stage. Flowers must last long enough after harvest to be transported from the grower to the packer, spend 2-3 days in the store, and still have a reasonable vase life after this for consumer satisfaction, usually a minimum of 5 days. Ideally therefore flowers need to last for 10-14 days overall. Previous work indicates that the dahlia flower may reach a vase life of up to 10 days (Dole *et al*., 2009) however, the commercial practicalities of this have yet to be reliably implemented.

There is considerable commercial potential for dahlias within the cut flower industry. Their close relative the chrysanthemum is already extremely popular as a cut flower; in 2013 Colombia exported $147m worth of chrysanthemum to the USA alone (PMA, 2015). In the EU the UK imports more chrysanthemums than any other country by a large margin, €128m worth compared to Germany's €39m worth in the same year (AHDB, 2015). In Japan there is considerable interest in dahlia as cut flowers, but the short vase life limits their appeal (Shimizu-Yumoto & Ichimura, 2013). However, there are differences in the way flowers are marketed in different countries, and the flowers that are most popular. The market in Japan consists of single flowers, often with short stems, whereas in Europe and the US it is more normal to market bunches of flowers, usually on long stems. Increased use of air freight makes quick transport much more viable from supplier countries in the developing world, however these raise concerns in the industry over cost and carbon footprint. Cut flowers are therefore an increasingly important cash crop in South America and East Africa, enabling farmers to move away from subsistence farming. The market in Ethiopia alone has grown up to 537% annually from 2002-2006 (CIBCO, 2007). However, the ability to grow dahlias in temperate environments, using only minimal protection, e.g. Spanish tunnels, makes them an attractive potential UK crop to supply locally-grown flowers. Due to their reduced carbon footprint locally grown

dahlias may be an attractive alternative to imported flowers. Despite the economic downturn of 2008, imports of cut roses to the EU continued to rise from $510 million in 2008 to $614 million in 2012 (CBI, 2013) showing that cut flowers remain popular even during an economic downturn. Europe is by far the largest cut flower market; in 2006 67% of global import value was accounted for by Europe (CIBCO, 2007), although the market in Japan is considerable and the US share is increasing. Furthermore, the UK cut flower industry has risen in value from £122m in 1998 to £691m in 2014, although the UK still lags behind other western European countries in per capita spending on cut flowers (Connolly, 2017). Per capita spending on flowers, plants and related goods in 2012 was €139 in Germany, €127 in France, and €126 in the Netherlands compared to just €78 in the UK (AHDB, 2015).

If commercialised, cut dahlias could be a significant source of income to UK flower growers. Currently just 10% of UK cut flowers are grown in the UK as they have been outcompeted by cheaper imports in past decades; 18% of UK cut flowers come from Africa and South America. However, the proportion may be even higher than that as some flowers from Africa and South America are exported to the Netherlands and then re-exported to the UK, thus becoming classed as European exports (Royal Flora Holland, 2017). In 2016 the UK exported £66m worth of ornamentals, a 20% increase on 2015, and imports of cut flowers to the UK in the same period grew 13% (DEFRA, 2017). This demonstrates the value of making the dahlia a commercially viable cut flower in the UK and how valuable it could be to UK based flower growers as a source of income.

Factors affecting the vase life of a cut flower include pre-harvest conditions such as temperature (Armitage & Laushman, 2003), photoperiod (Armitage & Laushman, 2003; Konishi & Inaba, 1964), and nutrition (Armitage & Laushman, 2003; Romer & Nelson, 2003; Ahmed *et al*., 2004). Postharvest treatments are also important and may include phytohormones or their inhibitors (Hashemabadi *et al*., 2009; Hashemabadi & Zarchini, 2010; Shimizu-Yumoto & Ichimura, 2013; Woltering & van Doorn, 1988) and nutrients including sugar (Lukasweska, 1983). Commercial growers may not be able to control all pre-harvest conditions due to financial and time constraints, and typical treatments to reduce ethylene sensitivity will not work on ethylene insensitive species. Ethylene sensitivity varies amongst different species and amongst cultivars of the same species, complicating the choice of treatment. Moreover, there are also issues surrounding the environmental toxicity of some commercial postharvest treatments including silver thiosulphate (STS) (Hunt *et al*., 1999). Unfortunately, much of the research on flower

longevity to date has been performed on plant species totally unrelated to dahlias, such as lilies, roses, and carnations. Within the Asteraceae family to which dahlia belongs, gerbera, sunflower, and chrysanthemum serve as the best models as they have a similar composite flower structure and are commercially popular (PMA, 2015). A better understanding of the physiological and molecular aspects of senescence in composite flowers might enable the design and marketing of specific preservatives to improve vase life in dahlias.

1.2. *Dahlia* physiology and genetics

The genus *Dahlia* is native to Mexico, of which it is the national flower. There are 42 accepted species of dahlia found endemically from Mexico through to northern South America (The Plant List, 2013; Kamenetsky & Okubo, 2012). It is a predominantly upland genus growing at elevations of 1500 to 3700 metres, perhaps partially explaining why it fares relatively well in lowland areas at cooler latitudes (Saar *et al*., 2003). Dahlias are frequently grown in gardens in temperate and tropical regions of the world.

Firstly, dahlias develop sequentially with the eldest outermost florets developing, expanding, and opening first, this appears as whorls but is a spiral pattern with florets circling inwards becoming younger and less mature towards the centre of the inflorescence (personal communication, Tony Stead). The flower heads of the dahlia are actually pseudanthia, ‘false flowers’, also sometimes referred to as a capitulum or a composite flower. A pseudanthium is an inflorescence in which anything from a small cluster to hundreds or sometimes thousands of florets (reduced flowers) are grouped together to form a single flower-like structure (Hutchinson, 1964). There are two main types of floret in Asteraceae species, ray and disc florets. Ray florets are generally female and zygomorphic (bilaterally symmetrical) whereas disc florets tend to be hermaphroditic and actinomorphic (radially symmetrical) (Fig. 1). Both floret types are generally small and often greatly reduced in size compared to normal flowers; however, the combination of these florets can cause the pseudanthium itself to be very large, e.g. in sunflowers.

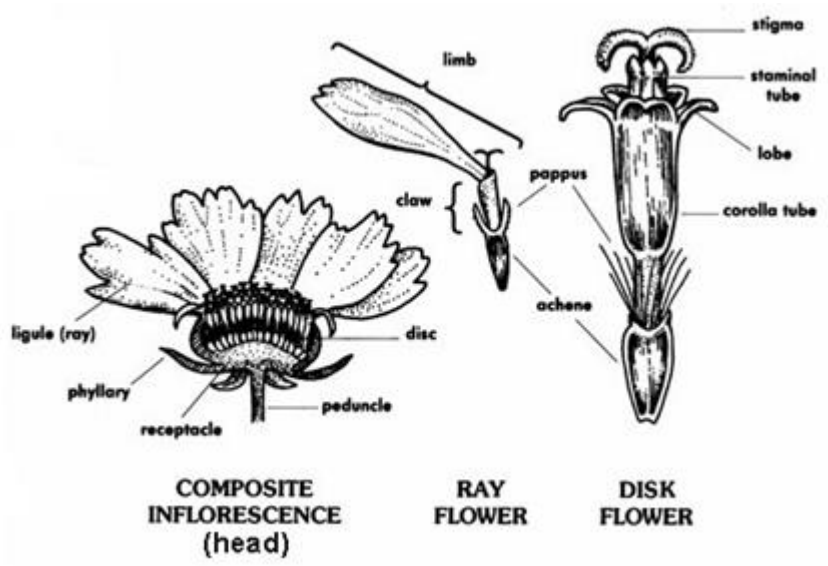


Figure 1: The structure of a composite inflorescence, demonstrating the difference between ray and disc florets (Swink & Wilhelm, 1994).

The modern garden dahlia, *Dahlia variabilis*, is a relatively new plant which is believed to have arisen following the introduction of dahlia into Europe from the New World in the late eighteenth century (Dean, 1897). Early observations of the chromosomes of *Dahlia coccinea* showed 2n = 32 (Lawrence, 1929) whilst garden dahlias all have 2n = 64 (Gatt *et al*., 1998). Wild *Dahlia* species can be divided into two groups depending on their flower colour. Group I dahlias produce magenta or ivory flowers and Group II dahlias bear orange-scarlet or yellow flowers (Lawrence, 1929, 1931; Lawrence & Scott-Moncrieff, 1935). The domesticated dahlia combines both colour series, leading these authors to conclude that the garden dahlia (2n = 64) is a hybrid derived from the crossing of two wild species (2n = 32), one belonging to Group I, the other to Group II.

More recently this view has been supported by analysing the ploidy level of the dahlia using SSR's (short sequence repeats). The number of SSR alleles per genotype exceeded four alleles in six out of the fourteen markers tested, indicating that the dahlia has a greater number of chromosomes than a tetraploid (Schie *et al*., 2014). As the number of chromosomes (2n = 64) in the domesticated dahlia cannot be divided by six to obtain a whole number and therefore not be hexaploid, it must be assumed *Dahlia variabilis* is octoploid (Schie *et al*., 2014). Although this does not confirm the hybridisation theory the authors also found that there was a low level of preferential pairing between chromosomes, suggesting that parts of the genome lack sufficient similarity for free pairing, thus suggesting hybridisation. The dahlia genome is also very large, up to 9.62 pg in size in some dahlia varieties, which corresponds to more than 9000 Mb (Temsch *et al*., 2008), in contrast to *Arabidopsis thaliana* the genome of which is only around 135Mb

in size (The *Arabidopsis* Genome Initiative, 2000). Due to the evidence on dahlia ploidy (Gatt *et al*., 1998; Schie *et al*., 2014) it was assumed that all dahlia cultivars in this study were octoploid, however, this was not investigated as it was not considered a factor in vase life.

The number of dahlia cultivars in existence is not certain but is in the order of tens of thousands (RHS, 2014). These cultivars can be classified either by size or by flower type. Sizes can range from micro dahlias with flowers less than 50 mm in diameter to cultivars that bear inflorescences over 250 mm in diameter (Romer & Nelson, 2003). However, it is more common for domesticated dahlia types to be defined by their inflorescence structure, not size. The most common types of dahlia flower type include cactus, waterlily, decorative, and ball; but there are many different types of dahlia, up to 14, though some of these are classed as miscellaneous types in some horticultural sources (Romer & Nelson, 2003; Vernon, 2014; Table 1). The variety in morphological form of dahlia inflorescences and the variety of colours they exhibit is the major reason for their popularity and the commercial interest in them as cut flowers.

Table 1 – A summary of domesticated dahlia flower types (types and descriptions: Vernon, 2014; cultivars and pictures: RHS, 2018)

Type	*Description*	*Example cultivar*	*Picture of example*
Anemone	Central disc florets are elongated leading to a protruding dome of long tubular florets surrounded by one or more whorls of ray florets.	‘Ryecroft Marge’	
Ball (pompom if <50 mm)	Many whorls of ray florets are tightly packed to form a globular shape. Pompoms are the name given to ball types under 50 mm in diameter.	'Mary's Jomanda'	

Cactus	Each ray floret is curled to form a pointed tubular like structure that gives the inflorescence a spiky appearance.	'Weston Pirate'	
Cactus (semi)	The same as the cactus except the ray florets are broader and flatter toward the base which make the appearance softer.	'Melody Gipsy'	
Collerette	A variation on single type dahlias, but between the centre of disc florets and outer whorl of ray florets is an intermediate collar of smaller ray florets, these may be of a different colour to the larger outer ray florets.	‘Clair de Lune’	
Decorative (Formal)	Many whorls of flat and broad ray florets which are usually blunt or only slightly pointed at the end. Formal types have more tightly packed neater arrangements of florets.	‘Fusion’	
Decorative (Informal)	Many whorls of flat and broad ray florets which are usually blunt or only slightly pointed at the end. Informal types have more loosely arranged ray florets with a more relaxed looking form.	'Hamari Gold'	

Fimbriated (a.k.a. Laciniated)	Each ray floret is split again towards its distal end leading each one to have a fork or fishtail like appearance. Often considered a subtype of cactus dahlia.	'Kenora Macop-b'	
Orchid (Single, a.k.a. Star)	A modified type of single dahlia. The single whorl of outer florets are elongated and curl inward.	'Honka Rose'	
Orchid (Double)	Ray florets are narrowly lance shaped and are rolled inward slightly along their length.	‘Pink Giraffe’	
Peony	More than one whorl of outer florets surrounding a centre of disc florets.	'Bishop of Llandaff'	
Single	A single outer ring of florets surrounding a centre of disc florets.	‘Happy Single Princess’	
Stellar	Often considered a subtype of decorative dahlia, the difference being the cupped shape of their ray florets which are slightly swept back as they spiral from the flower centre.	‘Camano Pet’	

Waterlily	Ray florets are broad, flat and roll inward slightly at the edges.	‘Le Castel’	

1.3. Floral senescence: a background

Floral senescence is the process by which floral tissue is actively degraded by the plant in a period of programmed cell death (PCD) in order for the plant to recover nutrients and reduce the energy demands of maintaining expensive floral organs, through nectar production and water loss through transpiration (Ashman & Schoen, 1994). However, flowers may also senesce because of pollinator activity, with flowers with more specific pollinators evolving longer lasting flowers to increase chances of pollination (Ashman & Schoen, 1994; van der Pijl & Dodson, 1966). Flowers are necessary for angiosperms’ sexual reproduction but are expensive to produce and maintain (Stead, 1992; Stead *et al*., 2006; Jones, 2002). During senescence, plants remobilize macronutrients including sugars and proteins, as well as micronutrients such as magnesium and iron (Jones, 2013). The breakdown and remobilisation of lipids, sugars and proteins are all an important part of plant senescence as it allows plants to reallocate nutrients to developing tissues instead of losing them if the flower abscises without any reallocation. Ethylene, a phytohormone frequently implicated in floral senescence, is considered particularly important in horticulture as it is common to split cut flowers into two categories, those that are ethylene sensitive and those that are not, and treatments based on this sensitivity are some of the major factors in improving postharvest value in these crops (van Doorn & Woltering, 2008; Iqbal *et al*., 2017). Inhibiting ethylene action is a way of improving vase lives in ethylene sensitive plants. However, this means that there are challenges in improving vase life in flowers whose floral senescence processes are ethylene independent. It is important to understand all major underlying aspects of floral senescence to allow us to further improve vase life in the future and to provide us with avenues of research into dahlia floral senescence, which does not respond to typical treatments as well as other cut flowers.

1.3.1. Autophagy in floral senescence

The role of autophagy in floral senescence is still unclear, however recent evidence suggests it does have a role in the breakdown of macromolecules during the process. In autophagy, autophagosomes, vacuole-like vesicles, breakdown the cytosol and cellular components and the process can be defined by the activation of autophagy genes, known as ATG's (van Doorn 2011; Jones, 2013; Yamada *et al*., 2009). In the ornamental species *Ipomoea nil*, homologs of the *Arabidopsis* autophagy related genes *ATG4b* and *ATG8a* were identified, and ethylene treatment which accelerated senescence was found to upregulate *ATG4b*. Autophagic structures have also been found during petal senescence in petunia (Shibuya *et al*. 2013), and ipomoea (Shibuya *et al*., 2009). Furthermore, during both pollination induced and developmental senescence in petunia there was an accompanied increase in transcript abundance of *PhATG8* homologs (Shibuya *et al*. 2013). Therefore, it is likely that autophagy plays a role in breakdown of macromolecules during flower senescence, however it remains to be well characterised.

1.3.2. Phytohormones in floral senescence

Senescence is regulated by multiple phytohormones though most research has focused on ethylene, which is thought to be the key modulator of flower senescence in many angiosperms. However, there are a number of cut flower species which have an ethylene independent process of flower senescence, and much less is known about this compared with ethylene dependent flower senescence (van Doorn & Woltering, 2008). Ethylene dependent flower senescence is usually associated with rapid increases in endogenous ethylene production, this upsurge in ethylene follows pollination and a short treatment with exogenous ethylene can replace pollination to trigger senescence (Nichols, 1966). In unpollinated flowers it is unknown what induces a rise in ethylene production.

There is abundant evidence for the role of ethylene in the flower senescence process of major cut flower species including rose (Hashemabadi *et al*., 2014), carnation (Asil *et al*., 2012; Yu & Bao, 2004), gentians (Shimizu-Yumoto & Ichimura, 2012), petunia (Whitehead *et al*., 1984), and *Alstroemeria* (Wagstaff *et al*., 2005). The receptor ETR1 is a key part of the ethylene recognition and response pathway. It has been found in numerous species that an *etr1-1* mutant unable to bind ethylene confers an ethylene insensitive phenotype, as the hormone is never recognised. The mutant receptor continues to behave as if ethylene is absent, and in ethylene's absence the receptor works to suppress ethylene responsive genes. This has been found in species as varied as *Arabidopsis*

(Wilkinson *et al*., 1997), kalanchoe (Sanikhani *et al*., 2008), and campanula (Sriskandarajah *et al*., 2007). This has great value for ornamental species, for example, petunia *etr1-1* mutants which lacked the correct ethylene receptor showed a delay in peak ethylene production and a near doubling in flower lifespan compared to controls (Wang *et al*., 2013a).

Usually in the presence of ethylene, ETR1 becomes inactive, thus stopping the phosphorylation of EIN2. This results in the proteolytic release of its C-terminal domain, which is targeted to the nucleus where it prevents degradation of the transcription factors EIN3/EIL1 (Wen *et al*., 2012; An *et al*., 2010). EIN3/EIL1 are then able to bind to the promoter region of the ethylene response factor (ERF) transcription factor genes, resulting in the activation of downstream ethylene response genes (Solano *et al*., 1998). Downstream genes include those involved in activating proteases, cysteine proteases implicated in senescence have been upregulated following upregulation of ethylene in carnation (Jones *et al*., 1994), rose (Tripathi *et al*., 2009) and petunia (Jones *et al*., 2005). Nucleases (Langston *et al*., 2005), DNases and RNases (Xu & Hanson, 2000; Langston *et al*., 2005), along with genes involved in nutrient remobilisation (Chapin & Jones, 2009; Hew *et al*., 1989) and cell wall breakdown (Hong *et al*., 2000) have also been found to be upregulated downstream of the ethylene response.

Outside of ethylene sensitive flowers, ethylene independent senescence is less well characterised. The hormone abscisic acid (ABA) is known to be involved in senescence in some species, for example iris (van Doorn *et al*., 2013). In daylily, ABA (abscisic acid) is thought to be the main hormonal regulator of flower senescence and senescence associated changes, including ion leakage, protease activity and activity of DNases and RNases (Panavas & Rubinstein, 1998). In species in which ethylene is the primary regulator of senescence ABA has been found to accelerate the process by promoting endogenous biosynthesis of ethylene (Ronen & Mayak, 1981), and in daffodils increases in ABA content coincided with visible signs of petal senescence (Hunter *et al*., 2004a). It has also been found that ABA and ethylene may positively regulate each other's biosynthesis (Ronen & Mayak, 1981; Li & Huang, 2011; Fig. 2).

In contrast to ABA and ethylene, cytokinins have been found to delay senescence in flowers. Cytokinins are known to delay flower senescence in a range of popular ornamental species including petunias (Chang *et al*., 2003), roses (Lukaszewska *et al*., 1994), and chrysanthemums (Guo *et al*., 2003). Inhibiting cytokinin degradation has also been found to delay senescence in carnation (Taverner *et al*., 2000). How cytokinins do

this remains unknown but has been suggested that they delay senescence through upregulation of genes involved in maintaining cellular integrity, defence against ROS and maintaining nutrient supplies to cells (Ehneß & Roitsch, 1997; Trivellini *et al.*, 2015). It has also been found during senescence in chrysanthemum that both cytokinins promoted gibberellic acid (GA) and that both cytokinins and GA inhibited ethylene production induced by ABA (Guo *et al.*, 2003; Fig. 2). Additionally, ethylene has been found to repress rises in GA (Wang *et al.*, 2013a; Fig. 2).

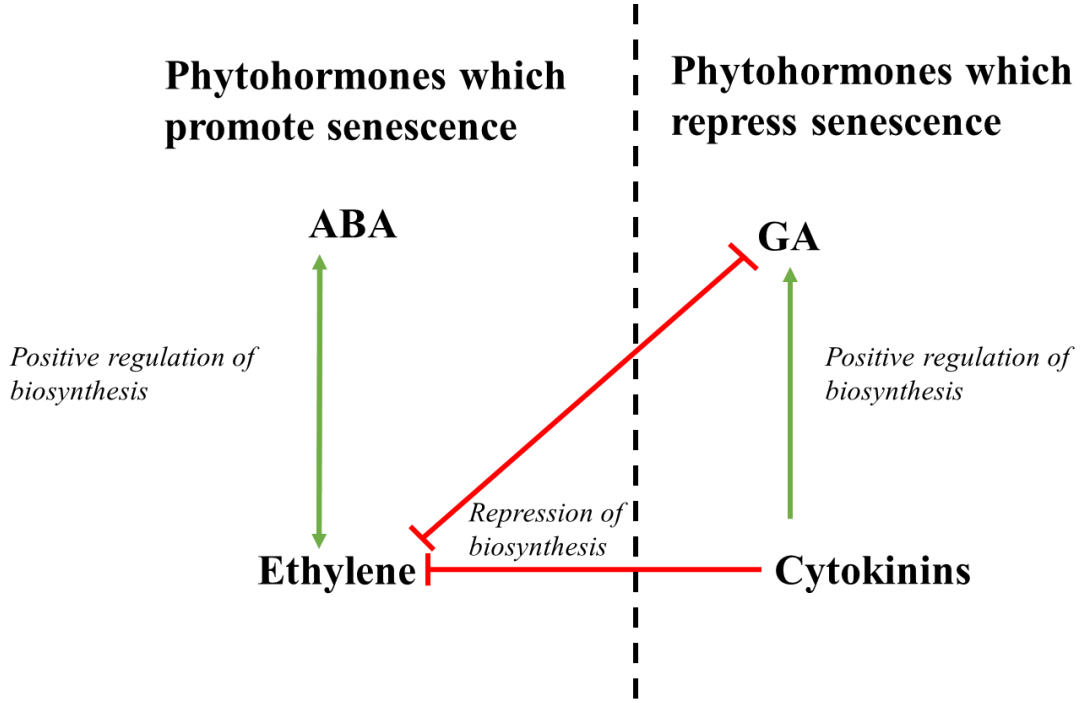


Figure 2: Summarised scheme of the phytohormones abscisic acid (ABA), gibberellic acid (GA), ethylene and cytokinins which have been implicated in either promoting or repressing senescence and their interactions with one anothers biosynthesis process.

1.3.3. Macromolecule breakdown in floral senescence

During senescence there is breakdown of nucleic acids, lipids and proteins. This is because senescence is partly a recycling process, during which the plant remobilises essential nutrients from senescing to developing tissues (Jones, 2013). Nucleic acids are broken down so the plant can recycle nutrients such as nitrogen and phosphates. During senescence it has been found in multiple ornamental species that nuclease activity increases, including in daylily (Panavas *et al.*, 1999), ipomoea (Yamada *et al.*, 2006) and petunia (Xu & Hanson, 2000).

There is rapid and sustained breakdown of phospholipids and an increase in phospholipid degradation products leading to cellular membrane breakdown during floral senescence. Leakage of ions and loss of differential membrane permeability follows the breakdown of phospholipids (Thompson *et al*., 1997; Phillips & Kende, 1980). These changes are mostly due to increased activity of acyl hydrolases and phospholipases. Increased activity of lipoxygenases, which oxidise membrane lipids leading to loss of membrane permeability, has been observed in daylily (Panavas & Rubinstein, 1998) and carnations (Sylvestre *et al*., 1989)

Protein breakdown may be proteasomal dependent or independent. Proteasomal systems of protein degradation have been found to be upregulated in carnation (Hoeberichts *et al*., 2007). Protein degradation is important in this process to retain nutrients, especially nitrogen. Targeted protein degradation plays a role in the flower senescence process of important ornamental species. This was demonstrated with transformed *Petunia hybrida* by silencing an endopeptidase component in the 20S core of the 26S proteasome of the plant (Stier *et al*., 2010). Transformed plants showed double the vase life of controls strongly suggesting that certain proteases play an important role in the progression of flower senescence.

Proteasome independent protease activity has also been shown to markedly increase during senescence. Proteases have been found to affect the process of senescence throughout plants, however only some proteases are senescence specific. Research on *Alstroemeria* tepals has shown that some proteases are upregulated throughout the flower senescence process (Wagstaff *et al*., 2002). There was a dramatic increase in the expression of the cysteine protease *ALSCYP1* indicating that this gene may encode an important enzyme for the proteolytic process in this species. Three papain class cysteine protease enzymes showing different patterns of activity during flower development were identified, one of which showed a similar expression pattern to the cysteine protease cDNA. This implies the importance of cysteine proteases in flower senescence. Use of cysteine proteases specific inhibitors such as E64 and antipain has demonstrated the importance of cysteine protease activity during flower senescence. Treatment with membrane permeable forms of these compounds has reduced total protease activity in petals of *Sandersonia*, petunia, iris, and *Hemerocallis* (Stephenson and Rubinstein, 1998; Jones *et al*., 2005; Pak & van Doorn, 2005; Eason *et al*., 2002).

1.3.4. Remobilisation in floral senescence

One of the main purposes of senescence is to remobilise nutrients. However, there are costs to remobilisation so only a portion of nutrients are recycled by the plant. Carbohydrates in the form of sucrose, nitrogen incorporated in amino acids, phosphorus as an inorganic ion, potassium, magnesium, and calcium, are all transported (Jones, 2013; Eason *et al*., 2000; van Doorn & Woltering, 2008). It 'costs' the plant to remobilize nutrients, therefore this cost must be equalled or surpassed by the benefits of remobilisation to another part of the plant. This may explain some of the differences seen in remobilisation profiles between cut and on-plant flowers. It has also been found that ethylene influences remobilisation, suggesting it may also in dahlia (Chapin & Jones, 2009). However, ethylene sensitivity is variable between cultivars indicating that remobilisation may also differ markedly between dahlia cultivars (Woltering & van Doorn, 1988; Dole *et al*., 2009; Shimizu-Yumoto & Ichimura, 2013).

Transporter proteins (TP's) are a critical part of the process of remobilisation. Extensive expression profiling in *A. thaliana* has found large numbers of TP's upregulated in senescing leaves (van der Graaf *et al*., 2006). Many TP's have been identified in leaves but fewer in petals, however some have been identified in senescing petals of *Alstroemeria* and carnation, including metal transport proteins involved in mobilisation of Fe and Mn (Breeze *et al*., 2004; Hoeberichts *et al*., 2007). In petunia, phosphate transport proteins (PT's) have been identified which may play a role in inorganic P transport during flower senescence and their expression accompanied the decline of P content in the senescing corolla (Wegmuller *et al*., 2008; Chapin & Jones 2009). This upregulation of PT's is regulated by ethylene, and *Petunia hybrida etr* (ethylene receptor) mutants have shown reduced transcript abundance of PT's, in contrast to treatment with exogenous ethylene for just 2 h which was found to upregulate transcripts of *PhPT1* (Chapin & Jones, 2009).

1.3.5. Reactive oxygen species (ROS) in floral senescence

An important role for reactive oxygen species (ROS) in floral senescence has also been proposed in many species including lily (Arrom & Munné-Bosch, 2010), chrysanthemum (Bartoli *et al*., 1997), and multiple species of orchid (Attri *et al*., 2008). In a study in chrysanthemum, concentrations of ROS increased during floral senescence (Chakrabarty *et al*., 2007). The activity of enzymes related to ROS scavenging such as superoxide dismutase, ascorbate peroxidase and catalase however declined during senescence. It was

suggested that the imbalance between an increase in ROS production and a failure of the scavenging enzymes results in oxidative damage and consequent petal deterioration (Chakrabarty *et al*., 2007). However, the causative link remains to be completely established.

1.4. Composite flower senescence

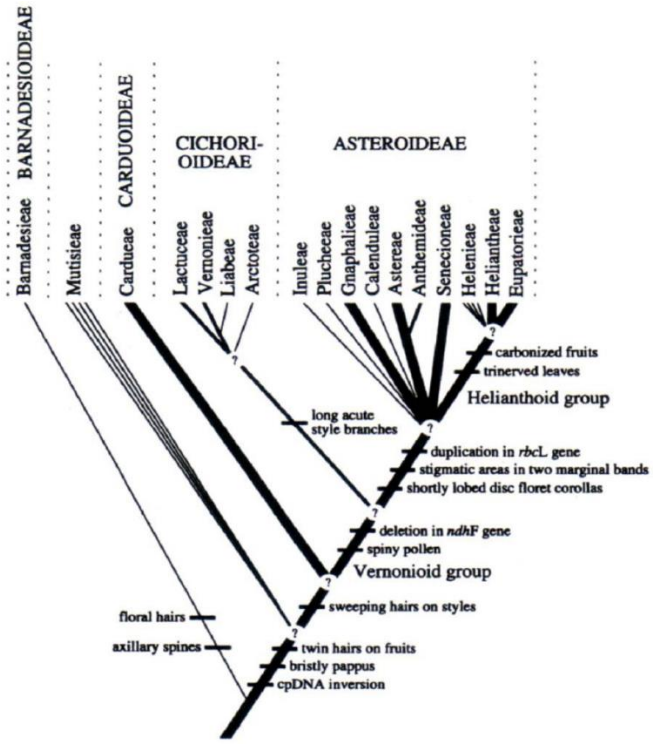


Figure 3: Phylogenetic tree of Asteraceae (Bremer, 1996)

Both sunflowers and chrysanthemum are members of the same subfamily as dahlias, Asteroideae, so it may be that these genera are closer to dahlia in their senescence process than gerbera, which is a member of the subfamily Mutisieae (also referred to as Mutisioideae) (Fig. 3; Panero & Funk, 2002). *Arabidopsis* is a member of the Eudicots, though it is part of the Rosid clade, not the Asterid clade (APG, 2016). Although evolutionary quite distant from dahlia *A. thaliana* is a well-studied plant, and until a member of the composite family is better annotated *A. thaliana* is a viable alternative, though any findings must be treated with caution. In this study comparisons are made between dahlia and other Asteraceae family members where possible, but in other cases comparisons have been made to less closely related ornamental species such as carnation (Asil *et al*., 2012; Hoeberichts *et al*., 2007; Ichimura *et al*., 2009; Yu & Bao, 2004; Jones, 2002) and petunia (Jones *et al*. 2005; Jones, 2008; Langston *et al*., 2005; Trivellini *et al*., 2015; Wang *et al*., 2013a; Wang *et al*., 2018; Change *et al*., 2003; Chapin & Jones, 2007).

Multiple genera in the Asteraceae were examined in one study, and it was concluded that sensitivity to ethylene was low: some dahlia and gerbera varieties were slightly sensitive to ethylene, but all others analysed were completely insensitive, including the varieties of sunflower and chrysanthemum assessed (Woltering & van Doorn, 1988). However, later studies indicated variation in sensitivity to ethylene within chrysanthemum (Doi *et al*.,

2003; Kumari *et al*., 2016). Furthermore, it has been found that transformation of the chrysanthemum ethylene receptor DG-ERS1 reduced the ethylene sensitivity of chrysanthemum plantlets and leaf yellowing of cut chrysanthemum was reduced in these lines. The transgenic chrysanthemum had green leaves for longer and better vase life compared to controls as a result (Satoh *et al*., 2006). All this suggests that there may be a great variation in ethylene sensitivity in dahlia, just as there is in chrysanthemum, and that multiple cultivars need to be assessed for more accurate conclusions to be drawn.

STS (silver thiosulphate) has been used extensively to lengthen the vase life of ethylene-sensitive cut flowers. The commercial value of STS was first demonstrated when it was found that pre-treatment of cut carnations with STS at concentrations of 0.1, 0.2 or 2.0 mM blocked the increase in ethylene production and prevented wilting within the experimental period (Veen, 1979). The treated flowers did not show a surge in ethylene production after 6-8 days, whereas the control flowers did. Treatments with STS therefore may indicate a sensitivity to endogenous ethylene in relation to floral senescence, and experiments on composite flowers using STS is a helpful indicator as to whether ethylene is relevant in their floral senescence. It has been found that both sunflower and chrysanthemum were unresponsive to STS treatments (Woltering & Van Doorn, 1988). Moreover, although treatment with ethylene biosynthesis inhibitors reduced ethylene production in sunflowers, this did not correspond with an improvement in vase life (Mensuali- Sodi & Ferrante, 2005). However, sunflowers treated with exogenous ethylene did have a reduced vase life (Mensuali- Sodi & Ferrante, 2005). This implies that ethylene inhibitors, effective in some species, are less effective in sunflowers, and perhaps also dahlias.

1.5. Transcriptomics and flower senescence

Understanding gene regulation of dahlia senescence would be helpful in assessing treatments to delay it. Unfortunately, as with the other Asteraceae, very little sequence data are available. The large and complex genomes of the Asteraceae, which most often have genomes in excess of 1 pg in size, make them difficult to work with compared to simpler model species such as *Arabidopsis thaliana*, the genome of which is just 0.16 pg in size (Bennett & Leitch, 2012). However, given the recent advances of next generation sequencing, new opportunities exist for examining changes in the transcriptome during senescence. This might identify changes in growth regulators, metabolic pathways or regulatory genes for which RNAi approaches might be contemplated.

Although there have been transcriptomic studies performed recently in the Asteraceae, notably in gerbera (Huang *et al*., 2017), chrysanthemum (Won *et al*., 2017; Wang *et al*., 2013b; Liu *et al*., 2016) and sunflower (Liang *et al*., 2017), these have not focused specifically on floral senescence. Two of these studies have focused on composite flowers, petal growth in gerbera (Huang *et al*., 2017) and development of ray and disc florets in chrysanthemum (Liu *et al*., 2016) respectively. The other studies in chrysanthemum and sunflower used stem, leaf or root tissue and investigated genomic evolution (Won *et al*., 2017), drought stress (Liang *et al*., 2017) or the acquisition of SSR markers (Wang *et al*., 2013b), showing that RNA-sequencing has varied applications that can be used to investigate multiple avenues of interest.

The transcriptomic studies carried out in dahlia have focused on multiple flower organs (stem, leaf, and flower bud) or on floral buds alone rather than on senescing florets and these have been limited by a lack of biological replication (Lehnert & Walbot, 2014) or fragmented coding sequences (Hodgins *et al*., 2014). The recent release of the annotated sunflower genome may help in future transcriptomic studies in the Asteraceae as it may provide a suitable reference genome, negating the need for *de novo* assemblies (Badouin *et al*., 2017).

In the Asteraceae, transcriptomic analyses have focused on floral development, confirming that development in these flowers is complex. For example, over 1500 sequences, unique to gerbera, were discovered in a transcriptome analysis of organ development in that species (Laitinen *et al*., 2005). This led to the discovery of specific TCP and MADS transcription factors that may determine differentiation of disc and ray florets (Laitinen *et al*., 2006; Broholm *et al*., 2008). Likewise, in chrysanthemum, progress is being made in understanding the regulation of flowering signals and early floral development through transcriptomic approaches (Wang *et al*., 2013b). Studies of transcriptomic changes during senescence of composite flowers are still needed and should be useful in giving a better insight into composite flower senescence on a molecular level.

1.6. Methods to improve floral vase life: pre-harvest and harvesting

Although studies on treatments for increasing vase life have often focused on post-harvest treatment of vase water, the effects of pre-harvest growth on post-harvest vase life has been established in many flowers (Pompodakis *et al*., 2005; Hashemabadi *et al*., 2009; Hashemabadi & Zarchini, 2010). These include levels of lighting and photoperiod,

temperature, growth medium, nutrition, and harvsting. Optimum environmental conditions are needed to produce large and healthy flowers in dahlia and photoperiod affects both their tuber development and flowering (Armitage & Laushman, 2003). In dahlia, long days induce flowering while a short-day length of eight hours inhibits flowering, the optimum day length being 13-15 hours (Armitage & Laushman, 2003). However, the number of hours required is not exact as different dahlia varieties show slight variation (Konishi & Inaba, 1964). Such a long day length is obviously not conducive to forcing flowering in the winter months in temperate regions, as the cost of artificial lighting would be prohibitive.

Temperature can also be a key factor influencing vase life. For example, in *Anthurium,* it was concluded that 53% of the variation in the vase life of cut flowers was positively related to the mean maximum temperature two months prior to harvest as well as post-harvest temperature (Paul *et al*., 1992). In addition, winter-harvested roses suffer a reduced vase life when compared to summer-harvested roses. It was suggested this was due to reduced photosynthesis and smaller carbohydrate pools (Pompodakis *et al*., 2005). For dahlia, an optimal day temperature of 23 ˚C - 26 ˚C was suggested however, this may be economically untenable in temperate regions (Armitage & Laushman, 2003). Nevertheless, frost damaged dahlia tubers may fail to produce flowering plants, so controlling winter temperatures is critical (Armitage & Laushman, 2003). When grown from seed it was concluded that a late September sowing date gave optimum seed germination in dahlia, this was when the average temperature was 25.9 ˚C (Afzal *et al*., 2000).

Unlike many other commercially grown species, dahlia flowers are currently often harvested semi open, as flower opening is inhibited if they are harvested at tight bud stage. Unfortunately, this increases the risk of damage during transport. In most dahlia cultivars flowers open in stages. Typically, first a sudden and relatively large opening movement is followed by an extended period where the flower remains static, finally followed by one or more short periods of further opening. The florets in these dahlias often show a slight closing movement during the period in which opening is stalled, the degree of which can vary depending on the cultivar (van Doorn & Kamdee, 2014). Flower opening may also be affected by water uptake and carbohydrate stores (van Doorn & van Meeteren, 2003).

It is claimed dahlias should be harvested when the flowers are 75% to fully open and before the outer petals reflex (Armitage & Laushman, 2003). It was found that the dahlia cultivar 'Purple Gem' could be picked semi-open (when only the outer 3 or 4 whorls of ray florets had opened) and still have a reasonable vase life if the following steps were taken (Lukaszewska, 1983). Vase life of up to 9 days, in contrast to control vase life of 4 days, was achieved by a treatment with 10% glucose solution as a nutrient source and 0.2 mM silver nitrate for 24h, suggesting a degree of ethylene sensitivity. This shows that picking at least semi-open buds may be an option commercially if the correct steps are taken. Removing all leaves when harvesting extended the vase life of dahlias (Nowak & Rudnicki, 1990) suggesting that water loss may be a factor limiting their vase life.

Overall pre-harvest conditions evidently can affect vase life but may be difficult to control. Control of pre-harvest conditions will depend in part on whether or not dahlias are grown indoors or outdoors and the costs of maintaining a constant environment for plants grown indoors.

1.7. Methods to improve floral vase life: post-harvest

1.7.1. Transportation and storage

The transportation of dahlias often decreases their subsequent vase life (Dole *et al*., 2009; Armitage & Laushman, 2003), but in the commercial supply-chain transportation is obviously unavoidable. Once in the supply chain dahlias are commonly stored wet. There is dispute over correct storage conditions, and contradictory data. It is likely that optimum storage conditions may vary depending on the cultivar in question. Cut flower handbooks suggest wet storage should be at an optimum temperature of 4˚C ± 1˚C (Nowak & Rudnicki, 1990; Armitage & Laushman, 2003) at 80% humidity (Armitage & Laushman, 2003). However, the results of several papers dispute this storage advice. The results of two studies challenge the wet storage view. One concluded that dry storage of the dahlia cultivars 'Primaner' and 'Red Cap' was a better technique than the use of wet storage (Balas *et al*., 2005). Another showed that both dry and wet storage at 2 ˚C for a week reduced the vase life of the 'Karma Thalia' flowers compared to non-stored flowers; in this study, however, there seemed to be no significant difference between dry and wet storage (Dole *et al*., 2009). A study in gerbera focused on the temperature effects of post-harvest life by dry storing fresh cut flowers packed in micro-perforated plastic for 7 days under different conditions (Berlingieri-Durigan & Mattuiz, 2009). They studied the cultivar of gerbera 'Suzanne' and found that low temperatures of 2 ˚C gave the longest

vase life of 13.8 days, compared to 8.6 days for flowers kept at 20 ˚C. However, these flowers were kept in dry storage, which the authors acknowledge may reduce vase life. Water quality postharvest may also affect vase life. In chrysanthemum, acidic solution led to vase life of 14.6 days compared to 6.1 days in flowers left in distilled water (Carlson & Dole, 2013). This may be because lower pH is associated with lower bacterial growth, and improved fluid uptake due to the reduced likelihood of bacterial blockages in vascular tissue (Conrado *et al*., 1980; van Doorn *et al*., 1989). Contrastingly, in zinnia, another member of the Asteraceae, pH had no effect on vase life but increased electrical conductivity of the solution reduced vase life; whilst in sunflower electrical conductivity made no difference to vase life but use of an acidic solution decreased vase life by 1.1 day, in contrast to the results in its close relative the chrysanthemum (Carlson & Dole, 2013). This suggests that within Asteraceae there is significant variation in the effect pH and electrical conductivity of holding solution have on vase life and trials from dahlia would need to be performed to assess its own response to varying pH and electrical conductivity.

1.7.2. Ethylene inhibition and its role in floral vase life

The ornamental flower species *Eustoma*, carnation, and petunia are all ethylene sensitive and ethylene production in these flowers increases during flower senescence (Wu *et al*., 1991; Whitehead *et al*. 1984; Ichimura & Goto, 2000). Furthermore, all of them show accelerated senescence when exposed to exogenous ethylene. In daffodils (*Narcissus pseudonarcissus*) it was found that ethylene also causes premature senescence (Hunter *et al*., 2004b). The level of sensitivity however varies, with some flowers, e.g. *Campanula medium*, only sensitised after a certain duration of exposure to ethylene (Kato *et al*., 2002). Experiments on carnations have shown marked variation in ethylene sensitivity amongst varieties (Onozaki *et al*., 2008), implying that there may also be marked variation amongst the tens of thousands of dahlia cultivars. The ethylene inhibitors aminooxyacetic acid (AOA) and aminoethoxyvinyl glycine (AVG) have been used as alternatives to STS and have enhanced vase life in carnation (Bichara & van Staden, 1993). In addition, tests on carnations, *Delphinium*, and Beard-tongue (*Penstemon hartwegii*) to examine the efficacy of ethylene inhibitors showed that STS was effective in preventing ethylene injury, whilst both AOA and AVG gave little or no protection against ethylene injury (Staby *et al*., 1993). This suggest that chemicals that have the same function may have varying effects depending on the species they are applied to. In addition, in many species it is possible to detect increased sensitivity to ethylene as

flowers mature and age. For example, in *Eustoma grandiflorum* ethylene sensitivity was found to increase as senescence progressed (Ichimura *et al*., 1998).

The role of ethylene in dahlia senescence remains unresolved. Earlier research concluded that dahlia flowers were slightly sensitive to ethylene (Woltering & van Doorn, 1988). However, it has been found that cv. 'Karma Thalia' dahlias were unaffected by ethylene by 16h of exposure with 1 μL L^{-1} (Dole *et al*., 2009). However, in research on *Campanula medium,* flowers were unaffected after exposure to ethylene at 10 μL L^{-1} for 16h but all flowers wilted after exposure to ethylene at 2 μL L^{-1} for 48h (Kato *et al*., 2002). Therefore, longer exposure to ethylene, at even lower concentrations, may affect dahlias as well. It was also reported that cut dahlia cv. 'Karma Thalia' stems were unaffected by treatments with STS or 1-MCP (1-methylcyclopropene), a chemical known to inhibit ethylene action through binding to its receptors, also suggesting these dahlias are insensitive to ethylene (Dole *et al*., 2009). However, other reports show contrasting results. More recently it was found that cut dahlia flowers (cv. 'Kokucho') continuously placed in 1 or 10 μL L^{-1} 2-chloroethylphosphonic acid (CEPA) solution, which generates ethylene, wilted earlier than those treated with distilled water (dH_2O) or citric acid (Shimizu-Yumoto & Ichimura, 2013). Furthermore, a pulse treatment with 1-MCP extended the vase life of dahlia florets (Shimizu-Yumoto & Ichimura, 2013).

1.7.3. Exogenous cytokinins and their role in floral vase life

Cytokinins are known to improve vase life in cut flowers and their exogenous addition or endogenous overexpression are consistently associated with prolonged flower life across a range of ornamental species. These include anthurium (Favero *et al*., 2015), petunias (Chang *et al*., 2003), iris (van Doorn *et al*., 2013, Macnish *et al*., 2010), carnations (Eisinger, 1977; Kelly *et al*., 1985; van Staden & Joughin, 1988), roses (Mayak & Halevy, 1970; Lukaszewska *et al*., 1994), *Eustoma* (Musembi *et al*., 2015), wallflowers (Price *et al*., 2008), and the dahlia's close relative chrysanthemum (Guo *et al*., 2003). In one study, petunias were transformed to overexpress the *ipt* gene from *Agrobacterium tumefaciens*, which encodes the cytokinin biosynthetic enzyme isopentenyl transferase, placed under the control of the senescence-associated promoter *SAG12* (Chang *et al*., 2003). The results showed that flower wilting in transformed plants occurred 6-10 days after control plants. Ethylene levels in transformed plants did increase after pollination, but in contrast to control plants, this increase was delayed. However, whether these delays in senescence

are additive or whether cytokinins and ethylene interact with the same pathway is not clear.

In addition, treatment with cytokinins does seem to be effective in delaying dahlia senescence as has been found in other ethylene-insensitive species (Shimizu-Yumoto & Ichimura, 2013). A pulse treatment with BA extended floret vase life and BA was more effective than 1-MCP when the flowers were exposed to ethylene via CEPA treatment (Shimizu-Yumoto & Ichimura, 2013). BA spray treatment also extended vase life of cut dahlia (cv. 'Kokucho', 'Kamakura', and 'Michan') (Shimizu-Yumoto & Ichimura, 2013). Therefore, dahlia flower senescence may be partially regulated by ethylene, and BA is likely to be more effective in delaying the senescence of some cut dahlia varieties than ethylene action inhibitors such as STS.

Treatment with BA (6-benzylaminopurine), a synthetic cytokinin, promoted endogenous GA (gibberellic acid) production helping to lengthen vase life of cut chrysanthemum (Guo *et al*., 2003). This all suggests that the hormonal regulation of senescence in these composite flowers is complex. In fact, at least four phytohormones affected senescence in cut chrysanthemum: GA, ABA (abscisic acid), ethylene, and two auxins, IAA (indole acetic acid), and IPA (indole-3-propionic acid) (Guo *et al*., 2003). ABA and IAA worked synergistically to promote ethylene production and reduce vase life. In contrast greater endogenous GA concentration following treatment with BA was found to increase vase life. Petal growth in *Gerbera hybrida* is promoted by GA and suppressed by ABA (Li *et al*., 2015). In addition, inhibition of GA and ABA biosynthesis by treatment with paclobutrazol (PAC) and fluridone (FLU) respectively resulted in the opposite effect of exogenously applied GA and ABA, suggesting that these hormones have a major impact on gerbera petal growth (Li *et al*., 2015).

Overall, cytokinins and ethylene may have a role in dahlia as they do in other related and unrelated ornamental species, this has been suggested through increases in vase life due to exogenous cytokinin treatment (Shimizu-Yumoto & Ichimura, 2013) and a degree of sensitivity to ethylene (Woltering & van Doorn, 1988). It may be that only certain cultivars are sensitive to ethylene and inhibiting ethylene will only work in those cultivars that are. Ethylene inhibition in conjunction with exogenous cytokinin treatments may be the most promising avenue postharvest to improve dahlia vase life and commercial viability. However, novel application methods may need to be found as spraying with BA

may not be a commercially viable alternative in the EU due to chemical regulation (personal communication, Martin Squire).

1.7.4. Carbohydrates and vase life

Sugars are known to prolong vase life in cut flower species like carnation (Hoeberichts *et al*., 2007). This is thought to be due to the suppressive effective sugars have on ethylene production as it has been found during ethylene sensitive flower senescence that high sugar levels promote the proteasomal degradation of the key transcription factor EIL3 which is responsible for activation of ethylene responsive genes (Yanagisawa *et al*., 2003; Hoeberichts *et al*., 2007). Research on dahlia found that the addition of 5% glucose, sucrose, or fructose decreased vase life of cv. 'Purple Gem' flowers picked semi-open (when only the outer 3 or 4 whorls of ray florets had opened), compared with using water (Lukasweska, 1980). This may be because the addition of sugars encouraged bacterial growth and led to vascular blockage, this has been found in dahlia's close relative *Zinnia elegans* in a previous study (Carlson *et al*., 2015). However, it was commented that sugar, particularly higher concentrations of glucose, did increase the number of flowers that opened and improved their size. In a later study cv. 'Purple Gem' dahlias treated with 5 or 10% glucose had better vase lives than those treated with sucrose (Lukasweska, 1983). A vase life of 9 days was achieved when a solution of 10% glucose and 0.2 mM of silver nitrate was used, compared to a control vase life of just 4 days (Lukasweska, 1983). More recently, it has been found that 'Karma Thalia' dahlias in solutions of 2 or 4% sucrose did not have a better vase life than control dahlias (Dole *et al*., 2009). In addition, the researchers found that treating dahlias with a 24-hour pulse of 10 or 20% sucrose at 20 ˚C, before returning them to plain water also had no effect on vase life. In sunflowers treatment with 150 mg L^{-1} of citric acid increased their vase life, whereas treatment with 2% sucrose combined with citric acid treatment reduced vase life (Mensuali-Sodi & Ferrante, 2005). Overall, these data suggest that carbohydrates would not be as effective in dahlias as they are in other cut flower species.

1.8. Summary

Overall, it is clear that the dahlia is relatively understudied. This is due to the domesticated dahlia being a marginal crop, especially within the cut flower market, and a lack of sequencing data in the past. Dahlia's sensitivity to ethylene remains uncertain and it is possible that the current methods of improving vase life will not be effective enough in dahlia to make it commercially viable. So far no research has found a 'key' to extending

dahlia vase life to an extent which would make them commercially viable in the UK market. Research needs to be performed assessing popular UK dahlia varieties with typical postharvest treatments and discover if novel applications of postharvest treatments can give a vase life long enough to satisfy consumer demands. Furthermore, there needs to be further investigation of the control of dahlia flower senescence on a molecular level, and whether the composite nature of dahlia is a major factor inhibiting its vase life potential.

1.9. Objectives

- To begin characterising senescence in multiple dahlia cultivars both on the plant and in cut inflorescences so that future researchers have a baseline for quantifying senescence in dahlia.
- Assess how some commercially popular UK dahlia cultivars respond to traditional postharvest treatments and develop novel ways to improve dahlia vase life.
- Assess ethylene and cytokinin sensitivity in a variety of dahlia cultivars to examine how variable their responses are and provide more evidence on the question of ethylene sensitivity in dahlia.
- Use qRT-PCR and RNA-sequencing to examine dahlia senescence on a molecular level and build a transcriptome to be of use to future researchers of dahlia flower senescence.

2. General Materials and Methods

2.1. Plant material growth and harvest

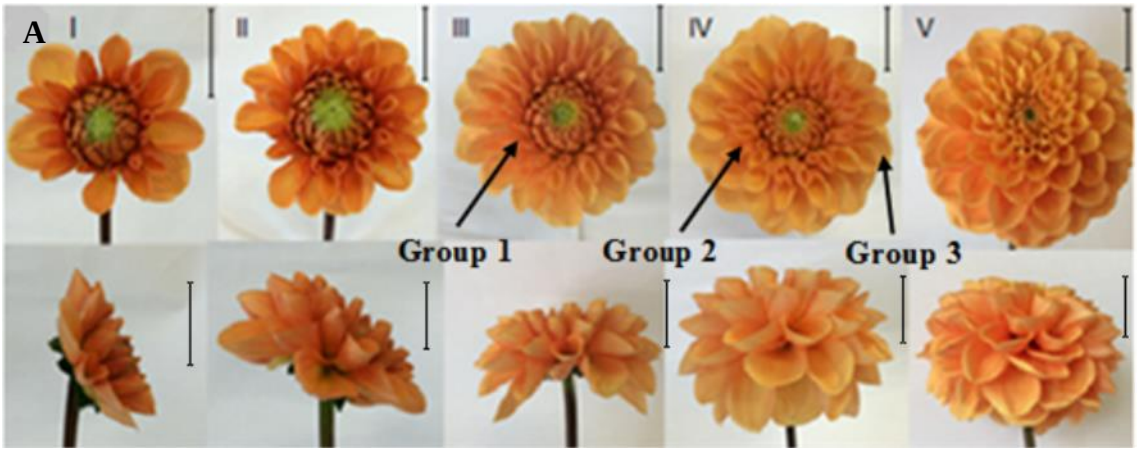
All dahlia tubers were bought from 'Rose Cottage Plants' (Essex, UK) and potted in multi-purpose peat-based compost (Longacres, Bagshot, UK) and grown in pots in a poly-tunnel until late May before being planted outside for the remainder of the growing season due to limitations on space, and to mimic industry conditions where dahlias would routinely be grown outside (personal communication, Martin Squire). The pots used were just large enough for tubers to fit in, as recommended by Vernon (2014), therefore pot size used varied depending on the size of the tuber. Holes dug for plants were lined with compost and a handful of bonemeal to ensure healthy growth and then monitored for the remainder of the season and given water and nutrients as and when needed. The nutrient solution given to plants was a balanced fertiliser of 20% N, 20% P, and 20% K with micro nutrients including Mn and trace elements ('Peters Professional Allrounder'). In the 2015 growing season the RHS Wisley research site (Deer Farm, Wisley, Surrey) was used, in the 2016 and 2017 seasons dahlias were planted at Royal Holloway University of London. Flowers were harvested throughout the growing season when possible, from July to October, though this varied due to variations in weather from year to year (Appendix I). The cultivars 'Gerrie Hoek', 'Karma Prospero', 'Onesta', and 'Sylvia' were used due to their commercial popularity and availability (Fig. 4). Flowers were harvested at Stage III for vase life trials, as it is recommended that dahlias are harvested 75% to fully open (Fig. 4; Armitage & Laushman, 2003). For RNA-sequencing the following material from cv. 'Sylvia' was harvested: inner florets of Stages III (group 1) or IV flowers (group 2) and outer florets of Stage IV (group 3) flowers (Fig. 5). These stages were picked for RNA-sequencing because they represent an intermediate of the flower staging, if the stages are too early the flower is still developing, too late and the flower will begin to senesce and the transcriptomic changes which may precede the visible signs of senescence could be missed. Furthermore, florets picked too late are difficult to extract quality RNA from, and poor RNA quality would affect the amount of data that can be obtained and the integrity of that data.

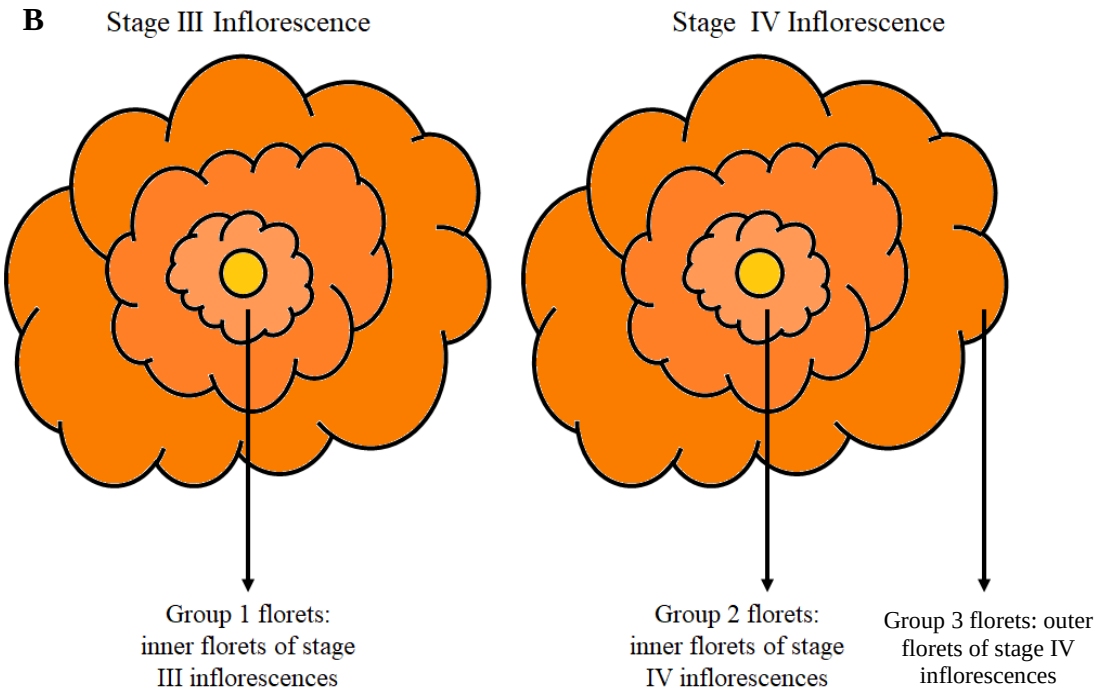
Flowers unusable due to pest damage or ageing were cut and discarded to encourage further flower growth on the plants and all flowers selected for experimentation had no 'daisy eye' features i.e. their disc florets were not visible or available to pollinators, this was to reduce the likelihood of pollination induced senescence (Jones, 2008). Flowers were transported in tap water to a constant temperature room set at 21 ^{o}C and a 12 h

photoperiod from cool white fluorescent tubes (15-20 µM m^{-2} sec^{-1}). All leaves were removed and stems cut to lengths of 5 cm. Although in industry typical stem lengths are 50 cm (personal communication, Paula Edgington), a length of 5 cm was used to maximise replication of flowers in a short growing season and obtain greater amounts of data. All glassware flowers were placed in was autoclaved to ensure it was sterile.

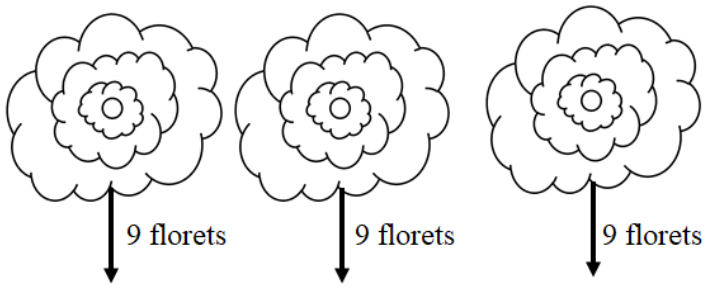
I **II** **III** **IV** **V**

Figure 4. Developmental Stages I-V of dahlia inflorescences (all scale bars represent 20 mm): A) cv. 'Gerrie Hoek' B) cv. 'Karma Prospero' C) cv. 'Sylvia' D) cv. 'Onesta'.





For each Group of florets:



= 27 florets total

9 picked at random = 1 biological replicate

This process was repeated for each Group of florets (1, 2, and 3), once in the 2015 season, and twice in the 2016 season, to make three biological replicates for each of the three Groups.

Figure 5a: Stages I-V of cv. 'Sylvia' flower development. Scale bars represent 20 mm. Arrows indicate the florets representing each of the three developmental stages used for the transcriptomic study.

5b: Scheme showing the different groups of florets taken from stage III and stage IV inflorescences for RNA-sequencing. Group 1 florets from the inner whorl of stage III flowers, Group 2 florets from the inner whorl of stage IV flowers and Group 3 florets from the outer whorl of stage IV flowers.

2.2. Postharvest treatments

The postharvest treatments used and the concentration they were applied at are shown below in Table 2. All solutions were dissolved in distilled water (dH_2O). All control treatments were dH_2O solutions, or in the case of sprayed flowers, a spray of dH_2O whilst placed in dH_2O. This is because this is what has been used as a control to compare treated dahlia flowers to in previous studies (Shimizu-Yumoto & Ichimura, 2013; Dole *et al*., 2009). Five whole flowers were used for each treatment.

The postharvest treatment STS was used because it has been shown to inhibit ethylene reception and extend floral vase life (Veen, 1979), a reaction to STS could suggest that endogenous ethylene is playing a role in flower senescence. CEPA was used to test sensitivity to exogenous ethylene, as exogenous ethylene has been found to reduce vase life in dahlia and their close relative chrysanthemum (Doi *et al*., 2003; Shimizu-Yumoto & Ichimura, 2013; Dole *et al*., 2009), as well as other ornamental species including *Alstroemeria*, *Cymbidium*, *Campanula*, carnation, geranium, rose, *Delphinium* and *Antirrhinum* (Woltering & van Doorn, 1988).

6-Benzylaminopurine (BA) and kinetin solution were used as exogenous cytokinins have been shown to extend vase life in other ornamental species (Favero *et al*., 2015; Chang *et al*., 2003; van Doorn *et al*., 2013, Macnish *et al*., 2010; Eisinger, 1977; Kelly *et al*., 1985; van Staden & Joughin, 1988; Mayak & Halevy, 1970; Lukaszewska *et al*., 1994; Musembi *et al*., 2015; Price *et al*., 2008) including the dahlia's close relative chrysanthemum (Guo *et al*., 2003), as well as the dahlia itself (Shimizu-Yumoto & Ichimura, 2013). Xanthine, linoleic acid and oleic acid were used as all of these chemicals have been implicated in the inhibition of N-glucosyltransferases (Hou *et al*., 2004; Won *et al*., 2007), enzymes implicated in cytokinin inactivation (Šmehilová *et al*., 2016).

Inhibition of these enzymes has been found to induce hypersensitivity to endogenous cytokinins in *A. thaliana* (Wang *et al*., 2011), thus they may represent an opportunity to enhance the role of endogenous cytokinins and delay senescence.

Table 2 – List of chemicals used and the concentration(s) of them used in postharvest treatment in this study (All sourced from Sigma Aldrich)

Chemical	*Concentration(s) [mM]*
6-Benzylaminopurine solution 1 mg/mL, plant cell culture tested	0.05, 0.1, 0.5
(Ethephon Chloroethylphosphonic acid or CEPA)	0.01, 0.02, 0.05
Kinetin solution 1 mg/mL, BioReagent, plant cell culture tested	0.05, 0.1, 0.5
Linoleic acid ≥99%	0.5, 2, 5
Oleic Acid-Water Soluble powder, BioReagent, suitable for cell culture	0.5, 2, 5
Silver thiosulphate (STS) [32 mM silver thiosulphate & 8 mM silver nitrate]	4
Xanthine	2, 5

Sucrose was dissolved in dH_2O at concentrations of 2% or 5%.

Flowers were either trialled in continuous solutions, pulsed for a specified time period and then placed in distilled water or a specified treatment, or sprayed. Sprayed flowers were sprayed from a distance of 30 cm in a fume hood until the solution had been applied to the whole flower's surface, and left to dry, before being removed to the constant temperature room.

All flowers were placed in autoclaved individual containers in 10 ml of dH_2O or relevant treatment solution. Flowers were checked daily, so they were never without water or solution and if required the relevant solution was added to prevent containers from drying out.

Exogenous ABA and GA were not used as postharvest treatments in this study as the existing studies on dahlia postharvest quality focus on ethylene and cytokinin (Shimuzu-Yumoto & Ichimura, 2013; Dole *et al.*, 2009; Woltering & van Doorn, 1988). Therefore, it was decided that in the limited growing season when dahlias could be grown, it would be better to investigate fewer treatments and gain better replication of flowers and number of treatments to build more knowledge on the role of cytokinins and ethylene in dahlia.

2.3. Physiological Measurements

2.3.1. Vase Life

Vase life was considered finished when the outer two whorls of florets showed visible signs of senescence. Signs of senescence include petal wilting, curling, discolouration, and abscission as outlined by van Doorn & Woltering (2008). Five whole flower replicates were used in each vase life experiment for each treatment. Statistical analysis was carried out using RStudio Desktop (version 1.1) on R (version 3.5), 2 sample t-tests were carried out to compare control flowers (dH_2O) to treated groups of flowers.

2.3.2. Floret Mass

All mass measurements in this study were conducted on fresh material, not dry material, and the results are fresh weight, not dry weight. Floret mass was measured and noted to the nearest mg. Floret mass was measured 1, 4, and 7d after flowers were cut, as untreated flowers usually senescence after 7d. For each time point of each cultivar of each treatment five whole inflorescences were used, and six florets from each of those five inflorescences were removed from the middle whorl and weighed individually. The weight of six florets was then averaged, and that average used as a single biological replicate. Therefore, there were five replicates per category. This level of replication was used in order to cover to ensure a large number of technical and biological replicates to account for natural variability in outdoor grown flowers where pre-harvest conditions were difficult to control. A category represents for example, measurements from cv. 'Sylvia' flowers treated with 4 mM STS 1d after cutting. A different group of five flowers were used for each timepoint, so that flowers injured 1 day after cutting due to florets being removed were not then used 4 or 7 days after cutting. All statistical analysis was carried out using RStudio Desktop (version 1.1) on R (version 3.5). 2-way ANOVAs were carried out to compare control groups to treated groups (e.g. dH_2O control to flowers treated with a 1h pulse of 4 mM STS) and to compare different timepoints of the experiment (e.g. 1 and 4 days after cutting).

2.3.3. Conductivity

After the six florets for reach of the five whole flowers sampled were weighed, they were separated into three pairs. Each pair, representing a technical replicate, was placed in 15 ml of dH_2O and the conductivity (used to determine membrane permeability) of the solution measured. They were measured at 3 h and then measured again after having been autoclaved. The conductivity after 3 h was then expressed as a percentage of total conductivity of petals indicated by the post-autoclave results (Whitlow *et al*., 1992). The average of the three pairs taken from a single whole flower was used as a biological replicate, giving five biological replicates, each representing florets from a single whole flower. Assessing membrane permeability can be used to indicate senescence as membrane breakdown is a major symptom of flower senescence (van Doorn & Woltering, 2008). Conductivity was measured using an Accumet AP75 data meter (Fisher Scientific). Statistical analysis was carried out as described in 2.3.2.

2.3.4. Protein content: extraction and assay

Material for analysis was collected 1, 4, and 7 days after cutting, or in the case of on-plant flowers, after they were tagged. Protein content was measured as decline in protein content is considered a hallmark of senescence and occurs prior to visible signs of senescence (van Doorn & Woltering, 2008; Jones *et al*., 2005). Five whole inflorescences were used for each day and florets were collected from the middle whorl of the inflorescence. Twelve florets were collected from each inflorescence and mixed together. The sixty florets were then split into three groups of 20 and each of these groups represented a biological replicate. Samples were placed in 15 ml Falcon tubes and flash frozen in liquid nitrogen. Therefore, for each category, e.g. 'Karma Prospero' flowers treated with a 1h pulse of 4 mM STS 4 days after cutting and treatment, there were three biological replicates. Statistical analysis was carried out as described in 2.3.2.

A native protein extraction was performed using an extraction buffer containing 0.2 M sucrose, 10 mM MOPS (3-(N-morpholino)propanesulfonic acid), 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, at a pH of 7.2. The buffer was stored at 4 ^oC until used. Floret samples were ground in liquid nitrogen using a mortar and pestle. Ground samples were then suspended in a 1:5 powder: cold isolation buffer in a 1.5 ml Eppendorf tube and centrifuged at 15000 x g for 10 minutes. Supernatant was withdrawn and stored at -20 ^oC.

Protein content was measured using the Bradford assay (Bradford, 1976). Final samples were diluted such that they contained sufficient protein to be within the linear range of the assay (approximately 0.1-1.8 mg RUDP equivalents/ml). Samples were agitated for 3 sec on a vortex mixer and left to react for 30 min at room temp.

For the assay, 1:4 Bradford reagent was made up fresh for each day of experiments and left for a minimum of 5 minutes at room temperature. A 1:4 dilution of Bradford reagent (1 ml) was used to calibrate the spectrophotometer (Novaspec Plus, Amersham Biosciences). Sample (5 µl) was added to 1 ml of 1:4 Bradford reagent and the cuvette inverted before measurement at 595 λ. Three technical replicates of each biological replicate were used. Concentration of protein was ascertained using a calibration curve made using know concentrations of BSA.

2.4. RNA extraction from dahlia florets

Two different methods were used for RNA extraction, due to the difficulty in obtaining quality RNA from senescent floral tissue, however the same extraction method was always used for samples in the same experiment. Concentrations have been clarified where possible, manufacturers' instructions of the kits used should be followed unless otherwise specified. RNA for each biological replicate in RT-qPCR was extracted from a ground mix of ten florets from five flowers. Five flowers were used for each day point of each cultivar of each treatment (e.g. d1, 'Sylvia', dH_2O), and six florets taken from the middle whorl of each these flowers, thirty in total. These thirty were mixed and split into three groups of ten, and each group placed in a different 15 ml Falcon tube. Each group of ten florets made up a single biological replicate. The five whole flowers used for RNA extraction were the same as those used for the floret mass and conductivity measurements, however different florets were picked, as florets used for mass measurement went on to conductivity measurement and had to be autoclaved.

2.4.1. Extracting RNA using RNEasy Plant MiniKit (QIAGEN)

A maximum of 100 mg floral tissue was ground in liquid nitrogen. The protocol was performed according to the manufacturer's instructions.

Buffer RLT supplemented with 10 µl/ml 14.3 M β-mercaptoethanol (to denature RNases) was used to resuspend the ground powder before loading onto a QIAshredder spin column to lyse and homogenise samples. The buffer also contained guanidine isothiocycanate, which aids in the binding of RNA to the silica membrane. This was centrifuged for two

minutes at 9000 x g and the flow through collected. The flow through was mixed with 0.5 volume 100% ethanol and loaded onto an RNeasy spin column and centrifuged again to bind RNA more effectively to the silica membrane. Following this step, when the RNA was bound to the column membrane, samples were washed with the RW1 buffer (to remove macromolecules such as carbohydrates, proteins and fatty acids) and then RPE buffer (to remove salts present from buffers used earlier in the protocol) provided in the kit to wash away contaminants. RNA was eluted in 40 µL RNase-free water.

2.4.2. Extracting RNA using NucleoSpin® RNA Plant (Macherey-Nagel)

This method was later used as an alternative to the RNEasy Plant MiniKit (QIAGEN) because it was more consistent in prodcing higher concentrations of better-quality RNA, as determined by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) (Chapter 2.4.3.).

A maximum of 100 mg floral tissue was ground in liquid nitrogen. The protocol was performed according to the manufacturer's instructions. Buffer RA1 (350 µl) and 3.5 µL ß-mercaptoethanol (ß-ME) were added to 100 mg tissue and vortexed vigorously.

To reduce viscosity and clear the lysate, samples were filtrated through NucleoSpin® Filter (violet ring) tubes placed in a 2 ml collection tube. Samples were placed in the NucleoSpin® Filter (violet ring) tube and centrifuged for 1 min at 11,000 x g. The remaining supernatant was then transferred to a new 1.5 mL microcentrifuge tube. 350 µL ethanol (70 %, 350 µL) was then added to the homogenized lysate and mixed by pipetting 5 times. Each sample was then loaded into a NucleoSpin® RNA Plant Column (light blue ring) placed in a 2 ml Collection Tube. The sample was centrifuged for 30 s at 11,000 x g and then the column was placed in a new 2 ml collection tube and centrifuged at 11,000 x g for 30 s. MDB (Membrane Desalting Buffer, 350 µL) was added to the lysate and the column was centrifuged at 11,000 x g for 1 min to dry the membrane. DNase reaction mixture was prepared in a sterile 1.5 mL microcentrifuge tube. For each sample, 10 µL of reconstituted rDNase was added to 90 µL Reaction Buffer for rDNase and 95 µL of DNase reaction mixture was applied directly onto the centre of the silica membrane of the column and incubated at room temperature for 15 min. Following DNA digestion, 200 µL Buffer RAW2 was added to the NucleoSpin® RNA Plant Column in order to deactivate the rDNase. The column was then centrifuged for 30 s at 11,000 x g before being placed in a new 2 ml collection tube. Buffer RA3 (600 µL) was then added

to the NucleoSpin® RNA Plant Column and centrifuged for 30 s at 11,000 x g. Flowthrough was discarded and the column placed back into the same collection tube. Finally, 250 µL of Buffer RA3 was added to the NucleoSpin® RNA Plant Column, which was centrifuged for 2 min at 11,000 x g to dry the membrane completely. The column was then placed into a 1.5 ml nuclease free collection tube. RNAse free water (40 µl) was pipetted directly onto the column membrane and centrifuged at 11,000 x g for 1 min. This is the elution volume recommended by the manufacturers in order to produce higher concentrations of RNA, this option was used to optimise the RNA concentration for the reverse transcription reaction and because of the relatively low amounts of RNA in senescent tissue.

2.4.3. RNA yield and quality

The RNA yield was quantified by measuring the absorbance at 260 nm of 2 µl of extract using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The 260/280 nm absorbance ratio gave an indication of purity of the RNA sample, with a ratio above 2.0 being optimal, and a minimum concentration of 50 ng/µL.

2.5. Polymerase chain-reaction

2.5.1. Primer Design

A summary of primers used, their products and homology to known sequences can be found in Appendix II.

Primers were designed manually using sequences obtained from the transcriptomic data performed for this project. Manual design was used to ensure sequences amplified would be as specific as possible to genes targeted during PCR which were members of large gene families with much shared homology.

2.5.2. Removal of genomic DNA and cDNA synthesis by reverse transcription

cDNA was prepared using a Quantitect Reverse Transcription kit (QIAGEN) according to the manufacturer's protocol, but with 0.5 µg of RNA instead of 1 µg, from the senescent petal material due to the poor recovery of RNA from this tissue. RNase free water was added to 1 or 0.5 µg RNA to a volume of 12 µl and 2 µl of the gDNA Wipeout buffer was added prior to incubation at 42 °C for five minutes, to ensure eradication of contaminating genomic DNA. The sample was then placed on ice and 1 µl Quantiscript

reverse transcriptase, 1 µl dNTPs ('Primer Mix') and 4 µl Quantiscript reverse transcription (RT) buffer were added per sample. The sample was incubated at 42 °C for 20 mins before the reaction was stopped by incubation at 90 °C for three mins.

2.5.3. Reverse transcription polymerase chain-reaction (RT-PCR) and gel electrophoresis

cDNA was prepared for RT-PCR by diluting by a factor of 5. This reduced the concentration of template to 5 ng/µL. BioMix™ Red, containing a *Taq* DNA polymerase, (Bioline) was used as per the manufacturer's instructions. Each sample contained 25 µL of BioMix™ Red, 5 µL each of the forward and reverse primer with a final concentration of 2 µM, 6 µL of template, and 9 µL of RNAse free water.

Agarose gels were made using to a concentration of 1% in TBE electrophoresis buffer (10X), electrophoresed at 70 V for 45 min using SYBR Safe DNA gel stain (Invitrogen by Thermo Fisher Scientific); size of product was estimated using HyperLadder™ 25bp (Bioline).

2.5.4. Sequencing of PCR products

If PCR results on the agarose gels were positive, products were sent to Eurofins for sequencing. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and according to the protocol provided. Purified products were checked on an agarose gel as described above and their concentration estimated. These were then diluted to the appropriate concentration according to Mix2Seq Kit (Eurofins Genomics) instructions and sent for sequencing. A summary of sequences returned by Eurofins can be found in Appendix II (Table 19).

2.5.5. Quantitative reverse transcription polymerase chain-reaction (qRT-PCR) and analysis

qRT-PCR was carried out according to a Rotor-Gene SYBR Green PCR Kit protocol (QIAGEN) and using a Rotor-Gene Q qPCR machine (QIAGEN). The qRT-PCR reaction was prepared by adding 25 ng of the prepared cDNA in 5 µl (5 ng/µL), assuming equal efficiency of reverse transcription in all samples), 0.6 µl of 10 µM forward and reverse primers and 10 µl 2x PrecisionPlus SY master mix (Primerdesign). The PCR program included a 2 min hold at 95°C and 35 cycles of 14 s at 95°C then 60 seconds at 60°C for annealing and extension. To check for the PCR primer specificity based on the detection

of a single amplicon of expected size, a melting curve was performed by ramping the temperature from 65-95 °C. The QIAGEN Rotorgene Q software reports the CT value ("Take off point"), the number of cycles required for the SYBR green fluorescence to reach a threshold above background fluorescence, and the efficiency (E) of the primers in each sample. Three biological replicates (as described in 2.4.) were used for each sample. Three technical replicates of each biological replicate were used. Only technical replicates with 'take off' values within 0.5 cycles of one another were used. Two reference genes were tested during experiments, R18S and β-tubulin. β-tubulin was used for the calculations due to its Ct values being much closer on average to target genes compared to R18S. The relative levels of cDNA were calculated using the following formula from Pfaffl (2001; Fig. 6).

$$ratio = \frac{(^{E}target)^{\Delta CTtarget\,(control^{-}sample)}}{(^{E}ref)^{\Delta CTref\,(control^{-}sample)}}$$

Figure 6: The Pffafl the equation used to calculate the ratio of target genes and reference genes used in rt-qPCR. E refers to the efficiency of the PCR for each set of primers used in the PCR (the amplification). Primers used are specified in the appropriate chapters. ΔCT is the difference in number of PCR cycles required for fluorescence to reach 'take off (CT)' point, defined as being above background levels of fluorescence between treated and control samples (Pfaffl, 2001).

3. RNA-sequencing and *de novo* assembly of the *Dahlia* floret transcriptome

3.1. Introduction

RNA-sequencing is a method which utilises next generation sequencing (NGS) so a transcriptome of a biological sample can be profiled and analysed (Wang *et al*., 2009). This can be utilised in different ways, for example for comparison of different tissue types or different treatment groups. In members of the Asteraceae family, the world's largest plant family, to which dahlia belongs, RNA-sequencing data has been used to investigate a number of areas of interest. These include establishing a more accurate phylogenetic tree in the *Flaveria* genus (Lyu *et al*., 2015), comparison of nickel accumulation in different populations of *Senecio coronatus* (Meier *et al*., 2018), evolution and genome duplication events in *Chrysanthemum* (Won *et al*., 2017), and changes in gene expression due to drought stress in *Helianthus* (Liang *et al*., 2017). In composite flowers it has been used to examine brassinosteroid mediated regulation of petal growth (Huang *et al*., 2017) and floral organ development (Laitinen *et al*., 2005) in *Gerbera.*

A number of recent studies have also used RNA-sequencing to study floral senescence, though none of these have focused on members of the Asteraceae. It has been used to investigate the effect of exogenous cytokinins in the popular ornamental petunia (Trivellini *et al*., 2015), gene expression changes in ethylene insensitive species such as *Gardenia jasminoides* (Tsanakas *et al*., 2014), the role of auxins in tepal senescence and abscission in lily (Lombardi *et al*., 2015), and has also been used to look at the mechanisms underlying pollination induced corolla senescence in petunia (Broderick *et al*., 2014; Wang *et al*., 2018). These have given insight into how fast gene changes occur following pollination (Broderick *et al*., 2014) the role of phytohormones such as auxin and ethylene in floral senescence (Broderick *et al.*, 2014; Lombardi *et al*., 2015), and which transcription factors are most important in the process (Tsanakas *et al*., 2014; Wang *et al.*, 2018). Thus, there are many different applications for this type of data, including in investigation of gene expression patterns in senescing flowers and in complex composite flowers closely related to dahlia. This demonstrates the versatility of RNA-sequencing as a tool to compare gene expression between tissues

Although RNA sequencing has now been applied widely, few studies so far have focused on dahlia. In one study in dahlia, RNA-sequencing has been used to identify differences in expression between different tissue types to assess the evolutionary and genomic

consequences of plant domestication in Asteraceae species including dahlia (Hodgins *et al*., 2014). Very recently, whole reference transcriptomes have also been generated for dahlia but have been limited by fragmented coding sequences (Hodgins *et al*., 2014) or by lack of biological replication (Lehnert & Walbot, 2014). Neither of these studies focused on on senescing flowers, instead they investigated either stem, leaf or floral bud so tissues could be compared (Lehnert & Walbot, 2014) or leaves and young flowers to investigate evolution within the Asteraceae family by comparing a dahlia transcriptome assembly to assemblies from other Asteraceae crop species (Hodgins *et al*., 2014). However, they have shown that *de novo* assemblies of dahlia transcriptomes are possible and can be used to study gene expression changes amongst different tissues types, e.g. flower bud and leaf. Previous studies of gene expression in dahlia have largely focused on floral colour. Loci implicated in flower colour have been identified and found to generally behave as diploid factors (Bate-Smith *et al*., 1955). This is unexpected since cultivated dahlias are thought to be octoploid (Schie *et al*., 2014). Genes implicated in flower pigmentation, such as anthocyanin pathway enzymes, are among the only ones to have been cloned (Suzuki *et al*., 2002; Ohno *et al*., 2011, 2013) and characterised (Yamaguchi *et al*., 1999; Ogata *et al*., 2001).

De novo assembly transcriptomics is a relatively cheap way of gathering large amounts of novel information about an organism's gene expression. The Trinity *de novo* assembly method has been widely used since its inception to assemble transcriptomes of non-model species without a reference genome, including in plants (Grabherr *et al*., 2011; Ward *et al*., 2012), and to examine transcriptomic changes in flowers (Broderick *et al*., 2014). *De novo* assemblies have been generated and used in transcriptomic studies of other Asteraceae species including diploids like *Senecio coronatus* (Meier *et al*., 2018), and those with high ploidy comparable to dahlia such as the decaploid *Chrysanthemum crissum* (Guan *et al*., 2017). Until very recently no genome of an Asteraceae member had been fully sequenced and available to be used as a reference genome for reads to be mapped against, however the diploid genome of *Helianthus annuus* was sequenced in 2017 and may provide a resource for other Asteraceae members (Badouin *et al*., 2017). In this study the *Helianthus annuus* genome could not be successfully used as a reference during the transcriptome assembly process.

Once the sequences obtained by RNA sequencing have been assembled into representative genes, the whole data sets can then be analysed to identify changes in expression of genes relating to whole metabolic pathways or regulatory networks.

Various tools have been developed to facilitate this process including the Kyoto Encyclopaedia of Genes and Genomes (KEGG) where metabolic pathways with up or downregulated genes can be identified (Kanehisa & Goto, 2000).

The application of pathway analysis allows for the examination of the possible roles of potentially senescence associated genes in dahlia without speculating by using postharvest treatments. Pathway analysis could be used to investigate changes in the biosynthesis and signalling of hormones implicated either in the promotion of senescence, such as ethylene, or the repression of senescence, such as cytokinins. Inhibition of endogenous ethylene action or of ethylene biosynthesis has been found to delay senescence (Yu & Bao, 2004; Veen, 1979; Woltering & van Doorn, 1988), whereas addition of exogenous ethylene has been found to accelerate senescence (Woltering & van Doorn, 1988). Therefore, if pathway analysis shows increased expression of genes implicated in the biosynthesis of ethylene, or of promotion of ethylene signalling as florets mature, this may suggest that ethylene is playing a role in dahlia floral senescence. Conversely, addition of exogenous cytokinins or increased biosynthesis of endogenous cytokinins has been found to delay senescence in ornamental species such as petunia, chrysanthemum and dahlia (Chang *et al*., 2003; Shimizu-Yumoto & Ichimura, 2013; Guo *et al.*, 2003) Without treatment we would expect a decline in the expression of genes involved in the promotion of cytokinin signalling or biosynthesis, and changes in expression of genes implicated in biosynthesis and signalling of cytokinins as florets mature may shed light on why exogenous cytokinins extend dahlia vase life (Shimizu-Yumoto & Ichimura, 2013).

Pathway analysis could also be used to examine the role of ABA, a phytohormone known to be involved in plant stress and pathogen response, much like ethylene (Finkelstein, 2013). Its role in senescence is less clear compared to ethylene but it has been found that treatment with exogenous ABA accelerates flower senescence in some plant species, such as daylily (Panavas & Rubinstein, 1998) gladiolus (Kumar *et al*., 2014), carnation (Mayak & Dilley, 1976), rose (Mayak & Halevy, 1972) and cocoa (Aneja *et al*., 1999). This includes species which are known to be ethylene insensitive, for example gladiolus, and it has been suggested that ABA has a more prominent role in species where the senescence process is ethylene independent. In gladiolus it was found that ABA accumulated in senescing petals and exogenous ABA treatment accelerated floral senescence (Kumar *et al*., 2014). Additionally, in *A. thaliana*, it has been found that thiol proteases, known to be upregulated in wilting tissue, are upregulated in response to ABA suggesting that it

has a role in upregulating senescence associated proteases (Williams *et al*., 1994). Moreover, ABA has been found to activate phospholipase D which is implicated in membrane breakdown in barley (Ritchie & Gilroy, 1998) and suppression of phospholipase D delayed ABA mediated senescence in *Arabidopsis* (Fan *et al*., 1997). In one study ABA was found to promote endogenous biosynthesis of ethylene (Ronen & Mayak, 1981), in another study it was found that ABA and ethylene positively regulate each other's biosynthesis (Li & Huang, 2011), suggesting that interactions between them are more important than each of them acting independently. Additionally, ABA treatment led to increase in transcriptional activity of ethylene biosynthetic genes ACO and ACS in the ornamental *Lupin luteus* suggesting that even if ABA does not directly cause senescence its accumulation may be necessary to activate ethylene which subsequently upregulates senescence associated genes (Wilmowicz *et al*., 2016). All this suggests that ABA may have a role in dahlia flower senescence, perhaps through protease or lipase upregulation or interaction with ethylene. If the dahlia flower senescence process is ethylene independent, then it is possible that ABA is a phytohormone which may have a role in accelerating senescence instead.

In this study the aim was to get a better insight into how gene expression changed throughout the dahlia flower senescence process by the analysis of transcriptomes from different stages of floret senescence. Extracting RNA from individual dahlia florets rather than the whole capitulum provides a clearer overview of the molecular processes underlying dahlia flower senescence. The overall aim was to provide a resource of contigs assembled from these transcriptomes that could be used in the future to investigate flower senescence in dahlia to a much more detailed level. The data could also be used specifically to examine the role of ethylene and cytokinins in dahlia floral senescence in a more comprehensive manner by looking at whole pathways rather than selected genes through RT-qPCR.

3.2. Aims

- To use RNA sequencing and the assembly of novel dahlia floret transcriptomes to identify transcripts significantly up or down regulated amongst different stages of dahlia floret senescence.
- To assess the usefulness of *Helianthus* as a reference genome for dahlia and as a source of gene annotations.
- To perform differential expression analysis and use the data to examine metabolic pathways implicated in floral senescence in dahlia.

3.3. Materials and Methods

3.3.1. Harvesting material

Plants were obtained and grown as described in 2.1., over the 2015 and 2016 seasons. The cultivar 'Sylvia' was used for floret harvesting. It was chosen due its ball type morphology, which made identifying whorls easier, its commercial popularity and because it was known from previous experiments to yield good quality RNA. Whole dahlia flowers were classed according to five developmental stages, as shown in Fig. 4. Florets were separated into three groups, 1, 2 and 3. Group 1 florets consisted of florets picked from the inner whorls of Stage III flowers, group 2 florets from the inner whorls of Stage IV flowers, and group 3 florets from the outer whorl of Stage IV flowers (Fig. 5; Table 3). Twenty-seven florets were collected for each replicate, nine each from three whole flowers. These florets were then mixed and then nine picked at random to serve as a single biological replicate. The first replicates for each stage were harvested during the 2015 season, the second and third replicates were harvested during the 2016 season. Florets were flash frozen in liquid nitrogen and stored at -80 ^{o}C.

Table 3 – Summary of the biological replicates used in the RNA-sequencing in this study and what part of and stage of the inflorescence the sample groups represent

Stage III Inflorescence	**Stage IV Inflorescence**	
Inner whorl florets		**Outer whorl florets**
Group 1	**Group 2**	**Group 3**
Biological replicate a (1a)	Biological replicate a (2a)	Biological replicate a (3a)
Biological replicate b (1b)	Biological replicate b (2b)	Biological replicate b (3b)
Biological replicate c (1c)	Biological replicate c (2c)	Biological replicate c (3c)

3.3.2. RNA-extraction

The RNeasy Plant Mini Kit (QIAGEN, 2016) was used for the RNA extraction process and RNA was extracted according to the protocol accompanying the RNeasy Plant Mini Kit (QIAGEN, 2016) as described in 2.4.1. The samples were then flash frozen in liquid nitrogen and stored at -80 ^{o}C. RNA yield and quality were assessed as described in 2.4.3.

3.3.3. Genomic DNA removal

Genomic DNA was broken down using the gDNA Wipeout buffer from the Quantitect Reverse Transcription kit (QIAGEN). RNA samples were then run on a 1% agarose gel to check that genomic DNA contamination was removed. The RNA yield was quantified by measuring the absorbance at 260 nm in 2 µl of extract using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The 260/280 nm absorbance ratio gave an indication of purity of the RNA sample, with a ratio above 2.0 being optimal, and a minimum concentration of 50 ng/µL.

3.3.4. RNA-sequencing

All RNA samples were quality tested using a Qubit fluorometer and then sequenced using an Illumina NovaSeq 5000 to produce paired-end reads for each sample. These steps, as well as the library preparation, were completed at Cardiff University by Angela Marchbank in the Genomics Research Hub, School of Biosciences. Quality control was

performed using the FastQC tool (version 0.11.5) to assess base and sequence quality (Andrews, 2010).

3.3.5. *De novo* assembly and analysis of transcriptome

A *de novo* transcriptome assembly was performed. This was carried out using the Advanced Research Computing at Cardiff (ARCCA), a Cardiff University based computer cluster which was accessed remotely using MobaXterm Personal Edition (version 10.2). Unix scripts were written with the help of Dr Katherine Tansey and Dr Robert Andrews of the Cardiff University, College of Biomedical and Life Sciences, data support team, and were edited using a combination of Notepad++ (version 7.3.3). Files were uploaded and managed through FileZilla Client (version 3.25.1). Forward and reverse reads from each of the samples were assembled into a reference transcriptome using the Trinity software package (version 2.3.2) with default settings (Grabherr *et al*., 2011; Fig. 7). The Trinity pipeline consists of three separate software stages: Inchworm assembles reads into unique transcripts; Chrysalis clusters transcripts made in Inchworm and constructs de Bruijn graphs for each cluster; Butterfly processes the de Bruijn graphs into full length transcripts, including transcripts for alternatively spliced isoforms and paralogous genes (Grabherr *et al*., 2011; Fig. 7). Trimmomatic (version 0.35) was run alongside Trinity to remove low quality reads, low quality bases (including N bases) and adapter regions from the data (Bolger *et al*., 2014). Trinity was used here as it has been shown to be more effective than other *de novo* assemblers, such as TransAByss, when looking at polyploids (Chopra *et al*., 2014).

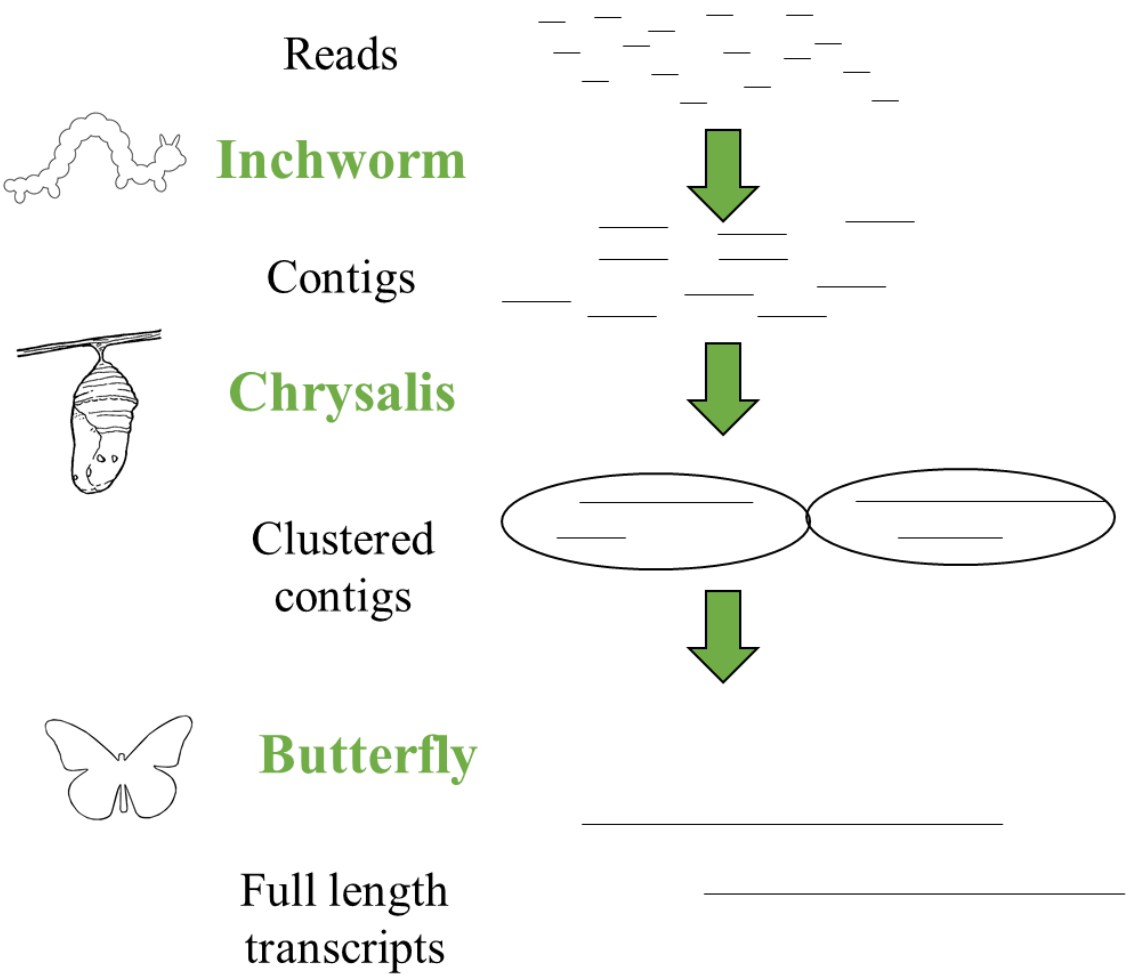


Figure 7: Flow chart summarising the steps in the Trinity de-novo *transcriptome assembly pipeline (Grabherr* et al.*, 2011).*

The trimmed paired reads were uploaded to the Galaxy online platform (Galaxy version 0.2.01) and the public server at *usegalaxy.org* was used to analyse the data. Each sample was mapped to the Trinity reference transcriptome or the *H. annuus* reference genome using the TopHat2 alignment software on default settings, which generated 'accepted hits' files of mapped reads which were inputted into the Cufflinks pipeline for differential expression analysis (Galaxy Version 2.1.1) (Afgan *et al.*, 2018; Kim *et al.*, 2013). A pipeline of the assembly and mapping programs can be found in Fig. 8.

Blastx alignment queries were performed using NCBI BLAST+ Blastx (Galaxy version 0.2.01) on the Galaxy online platform (Camacho *et al.*, 2009; Cock *et al.*, 2015; Afgan *et al.*, 2018) and using default settings and an e-value equal to or less than $1e^{-5}$, as performed by Lehnert & Walbot (2013). Blastx alignments were run against *Arabidopsis thaliana*

TAIR10 (Lamesch *et al*., 2012) or *Helianthus annuus* (Badouin *et al*., 2017) as subject sequences and the Trinity assembly as query sequences. Both the *A. thaliana* and *H. annuus* protein sequences were sourced from the EnsemblPlants site (EnsemblPlants, 2018). Blastx alignments with a bit score of less than 50 were filtered and removed using Microsoft Excel, as a bit score of over 50 is significant for homology searches in databases with fewer than 7 million entries (Pearson, 2014).

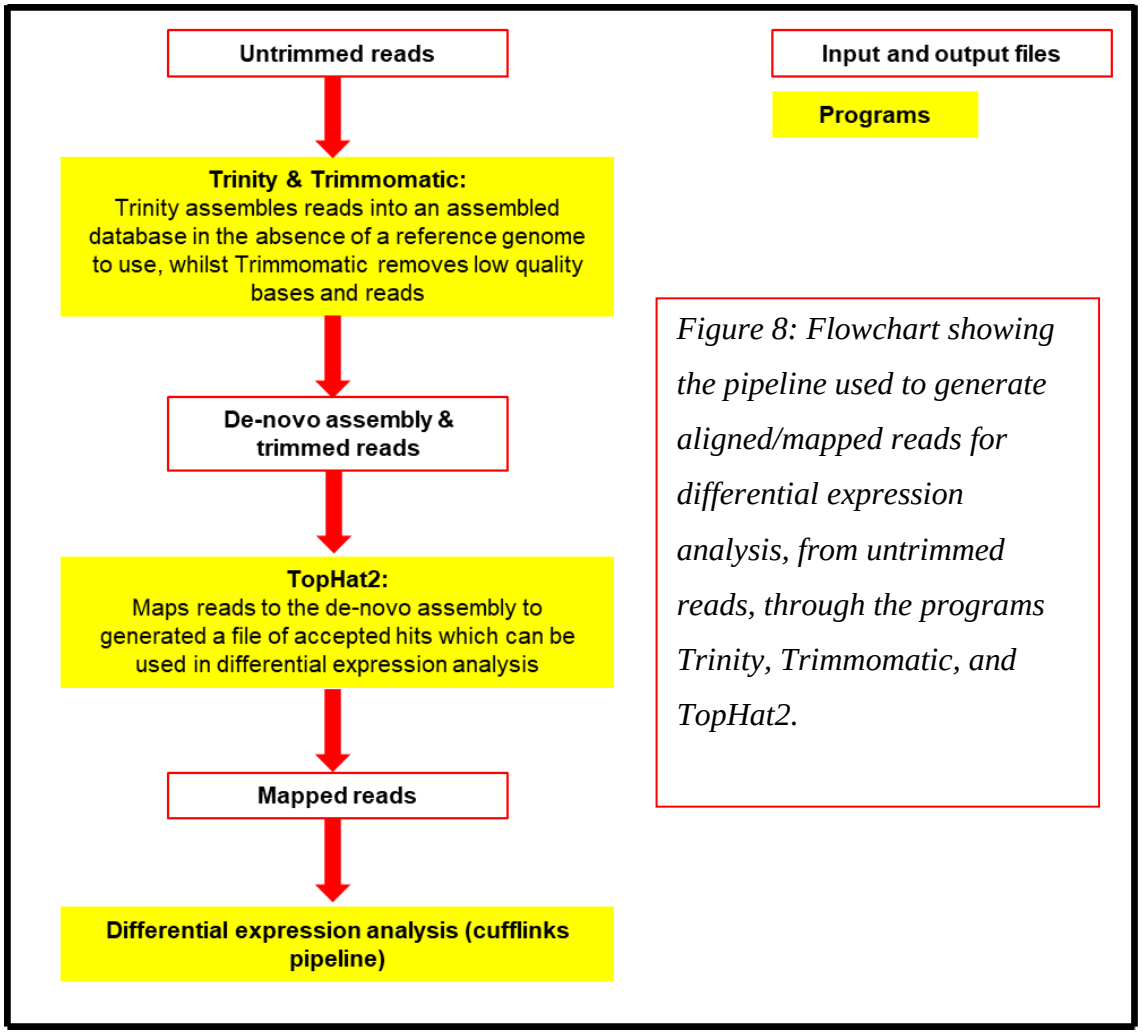


Figure 8: Flowchart showing the pipeline used to generate aligned/mapped reads for differential expression analysis, from untrimmed reads, through the programs Trinity, Trimmomatic, and TopHat2.

3.3.6. Differential expression analysis

Differential expression analysis was carried out using the Cufflinks pipeline on the public server at *usegalaxy.org* (Afgan *et al*., 2018). The Cufflinks pipeline was used because it is a relatively accessible, quick and simple way of generating output that can undergo differential expression analysis, from a database of reads mapped to a *de-novo* assembly or existing reference genome (Trapnell *et al*., 2010). The Cufflinks (Galaxy version 2.2.1.2), Cuffmerge (Galaxy 2.2.1.1 version) and Cuffdiff (Galaxy version 2.2.1.5) programs were used (Trapnell *et al.*, 2010). A diagram of the pipeline demonstrating the input files and programs used in each step can be found in Fig. 9. Cufflinks was used to assemble and estimate the abundance of the aligned reads generated using TopHat2 and was performed using the *H. annuus* annotation (EnsemblPlants, 2018) as a guide and otherwise default settings (Badouin *et al*., 2017). Using an annotation as a guide, instead of as a strict reference, means that the reference transcripts will be matched with faux-reads to provide additional information in assembly and the Cufflinks output will include all reference transcripts as well as any novel genes and isoforms that are assembled. Using an annotation as a guide means that these novel genes and isoforms are not lost during this assembly and will be included later in Cuffmerge and Cuffdiff so their differential expression can be analysed. Using a reference annotation in a strict manner means novel genes and isoforms are not reported, as the program will only include those genes which already exist in the reference, and so was not recommended when working on a species different to the reference, as was the case in this study (Trapnell *et al*., 2010).

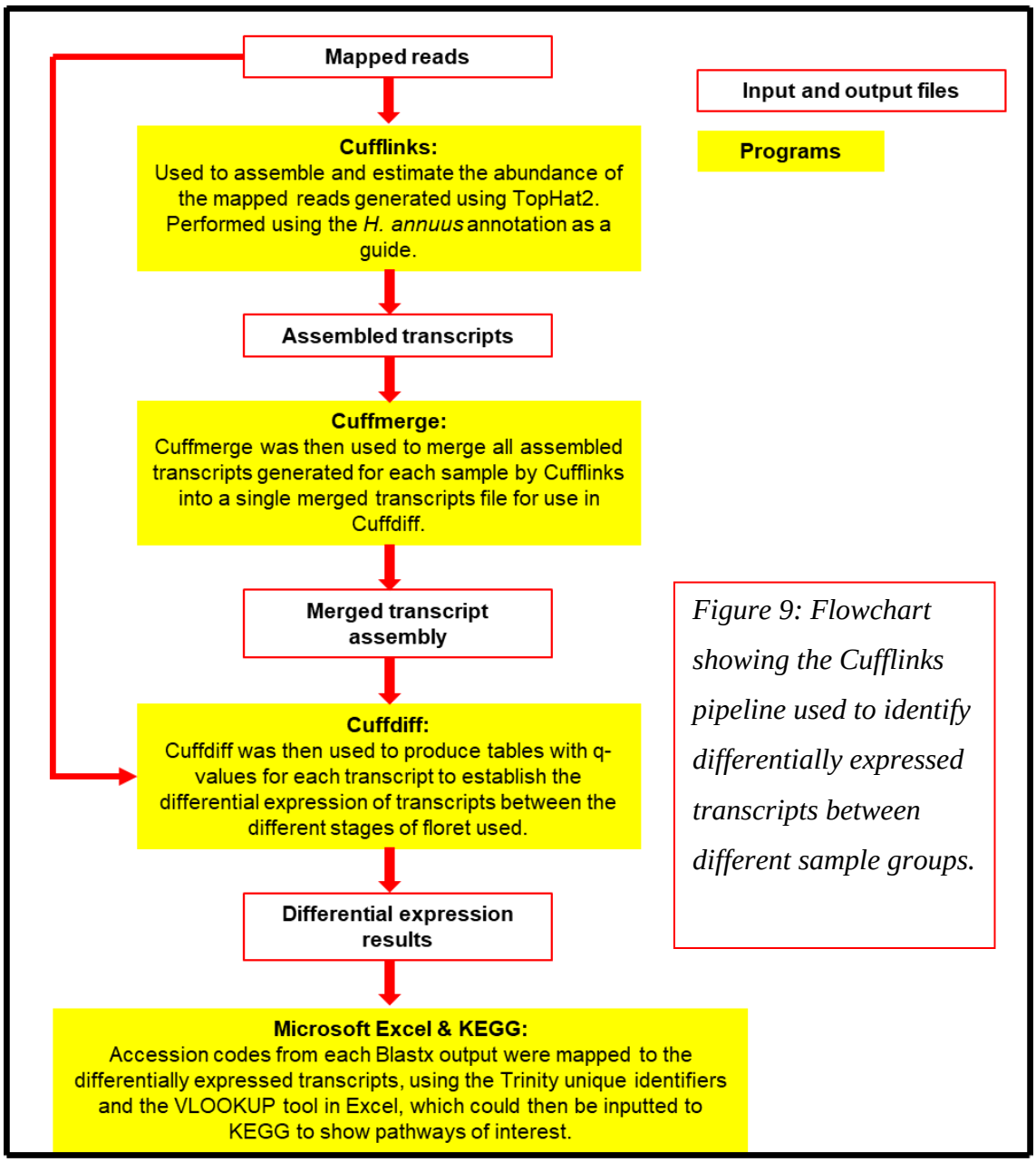


Figure 9: Flowchart showing the Cufflinks pipeline used to identify differentially expressed transcripts between different sample groups.

Cufflinks assembles the aligned reads into a parsimonious set of transcripts (i.e. those that require the simplest or fewest steps to be assembled together), the abundance of which is estimated based on how many reads support each transcript. Cuffmerge was then used to merge all assembled transcripts generated for each sample by Cufflinks into a single merged transcripts file for use in Cuffdiff. The Trinity assembly and the *H. annuus* genome were both used as sequence data in Cuffmerge to compare their usefulness, and otherwise default settings were used. Cuffdiff was then used to produce tables with q-values (p-adjusted values) for each transcript to establish the differential expression of transcripts between the different stages of floret used. The merged transcripts file and

'accepted hits' files of aligned reads generated by TopHat2 were used as input for Cuffdiff and used to compare each floret stage as a pair, e.g. groups 1 (inner whorl florets from stage III inflorescences) and 2 (inner whorl florets from stage IV inflorescences), 1 and 3 (outer whorl florets from stage IV inflorescences), or 2 and 3 (Fig. 5, Table 3). This is done by inputting the three files of accepted hits, 1 for each of the biological replicates of the relevant group, so they can be used by the Cuffdiff program as a single factor. The same is done with the other group for comparison, and they will be used as a second factor by the program, which when run will compare the two factors inputted, in this case the two groups of interest, using the three biological replicates for each group. Cuffdiff will output differentially expressed transcripts, by comparing the abundance of them in each group, giving q-values (p-adjusted value) to indicate statistical difference and fold change to indicate whether there was up or downregulation between the groups.

Differentially expressed transcripts were then inputted to Microsoft Excel, organised by q-value (p-adjusted value) to separate those significantly up or down regulated between samples, and the differentially expressed transcripts organised by log fold change to separate upregulated (positive fold change) from downregulated (negative fold change) transcripts. In Microsoft Excel accession codes from each Blastx output were mapped to the differentially expressed transcripts, using the Trinity unique identifiers and the VLOOKUP tool. Duplicated identifiers were removed using the 'Consolidate' function, and the total number of unique accession codes were calculated for each alignment. This approached was used because it was an easy way of displaying differentially expressed genes in each sample comparison, which could then easily be matched to identifiers from Blastx and then inputted to programs where pathways of interest could be identified, which was the aim of this experiment. As the differential expression analysis had been performed by Cuffdiff to produce adjusted p-values and fold change values for each gene (Trapnell *et al.*, 2010), Microsoft Excel merely acted as a platform to view and arrange data which could be inputted to programs such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG; Kanehisa & Goto, 2000).

The lists of up and down regulated transcripts, now aligned with an *A. thaliana* or *H. annuus* identifier, could then be inputted using the appropriate identifiers into KEGG where pathways could be identified. Both of these species were used so that the number of aligned transcripts could be compared and to assess the differences between using the new *H. annuus* database to the existing model *A. thaliana*. The *A. thaliana* genome is also

better annotated, and more programs are available when using *A. thaliana* identifiers, for example the TAIR database (Nakabayashi *et al*., 2005; Schmid *et al*., 2005; Winter *et al*., 2007; Waese *et al*., 2013).

Pathway analysis was carried out using KEGG (Kanehisa & Goto, 2000). *H. annuus* accessions were inputted into Blastp to generate NCBI protein ID's compatible with KEGG analysis. KEGG was used due to its compatibility with recently released *H. annuus* NCBI protein identifiers, and its capability in displaying a wide range of metabolic pathways by using lists of gene identifiers which can be colour coded depending on whether the relevant gene is significantly up or down regulated. A heatmap of genes present in all three sample comparisons was produced using the heatmap.2 function in R, in order to visualise which sample comparisons produced the most highly up or downregalated genes and how many genes upregulated there were compared to downregulated.

3.4. Results

3.4.1. Sequencing and *de novo* transcriptome assembly

Across all nine samples a total number of 345,038,365 reads were generated, a summary of these reads by sample can be viewed in Table 4. Details on how they were harvested can be found in Fig. 5 and Table 3. For the first of the three replicates of each group a higher read depth of sequencing was opted for to ensure a greater coverage of the transcriptome and to give a greater chance of finding rare transcripts. The quality of the forward and reverse reads was assessed using a sequencing quality score using FastQC, which measures the probability that a base is called incorrectly. Each base in a read is assigned a quality score, referred to as 'Q', which is defined as:

$Q = -10\log_{10}(e)$

where e is the estimated probability of the base being called wrong, the higher the score, the lower the probability of error (Babraham Bioinformatics, 2018). A quality score of 30 indicates an error rate of 1 in 1000, and an inferred accuracy of 99.9%, which is considered high. The output showed that mean sequence quality of reads for each sample was >34 showing that quality was high (Fig. 10; Andrews 2010; Babraham Bioinformatics, 2018). A warning is raised if the most frequently observed mean quality

is below 27 (0.2% error rate) and failure if the most frequently observed mean quality score of sequences is below 20 (1% error rate) (Babraham Bioinformatics, 2018).

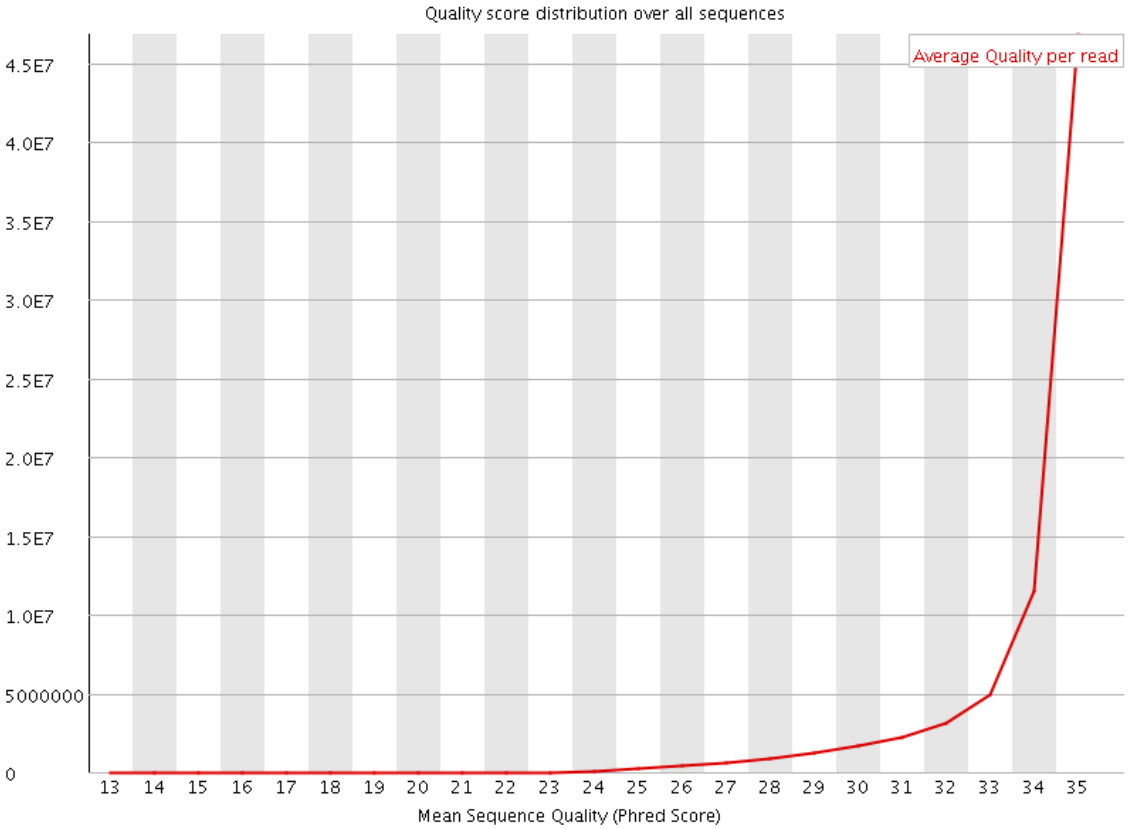


Figure 10: Mean sequence quality of reads from forward reads of sample 'a' from group 2 florets, used as an example because it generated more reads than any other sample, however all samples produced very similar graphs showing high quality reads (Table 4).

Table 4 – Number of reads produced by each sample, each group, and total produced across all samples

Sample	*Number of reads*	*Year samples were harvested*
1a	59,026,752	2015
1b	21,389,398	2016
1c	19,315,558	2016
Group 1 Total	**99,731,708**	
2a	74,777,274	2015
2b	24,886,223	2016
2c	25,815,275	2016
Group 2 Total	**125,478,772**	
3a	67,972,026	2015
3b	29,137,977	2016
3c	22,717,882	2016
Group 3 Total	**119,827,885**	
Total across all 9 samples	**345,038,365**	

From these data Trinity produced 289,538 contigs. The *de novo* assembly was used as the reference in TopHat2 and as a sequence source in Cuffmerge as the TopHat assemblies using *A. thaliana* or *H. annuus* were unsuccessful.

After processing through TopHat2 the number of transcripts was reduced to 137,376 contigs of high enough quality to be successfully mapped using the *de novo* assembly produced by Trinity.

3.4.2. Differential expression analysis

Following the Cufflinks pipeline, it was found that among these contigs between 1.9% and 11.5% showed differential expression between groups depending on the sample comparison, however this number was reduced when they were matched with Blastx ID's from *A. thaliana* and *H. annuus*.

Across all sample comparisons more than half of contigs were removed from the analysis as following Blastx (which compares predicted translations of each contig to the NCBI protein database) they did not show significant homology to a known protein or because

the homology was unreliable i.e. they had a bit score <50. However, less were discarded when matched with *H. annuus* ID's than with *A. thaliana* ID's.

Running Blastx using the Trinity assembly gave differing results depending on whether the *A. thaliana* or *H. annuus* databases were used as references, with 21,492 unique *Arabidopsis* proteins represented, and 27,842 unique *Helianthus* proteins represented. A summary of the number of transcripts generated by Cuffdiff and Blastx can be found in Table 5.

Table 5 – Number of genes which showed differential expression in each sample comparison and significant homology to a known A. thaliana *or* H. annuus *protein*

	Group 1 florets vs. Group 2 florets	*Group 1 florets vs. Group 3 florets*	*Group 2 florets vs. Group 3 florets*
Number of transcripts following TopHat2	137376	137376	137376
Genes which showed significant differential expression using Cuffdiff (q<0.05)	2588	15750	12427
Genes which showed significant differential expression using Cuffdiff (q<0.05) and significant homology to an A. thaliana *protein*	1045	7126	5230
Genes which showed significant differential expression using Cuffdiff (q<0.05) and significant homology to an H. annuus *protein*	1098	7866	5745

The transcripts which showed both significant differential expression and homology to a known protein, were sorted by fold change and split into groups shown to be either up or down regulated (Fig. 11). More transcripts significantly homologous to a known protein were found when *H. annuus* was used as a reference than when *A. thaliana* was used, 53 more in 1 vs. 2, 740 in 1 vs. 3, and 515 in 2 vs. 3. The heatmap (Fig. 12) shows that more genes were upregulated (represented by red) rather than downregulated (represented by blue) and that the positive fold change in upregulated genes was greater than the negative fold change in the downregulated genes (as indicated by the deeper red compared to paler blue). Furthermore, it shows that the comparisons with Group 3 florets (outer whorl florets of Stage IV inflorescences) show greater fold change than the comparisons between just Groups 1 and 2 (inner whorl florets of stage III and stage IV inflorescences respectively).

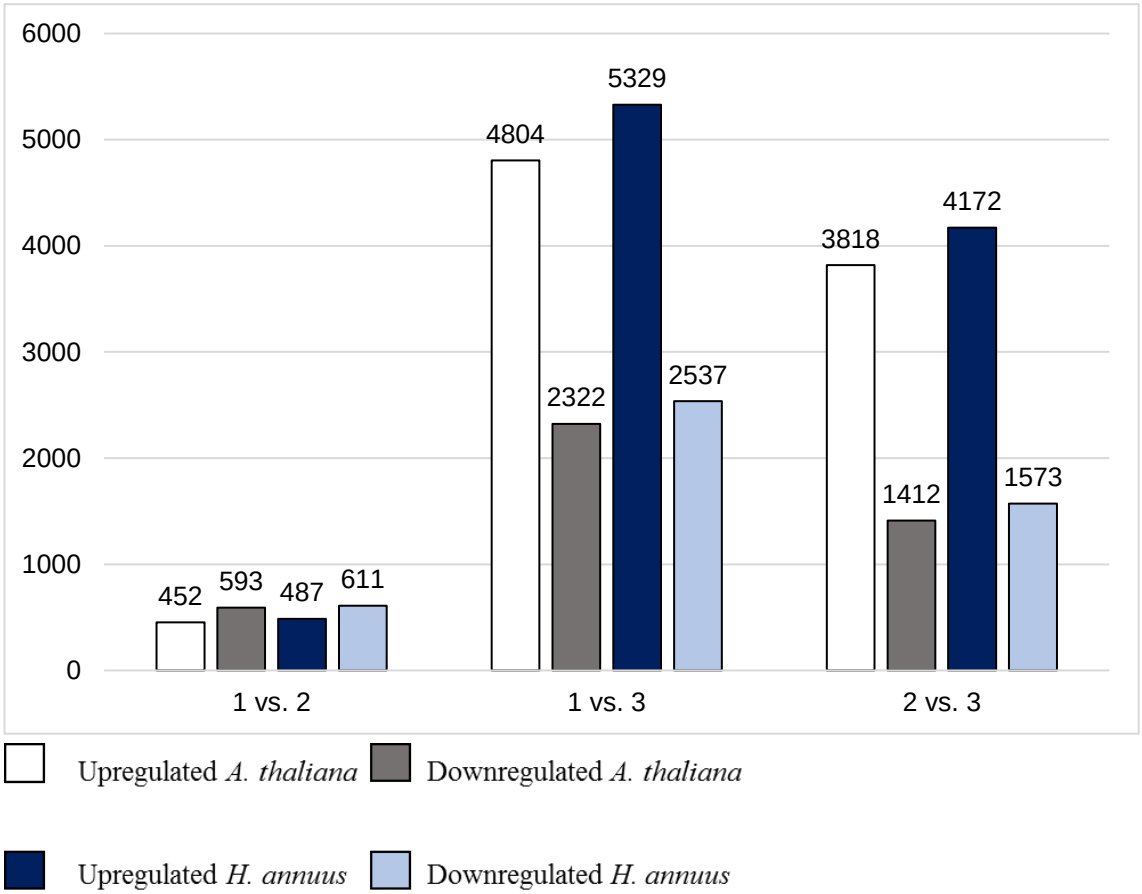


Figure 11: Number of up or down regulated sequences homologous to a known A. thaliana *or* H. annuus *protein in each sample comparison.*

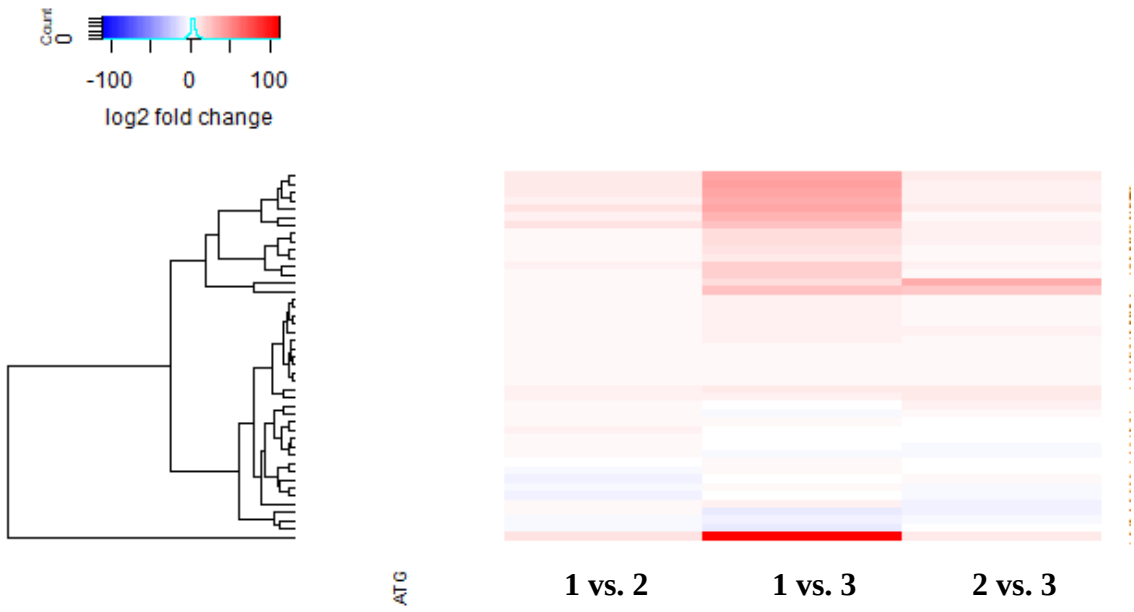


Figure 12: A heatmap showing the commonality of both up and downregulated genes, and which are present in all sample comparisons. Group 1: inner whorl florets of stage III inflorescences, Group 2: inner whorl florets of stage IV inflorescences, Group 3 outer whorl florets of stage IV inflorescences.

3.4.2.1. Differential expression analysis: abscisic acid (ABA) and senescence associated enzymes

Pathway analysis was used to examine the role of ABA, as it has been found that ABA is involved in plant stress and pathogen response (Finkelstein, 2013). The expression analysis found changes in the biosynthesis and signal transduction pathways of ABA between all floret groups tested. The putative dahlia gene identities here are based on homology to *H. annuus*. The stages represent group 1 (inner florets of Stage III), group 2 (inner florets of Stage IV flowers) and group 3 (outer florets of Stage IV flowers). Expression of the ABA receptor *PYR/PYL* was downregulated between samples 1 and 3 (Fig. 13B) but upregulated between 2 and 3 (Fig. 13C). There was no significant change in expression of *PYR/PYL* in the comparison between samples 1 and 2. No significant change of the protein phosphatase *PP2C*, implicated in repression of the protein kinase *SnRK2*, was found in sample comparisons 1 vs. 2 or 2 vs. 3 (Fig. 13A, C), but between 1 and 3 there was significant upregulation of *PP2C* (Fig. 13B). The protein kinase *SnRK2*, repressed by *PP2C* and involved in phosphorylation of ABA response genes, was

upregulated in the sample comparisons 1 vs. 2 and 2 vs. 3 (Fig. 13A, B) but there was no significant change between 2 and 3 (Fig. 13C), showing upregulation was highest earlier in the process but then plateaued. No significant up or downregulation of ABF, involved in transcriptional regulation of abscisic acid response genes, was found in any sample comparison (Fig. 13).

Examination of ABA biosynthesis showed that the ABA biosynthetic gene 9-cis-epoxycarotenoid dioxygenase (*NCED*), was upregulated in sample comparison 1 vs. 2 (Fig. 14A) but downregulated in samples comparisons 1 vs. 3 and 2 vs. 3 (Fig. 14B, C). The enzyme xanthoxin dehydrogenase (*AtABA2*) showed no significant change in expression in any sample comparison (Fig. 14). The final enzyme in the reaction, abscisic aldehyde oxidase (*AAO*) showed no change between 1 and 2 or 2 and 3 (Fig. 14A, C), but showed a significant decline in expression between samples 1 and 3, showing its expression was decreasing slowly during the stages examined (Fig. 14B).

The analysis also found changes in expression of potentially senescence associated enzymes between groups 1 and 3, and groups 2 and 3. A gene homologous to *SHM1* (serine transhydroxymethyltransferase 1) was found and was highly downregulated in the comparison 1 vs. 3, though was not found in any other sample comparison (Table 6). In *A. thaliana SHM1* is highly expressed throughout leaf development but is then downregulated in senescent leaves (Fig. 15A; Table 7). Between mature leaves and senescent leaves in *A. thaliana* there is a 71% decrease in expression of *SHM1* (Table 6).

Three genes homologous to *PSD3* (phosphatidylserine decarboxylase 3) were found, and all showed high upregulation with positive fold changes of over 23 (Table 6). Two of these sequences (TRINITY_DN88125_c0_g1_i1 & TRINITY_DN46270_c0_g1_i1) were found in both sample comparison 1 vs. 3 and 2 vs. 3, whereas the third sequence (TRINITY_DN100115_c0_g1_i1) was found in just 1 vs. 3. In *A. thaliana PSD3* is highly upregulated in senescent leaves (Fig. 15B; Table 7). Between mature and senescent leaves in *A. thaliana* there is a 116% increase in expression of *PSD3* (Table 7).

A dahlia gene homologous to *SERAT2* (serine acetyltransferase 2;1) was found to be upregulated in sample comparisons 1 vs. 3 and 2 vs. 3, with a fold change of +3.4 between groups 1 and 3 (Table 6). In *A. thaliana* leaves *SERAT2* is highly expressed in and specific to senescent leaves, it is also highly expressed in late stage petals and sepals (Fig. 15C; Table 7). Expression of *SERAT2* increase by 286% between mature and senescent leaves in *A. thaliana* (Table 7).

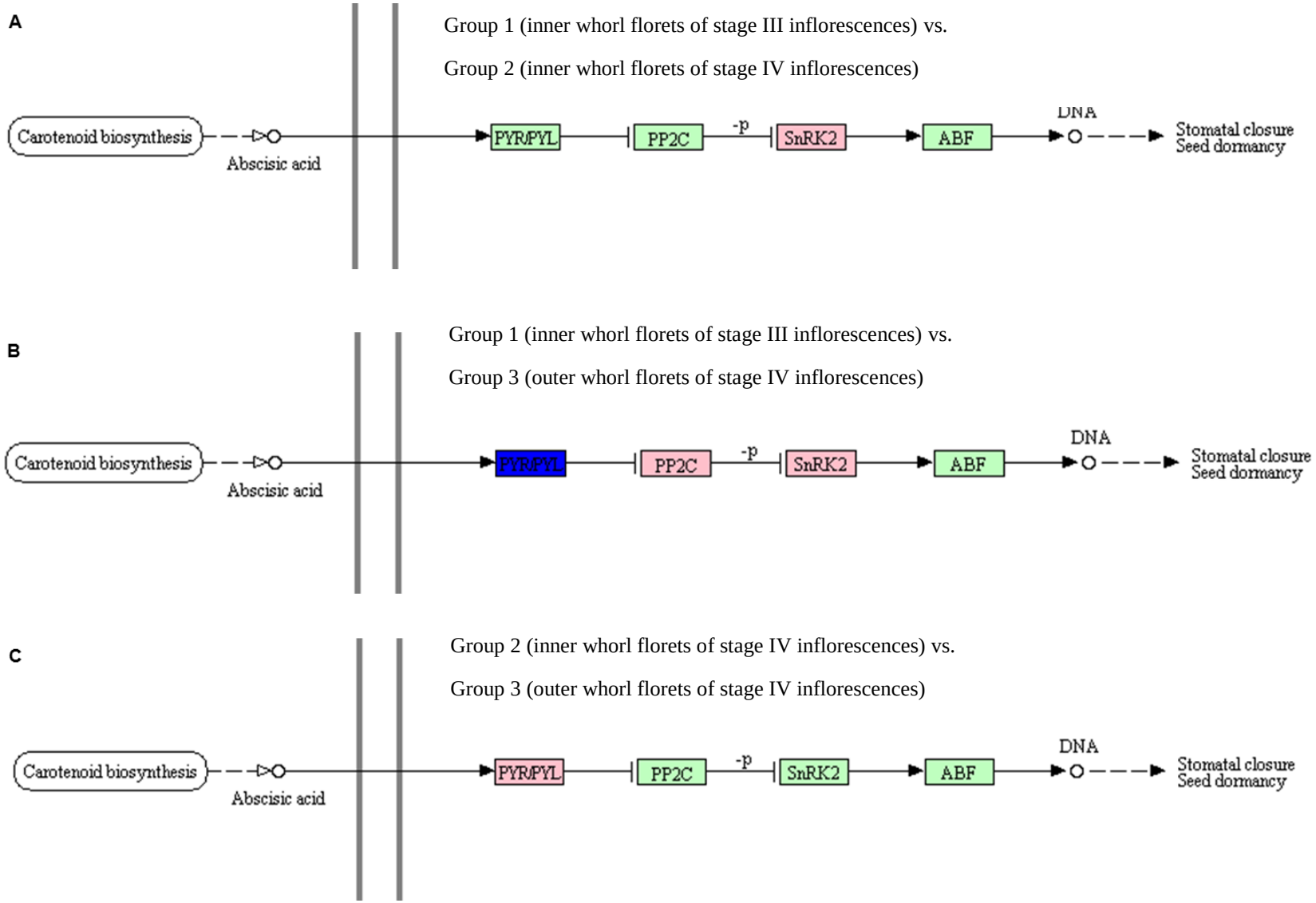


Figure 13: Signal transduction pathway of ABA in sample comparison 1 vs. 2 (A), 1 vs. 3 (B) and 2 vs. 3 (C) (Kanehisa & Goto, 2000).

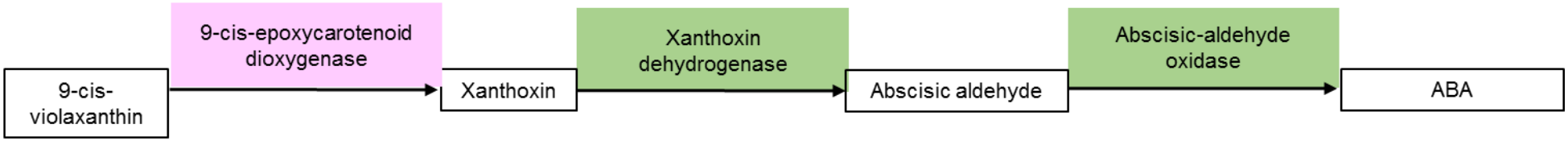
Green = no significant change

Pink = significantly upregulated

Blue = significantly downregulated

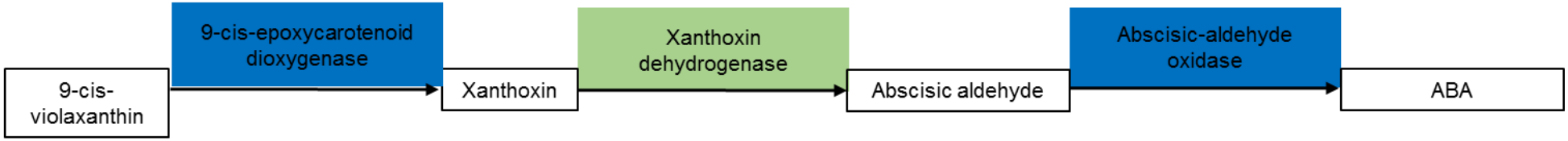
A Group 1 (inner whorl florets of stage III inflorescences) vs.

Group 2 (inner whorl florets of stage IV inflorescences)



B Group 1 (inner whorl florets of stage III inflorescences) vs.

Group 3 (outer whorl florets of stage IV inflorescences)



C Group 2 (inner whorl florets of stage IV inflorescences) vs.

Group 3 (outer whorl florets of stage IV inflorescences)

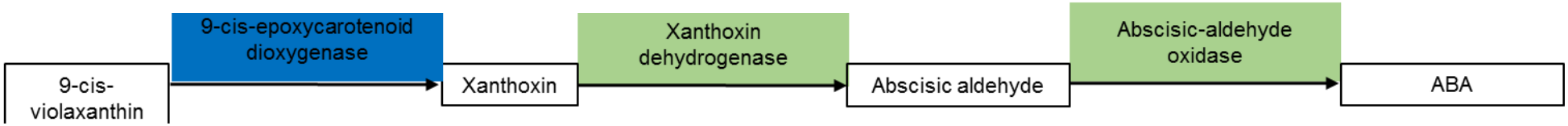


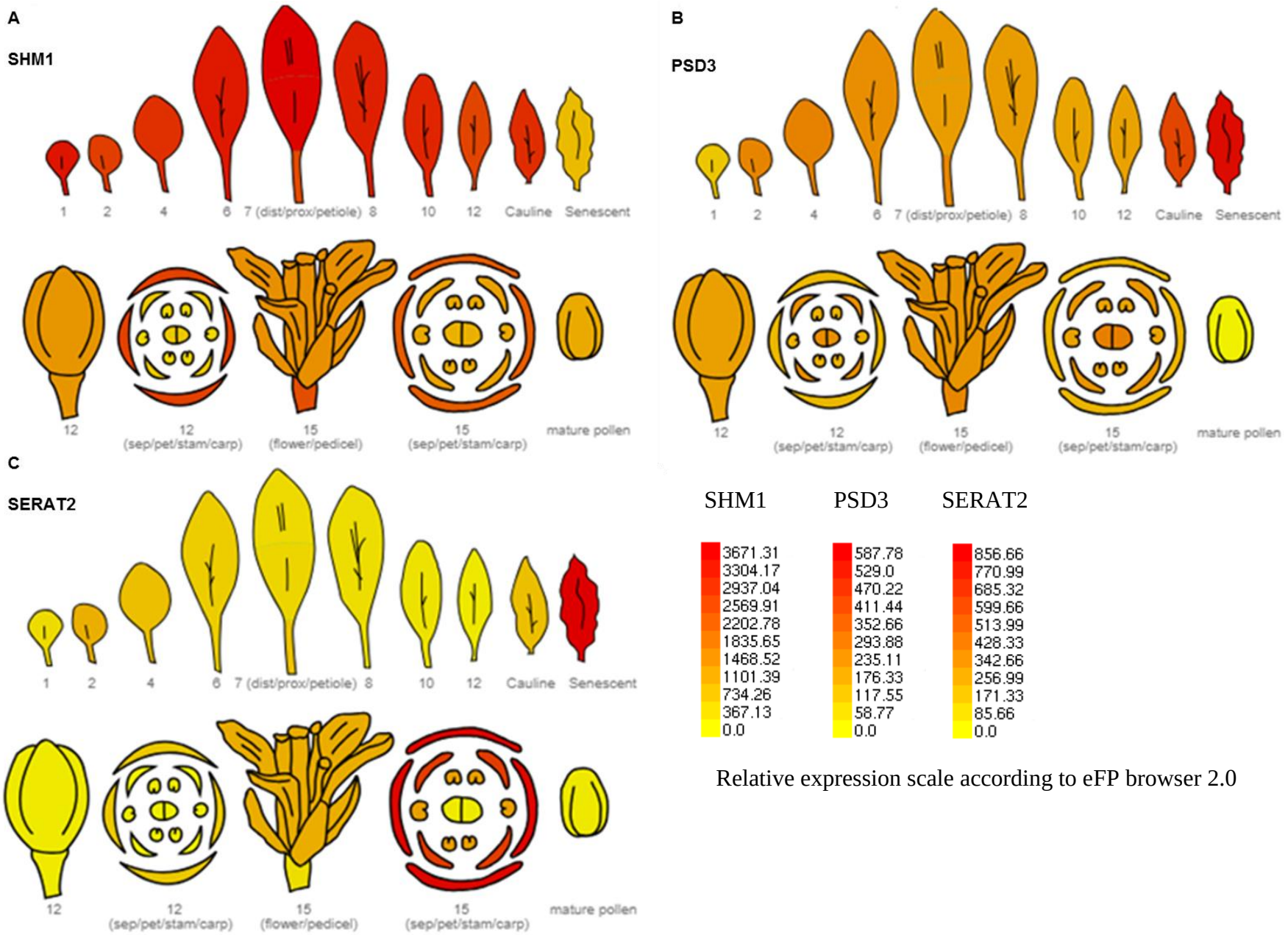
Figure 14: ABA biosynthesis in sample comparison 1 vs. 2 (A), 1 vs. 3 (B) and 2 vs. 3 (C) (Kanehisa & Goto, 2000). Green = no significant change, pink = significantly upregulated, blue = significantly downregulated.

Table 6 - Enzymes found in the differential expression dataset, the H. annuus *and* A. thaliana *homologs of those enzymes, and the fold change in the relevant sample comparison*

H. annuus *accession*	A. thaliana *accession*	A. thaliana *homolog name*	*Group 1 florets vs. Group 2 florets (Fold Change)*	*Group 1 florets vs. Group 3 florets (Fold Change)*	*Group 2 florets vs. Group 3 florets (Fold Change)*
OTG26891	AT4G37930	*SHM1*; serine transhydroxymethyltransferase 1	n/a	- 25.04	n/a
OTG30553	AT4G25970	*PSD3*; phosphatidylserine decarboxylase 3	n/a	+ 24.18	+ 24.15
OTG30553	AT4G25970	*PSD3*; phosphatidylserine decarboxylase 3	n/a	+ 23.72	n/a
OTG08082	AT4G25970	*PSD3*; phosphatidylserine decarboxylase 3	n/a	+ 24.2	+ 24.16
OTG31622	AT1G55920	*SERAT2;1*; serine acetyltransferase 2;1	n/a	+ 3.4	+ 2.75

Table 7 – Relative expression levels of A. thaliana *homologs (Nakabayashi et al., 2005; Schmid et al., 2005; Winter et al., 2007; Waese et al., 2013).*

		Relative expression level						
A. Thaliana *gene*	*ID*	*Flower*	*Sepals*	*Petals*	*Stamen*	*Carpel*	*Mature Leaf (whole rosette)*	*Senescent Leaf*
SHM1	AT4G37930	*1467*	*2267*	*1229*	*1088*	*964*	*2787*	*819*
PSD3	AT4G25970	*263*	*195*	*265*	*233*	*311*	*260*	*562*
SERAT2;1	AT1G55920	*250*	*831*	*643*	*300*	*36*	*222*	*857*

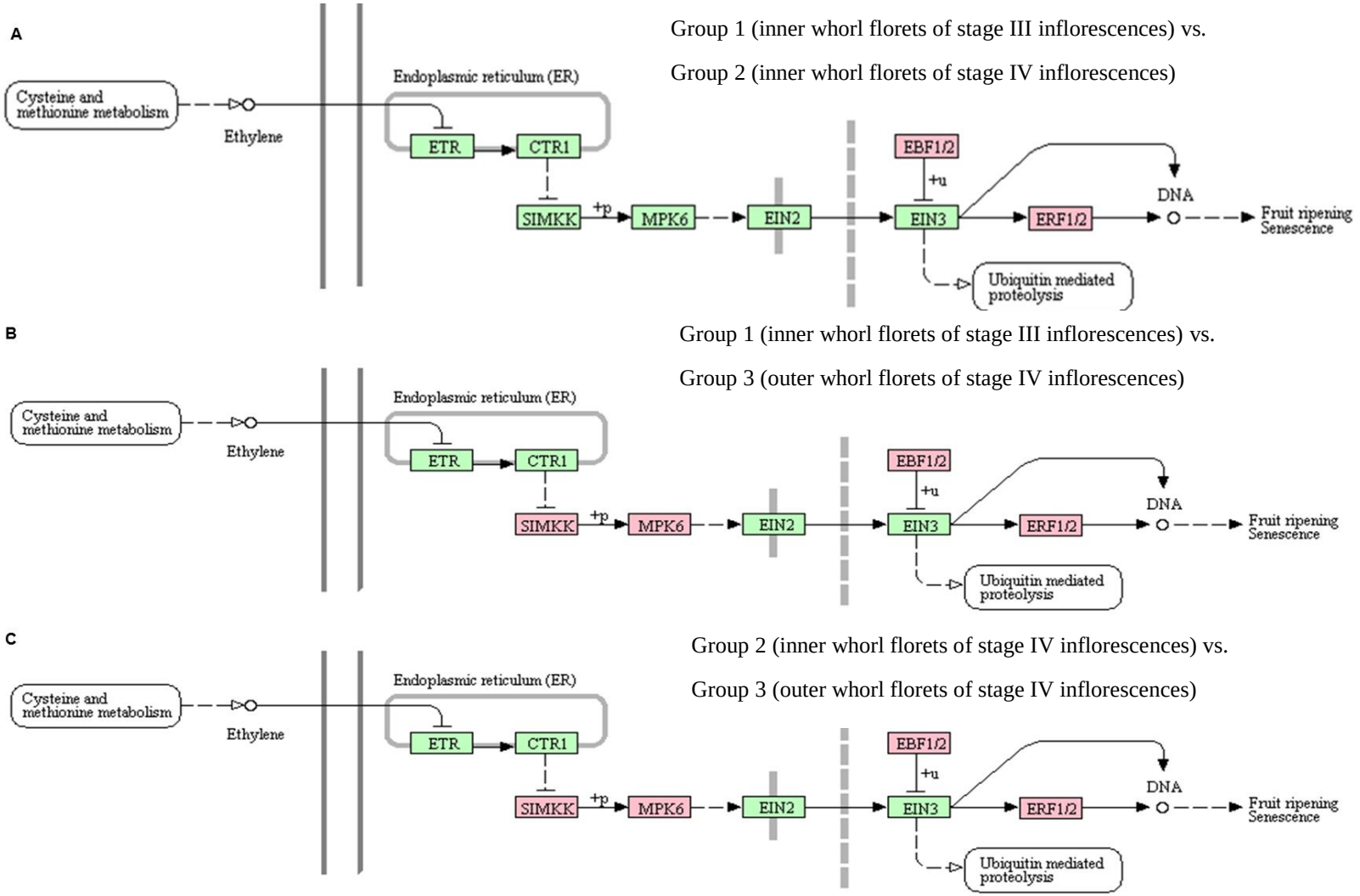


Relative expression scale according to eFP browser 2.0

Figure 15: Expression patterns of A. thaliana *genes listed in Table 7 according to eFP browser 2.0 in late stage floral and leaf tissue. Red colour indicates higher expression, yellow tissue indicates lower expression (Nakabayashi* et al.*, 2005; Schmid* et al.*, 2005; Winter* et al.*, 2007; Waese* et al.*, 2013).*

3.4.2.2. Differential expression analysis: ethylene

The stages used in analysis were: group 1 (inner whorl florets of stage III inflorescences), group 2 (inner whorl florets of stage IV inflorescences) and group 3 (outer whorl florets of stage IV inflorescences). More genes within the ethylene signal transduction pathway, *MPK6*, *SIMKK*, *ERF1/2* and *EBF1/2*, were upregulated later in the process, as shown by the 1 vs. 3 and 2 vs. 3 sample comparisons (Fig. 16B, C); however, in the earliest sample comparison, 1 vs. 2, only *EBF1/2* and *ERF1/2* were upregulated (Fig. 16A). In contrast the biosynthetic pathway showed downregulation of SAM synthetase between sample groups 1 and 3 which remained unchanged in the other comparisons (Fig. 17). The rate limiting enzyme ACC synthase was upregulated in all sample comparison, whereas ACC oxidase showed no significant change between any groups (Fig. 17).



Group 1 (inner whorl florets of stage III inflorescences) vs.

Group 2 (inner whorl florets of stage IV inflorescences)

Group 1 (inner whorl florets of stage III inflorescences) vs.

Group 3 (outer whorl florets of stage IV inflorescences)

Group 2 (inner whorl florets of stage IV inflorescences) vs.

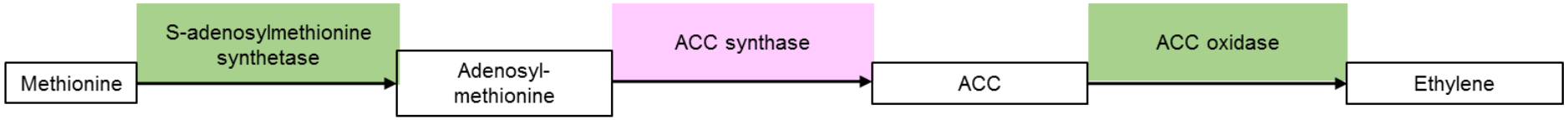
Group 3 (outer whorl florets of stage IV inflorescences)

Figure 16: Signal transduction pathway of ethylene in sample comparison 1 vs. 2 (A), 1 vs. 3 (B) and 2 vs. 3 (C) (Kanehisa & Goto, 2000).

Green = no significant change

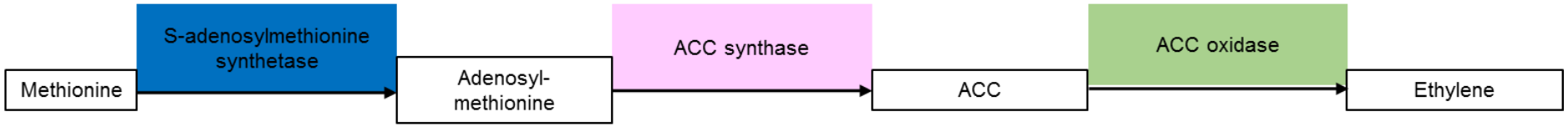
Pink = significantly upregulated

A Group 1 (inner whorl florets of stage III inflorescences) vs. Group 2 (inner whorl florets of stage IV inflorescences)



Group 1 (inner whorl florets of stage III inflorescences) vs. Group 3 (outer whorl florets of stage IV inflorescences)

B



Group 2 (inner whorl florets of stage IV inflorescences) vs. Group 3 (outer whorl florets of stage IV inflorescences)

C

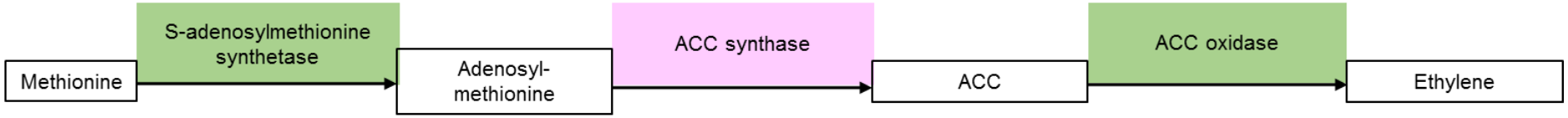


Figure 17: Ethylene biosynthesis in sample comparison 1 vs. 2 (A), 1 vs. 3 (B) and 2 vs. 3 (C) (Kanehisa & Goto, 2000). Green = no significant change, pink = significantly upregulated, blue = significantly downregulated.

The analysis also showed lipases differentially expressed between the stages examined. The gene encoding phospholipase *PLDP2* (phospholipase D P2) was highly upregulated between group 2 and 3 florets, the homolog of this enzyme in *A. thaliana* is also known to be highly upregulated in senescent leaves, its expression increases by 6467% between mature leaves and senescent leaves (Table 9, Fig. 18).

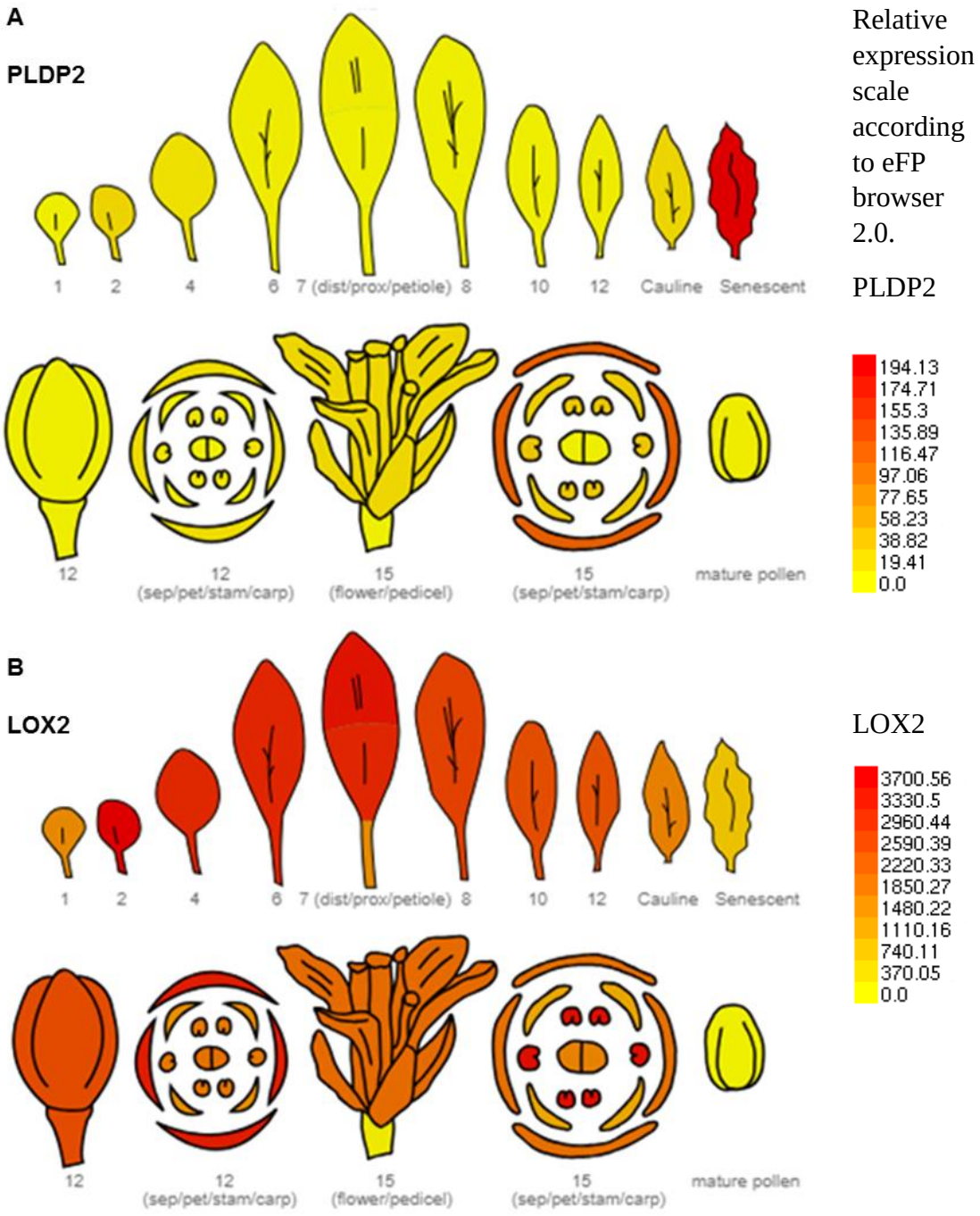
Two sequences homologous to the *A. thaliana* enzyme *LOX2* (lipoxygenase 2) were found, one (TRINITY_DN67684_c2_g1_i2) was upregulated in the sample comparisons 1 vs. 2 and 2 vs. 3 and other sequence was found only in the comparison 2 vs. 3 (TRINITY_DN67684_c2_g1_i5). In *A. thaliana* this gene is downregulated between mature and senescent leaves (Table 9; Fig. 18B), whereas in the present dataset the dahlia homologs were found to be slightly upregulated with fold changes of between one and two (Table 8).

Table 8 - Lipases found in the differential expression dataset, the H. annuus *and* A. thaliana *homologs of those lipases, and the fold change in the relevant sample comparison*

H. annuus *Accession*	A. thaliana *Accession*	A. thaliana *Gene Name*	*Group 1 florets vs. Group 2 florets (Fold Change)*	*Group 1 florets vs. Group 3 florets (Fold Change)*	*Group 2 florets vs. Group 3 florets (Fold Change)*
OTG02515	AT3G05630	*PLDP2*; phospholipase D P2	n/a	n/a	+(22.9)
OTG01649	AT3G45140	*LOX2*; lipoxygenase 2	n/a	+ (1.95)	+ (1.94)
OTG01649	AT3G45140	*LOX2*; lipoxygenase 2	n/a	n/a	+ (1.44)

Table 9 – Relative expression levels of A. thaliana *homologs (Nakabayashi* et al.*, 2005; Schmid* et al.*, 2005; Winter* et al.*, 2007; Waese* et al.*, 2013).*

		Relative expression level						
A. Thaliana gene	*ID*	*Flower*	*Sepals*	*Petals*	*Stamen*	*Carpel*	*Mature Leaf (whole rosette)*	*Senescent Leaf*
PLDP2	AT3G05630	*20*	*118*	*30*	*35*	*3*	*3*	*194*
LOX2	AT3G45140	*2067*	*1968*	*1129*	*3468*	*1705*	*2036*	*723*



Relative expression scale according to eFP browser 2.0.

PLDP2

LOX2

Figure 18: Expression patterns of A. thaliana *genes listed in Table 8 according to eFP browser 2.0 in late stage floral and leaf tissue. Red colour indicates higher expression, yellow tissue indicates lower expression (Nakabayashi* et al.*, 2005; Schmid* et al.*, 2005; Winter* et al.*, 2007; Waese* et al.*, 2013).*

3.4.2.2. Differential expression analysis: cytokinins

The stages used in analysis were: group 1 (inner florets of Stage III), group 2 (inner florets of Stage IV flowers) and group 3 (outer florets of Stage IV flowers). The cytokinin signal transduction pathways shows that in all sample comparisons multiple genes are being upregulated (Fig. 19). Type B-ARR was upregulated in all sample comparisons (Fig. 19A-C), *CRE1* and *A-ARR* were upregulated in sample comparisons 1 vs. 3 and 2 vs. 3 (Fig. 19B, C), and *AHP* was upregulated in sample comparison 1 vs. 3 only (Fig. 19B). No genes were found to be significantly downregulated in the cytokinin signal transduction pathway in any sample comparison (Fig. 19).

In sample comparisons 1 vs. 3 and 2 vs. 3 it was found that cytokinin biosynthetic genes, isopentenyl transferases (IPT's), were downregulated and cytokinin oxidases implicated in cytokinin catabolism were upregulated (Fig. 19). No significant changes in cytokinin oxidases or IPT's were found in sample comparison 1 vs. 2.

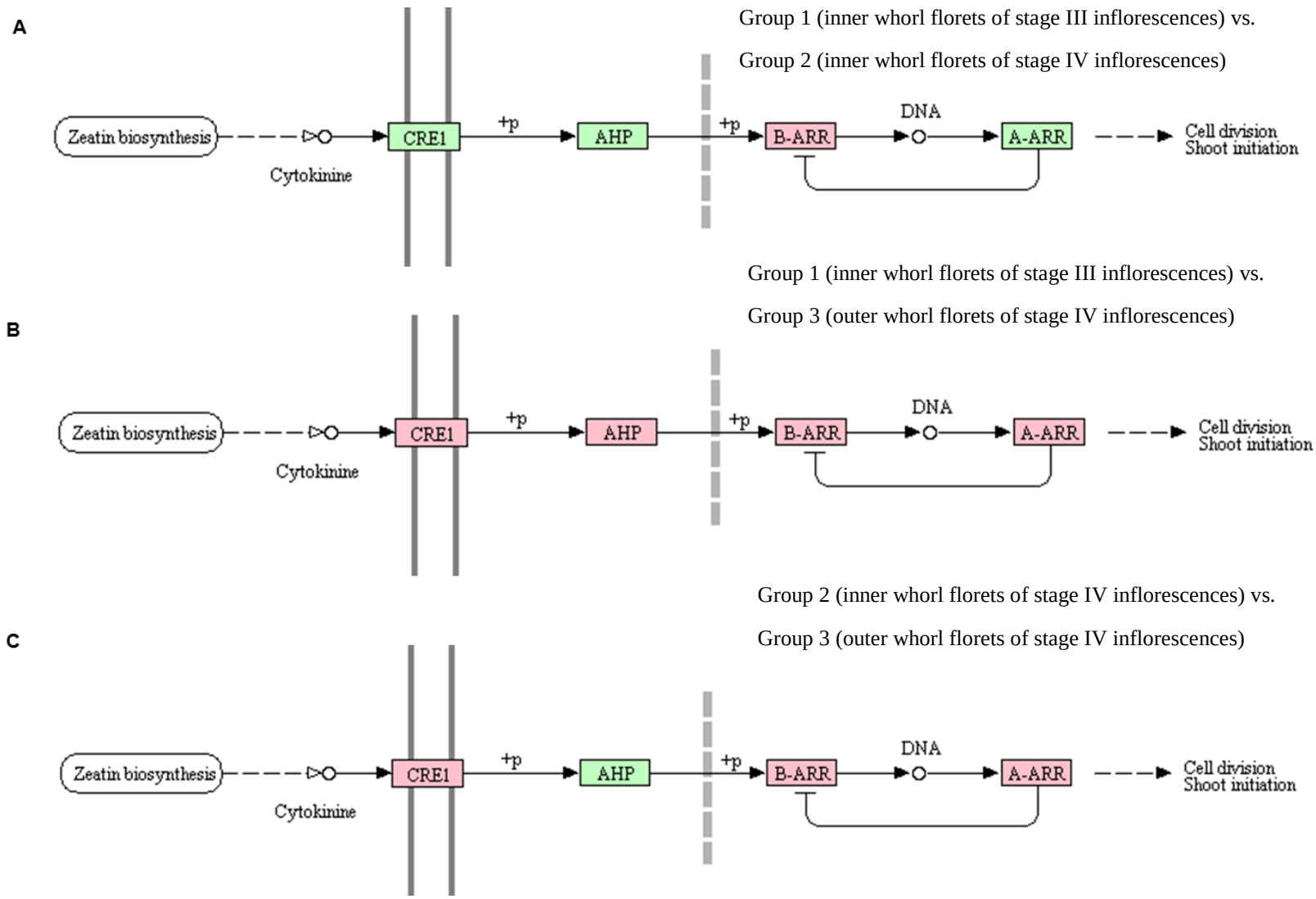


Figure 19: Signal transduction pathway of endogenous cytokinins in sample comparison 1 vs. 2 (A), 1 vs. 3 (B) and 2 vs. 3 (C) (Kanehisa & Goto, 2000).

Green = no significant change

Pink = significantly upregulated

Blue = significantly downregulated

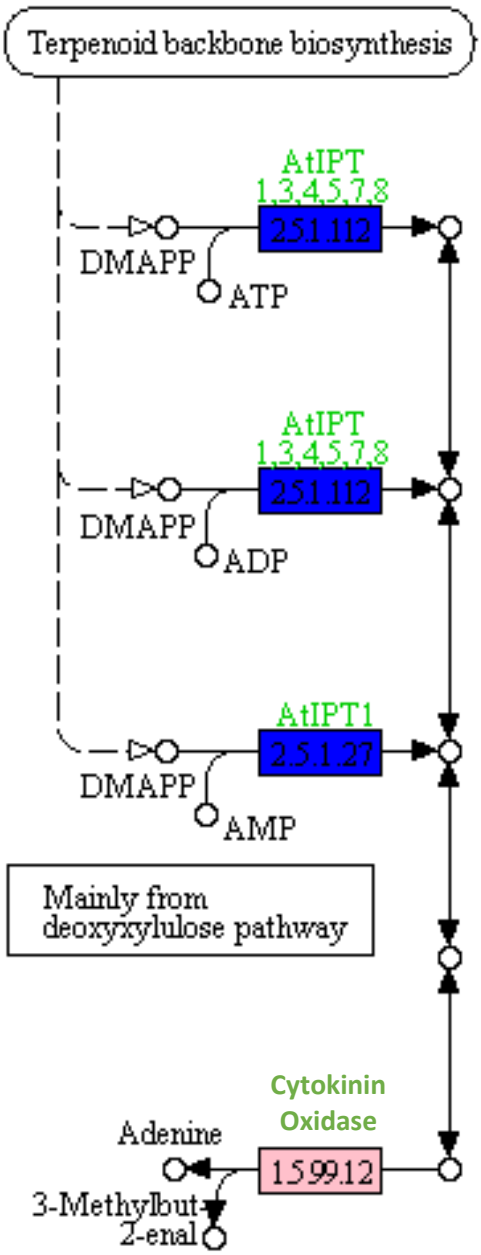


Figure 20: Cytokinin biosynthesis breakdown in 1 vs. 3 (Group 1, inner whorl florets of stage III inflorescences vs. Group 3, outer whorl florets of stage IV inflorescences) and 2 vs. 3 (Group 2, inner whorl florets of stage IV inflorescences vs. Group 3, outer whorl florets of stage IV inflorescences) sample comparisons. There were no significant changes in expression found in sample comparison 1 vs. 2 (Group 1, inner whorl florets of stage III inflorescences vs. Group 2, inner whorl florets of stage IV inflorescences) (Kanehisa & Goto, 2000).

Green = no significant change

Pink = significantly upregulated

Blue = significantly downregulated

3.5. Discussion

The aim of this experiment was to use RNA-sequencing and subsequent differential expression analysis of different stages of dahlia florets to examine genes and pathways, which were potentially relevant to flower senescence, and were differentially expressed. The RNA-sequencing was successful, and the generation of a *de novo* assembly and use of the Cufflinks pipeline to produce a database of differentially expressed transcripts matched to known proteins using Blastx can be used to show pathways involved in senescence, for example those relating to ABA biosynthesis and signalling. RNA-sequencing was successful with 345,038,365 reads being generated across all nine samples (Table 4). These reads were high quality enough (i.e. they had a base quality score of >30, see Chapter 4.4.1 for details) that following assembly and the Cufflinks pipeline 137,376 transcripts were assembled, and their level of expression examined (Table 4, 5; Fig. 10).

The Tophat2 assembly using reads mapped to *A. thaliana* or *H. annuus* reference genomes was unsuccessful. This may be because the assembly generated was too disparate. In a previous study on composite crops where *de novo* assemblies were used (Hodgins *et al*., 2014), it was found that the dahlia yielded more than 50% more contigs than the species with the next greatest number of contigs, which was the hexaploid *Helianthus tuberosus*, suggesting that higher levels of ploidy contribute to higher numbers of contigs, and high ploidy was given as a possible factor for the high contig numbers in that study (Hodgins *et al*., 2014). Given the disparity it is perhaps not surprising that the diploid *Helianthus annuus* did not serve as a close enough reference despite its close relation to dahlia. It may take assembly of a polyploid genome of another Asteraceae member before an assembly using a reference is useful in dahlia, although it is also possible that a lack of close enough homology at the level of individual genes was the reason for the unsuitability of a reference genome.

Another study concluded that in genera such as *Arachis* (peanut) which are poorly annotated, as is the case with dahlia, using lower quality assemblies, i.e. those against references which are not similar enough or using *de novo* assemblers that are not effective, led to problems in downstream processing and that high quality *de novo* assemblies, e.g. using Trinity, were better for optimal results and good coverage of the transcriptome (Chopra *et al*., 2014). Additionally, previous studies comparing reference assemblies to *de novo* assemblies have found that there is a high correlation between them in number

of isoforms found and expression patterns, suggesting that *de novo* studies are just as useful, especially if the available references are only of superficial similarity to the species of interest (Rodriguez *et al*., 2013). *De novo* assembly has also been successfully employed in the related species *Chrysanthemum crissum*, which as a decaploid, also has a very high level of ploidy and no reference genome (Guan *et al*., 2017), suggesting that for octoploid dahlia, *de novo* assemblies are also the best method to use when generating a transcriptome assembly until a suitable reference genome is available.

The mapping of reads to the *de novo* assembly using TopHat2 generated 137,376 contigs (Table 5). This is comparable to the number of contigs found in other studies where RNA-sequencing has been performed in dahlia. In cv. 'Thomas Edison' sequencing of transcriptomes from leaf and flower tissue using Illumina produced 135,229 contigs (Hodgins *et al*., 2014). The same study performed sequencing on many crops from the Asteraceae family, and the dahlia produced far more contigs than any other, the second greatest, 89,749, were produced by *Helianthus tuberosus*, a hexaploid, suggesting that high ploidy and contig number are linked, though more studies would need to be carried out confirm such a relationship. However, dahlia produced at least 50.7% more contigs than any other species in the study, all of which were close relatives and members of the same family (Hodgins *et al*., 2014). The authors also noted that amongst all the composite species analysed, the dahlia had the greatest number of unigenes and had the highest assembly length. It was suggested that this may be due to the multiple tissues sampled in the experiment (leaf and flower), or because of polyploidy (Hodgins *et al*., 2014). Additionally, RNA-sequencing performed on samples from cv. 'Rio Riata' dahlia using differing tissue samples, flower bud, stem and leaf, yielded an assembly with 122,053 contigs (Lehnert & Walbot, 2014). However, the high number of contigs found in this study using floret samples alone suggests that the high ploidy in dahlia (octoploid) is a major factor in the high number of contigs produced by these assemblies (Hodgins *et al*., 2014; Gatt *et al*., 1998). A very large number of contigs, 155,944, were also produced by a *de novo* assembly of the decaploid relative of dahlia, *Chrysanthemum crassum*, using only leaf samples, further suggesting that high ploidy is a major factor in the generation of large numbers of contigs following *de novo* assembly rather than the presence of multiple tissue types (Guan *et al*., 2017).

The aim of the study was also to compare gene expression across different floret stages. Between group 1 (inner whorl florets from stage III inflorescences) and group 2 (inner whorl florets from stage IV inflorescences) just 1.9% of transcripts were differentially

expressed, this rose to 9% between group 2 and group 3 (outer whorl florets from stage IV inflorescences), and 11.5% between group 1 and group 3 florets (Table 5). This suggests that the outer florets are showing quite different gene expression patterns compared to inner florets, at least in Stage IV inflorescences. The data also show that amongst genes which were significantly up or downregulated, in each of the three sample comparisons more were upregulated than downregulated, and fold change amongst those upregulated was greater than those that were downregulated (Fig. 12). This suggests that gene expression activity is increasing as florets approach senescence. The comparison between groups 1 and 3 showed the greatest fold change in both up and downregulating genes, further suggesting that outer florets have different expression patterns to inner florets, at least at the stages sampled.

The number of genes significantly up or down regulated in this study is high, compared with the figures found by Lehnert & Walbot (2014). In that study 2690 genes (2.2% of total genes) were elevated in expression in stems compared with leaves or buds, compared with 5329 genes (3.9% of total genes; Table 4; Fig. 11) found to be upregulated between groups 1 and 3 in this study, a 77.3% difference. This suggests that there is a large amount of gene expression change as petals age and programmed cell death occurs. Alternatively, it could be that the differences in the methods used or differences in levels of replication may be responsible for this disparity. Furthermore, the comparisons performed by Lehnert & Walbot (2014) were between different tissues types, rather than different stages of the same tissue, so we should be cautious in making conclusions from such comparisons.

Blastx using *A. thaliana* as a reference did not return as many hits as using *H. annuus* (Table 5; Fig. 11). This is unsurprising given the relatedness of dahlia to *H. annuus* compared with *A. thaliana*. This also suggests that there are dahlia genes which are represented when using the sunflower Blastx database but not when using the *Arabidopsis* Blastx database that would be missed when using analysis programs using only *A. thaliana* accessions. It is possible that these genes could be involved in the composite floral senescence process given that they are found in *H. annuus* and dahlia and not in *A. thaliana*. Analysis of transcripts which only show significant homology to an *Arabidopsis* protein may only be giving information about a portion of the floral senescence process in dahlia.

The results show that the changes in the signal transduction pathway (pictured in Fig. 13) of ABA during the stages of dahlia floral senescence examined are complicated.

PYR/PYL, responsible for ABA reception (Antoni *et al.*, 2012), was downregulated between groups 1 (inner whorl florets of stage III inflorescences) and 3 (outer whorl florets of stage IV inflorescences) (Fig. 13B), suggesting that reception of ABA is decreasing in importance between 1 and 3, but this is complicated by the upregulation of *PYR/PYL* between groups 2 (inner whorl florets of stage IV inflorescences) and 3 (Fig. 13C). It is possible that although reception of ABA may decrease during the stages assessed, the upregulation found in 2 vs. 3 may show the beginning of an increase in ABA reception due to an increase in *PYR/PYL*, this uptick would then lead to a greater repressive effect on PP2C which in turn would reduce the repressive effect on SnRK2 which activates ABA responsive genes. However, more samples from later stages of dahlia floral senescence would be needed to confirm this. *PP2C* which exerts a repressive effect on *SnRK2* was upregulated in samples 1 vs. 3 (Fig. 13B), but no change was found in the other sample comparisons. As PP2C is a negative regulator of ABA signalling and exerts a repressive effect on SnRK2 (Umezawa *et al.*, 2009) and therefore on ABA responsive genes, this suggests that ABA's importance is reduced in the later stages of senescence. *SnRK2*, a protein kinase which positively mediates ABA responses (Kobayashi *et al*., 2005), was upregulated in the sample comparisons 1 vs. 2 and 1 vs. 3 (Fig. 13A, B), however there was no significant change between 2 and 3 (Fig. 13C). This suggests that upregulation of this gene is greater earlier in the senescence process and that the ABA response may be being downregulated as senescence progresses, though more extensive analysis would be needed to confirm this. However, *ABF* transcription factors, genes directly responsible for transcriptional regulation of ABA responsive gene expression (Yoshida *et al*., 2010) show no significant change in expression any sample comparison (Fig. 13). This may be because expression of *SnRK2*'s is more important in the ABA response or because transcriptional activation of ABA response genes is not a major factor in the stages of dahlia floral senescence assessed.

As the ABA receptor *PYR/PYL* was being downregulated between 1 and 3 (Fig. 13B), this suggests that reception was also lower as senescence progresses, and this is compounded by the fact that *PP2C*, which is itself repressed by *PYR/PYL*, and represses *SnRK2* was also being upregulated (Fig. 13B). This would mean that as reception was being downregulated, repression of the kinases implicated in activating ABA responses are being upregulated. However, the picture is complicated by *PYR/PYL*'s upregulation between samples 2 and 3 (Fig. 13C). This may be the first sign of ABA being upregulated after a previous downregulation, though more sampling from more mature florets would

be needed to confirm this. Overall the differential expression data in this experiment suggests ABA is not playing a major role in dahlia floral senescence, however this is not certain and only sampling from later or earlier stages would be able to confirm this. Other studies have found that ABA triggers senescence indirectly through its activation of ethylene biosynthesis (Ronen & Mayak, 1981), therefore it is possible that ABA may have been upregulated at an earlier stage to activate ethylene biosynthesis.

The biosynthesis of ABA (pathway shown in Fig. 14) showed that in sample comparison 1 vs. 3 (inner florets of stage III inflorescences vs. outer florets of stage IV inflorescences) and 2 vs. 3 (inner florets of stage IV inflorescences vs. outer florets of stage IV inflorescences) no ABA biosynthetic enzyme was found to be significantly upregulated (Fig. 14B, C). However, in the earliest comparison, 1 vs. 2, significant upregulation of 9-cis-epoxycarotenoid dioxygenase (*NCED*) was found (Fig. 14A), however it was found to be downregulated in the other sample comparisons (Fig. 14B, C). Furthermore, xanthoxin dehydrogenase showed no change in any sample comparison (Fig. 14) and the only significant change showed by abscisic aldehyde oxidase was a significant downregulation in sample comparison 1 vs. 3 (Fig. 14B). This suggests that ABA biosynthesis is being downregulated as senescence progresses, however the upregulation of *NCED* in the earliest comparison, 1 vs. 2 (Fig. 14A), suggests that the downregulation is only beginning to occur in group 2, inner florets of a Stage IV inflorescence. This may be because ABA plays a triggering role in senescence (Kumar *et al*., 2014) and is not required to mediate later stages of senescence, or because its role in dahlia floral senescence is minimal, however far more data needs to be gathered to confirm such hypotheses.

The biosynthesis data also ties in with the downregulation of the signalling pathway seen in the comparison between 1 and 3 where receptor *PYR/PYL* is downregulated and the repressor *PP2C* is upregulated (Fig. 13B) and the lack of change in expression of *ABF* transcription factors. ABA's have been implicated in upregulation of thiol proteases at the onset of wilting (Williams *et al*., 1994), thiol proteases have also been implicated in ovary senescence in pea (Cercós *et al*., 1999) suggesting that ABA may upregulate senescence associated proteases.

Overall the data in this study shows a complex picture of ABA signalling (as shown by the pathways and sample comparisons in Fig. 13) and a possible overall downregulation of ABA biosynthesis (as shown by the pathways and sample comparisons in Fig. 14), suggesting that ABA's role in dahlia is minimal in the stages assessed, however further

sampling from younger and older florets would better establish if it has a role earlier or later in senescence. Treatment of flowers with exogenous ABA and RNA-sequencing on such samples would be helpful in further examining the physiological and transcriptional effects of ABA in dahlia floral senescence.

The dahlia homolog of the enzyme *SHM1* was found to be downregulated in the sample comparison, correlating with the data on this enzyme in *A. thaliana*, where it is found to be downregulated in senescent leaves compared to mature leaves (Fig. 15A; Table 6, 7). This suggests that downregulation of this gene could be used as a hallmark for the transition into senescence. The downregulation of this gene in sample comparison 1 vs. 3 suggests that this is happening in group 3 florets where it is found to be downregulated compared to inner florets. It would be interesting to examine in outer florets of Stage III flowers if it is also downregulated there. Loss of function *A. thaliana* mutants of *shm1* have been found to be prone to formation of necrotic lesions and more vulnerable to pathogen infection, suggesting that this gene acts to protect plants from such stresses, and in senescent leaves this is unneeded or undesirable. The authors also suggested it has a role in protection from oxidative damage and in controlling cell damage from both abiotic and biotic stresses (Moreno *et al*., 2004). Therefore, in dahlia its downregulation may be indicative of the onset of senescence where there is a decrease in protection from oxidative damage and cells may begin to senesce in part from such damage. It is also interesting to note that this pattern of expression has been found in both dahlia and *A. thaliana* suggesting that the expression pattern of *SHM1* is conserved.

Both *PSD3* and *SERAT2;1* are known to be upregulated in senescent leaves of *A. thaliana* compared with mature leaves, and they were also found to be upregulated in later sample comparisons in this study (Fig. 15; Table 6, 7). Three genes homologous to *PSD3* were found to be upregulated between groups 1 and 3 and 2 and 3 (Table 6). The upregulation was more than 23-fold, suggesting high upregulation. In *A. thaliana PSD3* has been found to be implicated in synthesis of endomembrane phospholipids and is localised to the endoplasmic reticulum (Nerlich *et al*., 2007), suggesting that upregulation of *PSD3* in senescence is indicative of maintenance of the ER which is involved in protein synthesis and transport, and lipid synthesis (Hawes *et al*., 2015). The differential expression results support this as in sample comparison 1 vs. 3 it was found that over 5000 genes were upregulated (Fig. 11; Table 5) showing that gene expression and likely subsequent protein synthesis is occurring in these senescent cells.

The upregulation of *SERAT2;1* was less drastic than that of *PSD3* with a positive fold change of just 3.4 in the sample comparison 1 vs. 3 (Table 6). *SERAT2;1* has been implicated in acclimation to stress response in *A. thaliana* (Park *et al*., 2013). Its primary role is in sulphur assimilation and in the sulphur containing amino acid cysteine's biosynthesis (Wirtz & Droux, 2005). This suggests its upregulation is due to increased need for the amino acid cysteine and therefore synthesis of proteins containing cysteine. This suggests that protein synthesis remains important for cells at this point during senescence even as the cell is preparing to degrade tissue and genes involved with protection from oxidative stress, such as *SHM1* are being downregulated (Table 6). The results suggest that genes up or downregulated in senescence in *A. thaliana* may follow broadly similar patterns of expression in dahlia flower senescence. However, caution is needed in making comparisons as most details on senescence in *A. thaliana* are from leaf tissue not flower tissue. Moreover, many more genes would need to be examined from the differential expression dataset of this study to be able to confirm that the expression patterns are similar.

The gene *ACS*, which encodes the rate limiting enzyme of ethylene biosynthesis (Yang & Hoffman, 1984; Fig. 21) was found in the differential expression analysis of cv. 'Sylvia' flowers to be upregulated in every sample comparison, suggesting that ethylene biosynthesis is being upregulated as florets age on the plant (Fig. 17). In contrast, *ACO* was not upregulated in the differential expression analysis (Fig. 17), suggesting that it may have been upregulated before or after the floret stages encompassed in our sampling. This further suggests that ethylene is playing a role in dahlia floral senescence at least in cv. 'Sylvia'. In a different study in dahlia, ethylene production was found to be relatively high the day of harvesting in ray florets, ovaries, and receptacles, however over the 5 days following harvesting ethylene production fell and did not significantly change for the rest of the experimental period (another 5d) (Shimizu-Yumoto & Ichimura, 2013). This suggests that ethylene production is occurring in dahlia flowers when they are on the plant. This may have occurred in the cv. 'Sylvia' flowers used for RNA sequencing in this study which showed upregulation of *ACS* (Fig. 17) as florets matured.

In the differential expression analysis, no genes in the ethylene signal transduction pathway were found to be downregulated, though some were upregulated (Fig. 16). The nuclear-localised *EBF* and *ERF* genes were upregulated in every sample comparison (Fig. 16, 21), whereas the genes *SIMKK* and *MPK6* were upregulated only in sample comparisons 1 vs. 3 (Fig. 16B) and 2 vs. 3 (Fig. 16C), showing these genes are

upregulated in outer florets of Stage IV flowers compared to inner florets of Stage III or IV flowers (Fig. 5) indicating an increase in expression as florets mature. EBF1 and 2 are part of a mechanism which helps regulate ethylene in a negative feedback loop to ensure the ethylene response is dampened (Merchante *et al*., 2013). In contrast, transcription factors ERF1 and 2 are responsible for the activation of downstream genes which lead to a transcriptional cascade leading to the ethylene response (Breeze *et al*., 2011; Buchanan-Wollaston *et al*., 2003). The upregulation of these genes suggests that in ageing dahlia florets ethylene responsive genes downstream of the transduction pathway are being activated by the increased expression of *ERF1/2* but this response is being dampened by *EBF 1/2*, perhaps to ensure senescence does not occur too quickly (Fig. 21). Senescence occurring too quickly would be negative for the plant as senescence must occur at a pace that allows the plant to remobilise nutrients back to developing tissues. This also suggests that endogenous ethylene is having a role in dahlia floral senescence as *ERF1/2* are being upregulated to mediate the transcriptional activation of ethylene responsive genes, such as those implicated in protease and lipase activity which degrade tissues (Hong *et al*., 2000; Tripathi *et al*., 2009). The upregulation of *MAPK6* and *SIMKK* suggests that ethylene signalling is being positively upregulated as these genes are upregulated in both the inner and outer florets of Stage IV flowers compared to the inner Stage III florets and have been implicated in the positive regulation of ethylene responses (Yoo *et al*., 2008; Ouaked *et al*., 2003). MAPK6 has also been implicated in ethylene biosynthesis, furthering the idea that its upregulation is part of ethylene's role in the senescence process (Xu *et al*., 2008). This further suggests that ethylene activity and induction of downstream genes activated by ethylene are important in dahlia floral senescence.

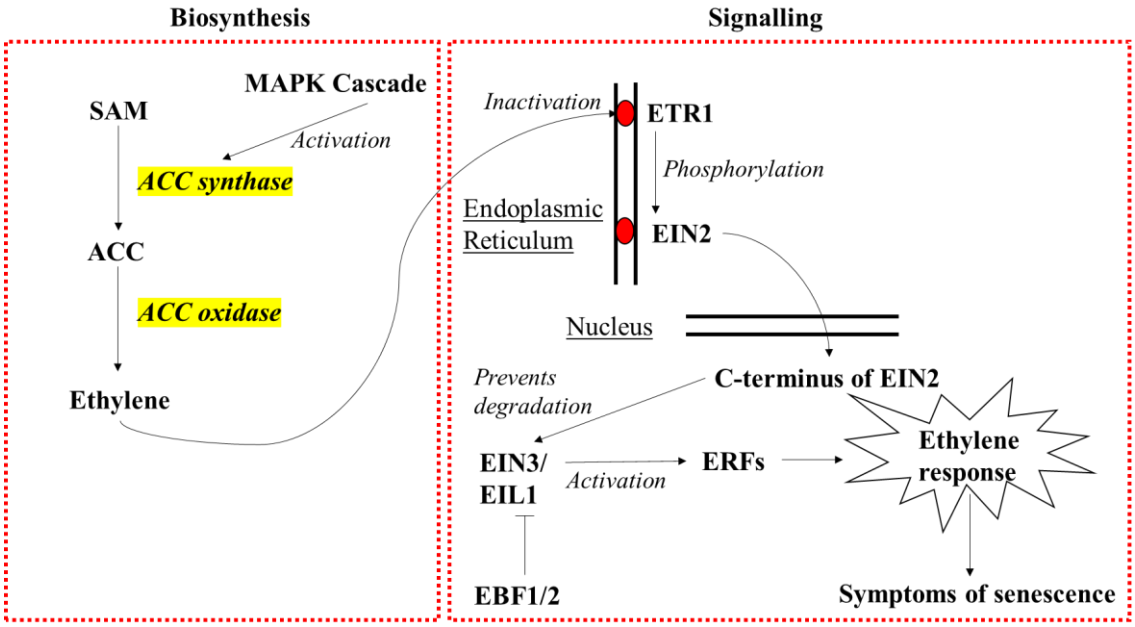


Figure 21: A simplified model of ethylene biosynthesis and signalling (Yang & Hoffman, 1984; Ouaked et al.*, 2003; Joo* et al.*, 2008; Liu & Zhang, 2004; Chen* et al.*, 2002; Wen* et al.*, 2012; An* et al.*, 2010; Solano* et al.*, 1998; Merchante* et al.*, 2013; Breeze* et al.*, 2011; Buchanan-Wollaston* et al.*, 2003).*

Type B-ARR was found to be upregulated as senescence advanced in the differential expression analysis (Fig. 19). No gene in the signal transduction pathway was found to be significantly downregulated in any of the sample comparisons (Fig. 19). *CRE1* (cytokinin response 1), which is involved in cytokinin reception (Inoue *et al*., 2001; Fig. 22) was upregulated in the comparisons 2 vs. 3 and 1 vs. 3 (Fig. 19B, C), showing that it was upregulated in the outer florets of Stage IV flowers compared with inner florets of Stage III or IV flowers. This suggests that cytokinin sensitivity is increasing, as receptors are upregulated, perhaps giving a boost to the downstream effects of cytokinins or compensating for the reduced endogenous cytokinin levels as florets senesce. *AHP* (*Arabidopsis* histidine phosphotransfer) protein was also upregulated in the comparison between inner Stage III and outer Stage IV florets (Fig. 19B). However, *AHP* is known to be a positive regulator of cytokinin signalling (Hutchinson *et al*., 2006; Fig. 22), again suggesting that preceding signs of senescence, at a stage where cytokinin biosynthesis is being downregulated and its breakdown accelerated (Fig. 20), the downstream effects of cytokinin implicated in extending vase life are still being positively mediated. However, it should be noted that A-ARR's, implicated in the negative regulation of cytokinin responses, are also being upregulated in the later sample comparisons (Fig. 19B, C). This

suggests that the negative feedback effect on cytokinin signal transduction is also becoming stronger as reception and positive regulation also increases, ensuring the cytokinin response does not become strong enough to delay senescence but also ensuring senescence does not occur too soon (To *et al*., 2004).

The downregulation of *IPT*'s in sample comparisons group 1 vs. 3 (inner whorl florets of stage III inflorescences vs. outer whorl florets of stage IV inflorescences) florets and group 2 vs. 3 (inner whorl florets of stage IV inflorescences vs. outer whorl florets of stage IV inflorescences) florets strongly suggest that cytokinin biosynthesis is being downregulated as florets age (Fig. 20, 22). Additionally, the upregulation of cytokinin oxidase shows that as their biosynthesis was being reduced their catabolism was being upregulated, suggesting that levels of endogenous cytokinins may be reducing as flowers age, and allowing senescence to progress (Fig. 20). Furthermore, as the *IPT*'s are not being downregulated until later stages of senescence it may be that the downregulation of signal transduction genes occurs at a more advanced stage of senescence than the sampling encompassed in this study (Fig. 19, 20). The upregulation of genes implicated in cytokinin signal transduction also suggests that whilst cytokinin biosynthesis is being downregulated and its breakdown upregulated, the effects of cytokinin are still affecting downstream processes and it may take sampling from later stages of florets to see a downregulation in genes involved in cytokinin signal transduction.

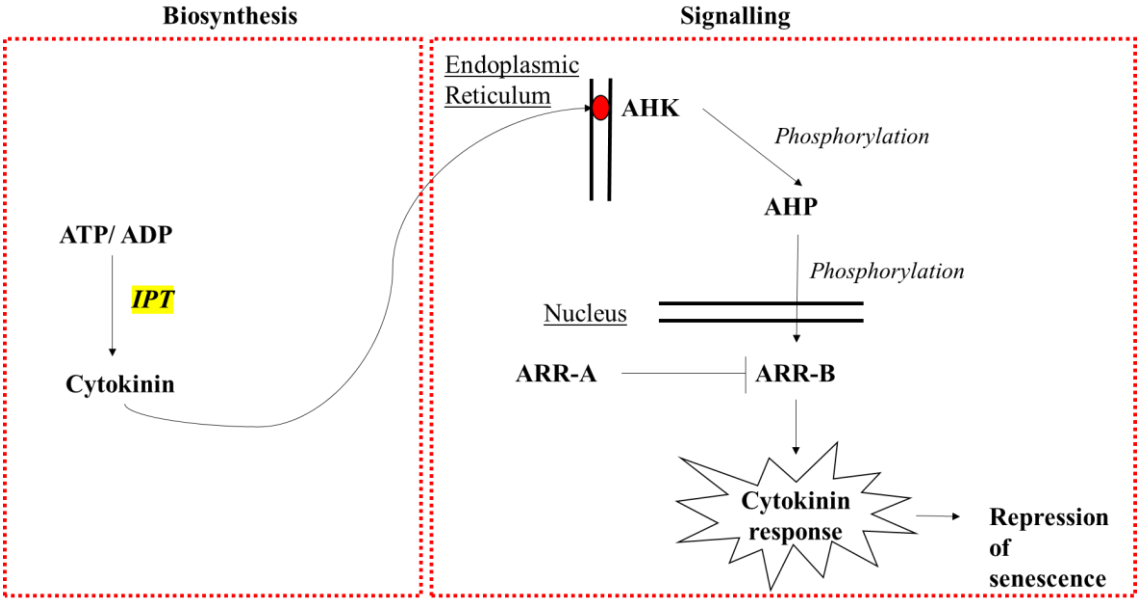


Figure 22: A simplified model of cytokinin biosynthesis and signalling (Kieber & Schaller, 2014; Inoue et al.*, 2001; Suzuki* et al.*, 2001; Ueguchi* et al.*, 2001; Yamada* et al.*, 2001; Hwang & Sheen, 2001; Sakai* et al.*, 2001).*

In the comparison between group 1 (inner whorl florets of stage III inflorescences) and 2 (inner whorl florets of stage IV inflorescences) florets no significant change in the expression of cytokinin biosynthetic or catabolic genes could be found, suggesting that this process does not begin to occur until whole inflorescences reach Stage IV, at least in the outer florets. This also suggests that the downregulation of IPT's and upregulation of cytokinin oxidases occurs days before the first signs of wilting occur as group 3 florets (outer florets of Stage IV flowers) are not yet wilted (Fig. 3C). In future studies, thidiazuron, a non-competitive inhibitor of cytokinin oxidases, could be applied to inhibit the enzymes and reduce the rate at which endogenous cytokinins are irreversibly inactivated by cytokinin oxidases (Schmülling *et al*., 2003; Werner *et al*., 2006). This could make the flowers' endogenous cytokinins last longer.

4. Physiology of *Dahlia* flower senescence on the plant

4.1. Introduction

Flowers vary greatly in lifespan, from one day to several months (Primack, 1985; van Doorn & Woltering, 2008). The cost of maintaining flowers is relatively high, therefore once the flower has been pollinated or if the stigma is no longer responsive, the corolla senesces (Stead, 1992; Stead *et al*., 2006; Jones, 2002), and may abscise (van Doorn & Woltering, 2008). The flower senescence process can take place in two different ways in the same species; the first is pollinator-induced senescence whereby a signal following pollination induces ethylene production to trigger senescence (Jones, 2008). The second is developmental senescence, where flowers senesce after remaining unpollinated and the stigma has become unresponsive (Jones, 2013). When flowers do not self-pollinate, cut flower senescence is most similar to developmental senescence. Much research has been performed in cut flowers, but such studies must be treated with caution when considering the senescence process in flowers left attached to the plant. Knowing how long dahlias last on their parent plant and the factors influencing their rate of senescence on the parent plant may shed light on why dahlias senesce so quickly once cut and why they are less responsive to traditional postharvest treatments compared to other cut flower species.

Most literature on developmental or pollinator-induced senescence is based on senescence of the corolla as this is the major factor on which postharvest quality of flowers is assessed in the ornamentals industry (personal communication, Paula Edgington). Although the corolla of a dahlia is technically made up ray florets rather than petals they serve the same function and within the ornamentals industry and amongst consumers there is not much differentiation between the two (personal communication, Martin Squire). Dahlia florets senesce sequentially, with the older outermost ray florets senescing first and the younger innermost florets senescing last, ray florets may abscise or wilt but remain attached to the inflorescence (personal communication, Tony Stead). Therefore, corolla senescence is also the major factor in postharvest quality in dahlia, suggesting much of the research on other plant species' developmental corolla senescence could be applicable to the dahlia. A summary of the types of senescence and senescence symptoms can be found in Fig. 23. The rate of senescence in flowers either cut or left uncut from their parent plants is affected by three main factors, though the timing of these factors can differ between the two groups (van Doorn & Woltering, 2008; Jones, 2008; Jones, 2013; Fig. 23):

1) Pollination success
2) Nutrient remobilisation
3) Cellular breakdown

It is important for plants to maintain their flowers until pollination is successful in order to propagate themselves. Once pollination has occurred, or if the stigma is no longer receptive to pollination, then senescence is activated, and the corolla begins to senesce (Jones, 2002). Senescence can be thought of as the final stage of flower development, whereby the plant remobilises the nutrients used to develop the flower for reuse in other parts of the plant, and the tissue breaks down.

Pollination is known to accelerate flower senescence in longer lasting flowers (Jones, 2008) and it has been suggested that pollination-induced ethylene production can drive nutrient transport (Jones, 2013; Verlinden, 2003; Chapin & Jones, 2007; Chapin & Jones, 2009). In petunia, it has been found that more nutrients are remobilised in pollinator-induced senescence compared with developmental senescence (Chapin & Jones, 2007). In most domesticated dahlia cultivars the density of the ray florets obscures, and often totally covers, the hermaphroditic disc florets which bear most if not all the nectar which attracts pollinators, making pollination in cut dahlias unlikely (Mani & Saravanan, 1999).

Nutrient remobilisation is vital to the plant as it allows it to reallocate important molecules to developing parts of the plant, such as new seeds, buds or leaves (Fig. 23). In cut flowers it is likely that nutrient transportation is reduced due to the absence of other developing tissues, assuming that remobilisation is driven by sinks in other parts of the parent plant (Jones, 2013). Differences have been found in nutrient remobilisation between cut flowers and attached flowers. Cut petunia flowers showed reduced levels of N and P remobilisation compared to flowers left attached to the parent plant (Jones, 2013). The higher levels of N and P remobilisation in on-plant flowers compared to cut flowers may be due to greater proteolytic activity and the expense of synthesising proteases and maintaining their activity (Jones, 2013). However, there was a reduction in Fe content in the cut flowers whereas this was not detected in the attached flowers (Jones, 2013). Additionally, studies in day-lily have shown that senescing tepals have a similar appearance in both detached and attached flowers but that the fresh weight, dry weight, sugar, and amino acid content of the two groups differed significantly (Bieleski, 1995). As in petunia the cut daylily flowers showed reduced nutrient remobilisation compared

to on-plant flowers (Bieleski, 1995). These studies show that in two unrelated genera, a dicot and a monocot, nutrient remobilisation is reduced in cut flowers compared to their on-plant counterparts. Therefore, it is likely that cut dahlia also show a difference in nutrient remobilisation between cut and on-plant flowers.

In petunia, which has been used as a model for this process, it has been found that in cut flowers there was a much greater reduction in Zn and Fe, whereas the attached flowers showed greater reduction in N, P, and Cu (Jones, 2013). It costs the plant energetically to remobilise nutrients, therefore this cost must be equalled or surpassed by the benefits of remobilisation to another part of the plant. This may explain some of the differences seen in remobilisation profiles between cut and on-plant flowers. It has also been found that ethylene influences remobilisation, suggesting it may also in dahlia (Chapin & Jones, 2009). However, ethylene sensitivity is variable between cultivars indicating that remobilisation may also differ markedly between dahlia cultivars (Woltering & van Doorn, 1988; Dole *et al*., 2009; Shimizu-Yumoto & Ichimura, 2013). Transporter proteins (TP's) are a critical part of the process of remobilisation. Expression profiling in *A. thaliana* has found large numbers of TP's upregulated in senescing leaves (van der Graaf *et al*., 2006). TP's have been identified in senescing petals of *Alstroemeria* and carnation, including metal transport proteins involved in mobilisation of Fe and Mn (Breeze *et al*., 2004; Hoeberichts *et al*., 2007).

Cellular breakdown occurs in one of two circumstances in on-plant flowers, after pollination or when the stigma is no longer receptive (Fig. 23). During this breakdown ethylene accelerates expression of senescence related genes, including ATG's, genes involved in autophagy, the process by which macromolecules are broken down in flower petals during senescence (Yamada *et al*., 2009). Membrane permeability increases during senescence as cell membranes disintegrate and ion leakage increases, and it is commonly used as a way of quantifying senescence (Whitlow *et al*., 1992). In petals protein content declines during senescence but not to the same degree as during leaf senescence (Price *et al*., 2008). This is mainly due to the fact that during leaf senescence it is the degradation of chloroplast proteins which constitutes the majority of remobilisation (Thomas *et al*., 2000). Proteolytic degradation is one of the main features of senescence and marked increases in protease activity during flower senescence have been observed in species as varied as *Hemerocallis*, *Iris*, and *Petunia* (Stephenson & Rubinstein 1998; Pak & van Doorn 2005; Jones *et al*. 2005). Cysteine proteases have been shown to be upregulated in various cut flower species including *Alstroemeria*, *Sandersonia*, *Petunia*, and *Narcissus*

(Wagstaff *et al*., 2002; Eason *et al*., 2002, Jones *et al*., 2005; Hunter *et al*., 2002). In tepals this upregulation occurs late in senescence.

Changes in mass during senescence vary between different plant species. In ethylene sensitive *Antirrhinum*, petals are abscised at their peak fresh weight (Goodwin *et al*., 2003). In contrast, ethylene insensitive daylilies abscise their petals after losing 95% of their sugar content and 65% of their dry mass, just 24 h after flower opening (Bieleski, 1995). Others, such as *Alstroemeria*, fall somewhere in between showing some signs of senescence before abscission but still retaining anywhere between 40% and 80% of their maximum fresh weight at abscission (Collier, 1997; Jones, 2013). Dahlia may use any of these strategies or it may be that different cultivars use different strategies.

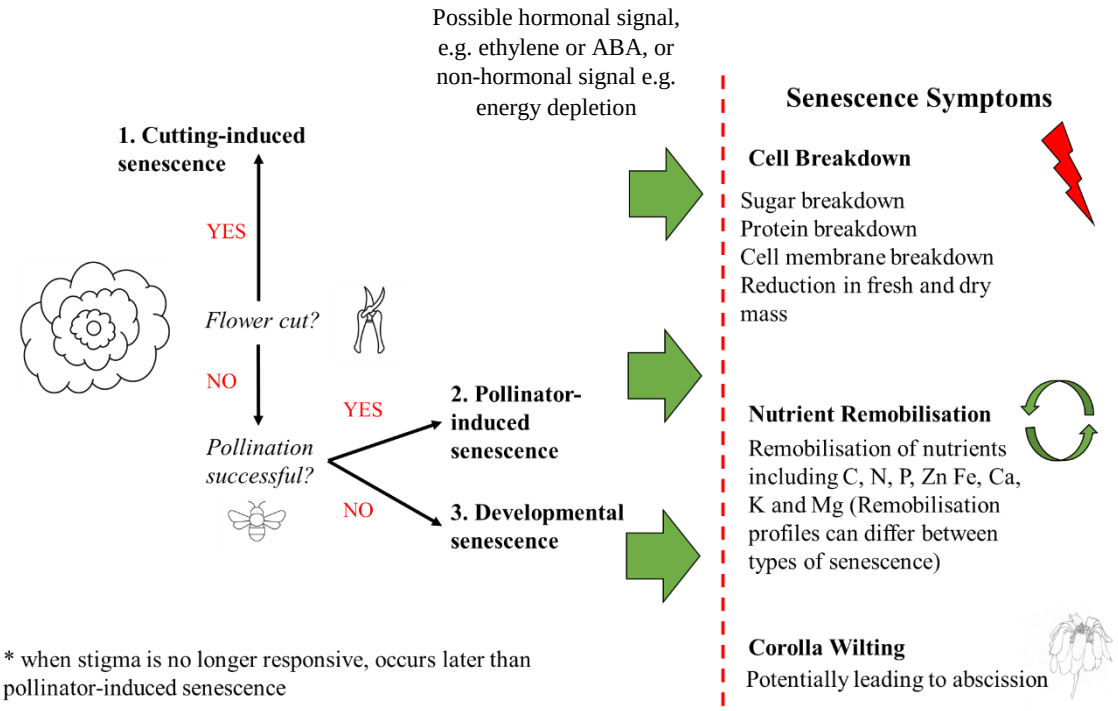


Figure 23: A summarised scheme of types of senescence, and the common processes that lead to symptoms of senescence. Depending on whether flowers are cut or pollinated they may undergo three types of senescence, which may be triggered by a hormonal or non-hormonal signal, which in turn leads to symptoms of senescence, including cell breakdown, nutrient remobilisation and subsequent wilting.

Important questions include how long dahlia flowers last before senescing when left on their parent plants, and whether there is significant variation between dahlia cultivars in this trait. Due to the low likelihood of pollinator-induced senescence in dahlia, this raises the question of whether flower senescence progresses in the same manner in cut flowers

compared to those left on the plant, which go through developmental senescence. Monitoring the loss of mass, the conductivity (and therefore cell membrane breakdown), and protein content loss in uncut flowers compared to cut flowers should help to identify differences in the senescence process between cut dahlia inflorescences and their counterparts left uncut. In cut dahlia, flower senescence is often too rapid a process for them to be sold as cut flowers commercially, therefore it is of interest to discover if dahlia flowers last longer on their parent plant compared to cut flowers. My hypothesis was that dahlia flowers left on the plant would senesce later and at a slower rate than those cut, especially given the lack of pollinator-induced senescence. A further question was whether cultivars which senesce earlier once cut compared to other cultivars, also senesce earlier when left attached to their parent plant.

4.2. Aims

- To examine the progress of senescence in dahlia flowers left on the plant compared to cut dahlia.
- Assess how different the on-plant senescence process is in different dahlia cultivars.
- Discuss the possible causes of any differences between cut and on-plant senescence.

4.3. Materials and Methods

4.3.1. Plant growth and harvesting

Plant material was bought from ‘Rose Cottage Plants’ (Essex, UK). The cultivars ‘Gerrie Hoek’, ‘Karma Prospero’, ‘Onesta’, and ‘Sylvia’ were used for on-plant comparisons. Plants for these experiments were grown at Royal Holloway University of London (Surrey, UK) in 2017 in the manner described in Chapter 2.1. Data for the weather during the growing season can be found in Appendix I. Flowers were tagged at the stage they would normally be cut at, Stage III (see Fig. 4), this was referred to as cutting stage. They were then cut from their parent plant at either 1, 4, or 7 days after being tagged. Five inflorescences were tagged for each time-point of the experiment.

4.3.2. Floret mass

Floret mass was measured as described in 2.3.2. Floret mass was measured 1, 4, and 7d after flowers were cut or in the case of uncut flowers, after they were tagged. Five biological replicates, each representing a single inflorescence, were used for each category, using the average produced by the six individual florets picked from each indivudal inflorescence (Chapter 2.3.2.).

4.3.3. Conductivity

Conductivity was measured as described in 2.3.3. Five biological replicates, each representing a single inflorescence, were used for each category, using the average produced by the three pairs of florets picked from each indivudal inflorescence (Chapter 2.3.3.).

4.3.4. Protein content: extraction and assay

Material for analysis was collected 1, 4, and 7 days after cutting, or in the case of uncut flowers, after they were tagged.

Protein extraction was performed, and relative protein content was measured as described in 2.3.4. There were three technical replicates of each of the three biological replicates for each category, e.g. uncut ‘Karma Prospero’ 4 days after tagging (Chapter 2.3.4.).

4.4. Results

4.4.1. Qualitative comparison of on-plant and cut flowers

Cv. ‘Gerrie Hoek’ (Fig. 24A) showed relatively little difference 7d after cutting stage between cut and uncut flowers. In contrast the cut ‘Karma Prospero’ (Fig. 24Bi) flowers showed significant wilting after 7d whereas uncut flowers (Fig. 24Bii) did not. In ‘Onesta’ the cut flowers (Fig. 24Ci) showed significant wilting after 7d whereas uncut flowers (Fig. 24Cii) showed minimal wilting and colour fading by comparison. In ‘Onesta’, the uncut flowers had also opened significantly more compared to cut flowers. Similarly, cv. ‘Sylvia’ cut flowers (Fig. 24Di) showed greater wilting and had opened less compared to the uncut flowers (Fig. 24Dii). Amongst cut flowers all cultivars except ‘Gerrie Hoek’ showed signs of senescence by d7, whereas amongst uncut flowers only ‘Onesta’ showed wilting after 7d (Fig. 24). This shows that in this study ‘Gerrie Hoek’ senesced the slowest and ‘Onesta’ the fastest, with ‘Karma Prospero’ and ‘Sylvia’ falling in-between.

Figure 24: Cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D) flowers 1, 4, or 7d after cutting stage (left to right) cut from the plant (i) or left uncut (ii). Scale bars represent 20 mm.

4.4.2. Conductivity

In all cultivars except ‘Gerrie Hoek’ the membrane permeability as determined by percentage conductivity of florets 7d after cutting stage was significantrly higher in cut flowers compared to uncut flowers (Fig. 25) However in ‘Gerrie Hoek’ flowers there was significantly higher conductivity between 7d uncut flowers and 1d uncut flowers whereas in cut flowers conductivity did not siginifcantly increase at all over the experimental period (Fig. 25A). In ‘Karma Prospero’ the reverse was found, with conductivity significantly increasing over time in cut flowers but not in uncut flowers over the same time period (Fig. 25B). In ‘Onesta’, cut flowers siginifcantly increased in conductivity after 4d but in uncut flowers there was only significantly higher conductivity 4d after cutting stage, with conductivity declining again after that (Fig. 25C). In ‘Sylvia’ conductivity of cut flowers increased consistently over the experimental period whereas uncut flowers showed the same pattern as in cv. ‘Onesta’, with conductivity increasing at 4d then declining again (Fig. 25D).

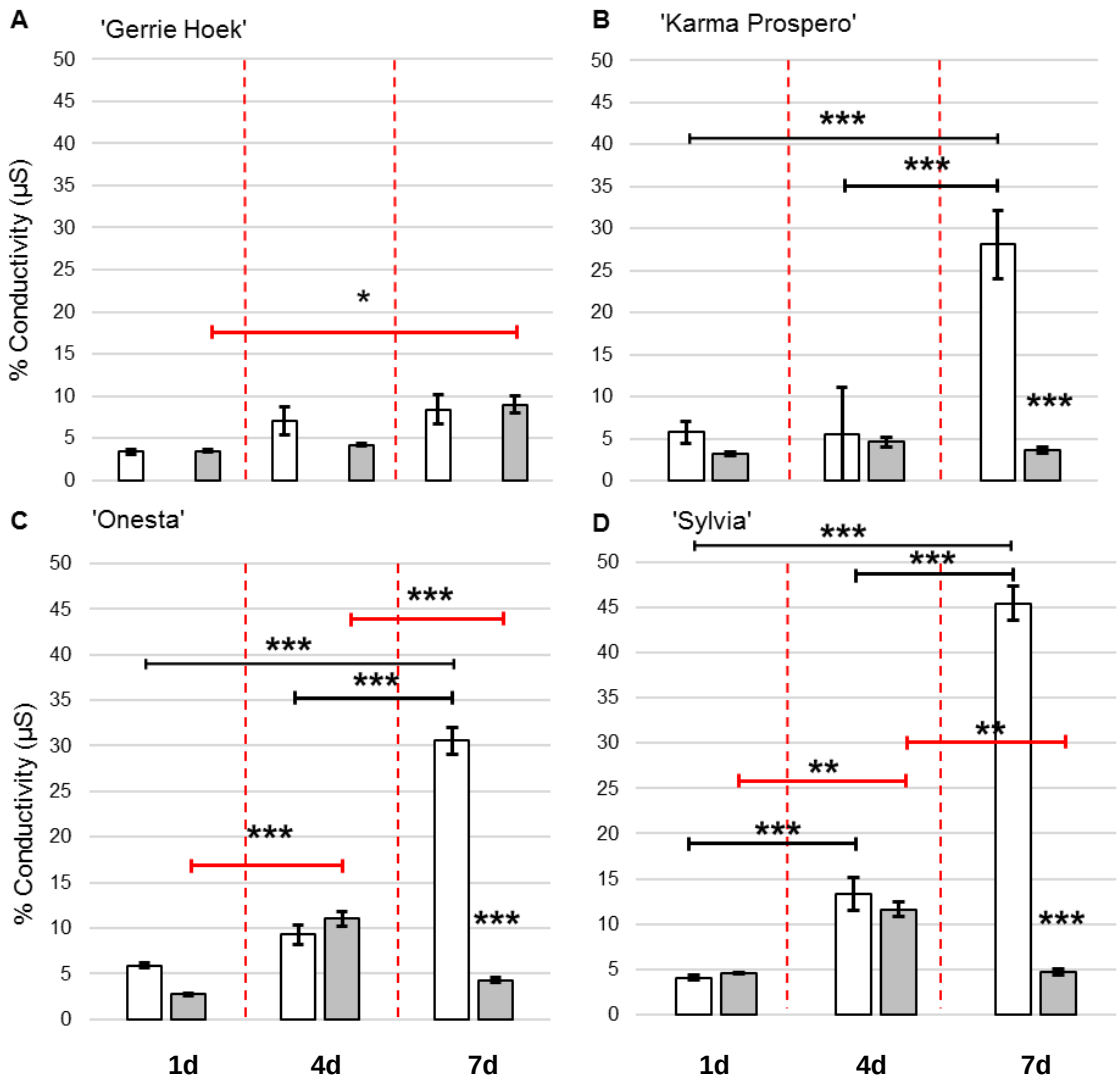


*Figure 25: Membrane permeability, as determined by conductivity of florets after 3 h submersion as a percentage of total conductivity of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D), cut flowers (white) compared to uncut flowers (grey), 1, 4, and 7d after cutting stage (mean ± S.E.; n=5). Black brackets represent significant differences between different timepoints of cut flowers, and red brackets between different timepoints of uncut flowers. Asterisks above bars represent comparison between cut and uncut flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=5).*

4.4.3. Floret Mass

In all cultivars assessed floret mass (mg) was significantly higher in uncut flowers compared to cut flowers 7d after cutting stage (Fig. 26). However, the day at which the mass of uncut flowers diverged from cut flowers varied between cultivars. In ‘Karma Prospero’ (Fig. 26B), ‘Onesta’ (Fig. 26C) and ‘Sylvia’ (Fig. 26D) significant differences were not found until 4d after cutting stage and in ‘Gerrie Hoek’ only 7d after cutting stage (Fig. 26).

Comparing cut or uncut flowers from one day point to the next showed that in ‘Gerrie Hoek’ cut flowers there was a significant decrease in mass between d4 and d7 for cut flowers but not d1 and d4. In uncut flowers of ‘Gerrie Hoek’ there was no significant change at all over 7d (Fig. 26A). In ‘Karma Prospero’, cut flowers showed a significant decrease in mass between 1 and 4d with no further change between 4 and 7d (Fig. 26B) whereas uncut ‘Karma Prospero’ flowers showed no change over 7d (Fig. 26B). In ‘Onesta’, both cut and uncut flowers showed a decline in mass between 4 and 7d (Fig. 26C). Mass of florets from uncut flowers showed a peak mass at d4, which then declined by d7, though at d7 the uncut florets remained significantly heavier than florets from cut flowers (Fig. 26C). In ‘Sylvia’ cut flowers showed a decline in floret mass from d1 onwards, whereas uncut florets showed no significant decline at all over 7d (Fig. 26D).

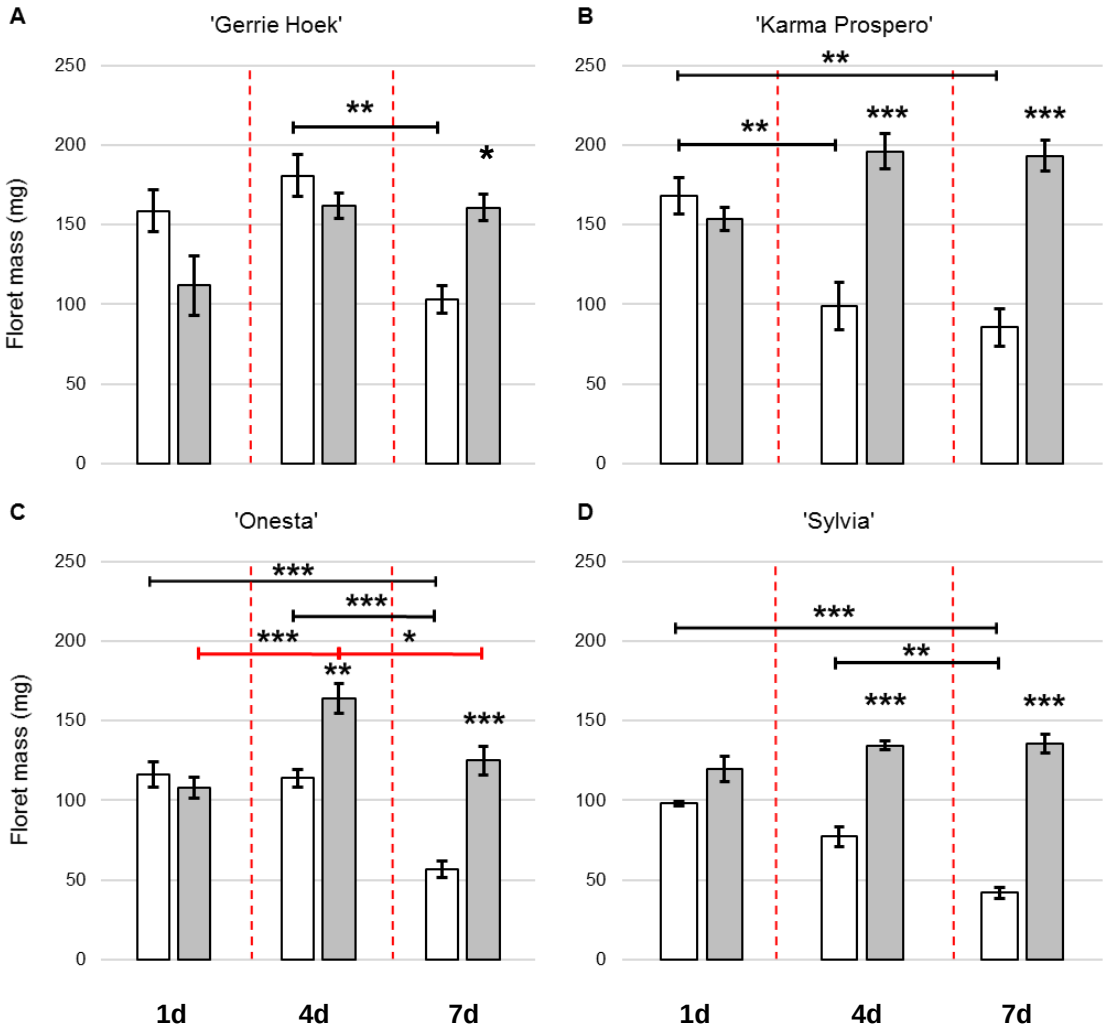


*Figure 26: Mass (mg) of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D) cut flowers (white) compared to uncut flowers (grey) 1, 4, and 7d after cutting stage (mean ± S.E.; n=5). Black brackets represent significant differences between different timepoints of cut flowers, and red brackets between different timepoints of uncut flowers. Asterisks above bars represent comparison between cut and uncut flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=5).*

4.4.4. Protein content

There were no significant differences found in protein content between uncut and cut flowers of cv. ‘Karma Prospero’ on any day and in ‘Onesta’ flowers 4 or 7d after cutting stage (Fig. 27). However, protein content in cut flowers of cv. ‘Onesta’ was significantly higher 1d after cutting stage compared to uncut flowers (Fig. 27B). In ‘Karma Prospero’ cut and uncut flowers’ relative protein content declined significantly between d1 and d7, (Fig. 27A). In ‘Onesta’ uncut flowers, protein content was retained more effectively compared to cut flowers. In ‘Onesta’ cut flowers protein content declined early, significantly decreasing between d1 and d4, but not between d4 and d7 (Fig. 27B). In uncut ‘Onesta’ flowers there was no significant decrease in relative protein content over the 7d experimental period (Fig. 27B).

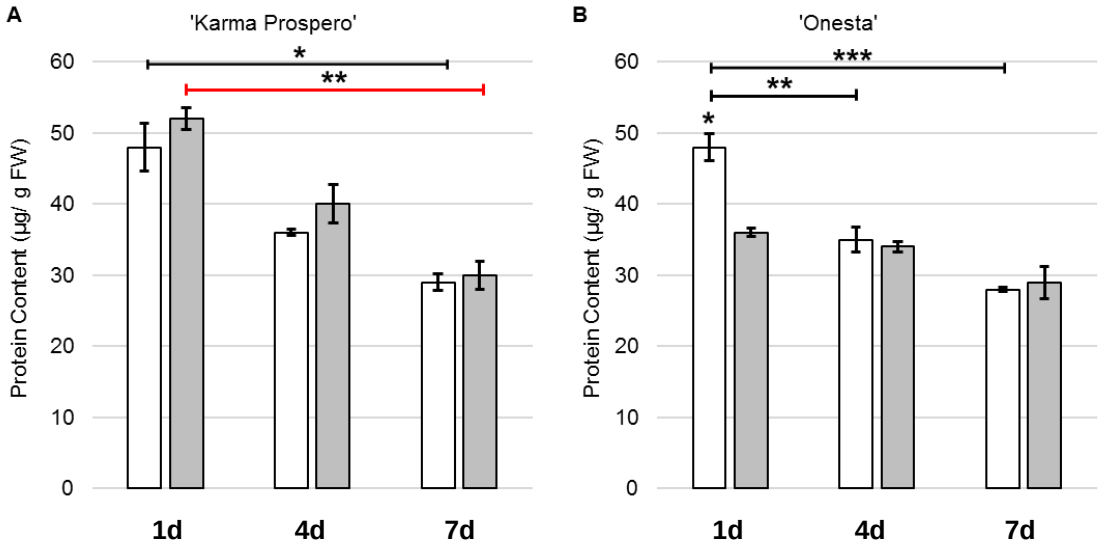


*Figure 27: Protein content (µg/ g FW) of cv. ‘Karma Prospero’ (A) and ‘Onesta’ (B) cut (white) and uncut flowers (grey) sampled 1, 4 or 7d day after cutting stage (mean ± S.E.; n=3). Black brackets represent significant differences between different timepoints of cut flowers, and red brackets between different timepoints of uncut flowers. Asterisks above bars represent comparison between cut and uncut flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (two-way ANOVA, followed by Tukey’s test, n=3).*

4.5. Discussion

The aim of these experiments was to evaluate how senescence on the plant differed from senescence in cut flowers, in particular, whether senescence occurred at the same rate and whether physiological assays could be used to quantify this effectively in dahlia. Measurements of conductivity (Fig. 25), floret mass (Fig. 26), and relative protein content (Fig. 27), along with photos for qualitative assessment (Fig. 24) show that cut flowers senesced at a faster rate compared to uncut flowers. However, there was variation between cultivars in the rate of senescence. Flowers of those cultivars with shorter life span on the plant also showed symptoms of senescence earlier as cut flowers. For example, uncut 'Gerrie Hoek' flowers showed no difference compared to their cut counterparts in wilting after 7 days, whereas cv. 'Onesta' flowers showed severe wilting when cut after 7 days and their uncut flowers were the only ones amongst all cultivars assessed to show wilting within the experimental period (Fig. 24). This suggests that assessments of flowers on the plant may also be helpful in assessing cultivars that will produce cut flowers with a good vase life. The cultivars used in this study, all were either ball, decorative, or water lily types, and all selected for experimentation had no 'daisy eye' features i.e. their disc florets were not visible or available to pollinators. In most domesticated dahlia cultivars, the density of the ray florets obscures and often totally covers the disc florets which bear most if not all the nectar which attracts pollinators (Mani & Saravanan, 1999). After 7 days the cut 'Onesta' flowers had developed daisy eye, as the disc florets had become visible, and would hence encourage pollinators to visit the flowers. However, these were in controlled conditions in the lab with no pollinators. Therefore, in this study it was assumed that pollinator induced senescence was not a significant factor, and all flowers used in the uncut studies progressed to developmental senescence, i.e. they senesced after they became unresponsive to pollinators (Jones, 2002).

The increases in conductivity shown in Fig. 25 indicates greater ion leakage as petals age, and that this increase occurred more quickly in cut flowers. This suggests that cellular membrane breakdown is occurring faster once flowers are cut from their parent plants. This may be because of a premature triggering of developmental senescence, e.g. involving endogenous ethylene, which was delayed in flowers left attached to the parent plant. It is also possible that ABA (abscisic acid) is triggering the senescence process instead as it has been found in ethylene-insensitive gladiolus that treatment with exogenous ABA accelerated senescence and that endogenous ABA accumulated as flowers senescence progressed (Kumar *et al*., 2014). It is possible that this occurs in dahlia

as they have been found to be only marginally sensitive to ethylene in other studies (Woltering & van Doorn, 1988), therefore ABA may be the senescence trigger in dahlia as it has been suggested to be in gladiolus (Kumar *et al*., 2014). In lilies, an ornamental with low sensitivity to ethylene, it has been found that cut flowers showed a 2.2-fold increase in ABA compared with attached flowers in outer tepals (Arrom & Munné-Bosch, 2012a), suggesting that it may possible that ABA is linked to accelerated senescence in cut flowers.

Increases in conductivity are due to cellular membrane breakdown and subsequent ion leakage. This membrane breakdown is due to a variety of lipases which degrade the phospholipids which underpin cellular membrane integrity. Phospholipases do this through the removal of head groups on phospholipids, which make up the cell membrane backbone (van Doorn & Woltering, 2008). Lipolytic acyl hydrolases remove fatty acids from the phospholipid bilayer, whilst lipoxygenases oxidise the double bonds in fatty acids (van Doorm & Woltering, 2008). Breakdown of phospholipids is due to enzymes, which include phospholipase D and C (PLD & PLC), lipolytic acid hydrolase (PLA1 & PLA2) and lipoxygenase (van Doorn & Woltering, 2008). It is known that phospholipases are upregulated during senescence and suppression of them can retard senescence (Jia & Li, 2015). ABA has been found to increase activity of PLD in barley (Ritchie & Gilroy, 1998), and it has been suggested that PLD is upregulated during both ABA and ethylene mediated senescence in *Arabidopsis* (Fan *et al*., 1997). The steep rise in conductivity in cut flowers of some of the dahlia cultivars between 4 and 7 days after cutting suggests that following cutting the change in conditions may have led to a signal which could have activated these enzymes downstream and triggered phospholipid breakdown, though more work needs carrying out to confirm if this is the case. Furthermore, ABA and ethylene have been shown to regulate this process and promote expression of PLA's, this suggests that following cutting there may be an increase in either ethylene or ABA (Jia *et al*., 2013). Suppression of PLA has been shown to suppress both ethylene and ABA promoted senescence suggesting that phospholipid breakdown is a major hallmark of senescence mediated by these phytohormones and that they could be implicated in the more rapid onset of senescence seen in the cut flowers in this study.

It may be that differences in the timing of upregulation of lipases may be the reason for the variation between cultivars, as cv. 'Onesta' (Fig. 25C), 'Sylvia' (Fig. 25D) and 'Karma Prospero' (Fig. 25B) flowers did not show a significant difference between cut and uncut flowers in membrane permeability until d7. In contrast, 'Gerrie Hoek' (Fig.

25A) flowers showed no significant difference regardless of whether they were cut or left attached, this may be because the upregulation of lipases occurred outside of the experimental period. In future studies of this cultivar an extended experimental period would be beneficial. Unfortunately, it is not yet known what the main trigger of dahlia senescence is but it is possible that one if not both ABA and ethylene are involved, and there is evidence to suggest ethylene does have a role in floral senescence in at least some dahlia cultivars (Dole *et al*., 2009; Shimuzu-Yumoto & Ichimura, 2013). It has been found in species including dahlia (Dole *et al*., 2009; Shimuzu-Yumoto & Ichimura, 2013; Woltering & van Doorn, 1988), gerbera (Woltering & van Doorn, 1988), and chrysanthemum (Doi *et al*., 2003) that there can be variation in ethylene sensitivity between different cultivars of these composite species. Whether or not variation in endogenous biosynthesis of ABA is present in dahlia is not known, but it could be the variation in sensitivity to ethylene or ABA that led to differences in timing of membrane breakdown.

It is worth noting that conductivity of uncut cv. 'Onesta' and 'Sylvia' (Fig. 25C, D) flowers was higher 4 days after cutting stage compared to flowers 1 and 7d after cutting stage. It is unlikely that cellular membranes were repaired between 4 and 7d after cutting stage as once membrane breakdown is occurring senescence is in a terminal phase and is irreversible (Thomas, 2013). This suggests that the flowers harvested 4d after cutting stage in the aforementioned cultivars were damaged by inclement weather relatively soon before harvesting, perhaps explaining why conductivity in 'Onesta' and 'Sylvia' uncut flowers rose and then fell again over the experimental period. Flowers damaged by pests were excluded from the study and this is not considered a likely factor.

However, it is still unknown what triggers senescence in ethylene insensitive flowers or what triggers ethylene sensitivity in flowers that remain unpollinated. It is possible that the developmental senescence of the dahlias in this study was independent of phytohormones and instead was triggered by nutrient deprivation. In the very early stages of tulip senescence it has been found that PCD is triggered by intracellular energy depletion which served as a signal for starting senescence rather than ethylene (Azad *et al*., 2008). This would suggest that the senescence trigger in the cut dahlia could have been caused by the depletion of energy stores which cannot be replenished as a cut flower, unlike those left attached to the parent plant. Moreover, it has been found in *Campanula medium* that ethylene is not as important in the senescence of unpollinated flowers compared to pollinated flowers. Instead, it was wilting of the pedicel in the unpollinated

C. medium flowers that reduced water absorption which in turn led to petal wilting (Kato *et al*., 2002). It is possible that the earlier increases in conductivity (Fig. 25) and the earlier wilting (Fig. 24) observed in cut dahlia was due to peduncle wilting and reduced fluid uptake compared with attached flowers. If the senescence process observed in this study was due to energy depletion or inhibition of nutrient or fluid uptake then cultivar variation may also be related to these factors. The rate of peduncle wilting may have varied or it may be because some cultivars had greater stores of carbohydrates than others to draw on after being cut from the parent plant.

Comparison of dahlia flower appearance (Fig. 24) shows that in all cultivars except 'Gerrie Hoek', outer florets began to wilt earlier in cut flowers compared to uncut flowers. Furthermore, in 'Gerrie Hoek' there was no significant difference in conductivity between uncut and cut flowers after 7 days and a difference in floret mass occurred later in 'Gerrie Hoek' than in any other cultivar (Fig. 25; Fig. 26). In all other cultivars assessed there were earlier differences in mass, conductivity, and wilting between cut and attached flowers (Fig. 24; Fig. 25; Fig. 26). From their appearance it appears that 'Gerrie Hoek' cut flowers (Fig. 24Ai) fared better than any other in postharvest quality, whilst 'Onesta' fared the worst with both cut and uncut flowers showing signs of wilting by day 7 (Fig. 24C). The variation in cultivars may be due to varying sensitivity to phytohormones mediating senescence or because of differences in rate of energy depletion or size of energy stores.

All cultivars showed no significant difference in conductivity between uncut and cut flowers until at least 7 days after cutting stage (Fig. 25). However, differences in mass between the two groups (cut and uncut) occurred earlier than differences in conductivity in all cultivars tested (Fig. 26). This suggests that the increase in remobilisation of macromolecules and/ or water loss in cut flowers compared to in uncut flowers occurs earlier than an increase in membrane breakdown. Furthermore, floret mass in cut flowers had decreased 7 days after cutting stage but there was no change in mass in uncut flowers in all cultivars except 'Onesta' which showed an increase up to day 4 after cutting stage before declining (Fig. 26). This suggests that membrane breakdown occurred particularly early in cut flowers of cv. 'Onesta', even before significant water loss or macromolecule loss. Furthermore, because uncut 'Onesta' flowers began decreasing in mass while all other cultivars continued to show increases, this suggests this cultivar has a more rapid floral senescence process compared with other cultivars.

Loss of mass is a commonly known feature of senescence. In cut flowers, significant decreases in mass occurred between 1 and 4 days in ‘Karma Prospero’ florets but ‘Sylvia’, ‘Gerrie Hoek’ and ‘Onesta’ began to decline in mass between days 4 and 7 following cutting (Fig. 26). However uncut flowers showed no significant decrease in mass up to 7 days after cutting stage, except for ‘Onesta’ where uncut floret mass declined between days 4 and 7, however at day 7 uncut mass was higher than in cut flowers (Fig. 26). This shows the expected trend that typical senescence processes such as cell breakdown, remobilisation and water loss are occurring sooner in cut flowers compared to uncut flowers.

Abscission was not observed during the experimental period, however mass continued to decrease in cut flowers as they senesced. By day 7 all cultivars had shown a decline in cut flower floret mass of between 35 and 57% compared to day 1 florets without abscission occurring (Fig. 26). In this study all dahlia cultivars followed a similar pattern to one another with floret mass in cut flowers declining significantly by day 7 in ‘Gerrie Hoek’, ‘Onesta’ and ‘Sylvia’ and by day 4 ‘Karma Prospero’ (Fig. 26). Hence, we can exclude, at least for the dahlia cultivars tested, that abscission occurs when their florets are at their maximum fresh weight. However longer time periods would be needed to assess how far loss of fresh weight goes at abscission, and whether at abscission differences between cultivars become evident. In cut flowers of ‘Karma Prospero’ this decline in mass plateaued between days 4 and 7, but in all other cultivars there was a significant decline between days 4 and 7 (Fig. 26). It is possible that they too would have plateaued in a longer timeframe, but this would require further work. Dahlias may fall into the intermediate category of fresh weight loss before abscission, similarly to *Alstroemeria*, which have been found to show some signs of senescence before abscission and lose 20-60% of their maximum fresh weight by the abscission stage (all dahlia cultivars in this study showed a decline in cut flower floret mass of between 35 and 57%), and given the relatively long time it takes dahlias to lose fresh weight compared to daylilies (Bieleski, 1995; Collier, 1997; Jones, 2013). In this study only uncut ‘Onesta’ showed a decrease in mass whilst still on the plant between 4 and 7 days (Fig. 26). The uncut ‘Onesta’ flowers also show slight wilting after 7 days which was not observed in any other cultivar (Fig. 24C). This suggest that senescence occurs earlier in uncut ‘Onesta’ than in other cultivars and that given enough time uncut flowers would also decline in mass in a similar manner to cut flowers, however this would require a longer experimental period to draw any conclusions.

Protein content declined more slowly in uncut flowers than in cut flowers in cv. 'Onesta', whereas in cv. 'Karma Prospero' both cut and uncut flowers declined in protein content between d1 and d7 (Fig. 27). This suggests, like the results in mass and conductivity, that macromolecule breakdown is occurring sooner in flowers that have been cut in cv. 'Onesta'. This suggests more than just a failure in cellular maintenance and that it is likely that proteases are being upregulated. Whether or not senescence specific proteases are being upregulated in dahlia is unknown, but it is likely that serine and cysteine proteases are implicated as they are known to be the proteases most heavily involved in the senescence processes of other cut flowers (van Doorn & Woltering, 2008). Protein breakdown is mostly due to protease activity and it is known that some senescence associated cysteine proteases are activated by ethylene responsive genes (Jones *et al*., 2005; Tripathi *et al*., 2009).

In both cut and uncut flowers of cv. 'Karma Prospero' there was only a significant difference in protein content between d1 and d7 flowers, suggesting a gradual drop in protein content as d7 flowers were still not significantly different from those on d4 (Fig. 27A). It also suggests that decrease in protein content don't necessarily lead to visible changes such as wilting, as uncut 'Karma Prospero' flowers did not show wilting 7d after cutting stage (Fig. 24Bii). The flowers may be remobolising other nutrients, including carbon incorporated as sugars, as well as phosphorus, potassium and magnesium (Jones, 2013; Eason *et al*., 2000; van Doorn & Woltering, 2008).

In 'Onesta' the uncut flowers showed no significant drop in protein content during the experimental period. In contrast, the cut flowers showed a significant drop between 1 and 4d which then levelled off between 4 and 7d (Fig. 27), suggesting that protein breakdown occurs early in cut 'Onesta' flowers, consistent with their rapid wilting (Fig. 24C), loss of mass (Fig. 26C) and rise in conductivity (Fig. 25C). The protein content decline accompanied with loss of mass in cut flowers could be due to the onset of senescence and increased protease activity (Jones *et al*., 2005; Tripathi *et al*., 2009), or differences in remobilisation profiles (Bieleski, 1995; Jones, 2013) between the two groups. Remobilisation of proteins has been shown to be more efficient in flowers left attached to the parent plant than in cut flowers and the more gradual reduction in protein content in uncut flowers in 'Onesta' may be reflecting this (Bieleski, 1995; Jones, 2013).

Overall it is possible that a signal, possibly ethylene or ABA (Jones, 2008; Kumar *et al*., 2014) or a non-hormonal signal such as stress induced by energy depletion (Azad *et al*., 2008) following cutting is leading to premature senescence in cut flowers compared to

uncut flowers, leading to earlier signs of senescence in cut flowers including loss of mass, protein loss, increases in membrane permeability, and wilting, though the onset of senescence symptoms varied between cultivars. In this study cut dahlia flowers senesced at a faster rate than those left on their parent plant suggesting there is potential for dahlias as cut flowers if their lifespan can be extended to match their attached counterparts.

5. The role of ethylene in *Dahlia* flower senescence

5.1. Introduction

The phytohormone ethylene is ubiquitous in the stress responses of higher plants and endogenous ethylene production modulates the processes of PCD and senescence following stress events (Wang *et al*., 2013c; Iqbal *et al*., 2017). These can include both abiotic and biotic stress, due to grazing or infection, or be generated in response to the plants' own life cycle and needs, as is the case in flower senescence where the plant remobilizes nutrients from defunct tissue to developing tissue.

As a simple molecule many compounds were originally hypothesized to be the precursor of ethylene, however it has been established that S-adenosylmethionine (SAM) and ACC (1-aminocyclopropane-1-carboxylic acid) are ethylene's molecular precursors (Yang & Hoffman, 1984; Fig. 21). Ethylene biosynthesis is initiated by the precursor ACC which triggers a mitogen-activated protein cascade, known as the MAPK cascade, which induces the activation and phosphorylation of the enzyme ACC synthase (ACS), which in turn converts the molecule SAM to ACC which is then converted by ACC oxidase (ACO) to ethylene (Ouaked *et al*., 2003; Fig. 21). This system functions as a positive regulator of the ethylene response pathway (Joo *et al*., 2008; Liu & Zhang, 2004). The phosphorylated ACC synthase is then stabilized and subsequently enhances the endogenous biosynthesis of ethylene (Liu & Zhang, 2004; Iqbal *et al*., 2013). The enzymes ACS and ACO are well known in their role as synthesizers of ethylene, which itself is strongly implicated in the flower senescence process of many cut flower species including rose (Hashemabadi *et al*., 2014), carnation (Asil *et al*., 2012; Yu & Bao, 2004), gentians (Shimizu-Yumoto & Ichimura, 2012) and petunia (Whitehead *et al*., 1984). The gene *ACS6* is known to be induced during leaf senescence in *A. thaliana* (Miller *et al*., 1999, Kim *et al*., 2008) and the gene encoding the ethylene forming enzyme *ACO4*, is most highly expressed in senescent leaves of *A. thaliana* (Winter *et al*., 2007; Schmid *et al*., 2005). However, the question of whether floral senescence in dahlia is ethylene-regulated remains unresolved (Woltering & van Doorn, 1988). ACS is the rate limiting enzyme of this reaction (Yang & Hoffman, 1984). Increased expression of *ACO4* and *ACS6* during dahlia senescence could indicate a role for ethylene during senescence that postharvest treatments have not adequately defined so far.

Ethylene binds to receptors on the endoplasmic reticulum membrane (Chen *et al*., 2002; Fig. 21). A family of ethylene receptors related to histidine kinase receptors (HSK's)

respond to ethylene, including ETR1. Ethylene induced inactivation of ETR1 leads to phosphorylation of EIN2 which in turn prevents degradation of the transcription factors EIN3/EIL1 necessary for the activation of ethylene response factors (ERF's) (Wen *et al*., 2012; An *et al*., 2010; Solano *et al*., 1998; Fig. 21). Activation of ERF's leads to ethylene responses which in senescence include protease upregulation, DNAse and RNase upregulation, nuclease upregulation, and upregulation of genes implicated in nutrient remobilisation and cell membrane breakdown (Jones *et al*., 2005; Langston *et al*., 2005; Chapin & Jones, 2009; Hong *et al*., 2000). It has been found in *A. thaliana* that ETR1 is the main receptor affected by STS, the chemical widely used to delay flower senescence, suggesting that it is this receptor that is most important in sensing ethylene in the senescence pathway (McDaniel & Binder, 2012).

In many ornamentals, inhibiting ethylene biosynthesis has been found to delay flower senescence. Inhibition of ACC oxidase in *Gentiana scabra* had no delaying effect on senescence but inhibiting ACC synthase, the rate limiting enzyme of the reaction, slightly delayed petal senescence in the plant (Shimizu-Yumoto & Ichimura, 2012; Yang & Hoffman, 1984; Fig. 28). Transgenic carnations with a transformed and non-functioning ACO, had a 95% reduction in ethylene production, and consequently a vase life 5 days longer than that of control flowers (Yu & Bao, 2004; Fig. 28). However, in the horticultural industry inhibiting ethylene reception is the most common method of delaying ethylene induced senescence. Two main chemicals are used commercially to inhibit ethylene reception: STS and 1-MCP (Veen, 1979; Serek *et al*., 1995). STS is a common postharvest treatment used in the cut flower industry to prolong vase life. The chemical functions as an inhibitor of ethylene by blocking the receptors (Veen, 1979). In ethylene sensitive species treatment with STS may greatly prolong vase life, whereas in other species treatment has little or no effect (Woltering & van Doorn, 1988).

Exogenous ethylene reduces vase life in ethylene sensitive flowers. Exogenous ethylene has been observed reducing vase life through wilting or abscission in many cut flower species including *Alstroemeria*, *Cymbidium*, *Campanula*, carnation, geranium, rose, *Delphinium* and *Antirrhinum* (Woltering & van Doorn, 1988; Fig. 28). This study found no Asteraceae species with ethylene sensitivity classified as intermediate or above, although later studies have contested that there is marked variation in sensitivity within species of this family including dahlia and chrysanthemum (Doi *et al*., 2003; Shimizu-Yumoto & Ichimura, 2013; Dole *et al*., 2009). This suggests that experiments need to assess a large range of cultivars for ethylene sensitivity. Modern dahlia cultivars are

hybrids of two wild ancestors, both tetraploids (Schie *et al*., 2014; Lawrence & Scott-Moncrieff, 1935) with different colour ranges. However, the dahlia's close relative chrysanthemum has also been shown to exhibit sensitivity to ethylene among different varieties (Doi *et al*., 2003). Another factor that may affect the assessment of ethylene sensitivity in dahlia is the dose, or duration of exposure to ethylene. In *Campanula medium*, exposure to ethylene needs to be more prolonged than has previously been carried out in dahlia to trigger wilting (Kato *et al*., 2002).

Experiment		***Species***	***Effect***
Exogenous ethylene	→	*Alstroemeria, Cymbidium, Campanula*, carnation, geranium, rose, *Delphinium, Antirrhinum,* dahlia, chrysanthemum	Premature wilting or abscission
Inhibition of ACC oxidase	→	*Gentiana scabra*	Slightly delayed petal senescence
Transformed and non-functioning ACC oxidase	→	Carnation	Reduced ethylene production by 95%, consequent 5 day increase in vase life

Figure 28: Scheme showing the reaction of relevant plant species to either exogenous ethylene, inhibition of an ethylene biosynthetic enzyme or a transformed and non-functional ethylene biosynthetic enzyme (Shimizu-Yumoto & Ichimura, 2012; Woltering & van Doorn, 1988; Yu & Bao, 2004).

Ethylene is known to regulate senescence partly through induction of proteases which carry out a key component of cellular breakdown (Jones *et al*., 2005). In petunia it has been found that an increase in ethylene correlates with an increase in senescence associated cysteine proteases (Jones *et al*., 2005). Furthermore, the transcript of cysteine protease *RbCP1* in *Rosa bourboniana* was found to accumulate just 30 min after ethylene treatment, showing its expression is ethylene responsive and that the response is very fast (Tripathi *et al*., 2009). Additionally, the gene's expression was reduced by treatment with 1-MCP leading to a delay in petal abscission, and a large decrease was observed in protein content during both ethylene-induced and developmental abscission (Tripathi *et al*., 2009).

We can hypothesize that treatment of dahlia with an ethylene inhibitor such as STS would delay protein content reduction compared to control flowers or those treated with exogenous ethylene in cultivars that are ethylene sensitive. This would provide additional information on ethylene's role in senescence of these cultivars if conducted alongside vase life measurements based just on observation of petal wilting. However, examining protein content in dahlia floral tissue of different ages and in flowers treated with CEPA (chloroethylphosphonic acid), an ethylene releasing compound, STS, or distilled water may allow us to identify differences in the rate of protein breakdown between cultivars. It has been found that both ethylene sensitive and insensitive petunias showed increases in proteolytic activity and a reduction in protein content during flower senescence, however ethylene was linked to the temporal regulation of these changes (Jones *et al*., 2005). The insensitive flowers' senescence was delayed by 8 days, and this delay was replicated in the proteolytic activity and protein content of these flowers (Jones *et al*., 2005). Therefore, it seems that in ethylene sensitive species or cultivars, ethylene triggers the anticipated proteolytic activity, presumably as a component of the senescence program that is activated.

Sugar treatment is known to prolong the vase life of many cut flowers including tulip (Iwaya-Inoue & Nonami, 2003), lily (Arrom & Munné-Bosch, 2012b), and gladiolus (Otsubo & Iwaya-Inoue, 2000), though is more effective in ethylene sensitive flowers compared to insensitive flowers (van Doorn, 2004). However, there are exceptions, in the ethylene insensitive species *Sandersonia aurantiaca* addition of exogenous sugars led to a delay in the expression of genes involved in remobilisation of proteins and fatty acids, markers of petal senescence (Eason *et al*., 2002), and in ethylene insensitive cut lily flowers sucrose treatment has also been found to delay flower senescence, though it did not affect tepal abscission (Arrom & Munné-Bosch, 2012b). In carnation sucrose delayed the increases in transcript abundance of almost all SAG's (senescence associated genes), in a manner similar to STS treatment (Hoeberichts *et al*., 2007). Studies have been carried out examining the effect of sugars on dahlia vase life. These have shown that sucrose treatments were ineffective in lengthening dahlia vase life, however each study only looked at one cultivar each (Lukasweska, 1980; Dole *et al*., 2009) so evaluation of more cultivars could give a better view of whether sucrose can improve vase life in dahlia. During ethylene sensitive flower senescence high sugar levels promote the proteasomal degradation of the key ethylene signalling transcription factor EIL3 (Yanagisawa *et al*., 2003). As discussed above, EIL3 is necessary for the translation of the ethylene signal to

an ethylene response by triggering expression of ethylene-responsive genes (Hoeberichts *et al*., 2007). In highly ethylene sensitive carnations, treatment with sugars suppressed the effect of exogenous ethylene (Yanagisawa *et al*., 2003; Hoeberichts *et al*., 2007), suggesting that the marginally ethylene sensitive dahlia may be less responsive to sugar treatments or that the response to sugars may vary in accordance with variation in ethylene sensitivity.

The reaction of cut dahlia flowers to treatments with exogenous ethylene or chemicals which inhibit ethylene production or signalling would allow us to assess whether any of these treatments might be useful commercially, or whether more novel approaches are required if such traditional methods are consistently unsuccessful. Questions also remain regarding the endogenous biosynthesis of ethylene. Does the reaction of a dahlia cultivar to a postharvest treatment correlate with the expression of ethylene biosynthetic genes and hence ethylene as an endogenous regulator of floral senescence, modulating cell breakdown and remobilisation of nutrients during senescence?

5.2. Aims

- Establish ethylene sensitivity across arrange of dahlia cultivars of differing floral morphologies.
- Establish the effectiveness of common postharvest treatments relating to ethylene in dahlias and the effects of these treatments on the senescence process.
- Investigate whether genes related to ethylene signalling or biosynthesis change in expression in dahlia flower senescence.

5.3. Materials and Methods

5.3.1. Plant Growth and Harvesting

Plant material was bought from ‘Rose Cottage Plants’ (Essex, UK). The cultivars ‘Boom Boom Yellow’, ‘Gerrie Hoek’, ‘Karma Prospero’, ‘Onesta’, and ‘Sylvia’ were used for experiments. Plants for these experiments were grown at Royal Holloway University of London (Surrey, UK) in 2017 in the manner described in Chapter 2.1. Flowers were cut at Stage III (see Fig. 4).

5.3.2. Postharvest treatments

Postharvest treatments included STS (silver thiosulphate), sucrose and CEPA (ethephon) (Chloroethylphosphonic acid). Chemical sources and treatments were carried out as described in Chapter 2.2. Inflorescences were pulsed with 4 mM STS for either 15 min, 30 min or 1 h before being removed to distilled water. Stems were cut to a length of 5 cm. All controls were placed in distilled water following cutting for the duration of the experimental period. Five whole flower replicates were used for each treatment.

5.3.3. Floret mass

Floret mass was measured as described in 2.3.2. Five biological replicates, each representing a single inflorescence, were used for each category, using the average produced by the six individual florets picked from each indivudal inflorescence (Chapter 2.3.2.).

5.3.4. Conductivity

Conductivity was measured as described in 2.3.3. Five biological replicates, each representing a single inflorescence, were used for each category, using the average produced by the three pairs of florets picked from each indivudal inflorescence (Chapter 2.3.3.).

5.3.5. Protein content: extraction and assay

Material for analysis was collected 1, 4, and 7 days after cutting.

Protein extraction was performed as described in 2.3.4. Protein content was measured as described in 2.3.4. There were three technical replicates of each of the three biological

replicates for each category, e.g. control (dH_2O) ‘Onesta’ flowers, 7 days after cutting (Chapter 2.3.4.).

5.3.6. RNA extraction

RNA extractions were performed using the NucleoSpin® RNA Plant kit (Macherey-Nagel) as described in 2.4.2. Quality of RNA and gDNA Wipeout was performed as described in 2.4.3. and 2.5.2. Three biological replicates were used, as described in 2.4.

5.3.7. Polymerase chain-reaction

Primer design, reverse transcription of RNA, gDNA Wipeout, PCR, sequencing of products and PCR analysis were all performed as previously described in Chapter 2.5. Three technical replicates of each of three biological replicates were used for each category, e.g. ‘Sylvia’ flowers treated with a 1h pulse of 4 mM STS 1 days after cutting with *DvACO4* primers.

The genes *DvACO4* and *DvACS6* were chosen for RT-qPCR from the differential expression analysis data because amongst *ACO* homologs in the dataset the *A. thaliana ACO* closest in homology to the dahlia *ACO*’s was the ethylene forming enzyme *ACO4*, which is most highly expressed in senescent leaves in *A. thaliana* (Winter *et al*., 2007; Schmid *et al*., 2005). Amongst dahlia sequences homologous to *ACS* those most highly upregulated were closest in homology to *A. thaliana ACS6*, a gene known to be induced during leaf senescence in *A. thaliana* (Miller *et al*., 1999, Kim *et al*., 2008). The *Dahlia variabilis* (domesticated dahlia) genes were referred to as *DvACO4* and *DvACS6* as in the manner of Suzuki *et al*., 2002, and Ohno *et al*., 2011 and 2013.

5.4. Results

5.4.1. Vase Life

Vase life was significantly improved compared to controls by a 1 h pulse with 4 mM STS in all cultivars tested except ‘Onesta’ (Fig. 29). A treatment with a combination of a 1 h pulse with 4 mM STS and a 100 µM BA spray improved vase life in all cultivars tested including ‘Onesta’, however ‘Onesta’ still did not show a vase life higher than any other cultivar (Fig. 29). A continuous solution of 20 µM CEPA made no significant difference to vase life any of the cultivars tested except ‘Gerrie Hoek’, which showed a significant reduction of vase life when treated (Fig. 30). Mean vase life of ‘Karma Prospero’ and ‘Onesta’ was also shorter with CEPA treatment, however the difference was not statistically significant compared to the control. A variety of different time pulses of 4 mM STS were tested on cv. ‘Onesta’ flowers but even a 1 h pulse used in conjunction with 5% sucrose solution following the pulse made no difference to ‘Onesta’ vase life (Fig. 31).

7 days ‘Gerrie Hoek’ flowers treated with CEPA were very wilted (Fig. 33B) compared with controls (Fig. 33A), STS pulsed (Fig. 33C) or STS pulsed and BA sprayed (Fig. 33D) flowers. After 7 days STS or STS and BA treated flowers were similar in appearance to controls, though STS pulsed flowers opened more fully compared to those pulsed with STS and sprayed with BA. After 7 days CEPA treated ‘Karma Prospero’ flowers showed the most wilting, and also showed floret browning, however control flowers were also significantly wilted by day 7 (Fig. 34 A, B). Flowers treated with STS or treated with STS and BA combination showed no wilting after 7 days (Fig. 34 C, D). CEPA treated ‘Onesta’ flowers were significantly wilted by day 7 and showed signs of wilting by day 4 and appeared more wilted and shrivelled compared with ‘Onesta’ flowers of any other treatment (Fig. 35B). Control flowers also showed wilting by day 7 but this was not as extensive compared with CEPA treated flowers (Fig. 35A). STS treated flowers were wilted by day 7, performing similarly to controls, but did not show the same level of wilting as CEPA treated flowers (Fig. 35C). ‘Onesta’ flowers treated with an STS pulse in conjunction with a BA spray showed no wilting after 7 days (Fig. 35D). CEPA treated ‘Sylvia’ flowers showed slight wilting 4 days after cutting, whereas controls only showed wilting after 7 days (Fig. 36A, B). CEPA treated ‘Sylvia’ flowers showed extensve wilting and browning and discolouration after 7 days (Fig. 36B). Flowers treated with an STS pulse with or without a BA spray showed no wilting after 7 days (Fig. 36C, D).

None of the cultivars tested showed a significant increase in vase life following treatment with sucrose (Fig. 32). In cv.'s 'Sylvia' and 'Karma Prospero' none of the sucrose treatments had any effect on vase life (Fig. 32B, D), whereas in cv. 'Gerrie Hoek' both 2% and 5% sucrose treatments had a negative effect on vase life (Fig. 32A). Sucrose treatments on cv. 'Onesta' cut flowers had a negative effect on vase life and this effect was more significant in flowers treated with 5% sucrose compared to those treated with 2% sucrose (Fig. 32C).

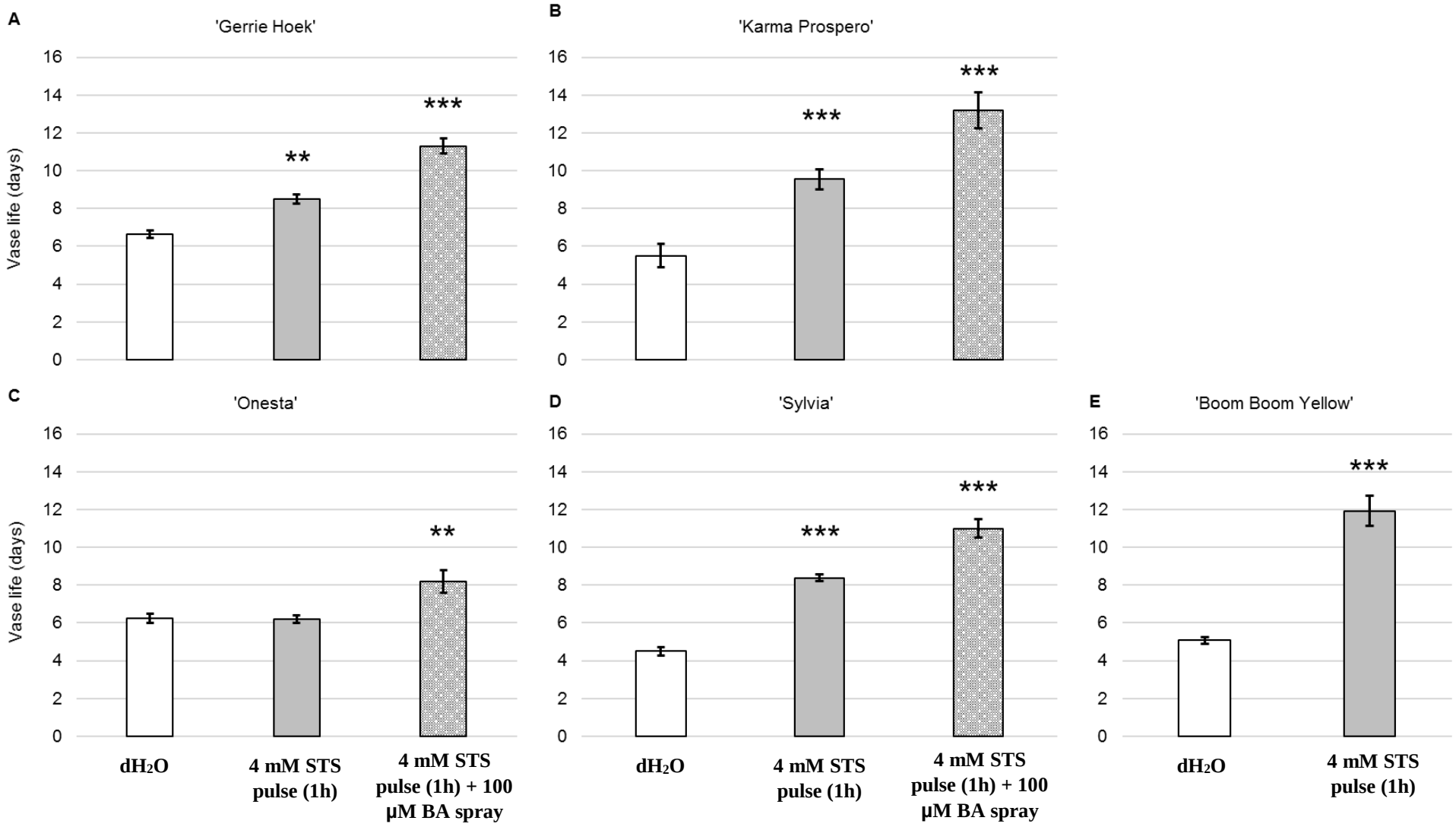

A
'Gerrie Hoek'
B
'Karma Prospero'
C
'Onesta'
D
'Sylvia'
E
'Boom Boom Yellow'
Vase life (days)
0
2
4
6
8
10
12
14
16
**
***
dH2O
4 mM STS pulse (1h)
4 mM STS pulse (1h) + 100 µM BA spray

*Figure 29: Vase life (days) of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), 'Sylvia' (D) and 'Boom Boom Yellow' (E), as control (dH₂O) flowers (white) compared to 1 h pulse 4 mM STS treated (grey) or 1 h pulse 4 mM STS combined with 100 µM BA spray treated flowers (patterned) (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * p < 0.05, ** p < 0.01, or *** p < 0.001 (2-sample t-test, n=5).*

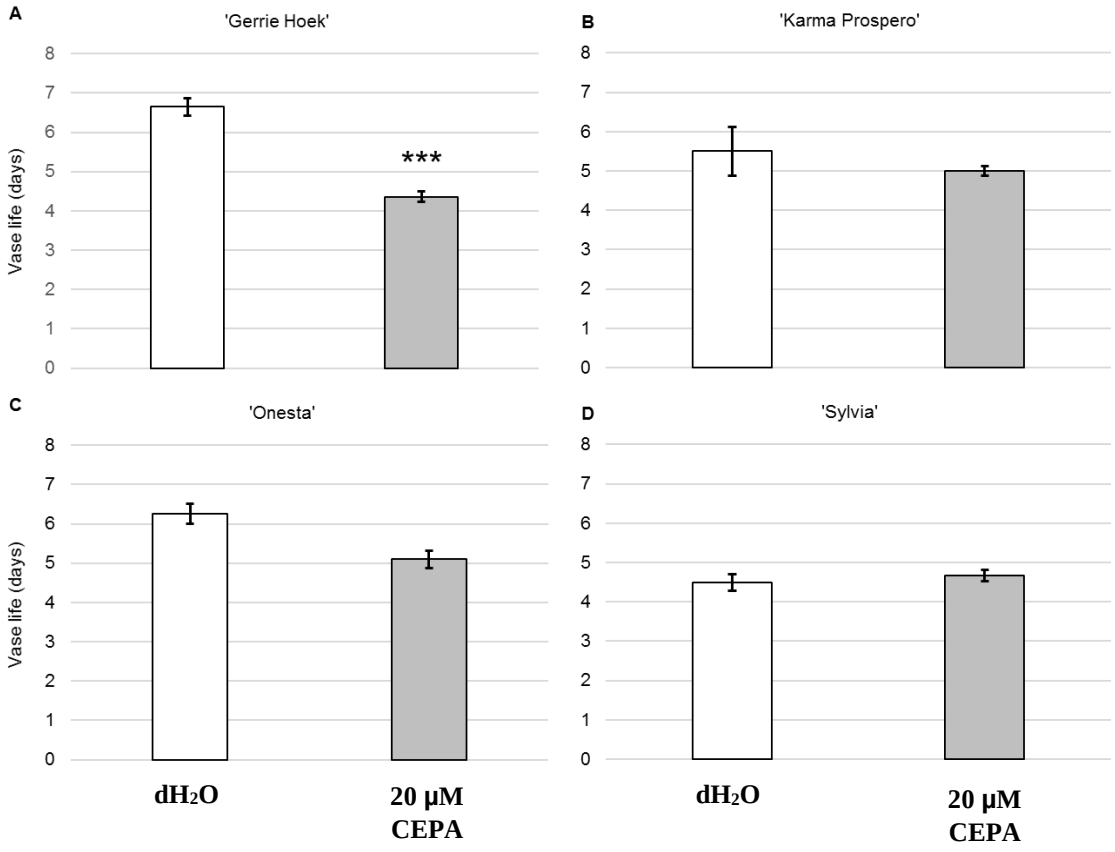


*Figure 30: Vase life (days) of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D) as control (dH₂O) flowers (white) compared to 20 µM CEPA (continuous solution) treated (grey) flowers (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * p < 0.05, ** p < 0.01, or *** p < 0.001 (2-sample t-test, n=5).*

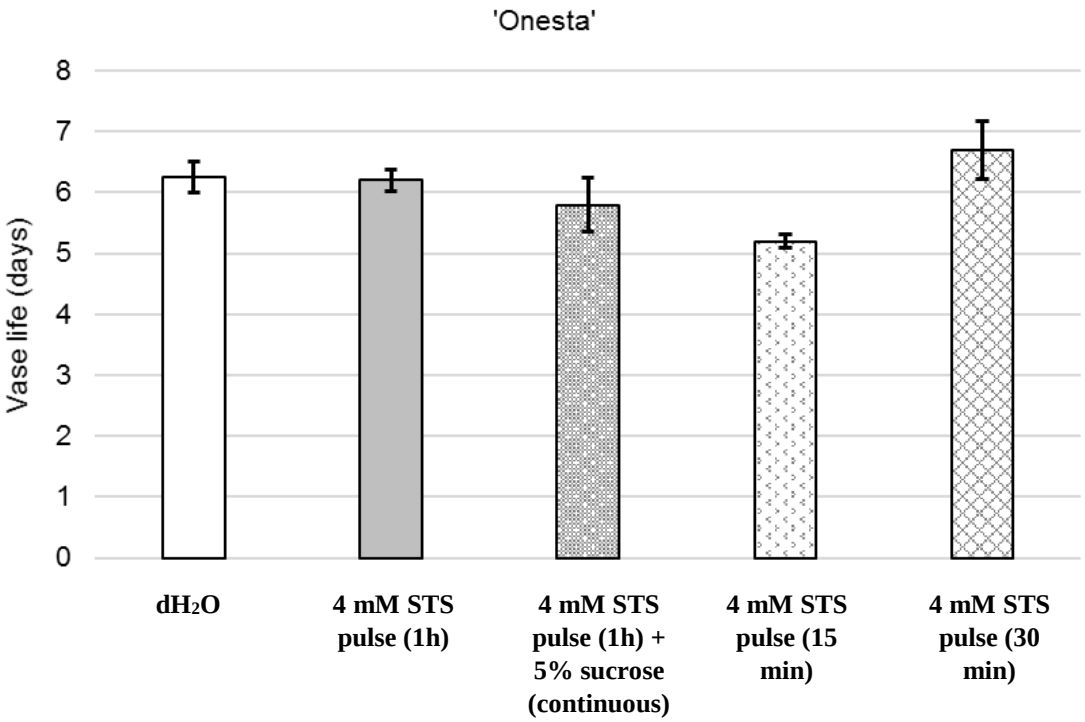


Figure 31: Vase life (days) of cv. 'Onesta' (C), control (dH_2O) flowers (white) compared to flowers treated with 1 h pulse 4 mM STS (grey), 1 h pulse 4 mM STS followed by a continuous solution of 5% sucrose (patterned), 15 min pulse 4 mM STS (dotted), or 30 min pulse 4 mM STS (diamond-grid) (mean ± S.E.; n=5). No significance was found (2-sample t-test, n=5).

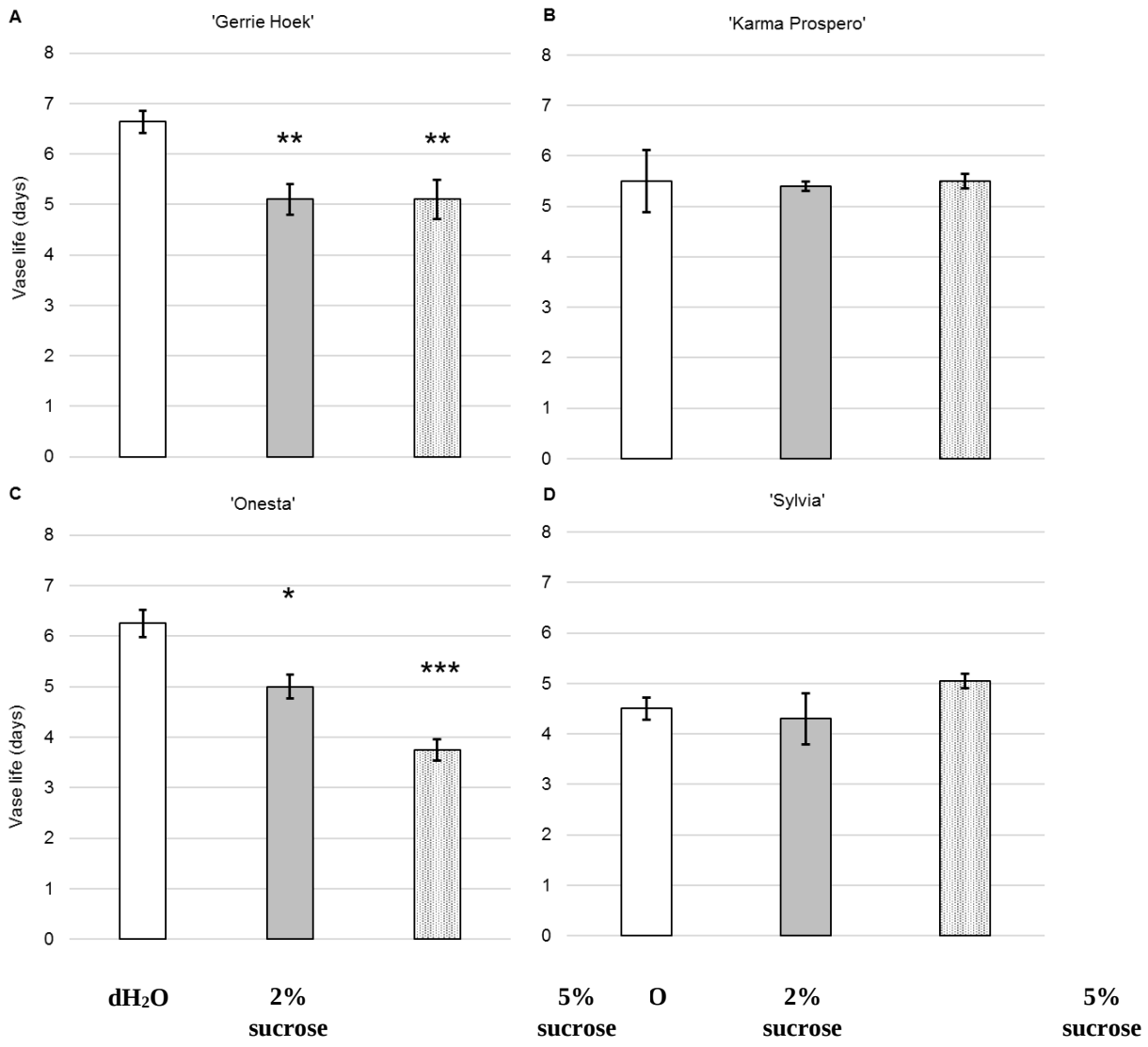


*Figure 32: Vase life (days) of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D) as control (dH2O) flowers (white) compared to flowers treated with 2% (grey) or 5% (patterned) sucrose (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * p < 0.05, ** p < 0.01, or *** p < 0.001 (2-sample t-test, n=5).*

Figure 33: Photos of cv. 'Gerrie Hoek' flowers 1, 4, or 7 d after cutting (left to right), treated as controls (dH_2O) (A), with 20 µM CEPA (B), a 1 h pulse of 4 mM STS (C), or a combination of a 1 h pulse of 4 mM STS and a 100 µM BA spray (D). Scale bars represent 20 mm.

Figure 34: Photos of cv. 'Karma Prospero' flowers 1, 4, or 7 d after cutting (left to right), treated as controls (dH_2O) (A), with 20 µM CEPA (B), a 1 h pulse of 4 mM STS (C), or a combination of a 1 h pulse of 4 mM STS and a 100 µM BA spray (D). Scale bars represent 20 mm.

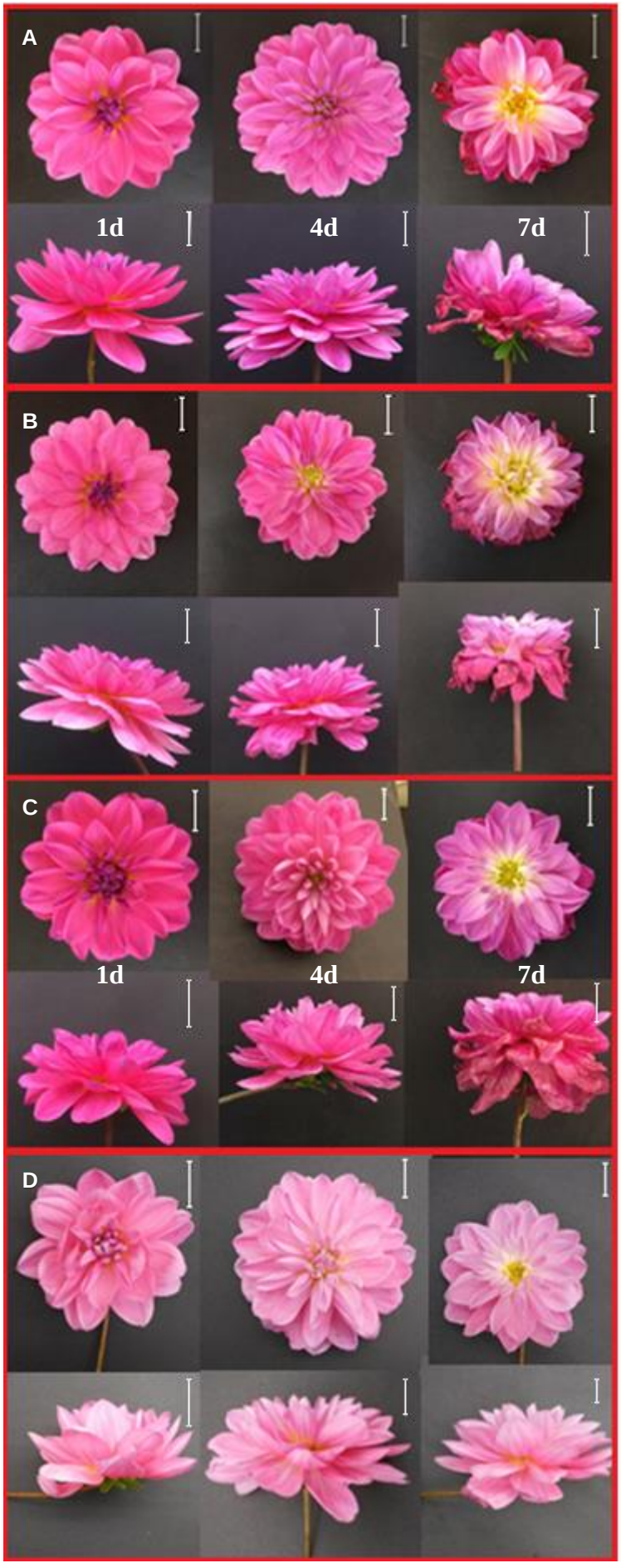


Figure 35: Photos of cv. 'Onesta' flowers 1, 4, or 7 d after cutting (left to right), treated as controls (dH_2O) (A), with 20 µM CEPA (B), a 1 h pulse of 4 mM STS (C), or a combination of a 1 h pulse of 4 mM STS and a 100 µM BA spray (D). Scale bars represent 20 mm.

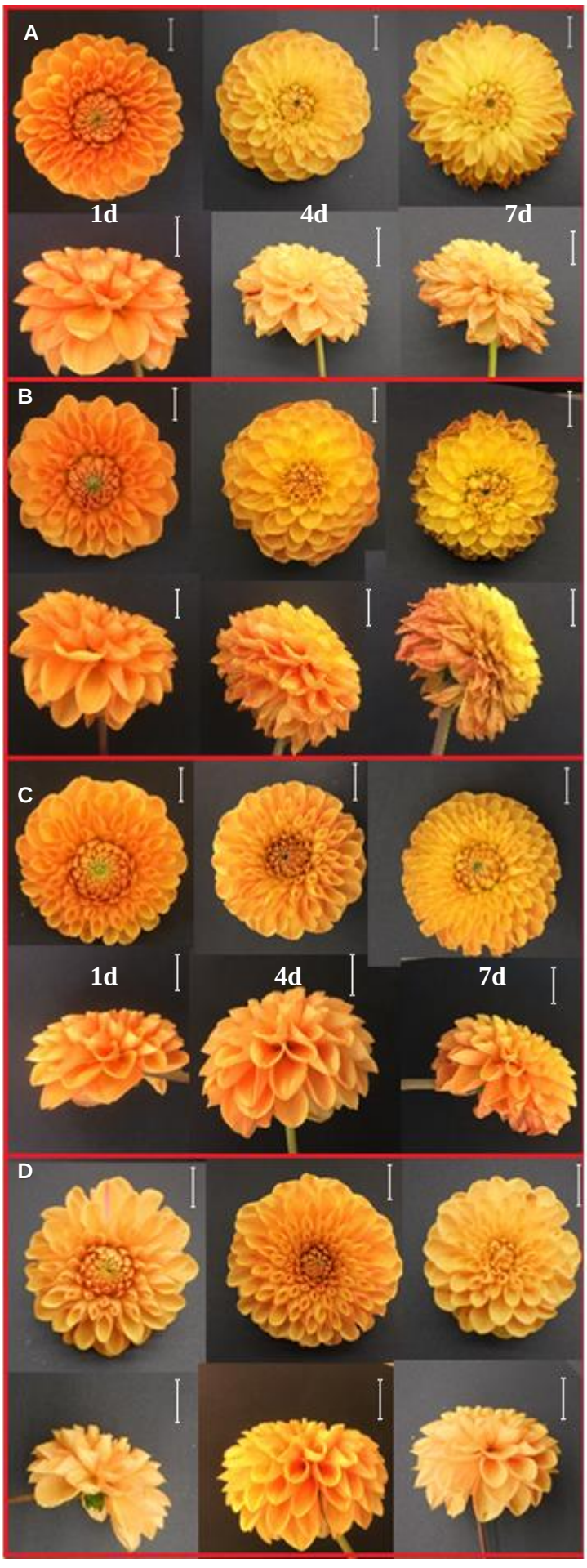


Figure 36: Photos of cv. 'Sylvia' flowers 1, 4, or 7 d after cutting (left to right), treated as controls (dH_2O) (A), with 20 µM CEPA (B), a 1 h pulse of 4 mM STS (C), or a combination of a 1 h pulse of 4 mM STS and a 100 µM BA spray (D). Scale bars represent 20 mm.

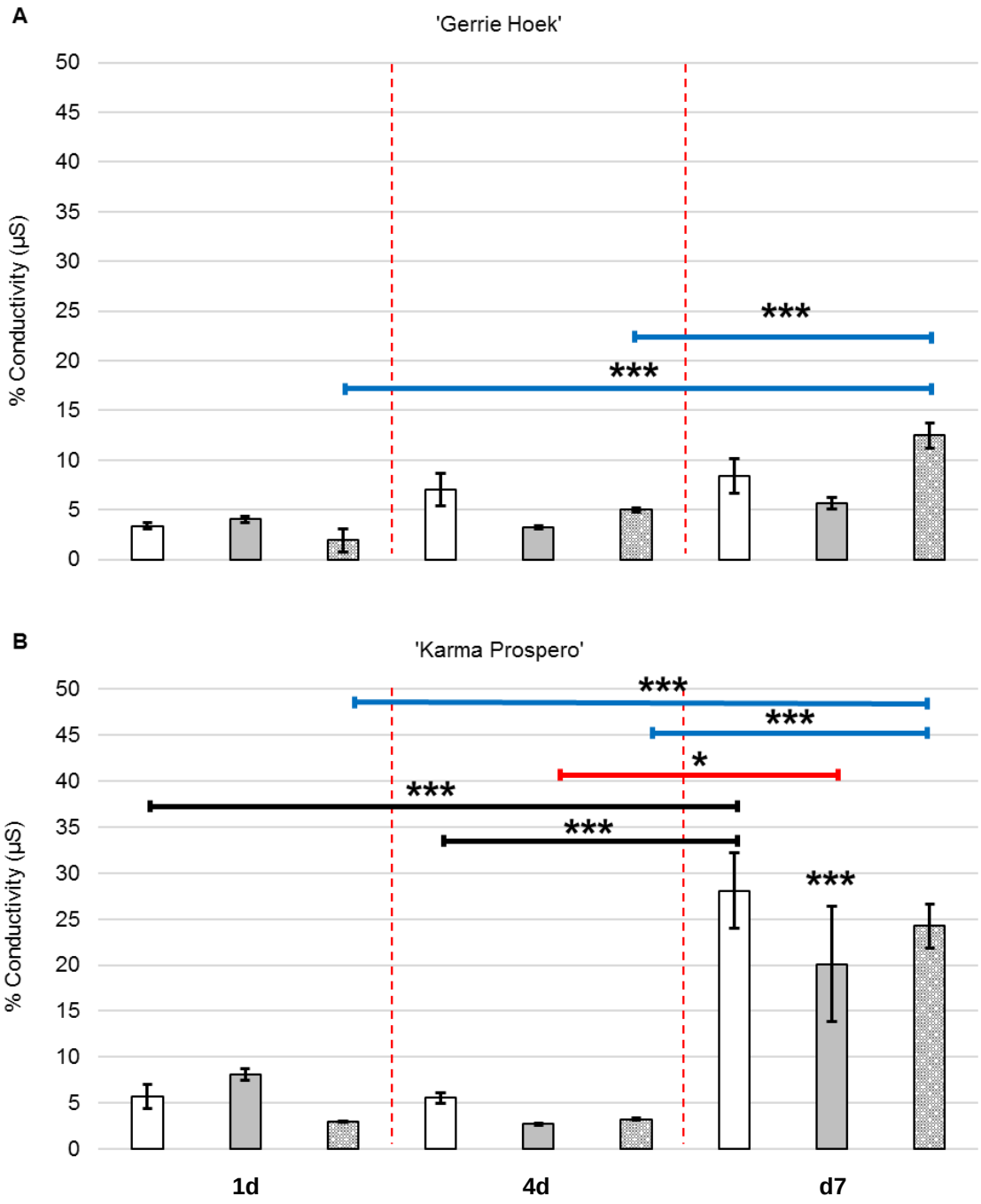
5.4.2. Conductivity

Membrane permeability as determined by percentage conductivity was significantly lower after 7 days from flowers treated with 4 mM STS in all cultivars except ‘Gerrie Hoek’, however percentage conductivity in control ‘Gerrie Hoek’ flowers was much lower after 7 days compared with the other cultivars assessed (Fig. 37). After 7 days CEPA treated flowers performed no worse or better than control flowers in all cultivars (Fig. 37A). Additionally, CEPA treated flowers showed a significant increase in conductivity over the 7-day experimental period in all cultivars (Fig. 37).

In cv. ‘Karma Prospero’ by 7 days only STS treated flowers showed significantly lower conductivity compared to controls (Fig. 37B). All treatment groups showed a significant increase in conductivity over the experimental period, though the increase was least significant in STS treated flowers (Fig. 37B).

In cv. ‘Onesta’ STS treated flowers showed lower conductivity after 7 days (Fig. 37C). and after 7 days conductivity of STS treated flowers was not significantly different to that of day 1 flowers, whereas both control and CEPA treated flowers showed significant increases (Fig. 37C).

Cv. ‘Sylvia’ STS treated flowers showed significantly lower conductivity at 4 and 7 days compared with controls, whereas CEPA treated flowers showed lower conductivity at 4 days but not at 7 days (Fig. 37D). CEPA treated flowers also showed a significant increase in conductivity between days 4 and 7, whereas STS treated flowers showed no change over the same period (Fig. 37D).


A
'Gerrie Hoek'
% Conductivity (µS)
50
45
40
35
30
25
20
15
10
5
0
***
***
B
'Karma Prospero'
% Conductivity (µS)
50
45
40
35
30
25
20
15
10
5
0
***
***
*
***
***
***
1d
4d
d7

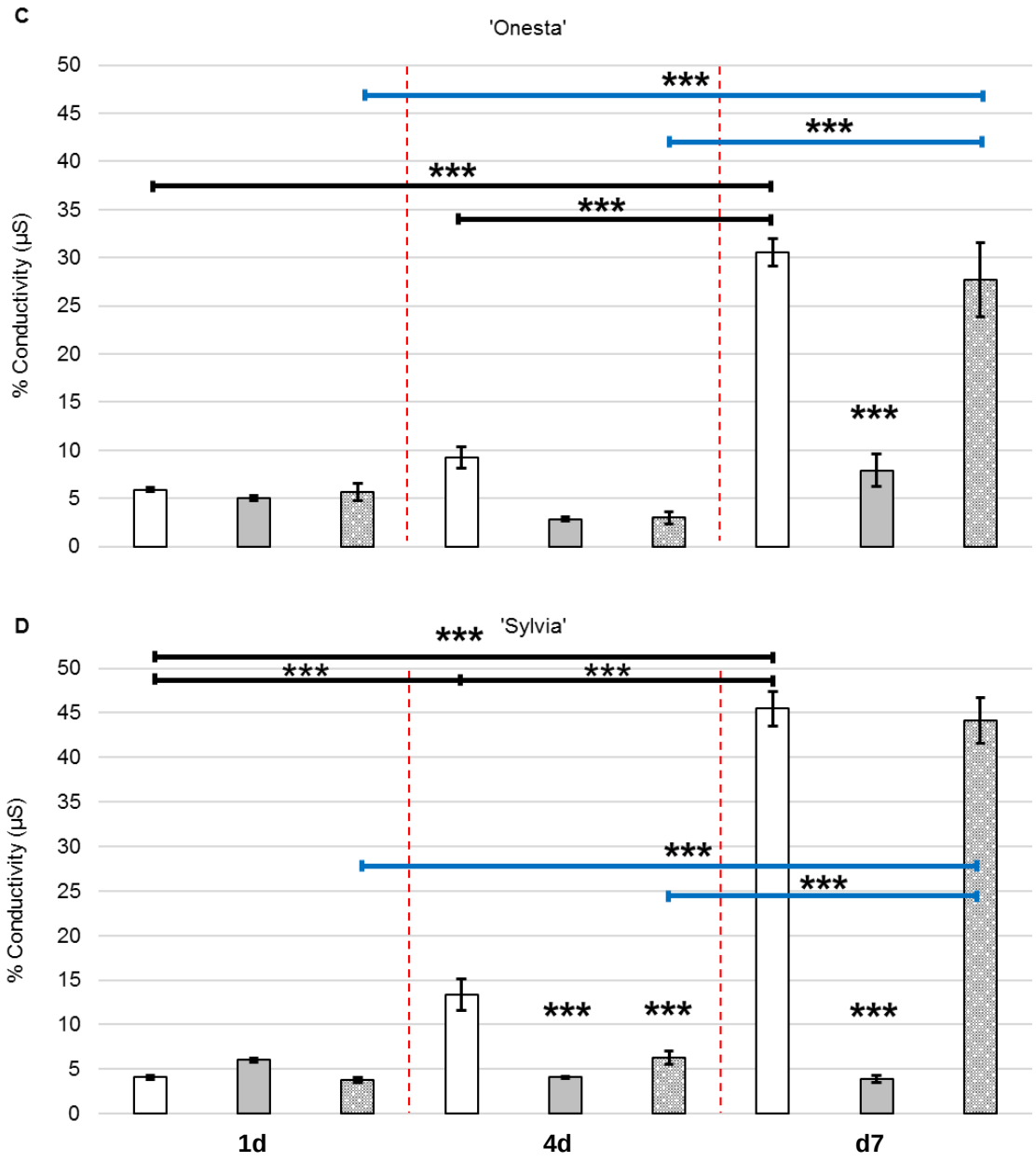
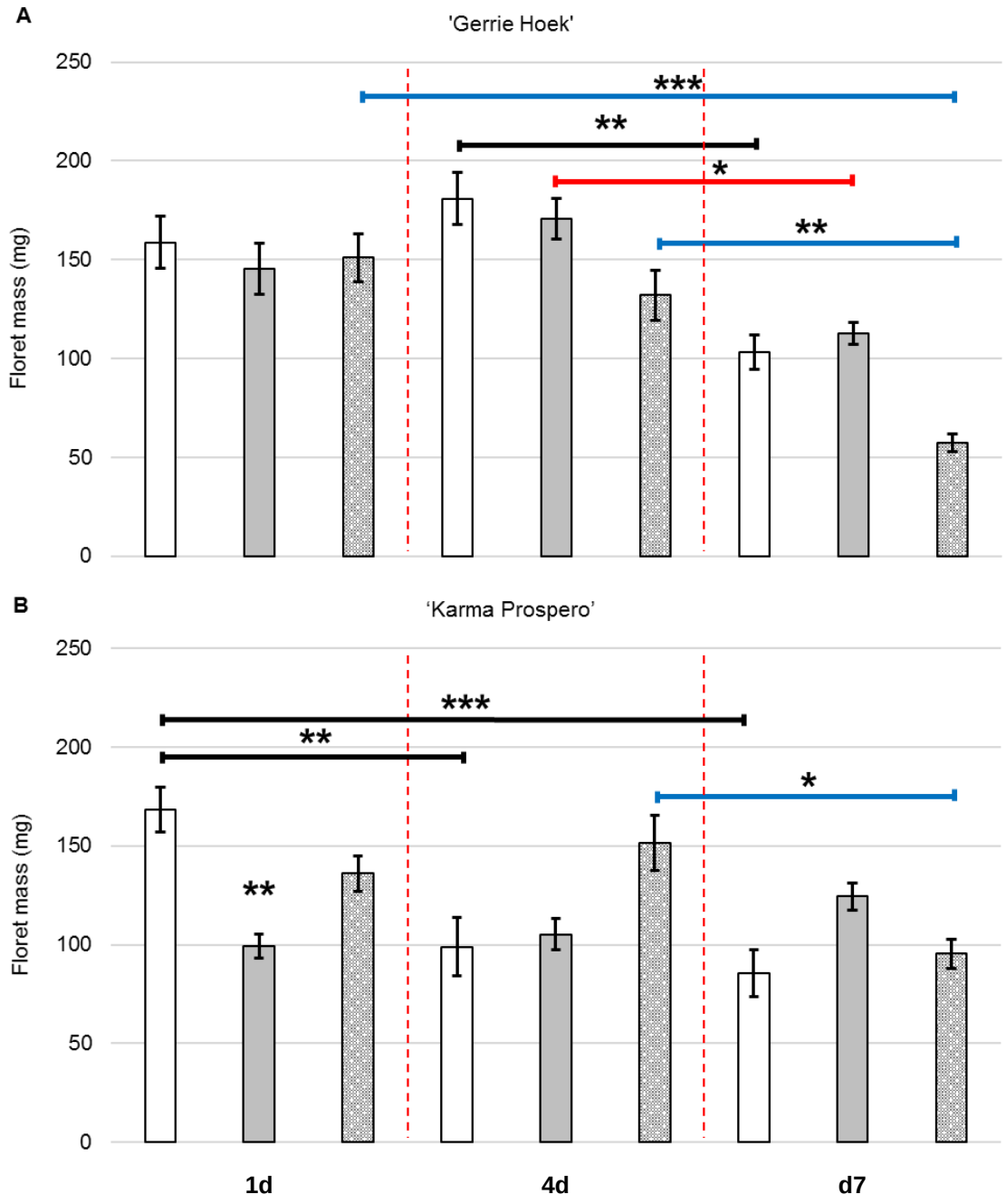


*Figure 37: Conductivity of florets after 3 h submersion as a percentage of total conductivity of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C) and 'Sylvia' (D), control (dH$_2$O) flowers (white) compared to 1 h pulse 4 mM STS treated (grey) and 20 µM CEPA treated (patterned) flowers, 1, 4, and 7 d after cutting (mean ± S.E.; n=5). Significance between different timepoints of the same treatment are represented by black (dH$_2$O), red (STS) or blue (CEPA) brackets. Asterisks above bars represent significant differences between control and treated flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=5).*

5.4.3. Floret Mass

After 7 days mass of florets from flowers pulsed with 4 mM STS were significantly heavier than controls or CEPA treated flowers in cv.'s 'Onesta' and 'Sylvia' (Fig. 38C, D). However, in cv. 'Karma Prospero', although STS treated florets were not heavier after 7 days compared to controls, they had not changed significantly over the experimental period, in contrast 'Karma Prospero' control florets showed a significant drop in mass over 7 days and CEPA treated florets showed a drop between day 4 and day 7 (Fig 38B). In cv. 'Onesta' CEPA treated and control flowers showed significant declines in floret mass over the experimental period, whereas STS treated flowers did not (Fig. 38C). After 1-day STS treated florets of cv. 'Karma Prospero' were significantly lower in mass compared to controls, but there were no other significant differences found in any other cultivars after 1 day (Fig. 38B). After 4 days the only significance found was in CEPA treated florets of cv. 'Sylvia' which were significantly heavier than controls, however by day 7 CEPA treated florets of cv. 'Sylvia' showed no significant difference compared to controls (Fig. 38D).


A
'Gerrie Hoek'
Floret mass (mg)
250
200
150
100
50
0
***
**
*
**
B
'Karma Prospero'
Floret mass (mg)
250
200
150
100
50
0
***
**
*
**
1d
4d
d7

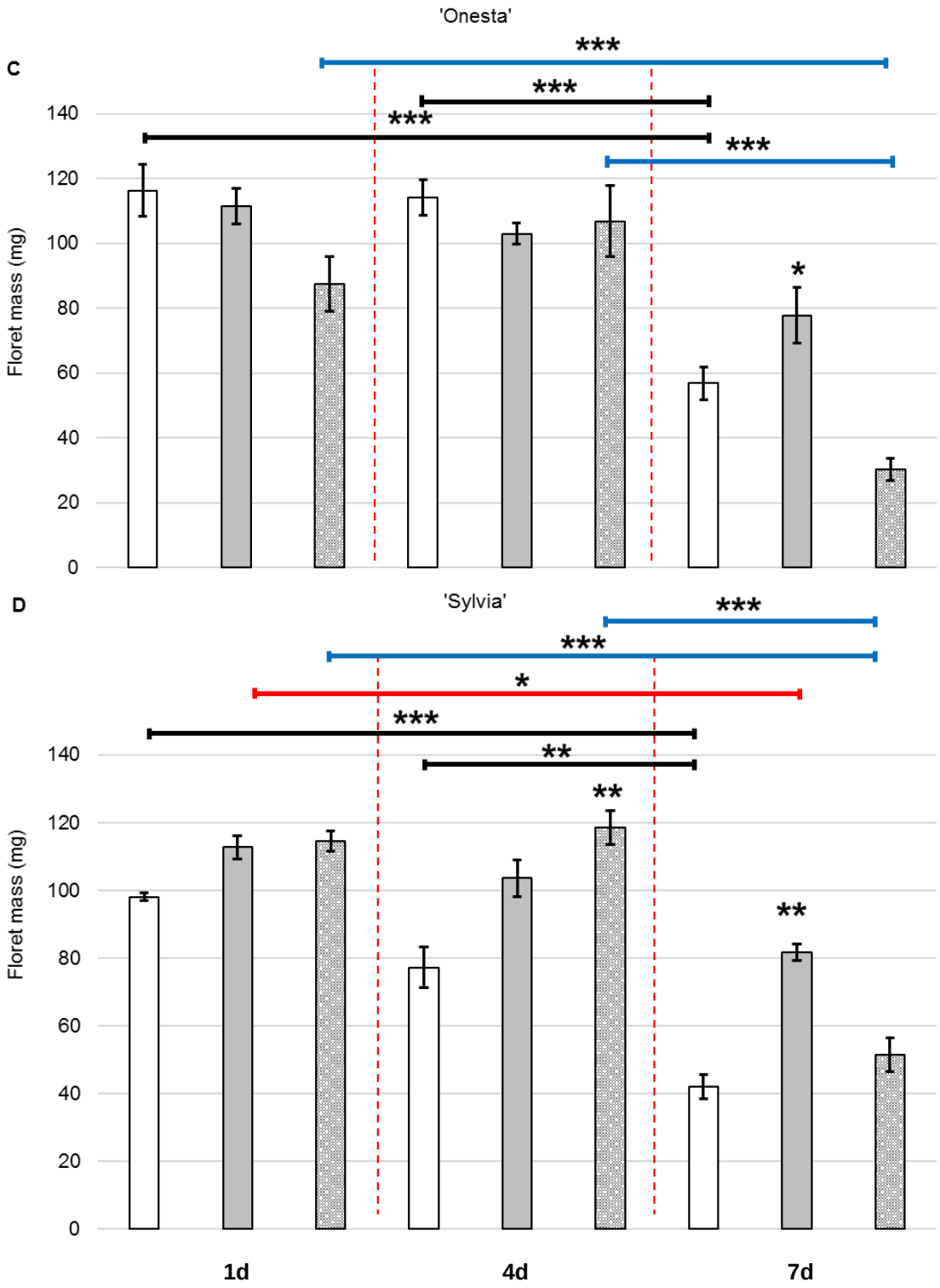

C
'Onesta'
***
***
***
***
***
*
Floret mass (mg)
140
120
100
80
60
40
20
0
D
'Sylvia'
***
***
*
***
**
**
**
Floret mass (mg)
140
120
100
80
60
40
20
0
1d
4d
7d

*Figure 38: Mass of florets (mg) of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C) and 'Sylvia' (D), control (dH_2O) flowers (white) compared to 1 h pulse 4 mM STS treated (grey) and 20 µM CEPA treated (patterned) flowers, 1, 4, and 7 d after cutting (mean ± S.E.; n=5). Significance between different timepoints of the same treatment are represented by black (dH_2O), red (STS) or blue (CEPA) brackets. Asterisks above bars represent significant differences between control and treated flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=5).*

5.4.4. Protein Content

At no timepoint was there a significant difference in protein content between controls and STS treated flowers in either cv. 'Onesta' or 'Karma Prospero' (Fig. 39). In cv. 'Karma Prospero' both control and STS treated flowers showed a decline in protein content over the experimental period and this decline was more significant in STS treated florets (Fig. 39A). In 'Onesta' controls and STS treated flowers protein content showed a significant decline between days 1 and 7 and days 1 and 4, before levelling off (Fig. 39B).

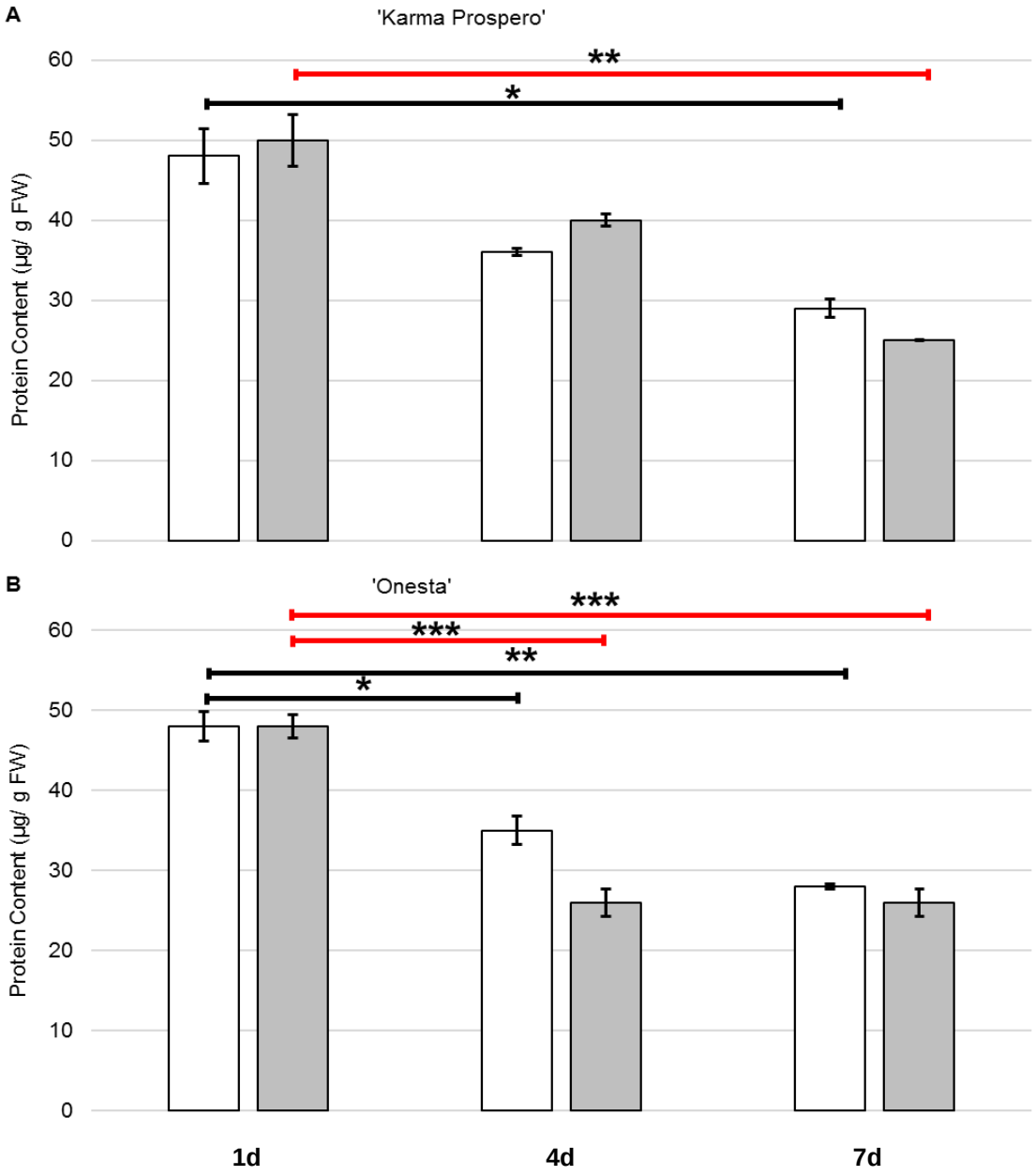


*Figure 39: Protein content (µg/ g FW) of cv. 'Karma Prospero' (A) and 'Onesta' (B) control (dH_2O) (white) or 1 h pulse 4 mM STS treated (grey) flowers sampled 1, 4 or 7d after cutting (mean ± S.E.; n=3). Significance between different timepoints of the same treatment are represented by black (dH_2O) or red (STS) brackets. Asterisks above bars represent significant differences between control and treated flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=3).*

5.4.5. Quantitative reverse transcription polymerase chain-reaction (RT-qPCR)

In cv. ‘Karma Prospero’ *DvACO4* expression was significantly lower in STS treated flowers 1 day after treatment but significantly higher after 4 days (Fig. 40A). *DvACS6* expression showed no significant difference between groups on either day in ‘Karma Prospero’ (Fig. 40B). In cv. ‘Onesta’ both *DvACO4* and *DvACS6* expression were significantly higher 4 days after treatment in STS treated flowers compared to controls but showed no significant difference compared to controls 1 day after treatment (Fig. 41).

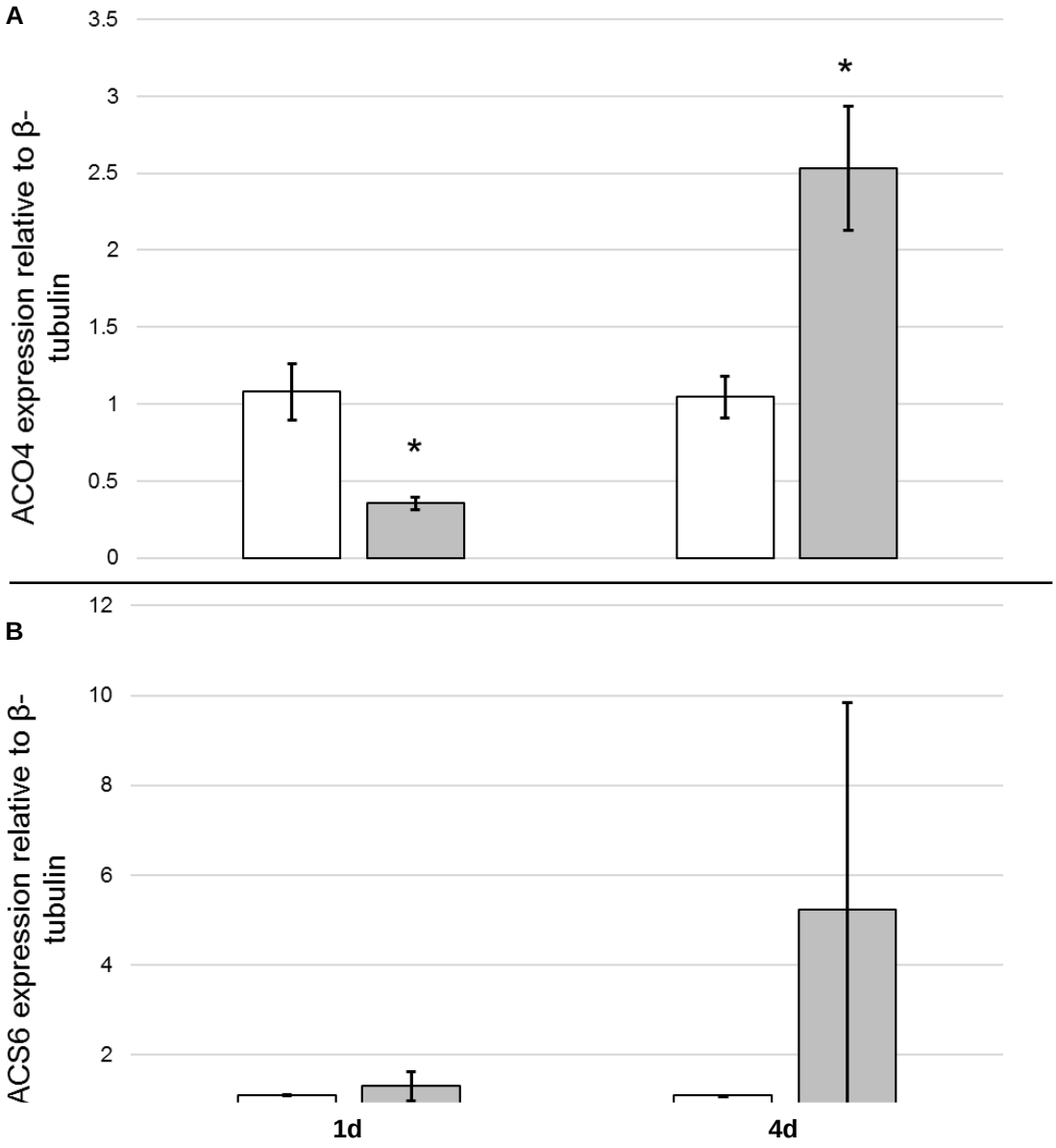
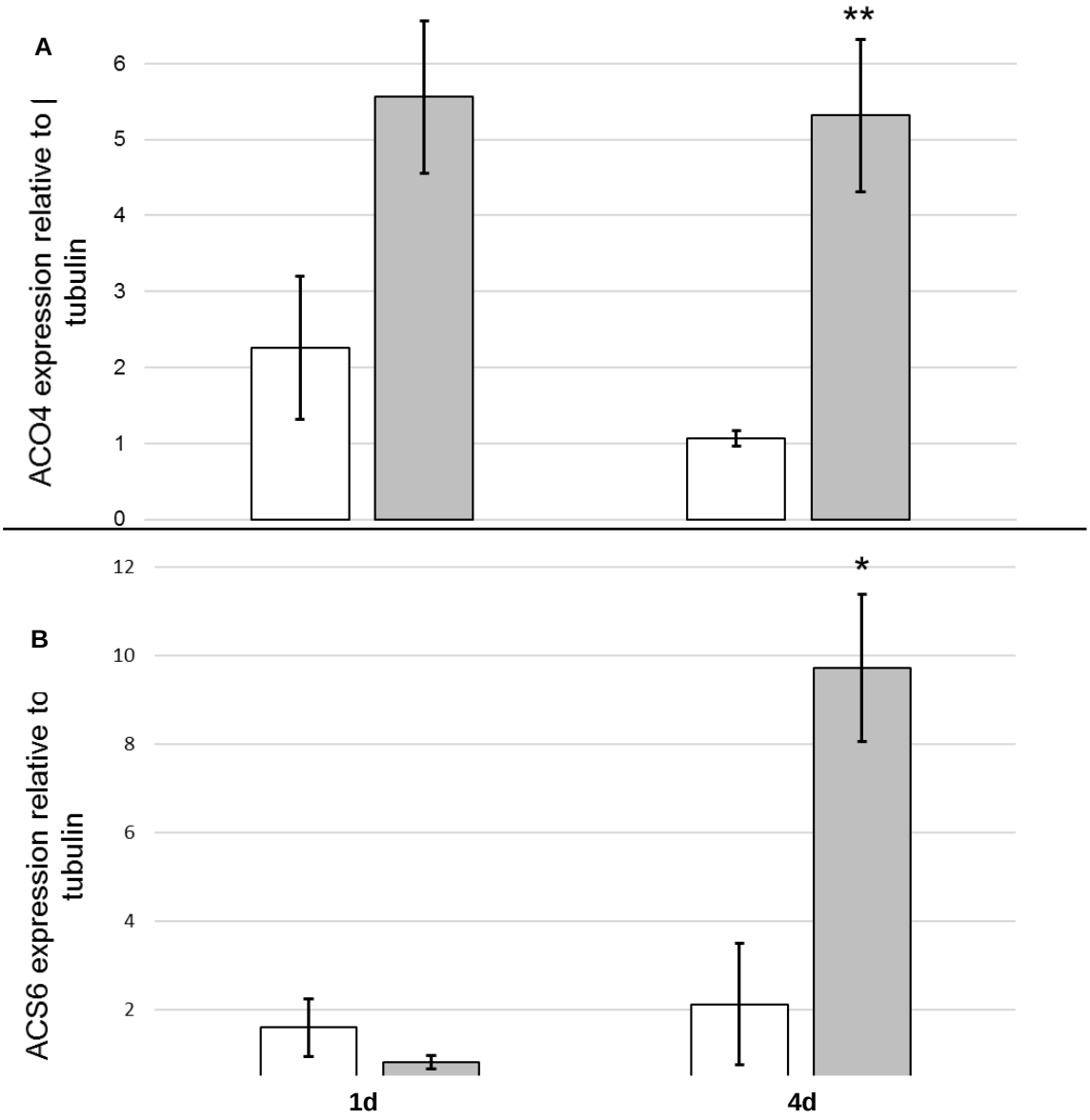


Figure 40: Relative expression of genes DvACO4 *(A) and* DvACS6 *(B) in cv. 'Karma Prospero' florets from flowers treated as controls (dH₂O) (white) or with a 1 h pulse of 4 mM STS (grey), 1 or 4 days after cutting using β-tubulin as a reference (mean ± S.E.; n=3). Significance is indicated by * p < 0.05, ** p < 0.01, or *** p < 0.001 (two-sample t-test, n=3).*



5.5. Discussion

Ethylene is the phytohormone most commonly implicated in plant senescence processes; however, its role in dahlia flower senescence has been uncertain. In this study, postharvest treatments and their physiological effects were analysed to gain further insight into ethylene's role in dahlia floral senescence. Differential expression analysis based on

RNA-sequencing and RT-qPCR were also used to examine how ethylene signalling and biosynthesis change during senescence or in response to postharvest treatment with STS.

The results show that vase life is significantly greater in the cultivars 'Boom Boom Yellow', 'Gerrie Hoek', 'Karma Prospero' and 'Sylvia' when they are treated with STS, which inhibits ethylene reception (Fig. 29A, B, D, E; Veen, 1979). However, in 'Onesta' there was no effect (Fig. 29C). This suggests that different dahlia cultivars vary in their response to ethylene inhibition. This has been found in dahlia before, and in the close relative chrysanthemum (Dole *et al.*, 2009; Doi *et al.*, 2003; Shimizu-Yumoto & Ichimura, 2013; Woltering & van Doorn, 1988), and suggests that only certain cultivars would be suitable for commercialisation as even with STS treatment some cultivars still show a vase life under 10 days (Fig. 29C). Furthermore, the highest vase life from a 1 h pulse of 4 mM STS was in the cultivar 'Boom Boom Yellow' (Fig. 29E). This was the only cultivar that showed a vase life of more than 10 days. This is the minimum needed to be commercially viable as the industrial supply chain demands a vase life of 10-14 days. This suggests that few cultivars are suitable for commercialisation using STS as a post-harvest treatment alone. The appearance of the flowers further showed that a pulse with STS is beneficial in all cultivars examined except 'Onesta' which showed significant wilting 7 days after treatment with STS (Fig. 33-36). However, a combination of an STS pulse with a BA spray gave longer vase lives in all the cultivars assessed (Fig. 29A-D). This suggests that a combination of the two could be suitable for the supply chain, however the cultivar 'Onesta' still showed a vase life of under 10 days suggesting it would not be suitable in a commercial setting (Fig. 29C). In all cultivars a combination of STS and BA prevented any wilting after 7 days (Fig. 33-36). This suggests that a combination of exogenous cytokinins and ethylene inhibition is beneficial to vase life, however whether the two are interacting is unclear, though cytokinins have been shown to repress endogenous ethylene biosynthesis (Guo *et al.*, 2003). In another study BA dip treatment of dahlia florets treated with CEPA showed that BA extended vase life of CEPA treated florets, suggesting that BA treatment aids in suppressing the effects of exogenous ethylene (Shimizu-Yumoto & Ichimura, 2013). In this study the STS pulse combined with BA spray could have extended vase life through two pathways; inhibition of ethylene reception by STS (Veen, 1979), or inhibition of endogenous ethylene biosynthesis by BA (Guo *et al.*, 2003).

Shorter 15 and 30 min pulses of STS were also used on cv. 'Onesta', as was a 1h pulse of 4 mM STS followed by continuous exposure to a solution of 5% sucrose (Fig. 31). These

pulses were used because STS can be toxic in high amounts (Veen, 1979), and it was hypothesized that 'Onesta' could be more sensitive to this toxicity and that a shorter pulse of STS may be of more benefit. However, no significant change in vase life was found with any of these treatments, suggesting that in this cultivar STS and sucrose are both ineffective. However longer pulses of STS were not tested and might be effective. More work using longer pulses and a greater variety of sucrose pulses in conjunction with STS would need to be performed to better establish whether these treatments could be effective in cv. 'Onesta'. The level of sensitivity to exogenous ethylene and the mild responses to STS in dahlia compared with highly sensitive species such as carnation, which can show vase lives of up to 16.1 days following STS treatment (Hashemabadi, 2014), suggests that it is possible that other phytohormones are more heavily involved in triggering dahlia floral senescence, for example ABA (Kumar *et al*., 2014; Panavas & Rubinstein, 1998; Mayak & Halevy, 1972; Mayak & Dilley, 1976).

To analyse sensitivity to exogenous ethylene, CEPA treatments were used. Vase life was unaffected by CEPA in all cultivars except 'Gerrie Hoek' where a significant decrease compared to controls was observed with a continuous solution of 20 µM CEPA (Fig. 30). The outer florets of cv. 'Gerrie Hoek' flowers 7 days after treatment were significantly wilted when treated with 20 µM CEPA whereas no wilting was seen in controls after 7 days (Fig. 33A, B). This suggests that there is a degree of ethylene sensitivity in this cultivar, and that it may be more ethylene sensitive than the other cultivars assessed in this study. In cultivars 'Karma Prospero' and 'Onesta' average vase life was lower in 20 µM CEPA treated flowers and CEPA treated flowers showed greater amounts of wilting 7 days after treatment than controls, however the difference in vase life was not significant (Fig. 30, 23, 24). Additionally, in cv. 'Sylvia', significant wilting was seen 7 days after treatment in both control and CEPA treated flowers, and there was no significant difference in vase life (Fig. 30D, 25). The data on CEPA and STS suggests there is a variation in response to exogenous ethylene and ethylene inhibition between cultivars. However, in most cultivars a 1 h pulse of 4 mM STS was not enough to increase vase life to a commercially acceptable level, furthermore the CEPA treatments showed low levels of sensitivity to exogenous ethylene, though inhibition of ethylene reception with STS was effective in all cultivars examined with the exception of 'Onesta'. This suggests there is a role for ethylene in dahlia floral senescence and is consistent with previous findings in dahlia, in that they show variation in ethylene sensitivity with different cultivars being

insensitive or mildly sensitive (Dole *et al*., 2009; Shimizu-Yumoto & Ichimura, 2013; Woltering & van Doorn, 1988).

In this study none of the sucrose treatments tested significantly improved vase life in all cultivars of dahlia of dahlia assessed (Fig. 32). In ethylene sensitive flowers it has been suggested that a sucrose treatment improves vase life by suppressing ethylene action through the degradation of the transcription factor EIL3, which is responsible for activation of ethylene responsive genes, and there is evidence for this in carnation (Yanagisawa *et al*., 2003; Hoeberichts *et al*., 2007), this suggested that in dahlia, if the cultivar was ethylene sensitive, that sucrose would improve vase life by promoting degradation of EIL3 and therefore indirectly repressing the activation of ethylene responsive genes. However, sucrose treatments also enhance vase-life in ethylene insensitive species such as lily (Arrom *et al*., 2012b). The results in this study agree with previous studies in dahlia that found sugar treatments to have no or a small effect on vase life (Lukasweska, 1980). Additionally, sucrose treatments decreased vase life significantly in 'Gerrie Hoek' and 'Onesta' flowers suggesting that in some cultivars the effects of sucrose are negative and would be harmful to vase life (Fig. 32). Similar results have been found in sunflower where treatment with citric acid combined with 2% sucrose decreased vase life (Mensuali-Sodi & Ferrante, 2005). It may be that sucrose treatments are encouraging bacterial growth, and this is counteracting the positive effects of sucrose. It has been found in the dahlia's close relative *Zinnia elegans* that higher levels of bacteria in vase solution reduced vase life through vascular blockage (Carlson *et al*., 2015). Overall the results of sucrose treatments suggest that this treatment would not be effective in improving dahlia vase life and may have a negative effect on dahlia vase life, thus would be a poor postharvest treatment in a commercial setting.

The physiological effects of CEPA and STS shed light on how they may be affecting dahlia floral senescence. Measurements of floret mass and conductivity showed that STS treatment was effective in slowing down either loss of floret mass or cellular integrity across all cultivars examined (Fig. 37). In 'Gerrie Hoek' neither the CEPA or STS treatment resulted in conductivity 7 days after treatment that significantly differed from controls (Fig. 37A), whereas in all other cultivars, after 7 days, STS treated florets showed lower conductivity compared to control and CEPA treated florets showed no significant difference. However, conductivity remained below 15% for all treatments in 'Gerrie Hoek', whereas in controls of other cultivars conductivity was over 25% after 7 days. This suggests that measurements later than 7 days after treatment may be needed in cv.

‘Gerrie Hoek’ to detect a difference, if any, in membrane permeability between CEPA or STS treated flowers and controls and indicates a possibly slower progression of senescence in this cultivar. In ‘Onesta’, which showed wilting after 7 days with STS treatment, conductivity was significantly lower in STS treated flowers (Fig. 37C). This shows that STS treatment is having a beneficial effect on cellular membrane integrity and this effect is present even if the flowers appear to be wilted. This suggests that inhibiting ethylene is having a physiological effect on flowers, suggesting that ethylene has a role in floral senescence of this dahlia cultivar, and that this role is in some way related to membrane breakdown. However, the lack of a response to CEPA in cultivars which respond to STS (Fig. 29, 19) suggests that addition of exogenous ethylene has little physiological effect, this may be due to limited ethylene receptors or because ethylene treatment needs to be given at an earlier stage to have an effect.

Floret mass was significantly higher compared to controls after 7 days of STS treatment in cv.’s ‘Sylvia’ and ‘Onesta’ (Fig. 38C, D). However, although there was no significant difference between controls and STS treated florets after 7 days in ‘Karma Prospero’, there was also no significant change in mass in STS treated florets over the experimental period whereas CEPA and control florets showed significant declines in mass (Fig. 38B). In ‘Onesta’ STS treated florets did not decline in mass, whereas control and CEPA treated florets did (Fig. 38C). This shows that STS treatment appears to be helping to maintain the mass or mitigate mass decreases compared to CEPA treated or control florets of all four cultivars tested. This is in agreement with the results of conductivity measurement and shows that across all cultivars STS delayed common physiological signs of senescence, loss of mass and membrane breakdown. The greater mass of STS treated florets after 7d compared with controls or the maintenance of floret mass over the experiment in STS treated flowers suggests that inhibition of ethylene action in some dahlia cultivars is effective and is inhibiting senescence leading to delayed senescence and longer-lived florets. Ethylene has been linked to remobilisation of nutrients in senescing tissue, along with breakdown of proteins, cellular membranes and RNA and DNA (Jones *et al*., 2005; Langston *et al*., 2005; Chapin & Jones, 2009; Hong *et al*., 2000). It is presumed that STS treatment is helping dahlia floral cellular integrity by delaying or inhibiting ethylene reception and ethylene responses, meaning ERF’s, transcription factors responsible for the ethylene response, are not being activated and the transcriptional cascade they induce which activates senescence associated genes, is not occurring, or is delayed (Breeze *et al*., 2011; Buchanan-Wollaston *et al*., 2003).

Protein breakdown is another common hallmark of senescence. In both cultivars tested ('Onesta' and 'Karma Prospero') no significant difference in protein content could be found between the treatments (Fig. 39). In controls, cv. 'Onesta' protein content decreased from d1 to d4 and then levelled out, whereas in 'Karma Prospero' a difference was only found after 7 days, suggesting protein content decreases may be slower in 'Karma Prospero' than in 'Onesta' (Fig. 39). The lack of difference seen between controls and STS treated florets suggests that ethylene is not playing a role or only has a minor one in protease activation in dahlia. The degree to which different proteases are involved in senescence varies between species, it may be that senescence-specific endoproteases in dahlia are not ethylene dependent, but more research would be needed to confirm this (Azeez *et al*., 2007; Jones *et al*., 2005).

Ethylene biosynthesis has been found to be upregulated prior to senescence in species such as petunia (Jones, 2008; Fig. 21). In this study it was found in both cultivars 'Karma Prospero' and 'Onesta' that a 1 h pulse of 4 mM STS leads to an increase in expression of the ethylene biosynthetic gene *DvACO4* 4 days after treatment compared to controls, however only in cv. 'Onesta' was expression of the rate limiting enzyme *DvACS6* higher compared to controls after 4 days (Fig. 40, 41). In 'Karma Prospero' mean expression of *DvACS6* was higher than controls, but this was not statistically significant. The upregulation of *DvACO4* in both cultivars and *DvACS6* in 'Onesta' 4d after STS treatment suggests that this change takes time as no difference, or even a decrease, was observed after 1 day. In rose, changes in expression after exogenous ethylene treatment have been found to occur just 30 min after treatment (Tripathi *et al*., 2009), suggesting that exogenous hormones cause quicker changes than treatments that inhibit endogenous enzymes. Alternatively, dahlia may react more slowly to postharvest treatments. More sampling would also need to be done to examine whether the changes in expression began 2 or 3 days after treatment and how long they continued after the 4-day sampling point. The upregulation of *DvACO4* in 'Onesta' and 'Karma Prospero' and *DvACS6* in 'Onesta' suggests that dahlias may produce more ethylene in response to its inhibition, though more experiments need to be carried out to confirm this, for example performing RNA-sequencing on material treated with STS or measurements of endogenous ethylene in treated and untreated flowers. Furthermore, this shows that in cv. 'Onesta', which showed no significant increase in vase life after STS treatment and showed wilting similar to controls (Fig. 29C; 24C), there is a molecular response to the treatment. This suggests that cultivars which appear superficially ethylene insensitive based on their response to

STS may produce more ethylene in response and perhaps are simply more effective at producing ethylene in quantities that counterbalance the inhibitory treatment. However, more examination of more than two cultivars, more time points following treatment, and measurement of ethylene production in flowers would give more evidence to this hypothesis. Shimizu-Yumoto & Ichimura (2013) found that ethylene production fell following harvesting, and this may also have been the case for control cut flowers of both cultivars tested in this study ('Karma Prospero' and 'Onesta', Fig. 40, 41). However, in cv. 'Onesta' *ACS6* expression was higher 4d after cutting in STS treated flowers (Fig. 41B), suggesting that STS treatment can stimulate ethylene production in dahlia post-harvest, though measurements of endogenous ethylene would need to be carried out to confirm this.

Overall the data suggest that ethylene does play a role in dahlia floral senescence, and differential expression analysis has shown ethylene biosynthetic genes to be upregulated in ageing florets (Fig. 17). However, although some dahlias do react to ethylene inhibition, treatment with the ethylene receptor inhibitor STS can lead to an increase in the expression of ethylene biosynthesis genes in some cultivars (Fig. 41). All this strongly suggests a role for ethylene in dahlia floral senescence. However, the weak response to treatments, for example, of the five cultivars assessed, only 'Boom Boom Yellow' showed a vase life in excess of 10 days, the industry threshold (personal communication, Martin Squire), following STS treatment on its own, suggests that dahlia is only mildly sensitive to ethylene.

6. Role of cytokinins in *Dahlia* flower senescence

6.1. Introduction

Cytokinins are a family of phytohormones implicated in many plant developmental processes, including senescence (Kieber & Schaller, 2014). Limited data are available on the role of cytokinins in dahlia senescence but research in other commercial cut flowers species allows us to gain an insight into how cytokinins may affect the process in dahlia. Cytokinins are known to extend the vase life of many flowers including anthurium (Favero *et al*., 2015) petunias (Chang *et al*., 2003), iris (van Doorn *et al*., 2013, Macnish *et al*., 2010), carnations (Eisinger, 1977; Kelly *et al*., 1985; van Staden & Joughin, 1988), roses (Mayak & Halevy, 1970; Lukaszewska *et al*., 1994), lisianthus (Musembi *et al*., 2015), wallflowers (Price *et al*., 2008) and the dahlia's close relative, chrysanthemum (Guo *et al*., 2003). Therefore, it is likely that dahlia flower senescence may also be partially regulated by cytokinins. It is possible that treatment with cytokinins such as 6-benzylaminopurine (BA) may be more effective in delaying the senescence of cut dahlia flowers as this has been found in a previous study (Shimizu-Yumoto & Ichimura, 2013).

IPT is well documented as a critical enzyme involved in cytokinin biosynthesis (Kieber & Schaller, 2014; Fig. 22). Petunias transformed to express the cytokinin biosynthetic gene *ipt*, which encodes the enzyme isopentenyl transferase, placed under the control of the promoter from the senescence associated gene *SAG12*, resulted in flower wilting in transformed plants occurring 6-10 days after control plants (Chang *et al*. 2003). This suggests that increased levels of endogenous cytokinins during senescence can delay the process (Chang *et al*. 2003). Additionally, overexpression of *ipt* delayed leaf senescence in rose (Zakizadeh *et al*., 2012), canola (Kant *et al*., 2015), and creeping bentgrass (Xing *et al*., 2009). The IPT family member *IPT3* is known to be upregulated in senescent leaves in *A. thaliana* (Winter *et al*., 2007; Schmid *et al*., 2005). *IPT3* is most heavily involved in nutrient signalling, suggesting it could be implicated in senescence by mediating signals associated with reducing nutrient transport to senescent tissues or in nutrient remobilisation as nutrients are transported from senescent tissues to growing tissues (Masclaux *et al*., 2000; Sakakibara, 2006; Kieber & Schaller, 2014). A model suggested by Kieber & Schaller (2014) proposes that it is the balance of cytokinins in the root and shoot that ultimately leads to expression of cytokinin-responsive genes in other parts of the plant that in turn modulates nitrogen metabolism depending on available resources. It is known that nitrogen is remobilised during senescence, and it may be that *IPT3* is involved in this as it is in other nutrient signalling (Hörtensteiner & Feller, 2002).

The cytokinin receptor family in *Arabidopsis* is composed of three histidine kinases: AHK2, AHK3, and AHK4 (Kieber & Schaller, 2014; Fig. 22). A combination of studies have supported the role of these three molecules as cytokinin receptors (Inoue *et al*., 2001; Suzuki *et al*., 2001; Ueguchi *et al*., 2001; Yamada *et al*., 2001; Fig. 22). Type-A ARR's (*Arabidopsis* response regulators) are associated with negative feedback of cytokinin regulation and serve to de-sensitise the tissue to cytokinins, whereas type-B's are involved in the transcriptional output of cytokinin perception (Kieber & Schaller, 2014). Type-B ARR's have also been shown to be transcription factors that positively mediate cellular response to cytokinins (Hwang & Sheen, 2001; Sakai *et al*., 2001; Fig. 22). The type B ARR, *ARR2*, has been implicated in senescence as its overexpression has been shown to extend leaf longevity in *Arabidopsis* (Kim *et al*., 2006). The results suggested that an AHK3-ARR2 phosphorelay was responsible for cytokinin–dependent regulation of leaf longevity by modulating downstream elements of the senescence pathway, for example hexose transporters, cell wall invertases and promoting sink activity (Kim *et al*., 2006).

Cytokinin oxidases are enzymes implicated in the breakdown and irreversible inactivation of cytokinins (Schmülling *et al*., 2003; Werner *et al*., 2006). These enzymes cleave the N6 side chains from cytokinins thus inactivating them. However, it has been found that the synthetic cytokinin BA could not act as a substrate to cytokinin oxidases in maize (Bilyeu *et al*., 2001). This suggests that exogenously applied BA, if targeted to floral tissue, may not be affected by some of the endogenous enzymes which breakdown the plant's endogenously produced cytokinins.

Spraying whole dahlia flowers with the synthetic cytokinin BA (50 µM) has been found to increase their vase life by 2 days (Shimizu-Yumoto & Ichimura, 2013). However, placing the stems of cut dahlias in BA solution at the same concentration as the spray did not improve vase life suggesting there is a problem with mobility of BA to floral tissues (Shimizu-Yumoto & Ichimura, 2013). Research in petunia found that 200 µM BA improved their vase life by 2.3-3.3 days, but petunias were placed in solution via their pedicels so the route from solution to target tissue was relatively short compared to a 30 cm stem which would be needed in industry (Trivellini *et al*. 2015). Furthermore, immersing the base of carnation flower stems into solution was not as effective as spraying due to low BA mobility (Kelly *et al*., 1985). After treatment with 14C-labeled BA solution it was found that most of the labelled BA and its metabolites remained localized in carnation stem tissue and did not advance to the floral tissue. In dahlia, this

may also be the case as similar work in the genus found that dipping of florets in 500 µM BA increased vase life by 3.1 days compared to controls, whilst immersing stem ends in 500 µM BA solution reduced vase life by 1 day and spraying with 500 µM BA only lengthened vase life by 1.7 days compared to controls (Shimizu-Yumoto & Ichimura, 2013). This demonstrates how the same chemical administered in a different way can give different results.

Following pre-treatment with 1 µL L-1 CEPA, treatment with 2 µL L-1 1-MCP or 500 µM BA extended vase life of cv. 'Kokucho' dahlia florets (Shimizu-Yumoto & Ichimura, 2013). However, BA offset the negative effect of CEPA on floret vase life to a greater extent than the ethylene inhibitor 1-MCP. CEPA treated florets had a 4-day vase life compared to 7.9 days for those then treated with 2 µL L-1 1-MCP and 8.6 days for those dipped in 500 µM BA. This shows that for dahlias the effects of cytokinin may be more beneficial than those of ethylene inhibitors. In dahlia's close relative chrysanthemum similar results have been found, with cut flowers being marginally responsive to ethylene inhibitors (Woltering & van Doorn, 1988; Guo *et al*., 2003) but showing significant improvement when treated with exogenous cytokinins (Guo *et al*., 2003).

It has also been found that increasing sensitivity of tissue to cytokinins through inhibition of cytokinin specific glucosyltransferases, which have been found to inactivate cytokinins (Šmehilová *et al*., 2016), may offset the low mobility of cytokinins from solution to floral tissue (Hou *et al*., 2004; Wang *et al*., 2011). Inhibition of N-glucosyltransferase, encoded by the gene *UGT76C2*, can induce hypersensitivity to exogenous cytokinins in *Arabidopsis thaliana* (Wang *et al*., 2011). Cytokinins inhibit lateral root development and it was found that *ugt76c2* loss of function mutants developed a phenotype with lower lateral root density compared to wildtype and overexpressing plants due to their enhanced sensitivity to cytokinins, perhaps because the N-glucosyltransferase is involved with cytokinin inactivation (Wang *et al*., 2011). Moreover, exogenous spraying of *A. thaliana* with xanthine inhibited glucosyltransferase activity, with xanthine acting as a competitive inhibitor (Hou *et al*., 2004). This suggests that inhibition of this enzyme with chemicals such as xanthine may maintain cytokinin activity for longer periods of time. The fatty acids oleic acid and linoleic acid may also act as inhibitors of glycosyltransferases, these molecules are non-competitive inhibitors, binding only to the enzyme-substrate complex rather than to the free enzyme, thus they may be less effective than xanthine at inhibiting N-glucosyltransferase (Won *et al*., 2007).

The beneficial effects of BA spray found by Shimizu-Yumoto & Ichimura (2013) varied between dahlia cultivars, with cv. 'Kamakura' exhibiting an average vase life of 6.6 days compared to cv. 'Michan' which showed a vase life of 9.3 days after spraying. However, cv. 'Kamakura' also performed significantly worse compared to 'Michan' in controls suggesting that such variation in response to BA treatment was mostly due to one cultivar performing better as a cut flower due to physiological reasons rather than because it was more sensitive to exogenous cytokinins. This demonstrates the importance in a commercial setting of using cultivars which are known to be better as cut flowers and screening many cultivars to detect differences in reaction to postharvest treatments.

Cytokinins may be used to increase vase life of many cut flower species, including dahlias, and the positive effects of this could outperform traditional ethylene inhibition treatments in dahlia. However, novel methods of applying cytokinins will need to be developed to make cytokinin treated dahlias commercially viable, due to the issue of chemical spraying of a consumer product in a commercial setting.

6.2. Aims

- Does the application of exogenous cytokinins improve dahlia vase life and does the effect on vase life vary depending on the type of cytokinin or method of application?
- Does the application of exogenous cytokinins affect expression of cytokinin response regulators?
- Does the expression of cytokinin biosynthetic genes vary between cut flowers and those left on the plant?
- Does expression of genes related to cytokinin biosynthesis, reception, breakdown, or signalling change as florets age?

6.3. Materials and Methods

6.3.1. Plant Growth and Harvesting

Plant material was bought from ‘Rose Cottage Plants’ (Essex, UK). The cultivars ‘Gerrie Hoek’, ‘Karma Prospero’, ‘Onesta’, and ‘Sylvia’ were used for experiments. Plants for these experiments were grown at the RHS Wisley research site (Deer Farm, Wisley, Surrey) in 2017 in the manner described in Chapter 2.1. Flowers were cut at Stage III (see Fig. 4).

6.3.2. Postharvest treatments

Postharvest treatments included xanthine, oleic acid, linoleic acid, BA (6-benzylaminopurine) and kinetin. Chemical sources and treatments were made as described in Chapter 2.2. Inflorescences were pulsed with BA for either 1, 3, 6, 18 or 24h before being removed to distilled water. Sprayed inflorescences were placed in a fume hood and sprayed from a distance of 30 cm until solution covered the entire inflorescence surface and left to dry before being removed to the constant temperature room (conditions of which are described in 2.1.). Inflorescences placed in continuous solutions were never moved to distilled water and were made sure never to be out of the relevant solution during the experimental period. Stems were cut to a length of 5 cm or in the case of ‘long stem’ flowers, 15 cm. All controls were placed in distilled water following cutting for the duration of the experimental period.

6.3.3. Floret mass

Floret mass was measured as described in 2.3.2. Florets were removed from the middle whorls of flowers. Five biological replicates, each representing a single inflorescence, were used for each category, using the average produced by the six individual florets picked from each indivudal inflorescence (Chapter 2.3.2.).

6.3.4. Conductivity

Conductivity was measured as described in 2.3.3. Florets were removed from the middle whorls of flowers. Five biological replicates, each representing a single inflorescence, were used for each category, using the average produced by the three pairs of florets picked from each indivudal inflorescence (Chapter 2.3.3.).

6.3.5. Protein content: extraction and assay

Material for analysis was collected 1, 4, and 7 days after cutting, or in the case of on-plant flowers, after they were tagged.

Protein extraction was performed as described in 2.3.4. Relative protein content was measured as described in 2.3.4. There were three technical replicates of each of the three biological replicates for each category, e.g. uncut 'Karma Prospero' 4 days after tagging (Chapter 2.3.4.).

6.3.6. RNA extraction

RNA extractions were performed using the NucleoSpin® RNA Plant kit (Macherey-Nagel) as described in 2.4.2. Quality of RNA and gDNA Wipeout was performed as described in 2.4.3. and 2.5.2.

6.3.7. Polymerase chain-reaction

Primer design, reverse transcription of RNA, gDNA Wipeout, PCR, sequencing of products and PCR analysis were all performed as previously described in Chapter 2.5. Three technical of replicates of each of three biological replicates for each category, e.g. 'Onesta' control flowers 7 days after cutting using primers *DvIPT3*, were used. The gene *IPT3* was chosen for a comparison of control (dH_2O) and on-plant because two sequences homologous to *IPT3* in *A. thaliana* were significantly upregulated in the differential expression dataset. *IPT3* is also known to be upregulated in senescent leaves in *A. thaliana* (Winter *et al*., 2007; Schmid *et al*., 2005) and is most heavily involved in nutrient signalling, suggesting that on-plant and cut flowers would differ in its expression as nutrient remobilisation would differ between them (Masclaux *et al*., 2000; Sakakibara, 2006; Kieber & Schaller, 2014).

ARR2 was chosen for comparisons of control (dH_2O) and exogenous BA (100 µM spray) treated flowers because a sequence homologous to the type-B ARR *ARR2* in *A. thaliana* was present in the dahlia transcriptome and was significantly upregulated. *ARR2* is implicated in senescence, its overexpression has been shown to extend leaf longevity (Kim *et al*., 2006). The results suggested that an AHK3-ARR2 phosphorelay was responsible for cytokinin–dependent regulation of leaf longevity by modulating downstream elements of the senescence pathway, for example hexose transporters, cell

wall invertases and promoting sink activity (Kim *et al*., 2006). Therefore, the hypothesis here is that exogenous BA could affect vase life through upregulation of positive mediators of cytokinin signalling, such as *ARR2*. In this study, the *Dahlia variabilis* (domesticated dahlia) genes were referred to as *DvIPT3* and *DvARR2* as in the manner of Suzuki *et al*., 2002, and Ohno *et al*., 2011 and 2013.

6.4. Results

6.4.1. Vase Life

None of the BA continuous solution treatments significantly improved vase life in cv. 'Gerrie Hoek' (Fig. 42A). In contrast BA sprays at both concentrations improved vase life by almost 2-fold compared to the control spray (Fig. 42B). BA pulses from 1h to 6h in length were also effective in improving vase life but pulses of 18h or 24h had no effect compared to controls (Fig. 42C). A combination of STS and BA also improved vase life, showing the highest mean vase life of 14 days (Fig. 42C).

In cv. 'Karma Prospero' continuous BA solutions had no effect on vase life (Fig. 43A). In contrast a BA spray of 100 µM improved vase life, but a 50 µM BA spray had no statistically significant effect (Fig. 43B). BA pulses of 1, 3, and 6h in length improved vase life, 18h had no effect, and 24h had a negative effect (Fig. 43C). A combination of STS and BA as a pulse resulted in the best mean vase life of over 13 days (Fig. 43C). Kinetin spray, oleic acid, and linoleic acid had no effect on vase life, whereas a 500 µM kinetin solution decreased vase life compared to controls (Fig. 44).

Vase life of cv. 'Onesta' inflorescences was significantly improved by treatment with a combination of STS and BA, or BA pulses of 1, 3, or 6h in length, while 18h pulses had no effect and 24h pulses negatively affected vase life (Fig. 45B, 46B). BA and kinetin sprays had no effect on vase life whereas BA and kinetin solutions negatively affected vase life (Fig. 45A). Oleic acid also had no effect on vase life whereas linoleic acid and xanthine significantly decreased vase life (Fig. 46A).

Vase life of cv. 'Sylvia' inflorescences was improved by BA sprays of 100 or 500 µM but not by spray treatment with 50 µM (Fig. 47A). Holding flowers continuously in BA solutions had no effect on vase life (Fig. 47A). All lengths of BA pulse used improved vase life, with the best performing BA pulse being 18h, a longer pulse than in any other cultivar assessed (Fig. 47B). A continuous kinetin solution of 50 µM slightly improved vase life whereas 500 µM kinetin solution decreased vase life, and 100 µM solution had no effect (Fig. 48A). Kinetin spray had no significant effect on vase life (Fig. 48A). Treatment with xanthine, linoleic acid or oleic acid had no effect on vase life (Fig. 48B), in contrast all combinations of BA and STS treatment improved vase life (Fig. 48C),

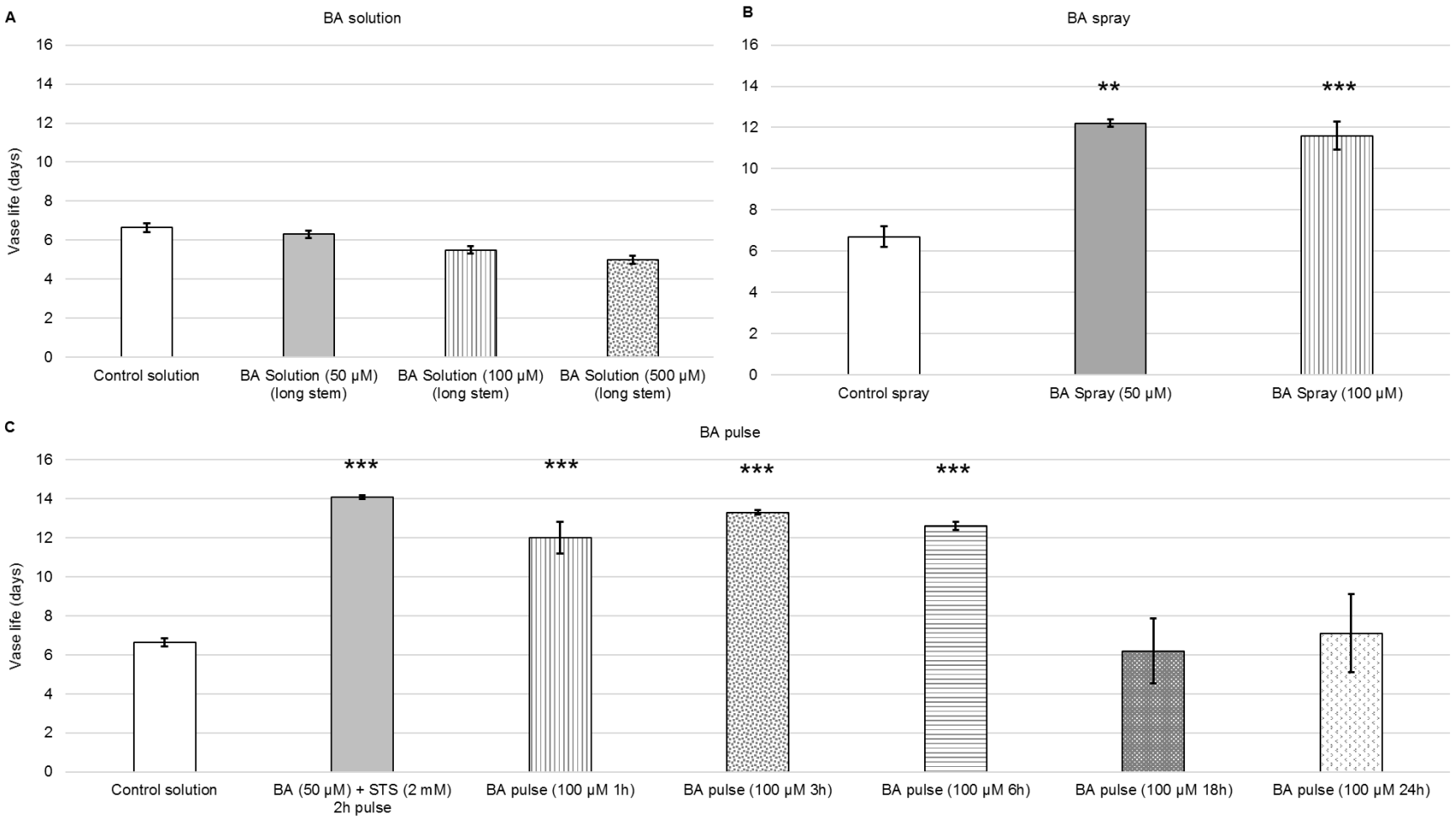


*Figure 42: Vase life (days) of cv. 'Gerrie Hoek' flowers treated with solutions (A), sprays (B), or pulses (C) of BA (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

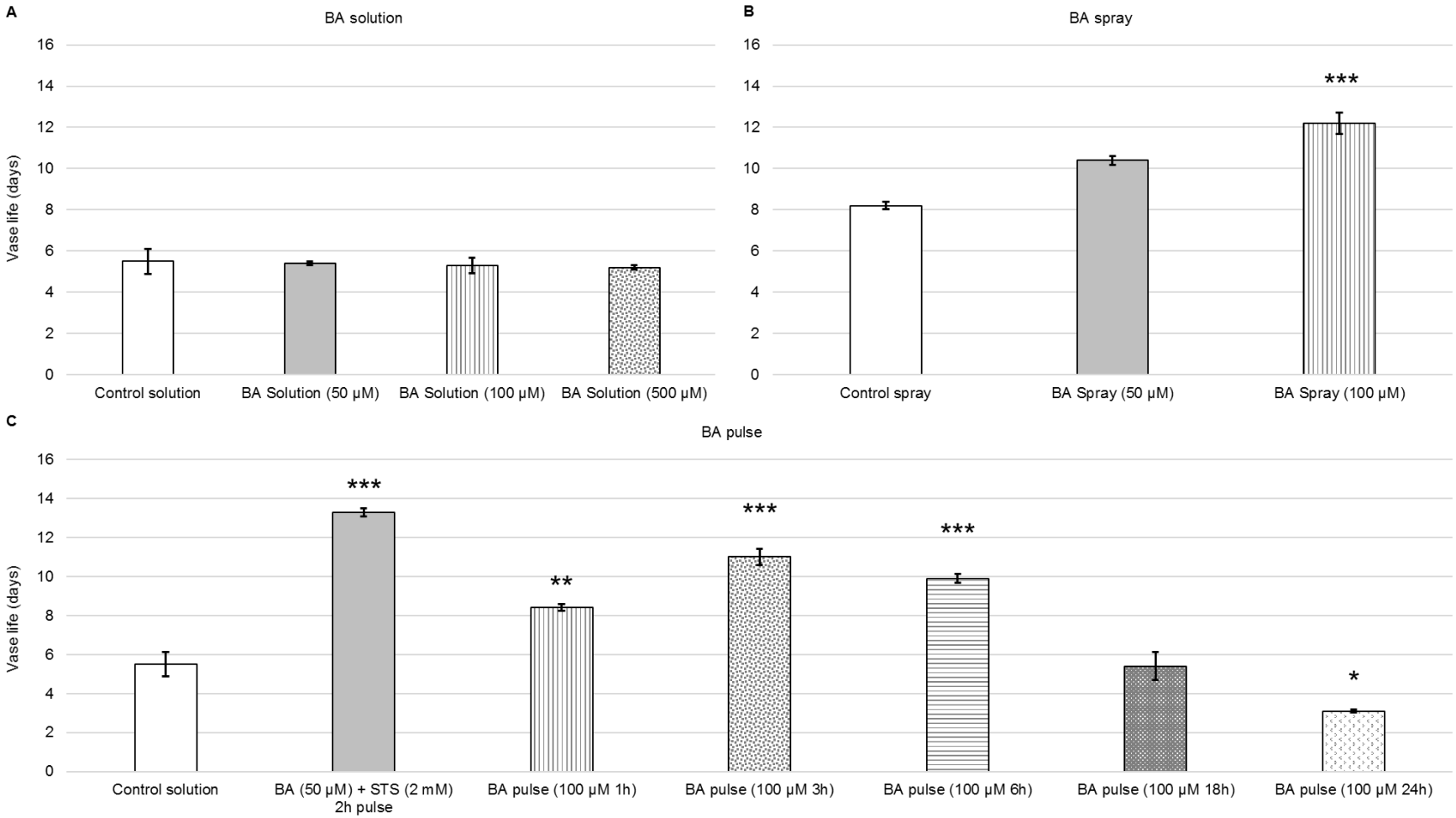


*Figure 43: Vase life (days) of cv. 'Karma Prospero' flowers treated with solutions (A), sprays (B), or pulses (C) of BA (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

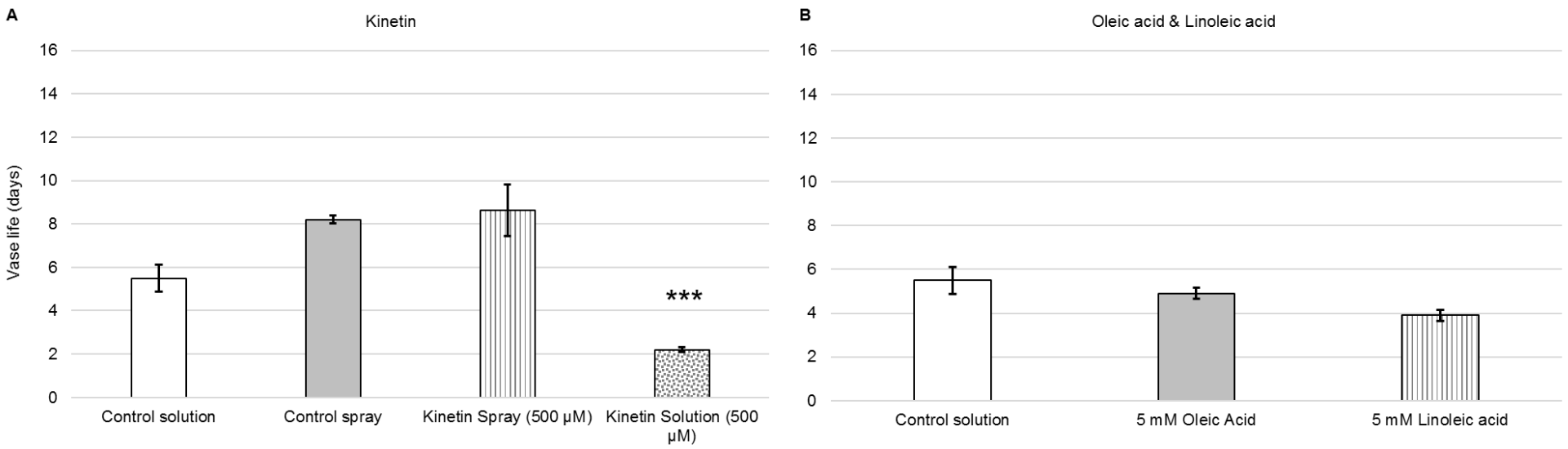


*Figure 44: Vase life (days) of cv. 'Karma Prospero' flowers treated with kinetin spray or solution (A), or with linoleic or oleic acid (B) (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

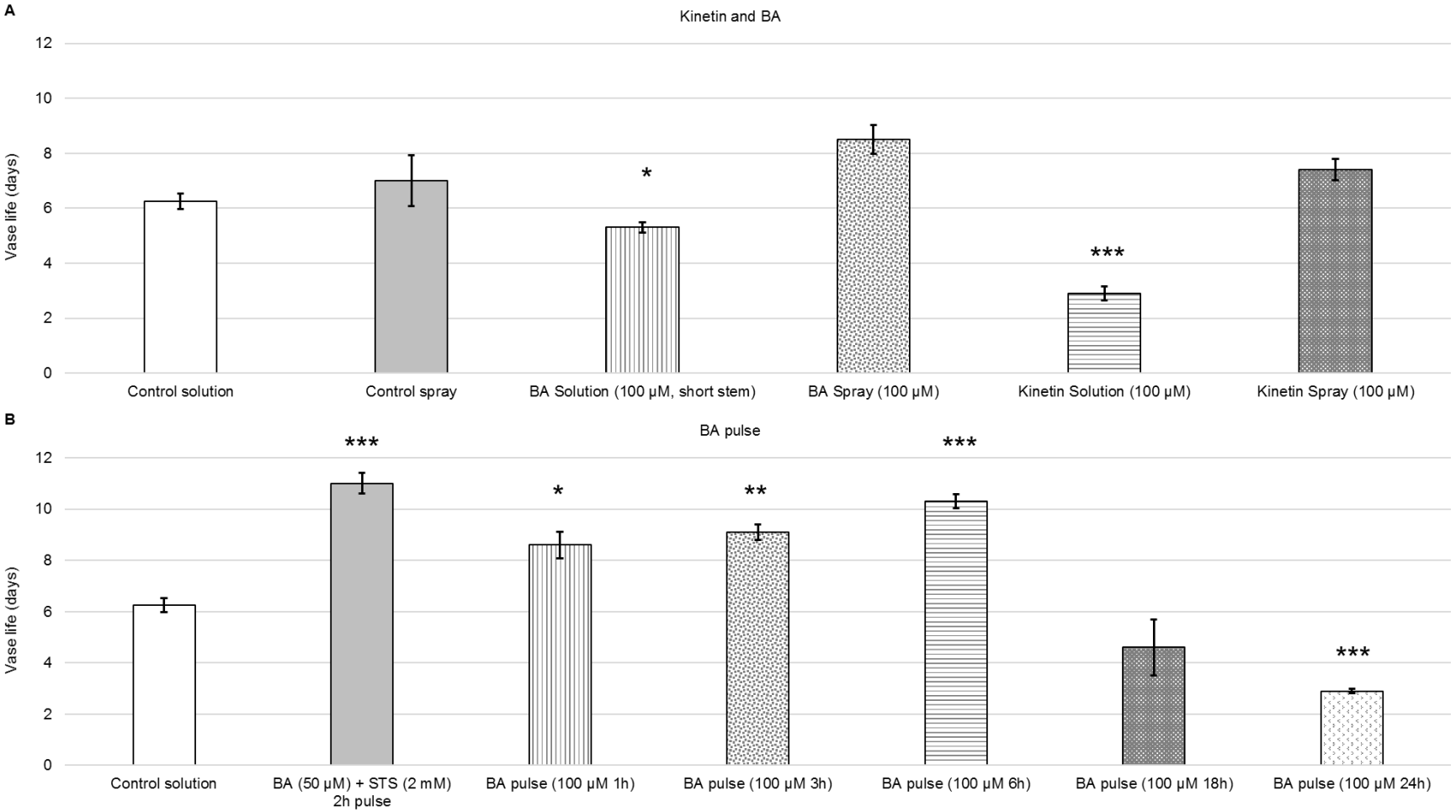


*Figure 45: Vase life (days) of cv. 'Onesta' flowers treated with BA spray or solution or kinetin spray or solution (A), or with a BA pulse (B) (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

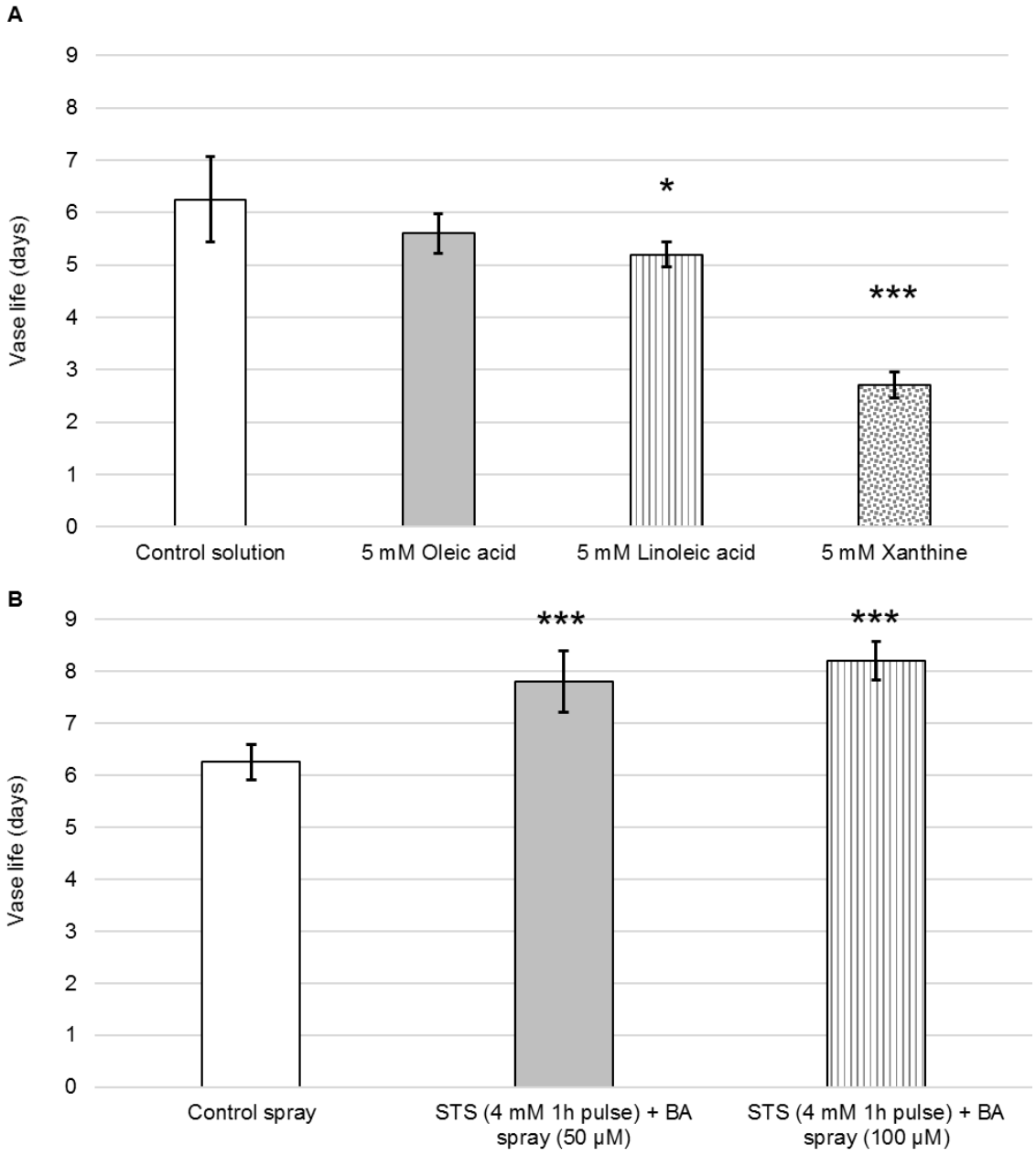


*Figure 46: Vase life (days) of cv. 'Onesta' flowers treated with oleic acid, linoleic acid or xanthine (A), or with an STS pulse in conjunction with a BA spray (B) (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

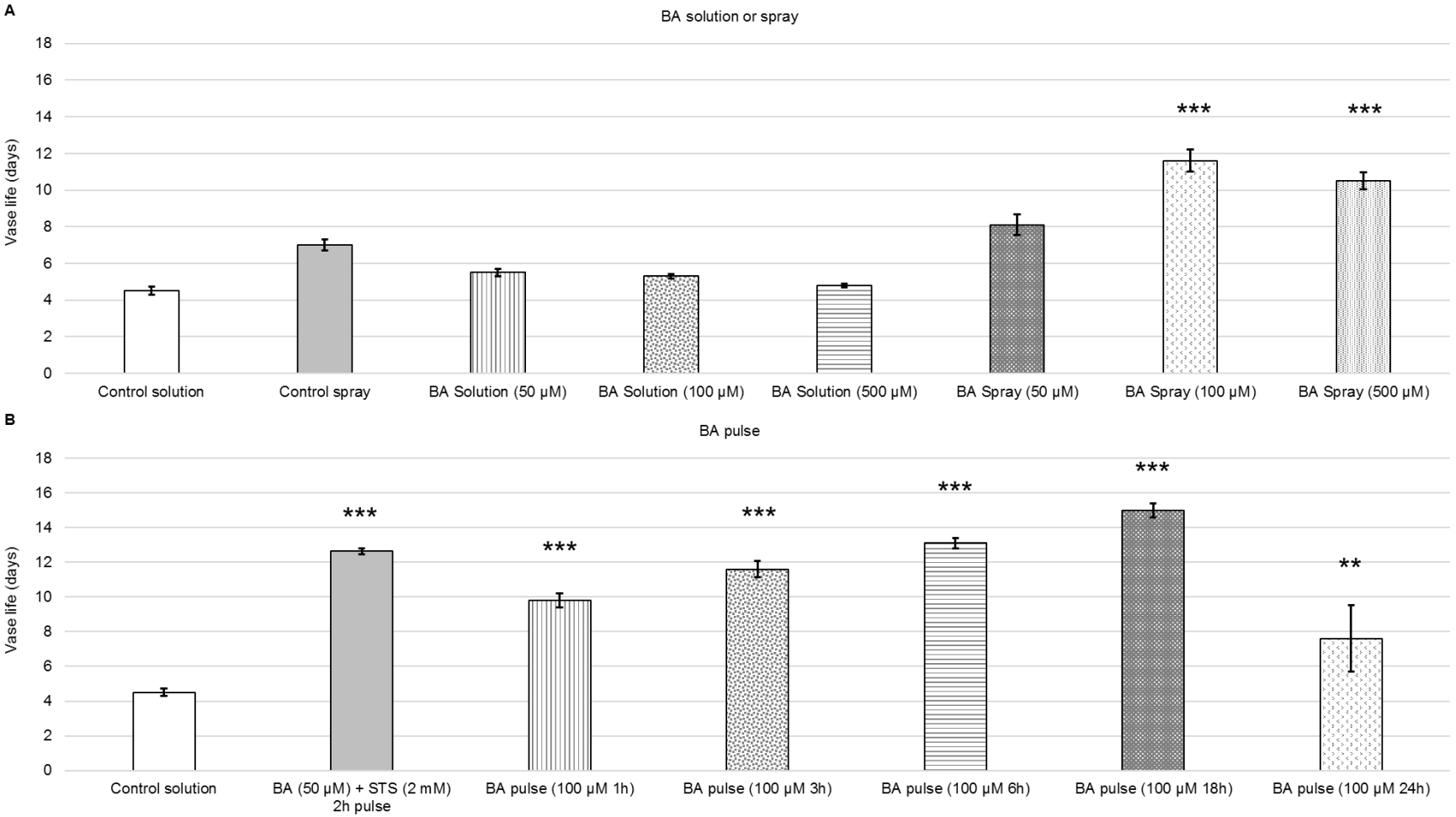


*Figure 47: Vase life (days) of cv. 'Sylvia' flowers treated with BA solutions or sprays (A), or with BA pulses (B) (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

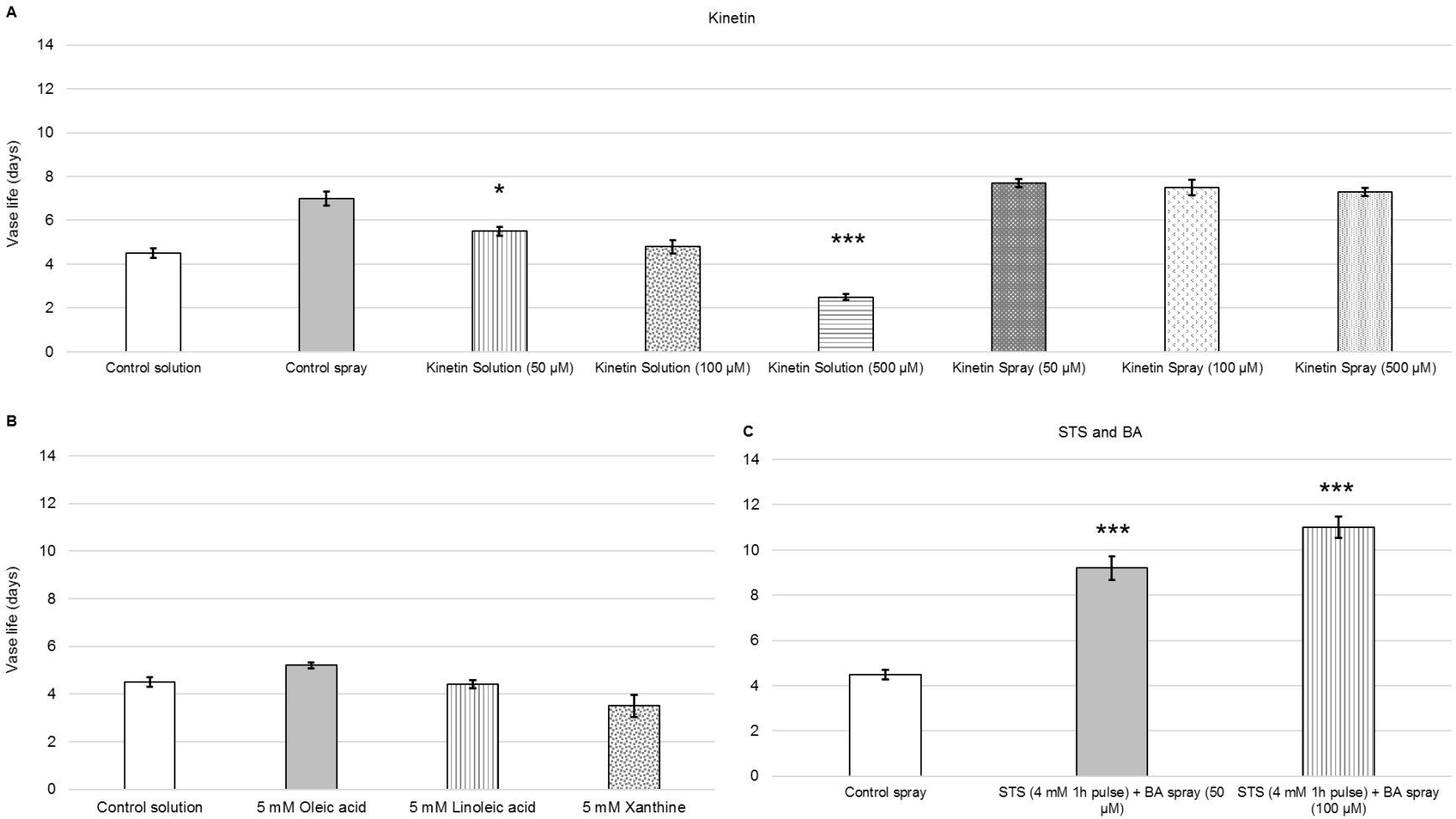


*Figure 48: Vase life (days) of cv. 'Sylvia' flowers treated with kinetin (A), or with oleic acid, linoleic acid, or xanthine (B), or an STS pulse in conjunction with a BA spray (C) (mean ± S.E.; n=5). Significance is comparison between control and treated flowers and is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (2-sample t-test, n=5).*

After 7 days control cv. ‘Gerrie Hoek’ flowers and those pulsed or sprayed with BA showed no wilting and retained colour for the whole experimental period (Fig. 49A, B, D). Those treated with a BA solution showed slight wilting by 7 days after cutting (Fig. 49C).

Both control flowers and flowers of cv. ‘Karma Prospero’ treated with a BA solution showed significant wilting 7 days after cutting, however the wilting was more extensive in those treated with BA solution (Fig. 50A, C). Flowers sprayed or pulse with BA showed no wilting after 7 days (Fig. 50B, D).

‘Onesta’ flowers treated as controls or with a BA solution showed extensive wilting by 7 days after cutting (Fig. 51A, C). Flowers sprayed or pulsed with BA showed no wilting 7 days after cutting, however, spray treated flowers opened significantly better compared with pulsed flowers (Fig. 51B, D).

In cv. ‘Sylvia’ control flowers showed the most extensive wilting and browning 7 days after cutting, however flowers treated with a BA solution also showed significant wilting and browning after 7 days (Fig. 52A, C). Flowers pulsed or sprayed with BA showed no wilting 7 days after cutting (Fig. 52B, D).

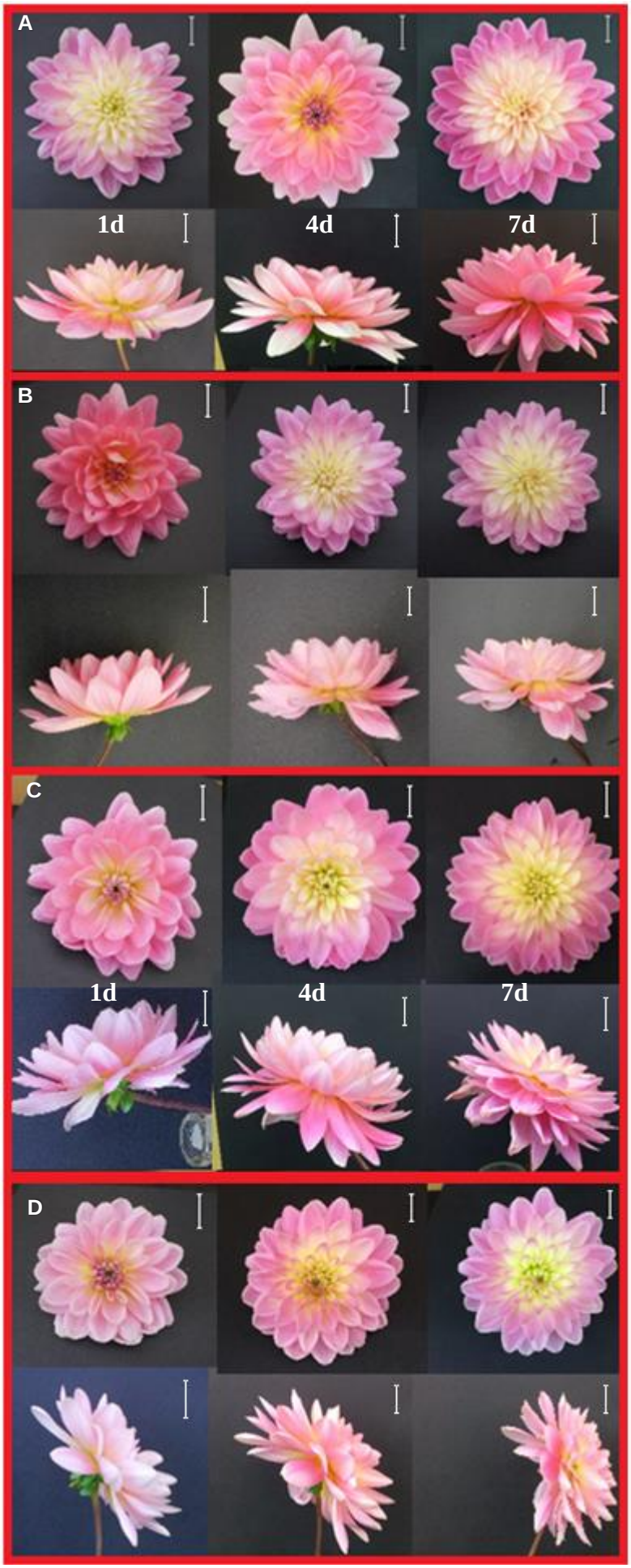


Figure 49: Photos of cv. 'Gerrie Hoek' flowers 1, 4, or 7 d after cutting stage (left to right) treated as controls (dH_2O) (A), with a 3 h BA pulse (100 µM) (B), a BA solution (100 µM) (C), or a BA spray (100 µM) (D). Scale bars represent 20 mm.

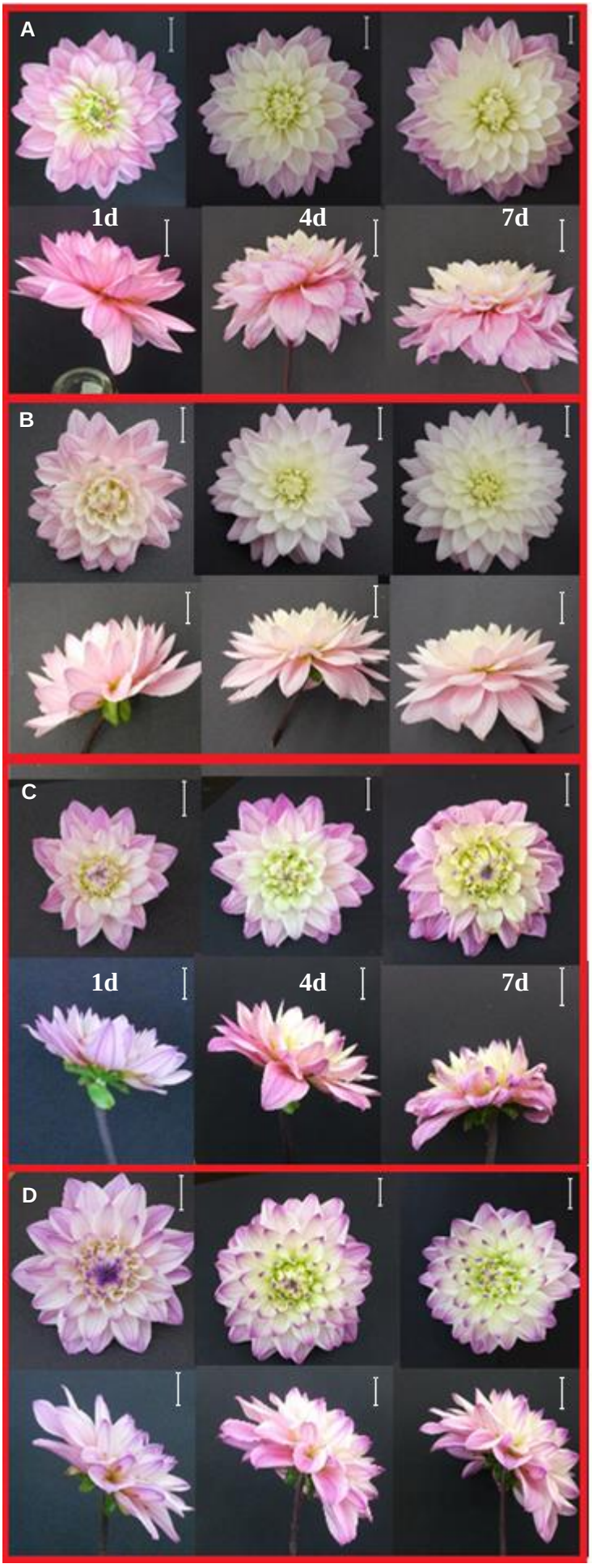


Figure 50: Photos of cv. 'Karma Prospero' flowers 1, 4, or 7 d after cutting (left to right) treated as controls (dH_2O) (A), with a 3 h BA pulse (100 µM), a BA solution (100 µM), or a BA spray (100 µM). Scale bars represent 20 mm.

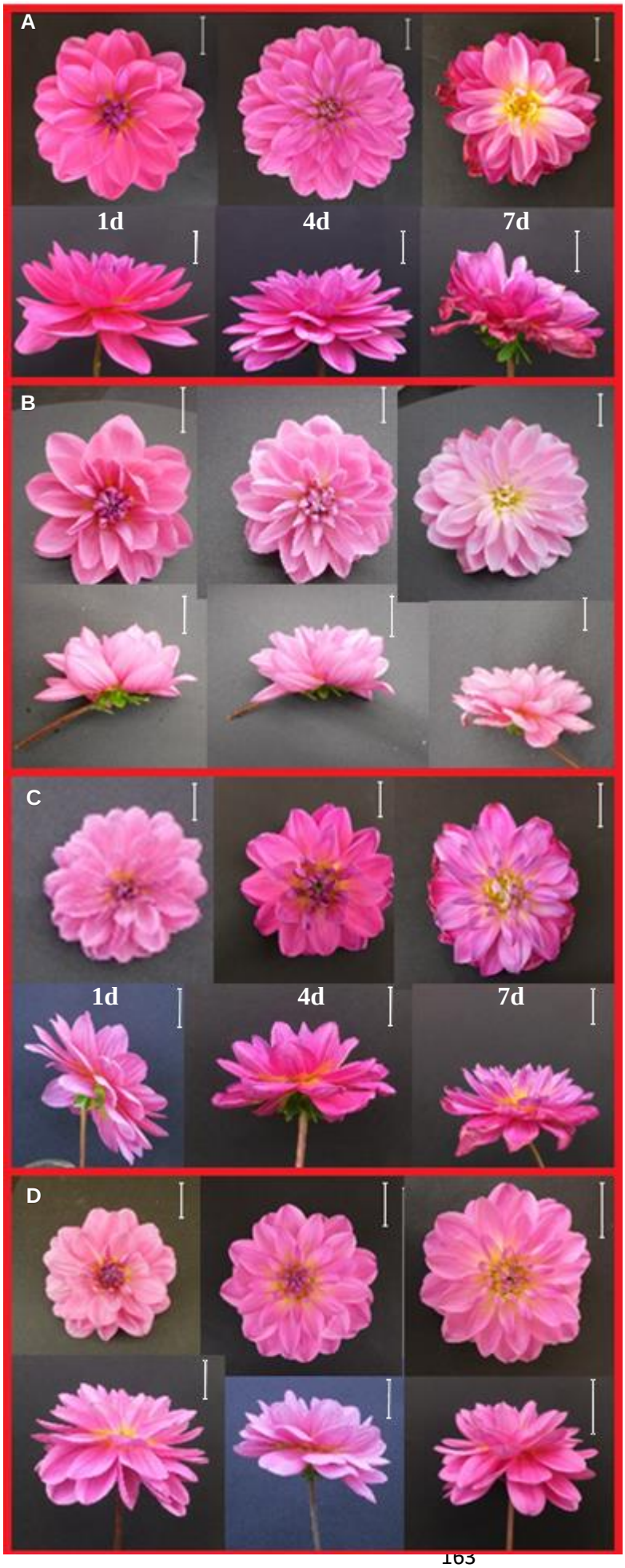


Figure 51: Photos of cv. 'Onesta' flowers 1, 4, or 7 d after cutting (left to right) treated as controls (dH_2O) (A), with a 3 h BA pulse (100 µM) (B), a BA solution (100 µM) (C), or a BA spray (100 µM) (D). Scale bars represent 20 mm.

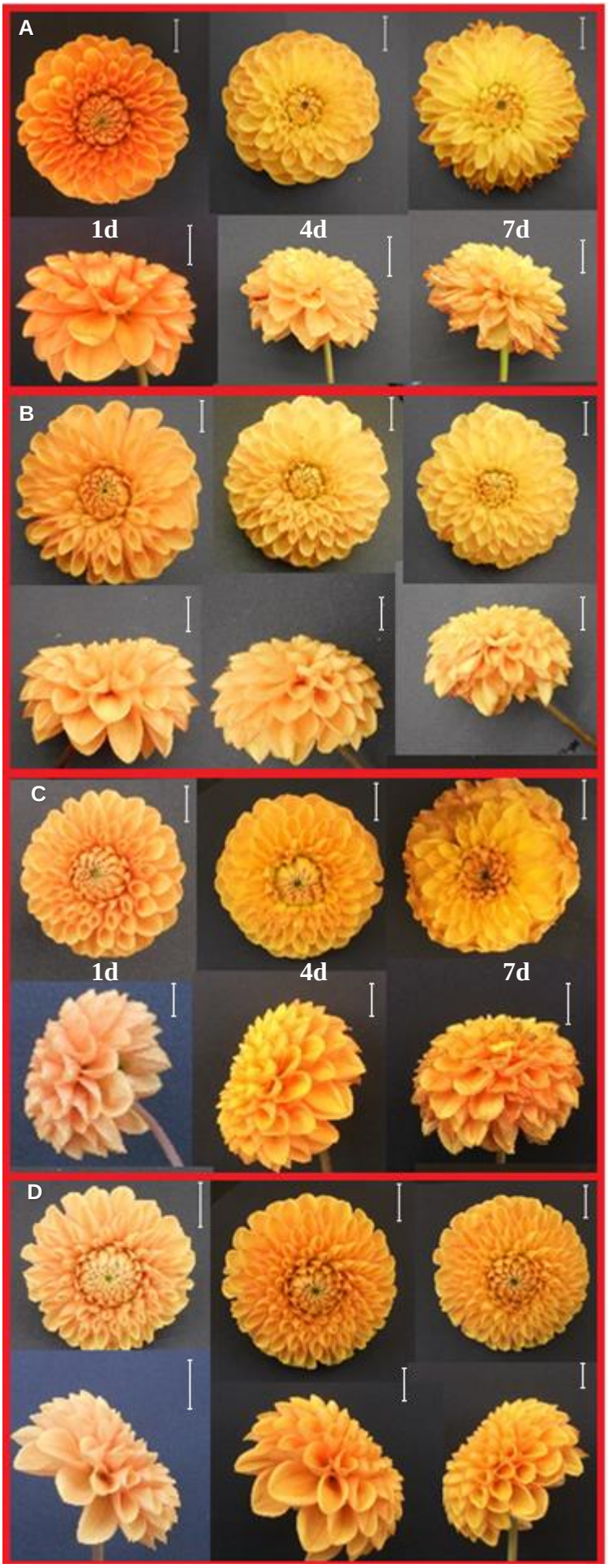


Figure 52: Photos of cv. 'Sylvia' flowers 1, 4, or 7 d after cutting (left to right) treated as controls (dH_2O) (A), with a 3 h BA pulse (100 µM) (B), a BA solution (100 µM) (C), or a BA spray (100 µM) (D). Scale bars represent 20 mm.

6.4.2. Conductivity

Membrane permeability as determined by percentage conductivity was significantly lower after 7 days in BA treated flowers in all cultivars except ‘Gerrie Hoek’ where conductivity was significantly higher compared to controls (Fig. 53). In ‘Onesta’, ‘Karma Prospero’, and ‘Sylvia’ conductivity of BA treated flowers was significantly lower compared to controls at 4 and 7 days after cutting (Fig. 53B-D). However, conductivity did rise in BA treated flowers in all three of those cultivars over the experimental period, though this rise was not as great as it was in control flowers (Fig. 53).

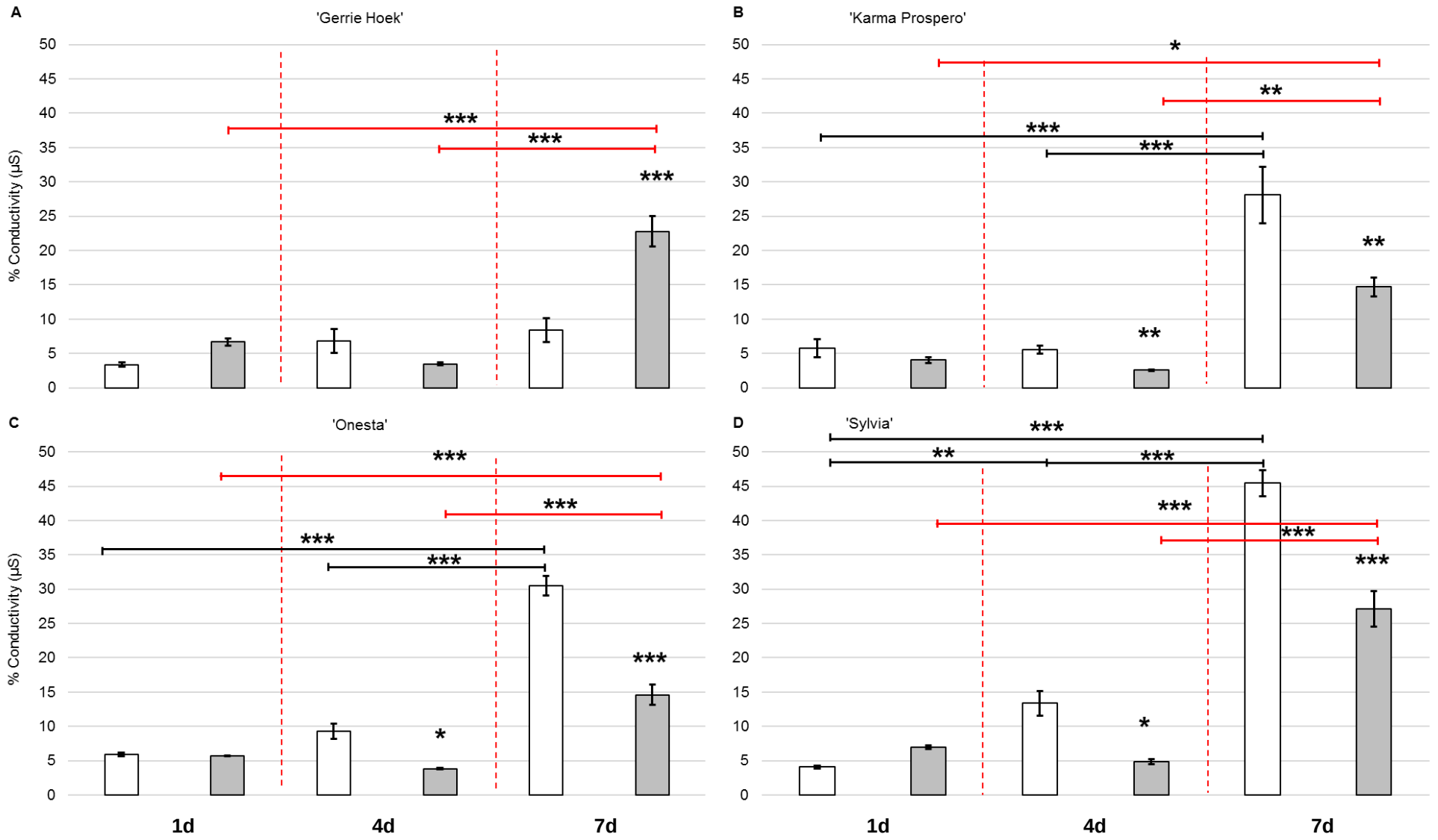

A
'Gerrie Hoek'
% Conductivity (µS)
B
'Karma Prospero'
C
'Onesta'
D
'Sylvia'
1d
4d
7d

*Figure 53: Conductivity of florets after 3 h submersion as a percentage of total conductivity of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D), control (dH_2O) flowers (white) compared to 100 µM BA spray treated flowers (grey), 1, 4, and 7 d after cutting (mean ± S.E.; n=5). Significance between different timepoints of the same treatment are represented by black (dH_2O) or red (BA) brackets. Asterisks above bars represent significant differences between control and treated flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=5).*

6.4.3. Floret mass

One day after treatment none of the cultivars tested showed any difference between controls and BA treated flowers (Fig. 54). Mass of BA treated flowers was significantly higher compared to controls (dH_2O) after 7 days in all cultivars except 'Gerrie Hoek' where no difference was observed (Fig. 54). In cv. 'Karma Prospero' and cv. 'Sylvia' this divergence in mass could be observed by day 4, where as in 'Onesta' it did not appear until day 7 (Fig. 54B-D). In all cultivars there was no significant decline in mass in BA treated florets over experimental period, whereas controls declined in mass significantly (Fig. 54).

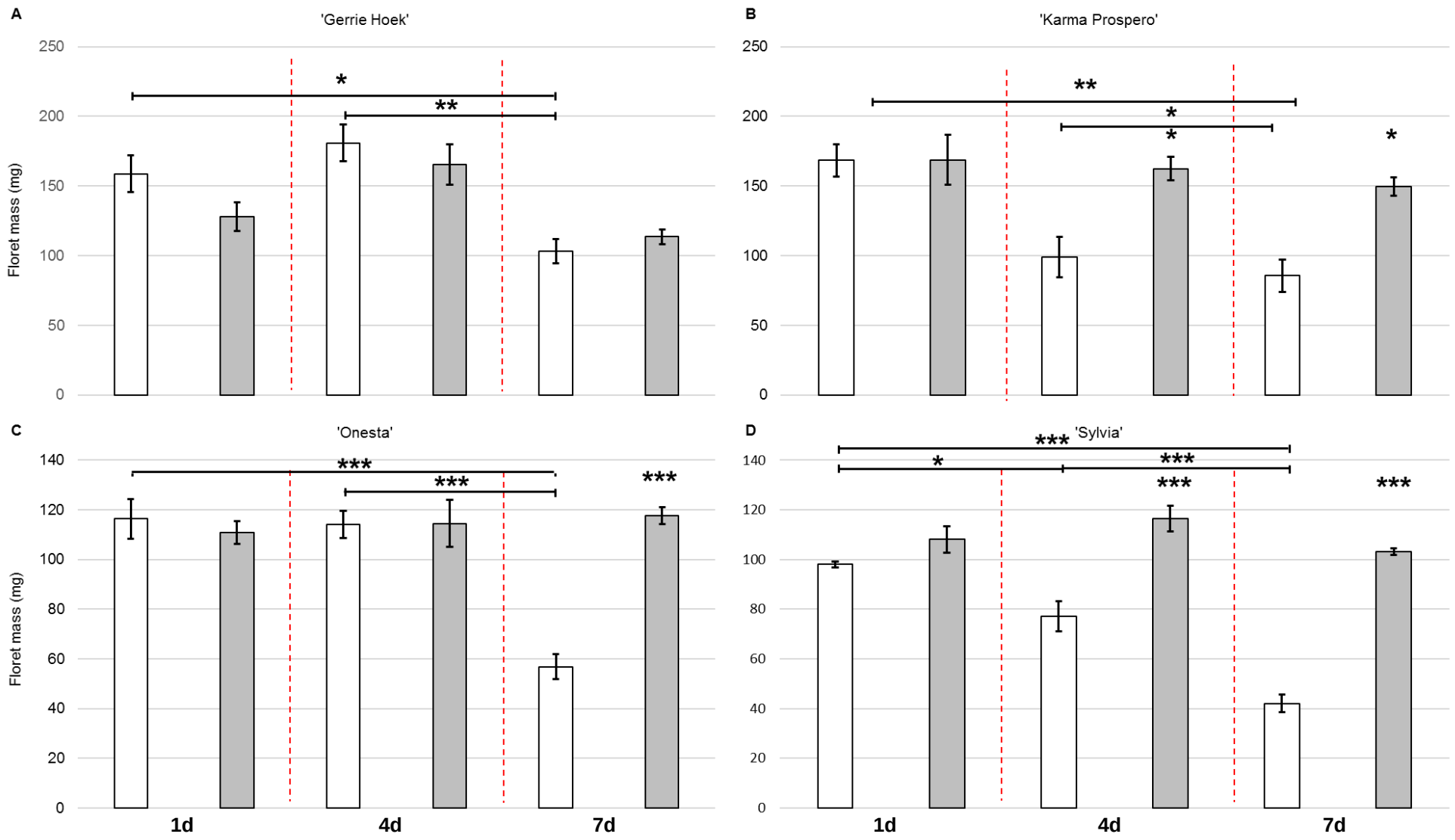

A
'Gerrie Hoek'
B
'Karma Prospero'
C
'Onesta'
D
'Sylvia'
Floret mass (mg)
Floret mass (mg)
250
200
150
100
50
0
140
120
100
80
60
40
20
0
*
**
***
1d
4d
7d

*Figure 54: Mass (mg) of cv. 'Gerrie Hoek' (A), 'Karma Prospero' (B), 'Onesta' (C), and 'Sylvia' (D) control (dH_2O) flowers (white) compared to flowers sprayed with 100 µM BA (grey), 1, 4, and 7 d after cutting (mean ± S.E.; n=5 Significance between different timepoints of the same treatment are represented by black (dH_2O) or red (BA) brackets. Asterisks above bars represent significant differences between control and treated flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=5).*

6.4.4. Protein content

There were no significant differences in protein content between control and BA (100 µM spray) treated flowers 1, 4 and 7 days after cutting in either cultivar (Fig. 55). However, in 'Karma Prospero' protein content declined in controls between 1 and 7 days, whereas in BA treated flowers there no significant change over the 7 days (Fig. 55A). In 'Onesta' controls showed a decline early, between days 1 and 4, after which the decline plateaued, whereas in the BA treated group content there was no significant decline in protein content over the 7 days (Fig. 55B).

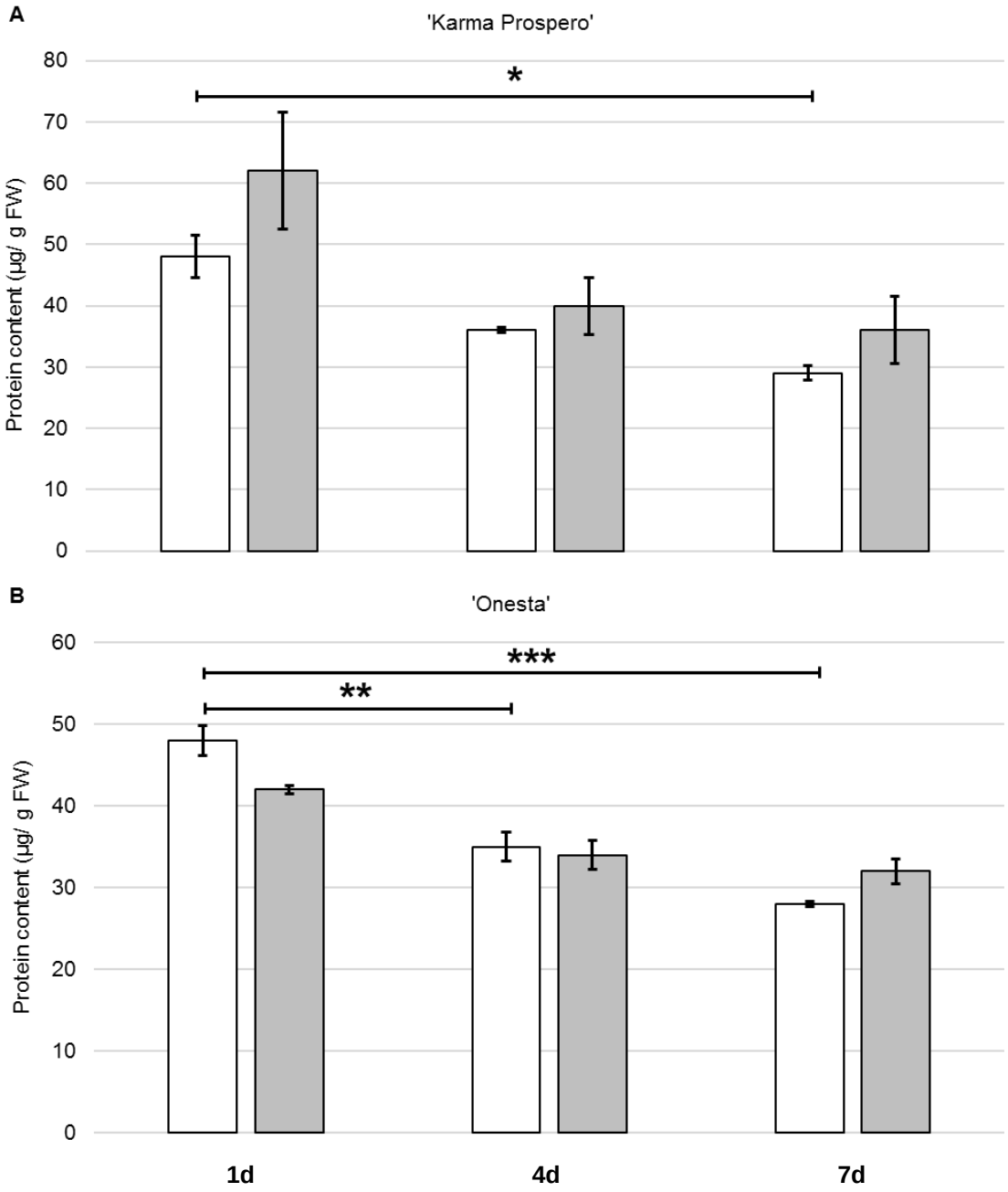


*Figure 55: Protein content (µg/ g FW) of cv. 'Karma Prospero' (A) and 'Onesta' (B) control flowers (dH_2O) (white) or flowers sprayed with 100 µM BA (grey) sampled 1, 4 or 7 d day after cutting (mean ± S.E.; n=3). Significance between different timepoints of the same treatment are represented by black (dH_2O) or red (BA) brackets. Asterisks above bars represent significant differences between control and treated flowers for each day point and are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA, followed by Tukey's test, n=3).*

6.4.5. Quantitative reverse transcription polymerase chain-reaction (RT-qPCR)

No significant differences in relative expression of *DvARR2* were found between any treatment or day group in cv. ‘Onesta’ (Fig. 56A). However, expression of *DvIPT3* was significantly lower in on-plant ‘Onesta’ flowers 4 days after cutting stage (Stage III, see Fig. 4) compared to flowers sampled 1 day after cutting stage (Fig. 56B).

The pattern of expression of *DvARR2* and *DvIPT3* in cv. ‘Sylvia’ was similar to that observed in cv. ‘Onesta’ (Fig. 56, 57). No significant differences in relative expression of *DvARR2* were found between any treatment or day group in cv. ‘Sylvia’ (Fig. 57A). However, expression of *DvIPT3* was significantly lower in on-plant ‘Sylvia’ flowers 4 days after cutting stage compared to flowers sampled 1 day after cutting stage (Fig. 57B). However, the large variation seen in some samples due to variation between biological replicate suggests more sampling would be helpful in better establishing expression of *ARR2* and *IPT3*.

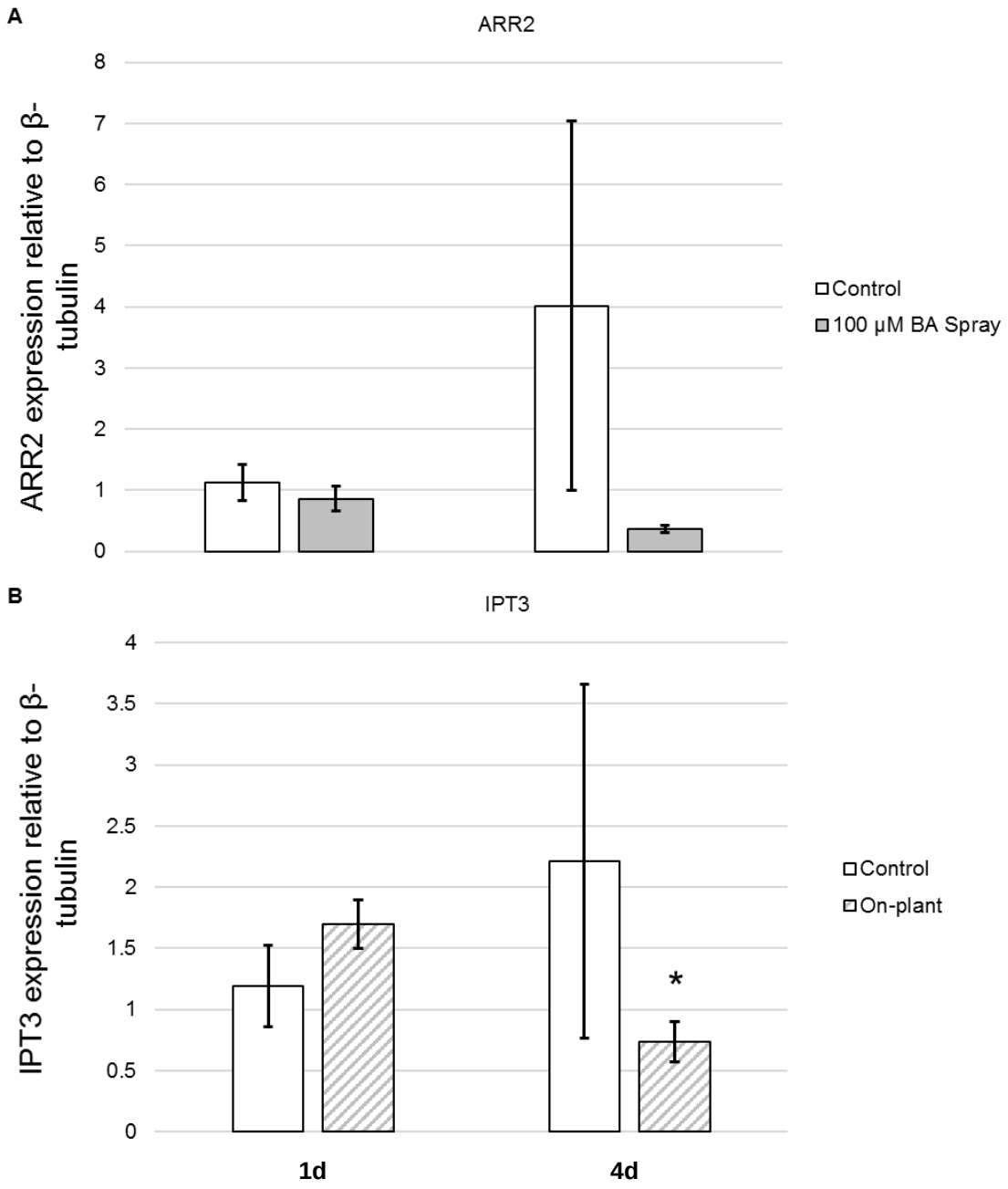


*Figure 56: Relative expression of ARR2 (A) in cv. 'Onesta' flowers treated as controls (dH_2O) (white) or with a 100 µM BA spray (grey), and IPT3 (B) in cv. 'Onesta' flowers treated as controls (dH_2O) (white) or left on the plant (striped), 1 or 4 days after cutting or cutting stage using β-tubulin as a reference (mean ± S.E.; n=3). Significance is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (two-sample t-test, n=3).*

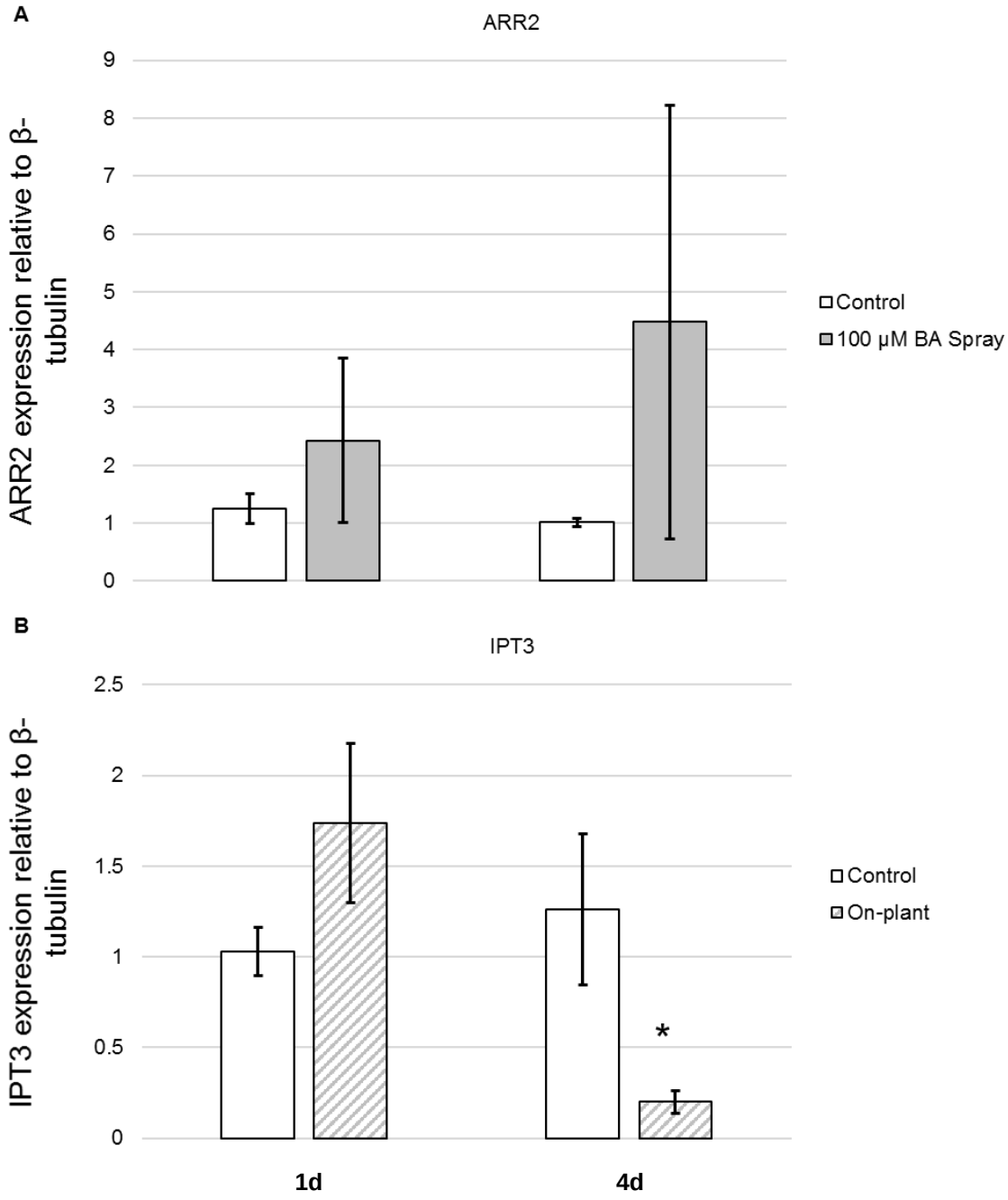


*Figure 57: Relative expression of ARR2 (A) in cv. 'Sylvia' flowers treated as controls (dH_2O) (white) or with a 100 µM BA spray (grey), and IPT3 (B) in cv. 'Sylvia' flowers treated as controls (dH_2O) (white) or left on the plant (striped), 1 or 4 days after cutting or cutting stage using β-tubulin as a reference (mean ± S.E.; n=3). Significance is indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ (two-sample t-test, n=3).*

6.5. Discussion

Cytokinins are known to extend vase life in many different ornamental plant species, including dahlia (Shimizu-Yumoto & Ichimura, 2013). In this study the response of dahlia cultivars popular in the UK to various cytokinin treatments was assessed, and genes involved in cytokinin biosynthesis and signalling were examined through RT-qPCR and differential expression analysis of transcriptomic data. The results show that exogenous cytokinin, if applied as a spray can slow down physiological deterioration of dahlia florets including maintaining their mass and cellular membrane integrity (Fig. 53, 54). It also showed that BA treated florets declined in protein content more slowly than controls and expression of the cytokinin biosynthetic gene *IPT3* differed between cut flowers and those attached to their parent plant (Fig. 55, 56, 57). Furthermore, differential expression analysis showed downregulation of IPT's in senescing dahlia tissue, and upregulation of cytokinin catabolic enzymes (Fig. 20). Furthermore, genes involved in cytokinin signal transduction were upregulated showing that cytokinins are still playing some role before visible signs of senescence (Fig. 19).

The work presented in this chapter was to elucidate the effect exogenous cytokinins have on cut dahlia flowers of different cultivars when applied at different concentrations and with different application methods. Across all cultivars, BA solutions were outperformed in vase life extension by BA pulses or sprays (Fig. 42, 43, 45, 47). The improvement in postharvest attractiveness 7 days after cutting in response to BA treatment strongly suggests cytokinins have a role in the control of the endogenous senescence process of at least some dahlia cultivars. These results are consistent with those found previously in other dahlia cultivars (Shimizu-Yumoto & Ichimura, 2013). In the present study spraying, pulses and continuous solutions were used as a method of BA application based on previous results with dahlia (Shimizu-Yumoto & Ichimura, 2013). In that study, on a single cultivar ('Kokucho'), dipping was found to be more effective than spraying. However, a 50 µM spray was more effective than a 100 µM spray, whereas in the present study the reverse was found in the cultivars 'Gerrie Hoek', 'Sylvia' and 'Karma Prospero' (Fig. 42, 43, 47). This suggests varying sensitivity to exogenous cytokinins amongst cultivars of dahlia. The success of dipping and spraying found by Shimizu-Yumoto and Ichimura (2013) compared to solutions, and the effects of sprays and pulses in this study compared to solutions, also suggests that the method of application is very important in

determining how effective BA treatments are, and that cytokinins have different effects depending on their localisation or concentration. However, BA treatment extended vase life in the semi-cactus type dahlia 'Kokucho', the decorative type dahlia cv. 'Kamakura' and the pom-pom dahlia cv. 'Michan' (Shimizu-Yumoto & Ichimura, 2013), in addition to the water lily type cultivars 'Gerrie Hoek', 'Karma Prospero', and 'Onesta' and ball type cv. 'Sylvia' used in this study, suggesting that BA treatment is effective and could produce similar results across many dahlia cultivars, including those which are morphologically disparate.

Visually, cv. 'Gerrie Hoek' flowers showed slight wilting on the outer florets 7 days after treatment with BA solution, whereas controls, sprayed and pulsed flowers all showed no wilting (Fig. 49). This indicates that these BA solutions as a treatment were accelerating senescence. Similarly, 'Karma Prospero' flowers treated with BA solution showed significant wilting compared with controls, which showed only slight wilting after 7 days (Fig. 50). The sprayed and pulsed flowers showed no wilting at all. All this suggests that BA in too great amounts, for example with a continuous solution, can accelerate senescence. Similar results are found in cv. 'Onesta' (Fig. 51). However, in cv. 'Sylvia', flowers treated with a BA solution showed only slight wilting after 7 days, which was not as extensive as in either 'Onesta' or 'Karma Prospero' (Fig. 52). Thus, overall, solutions of BA that give continuous exposure of the hormone to the flowers had a negative effect on vase life (Fig. 42, 43, 45, 47). This effect may be due to the induction of hypersensitivity to cytokinins, high concentrations of cytokinins have been found to induce PCD in both carrot and *A. thaliana* (Carimi *et al*., 2003). Additionally, in a study on tobacco it was found that high cytokinin levels induced a hypersensitive-like response which led to necrotic lesions forming on the tobacco's leaves, this is usually associated with pathogen attack (Novák *et al*., 2013). This suggests that high levels of cytokinin, such as those up taken by the dahlias placed in continuous BA solution, are causing a response similar to that caused by pathogenic attack leading to accelerated cell death. This was caused by an upregulation of *ipt* in the tobacco, however more work would need to be carried out in BA solution treated dahlias to examine whether this is also the case in dahlia (Novák *et al*., 2013). The sprays and pulses expose florets to exogenous cytokinin for a much shorter period. However, the effectiveness of BA pulses shows that in vase solution uptake is still efficient enough for the BA to be localised to the inflorescence and improve vase life and may avoid the need for spraying as a commercial

treatment. Although spraying was found to be very effective, making sure each inflorescence is given an equal volume of solution is harder to control.

A combination of STS and BA as a pulse was effective and gave the highest vase life in cv. 'Gerrie Hoek', 'Onesta', and 'Karma Prospero' of any treatment (Fig. 42, 43, 45, 47). In cv. 'Sylvia' the most effective pulses were much longer than those in other cultivars: 18h compared to 3h for 'Karma Prospero' and 'Gerrie Hoek' or 6h in cv. 'Onesta'. A high concentration 500 μM BA spray was also effective in 'Sylvia' (Fig. 47). This suggests that there is a variation in sensitivity to cytokinins between cultivars, and that cultivars like 'Sylvia' may require greater concentrations of BA to produce the same outcome as other cultivars which are more sensitive to exogenous cytokinins.

Treatment with BA consistently improved postharvest measurements of senescence such as maintenance of floret mass and cellular membrane integrity (Fig. 53, 54). This agrees with other studies, where exogenous BA has been found to delay membrane deterioration during the senescence processes in *Helianthus annuus* cotyledons (Naik *et al*., 2002) and rice leaves (Liu *et al*., 2015). In all cultivars except 'Gerrie Hoek' BA treated florets had significantly lower conductivity after 7 days compared to controls (Fig. 53). Similarly, all cultivars except 'Gerrie Hoek' showed greater floret mass after 7 days compared to controls (Fig. 54). In 'Gerrie Hoek' there were no significant differences, however floret mass in both controls and BA treated flowers did peak 4 days after cutting before decreasing after 7 days. This suggests that the positive effects of BA may not be seen until after 7 days in 'Gerrie Hoek' as controls of this cultivar performed better than those of the other cultivars examined. Experiments 8, 9, or 10 days after cutting may be helpful, however the performance of 'Gerrie Hoek' controls suggests they could be good cut flowers commercially and certainly as a future interest for research into dahlia flower senescence. The improved cellular integrity of dahlia flowers treated with BA may be due to upregulation of genes involved in cell membrane maintenance that have been found to be induced following exogenous cytokinin treatment, for example cell wall invertase in *Chenopodium rubrum* and phospholipase A1 (*PLA1*) in petunia (Trivellini *et al*., 2015; Ehneß & Roitsch, 1997). This would also explain the delayed wilting of BA sprayed or pulsed dahlias, as the exogenous cytokinins would be aiding in the maintenance of cell membranes, the breakdown of which is one of the main causes of wilting. Symptoms of senescence, including petal browning and wilting, are due in part to the degradation of proteins and phospholipids (van Doorn & Woltering, 2008). There was an evident delay of browning and wilting of petals in flowers of all cultivars treated with exogenous BA

as a pulse or spray compared with controls (Fig. 49-52), therefore the differences between treated and control flowers in this study may be attributable to exogenous cytokinins upregulating genes which code for 'protective' proteins, or downregulating genes which code for degrading proteases or lipases. The lower levels of conductivity 7 days after treatment in three of the four cultivars assessed also suggests that enzymes involved in membrane and cell wall breakdown are being repressed or downregulated due to exogenous BA treatment (Fig. 53).

Kinetin was a less effective postharvest treatment compared to BA, and kinetin solutions significantly decreased vase life in cv. 'Karma Prospero', 'Onesta', and 'Sylvia' (Fig. 44, 45, 47). This may be because kinetin is a naturally occurring cytokinin and can be catabolised by endogenous enzymes such as cytokinin oxidases and dehydrogenases (Bilyeu *et al*., 2001). In contrast it has been found that BA does not act as a substrate for cytokinin oxidases in maize (Bilyeu *et al*., 2001), suggesting that some catabolic enzymes may not be able to break BA down thus it may take longer for plants to break down exogenous BA. This may explain why BA was effective in improving vase life as its positive effects persisted as the BA took longer to be catabolised, however further work on catabolism of exogenous BA would be needed to confirm this.

In both cultivars tested ('Onesta' and 'Karma Prospero') protein content in BA treated florets did not significantly decline (Fig. 55). In contrast control flowers showed significant declines in protein content over the 7 days (Fig. 55). This suggests that BA is affecting protein breakdown, however it is possible it is doing this indirectly. It has been found that ethylene induces protease activity during senescence (Jones *et al*., 2005; Tripathi *et al*., 2009). If cytokinins repress ethylene biosynthesis, and there is evidence they do in chrysanthemum (Guo *et al*., 2003), it may be that BA treatment slows down protein breakdown indirectly through its repression of ethylene.

The exact reason for cytokinin's improvement of vase-life is not known but it could be due to various actions which help maintain cellular activity and repress the senescence process, e.g. by regulating other phytohormones, maintaining cellular integrity or helping in nutrient transport or protection against ROS (reactive oxygen species). Guo *et al*., (2003) found that BA promoted gibberellic acid and inhibited ethylene production induced by ABA, helping to lengthen vase life of cut *Chrysanthemum*. The researchers concluded that it was BA which was the main factor in retarding senescence in the chrysanthemum. It is possible that the results seen in this study, including improved postharvest attractiveness and retention of petal mass for a longer period of time, are due

to exogenous cytokinins affecting levels of other phytohormones, including promotion of gibberellins and inhibition of ethylene biosynthesis.

In *Chenopodium rubrum* plants, cytokinins can induce the accumulation of the mRNA encoding cell wall invertase, which has a major role in supplying carbohydrates and amino acids to cells via the apoplastic pathway (Ehneß & Roitsch, 1997). This suggests that the BA treatments in this study may have induced transcription of genes that delay senescence through such mechanisms thus enhancing supplies of nutrients to cells and consequently lengthening the amount of time they can remain viable. Furthermore, transcriptomic analyses in petunia have found that addition of exogenous cytokinins to petunia upregulates HSP's (heat shock proteins) thus increasing stress tolerance (Trivellini *et al*., 2015). Moreover, genes affecting lipid metabolism were upregulated after cytokinin addition, including *PLA1*, which encodes an enzyme whose function centres on membrane maintenance, indicating that cytokinins may work to prolong senescence by maintaining cellular integrity and thus floral tissue integrity for a longer period (Trivellini *et al*., 2015; Richmond & Smith, 2011). Similarly, the transcriptomic analysis in petunia showed that up-regulation of genes involved in the defence against reactive oxygen species (ROS), including catalase, ascorbate peroxidase and ascorbate oxidase occurred following cytokinin addition (Trivellini *et al*., 2015). Some, or all, of these factors could have contributed to exogenous BA's extension of vase life in dahlia.

Oleic acid, linoleic acid and xanthine were used in this study due to their implication in the inhibiton of glycosyltransferases which inactivate endogenous cytokinis (Hou *et al*., 2004; Won *et al*., 2007). In *Arabidopsis*, overexpressing the glycosyltransferase *UGT76C1*, resulted in higher levels of glycosides (inactive forms of cytokinins) showing that increased activity of this enzyme increased the rate of cytokinin inactivation (Hou *et al*., 2004). Conversely, inhibiting *UGT76C2*, resulted in a phenotype with lower lateral root density due to increased cytokinin sensitivity (Wang *et al*., 2011). Thus, inhibiting N-glucosyltransferase may be a method to increase cytokinin sensitivity. Xanthine, oleic acid, and linoleic acid have been implicated in inhibiting the enzyme U-glucosyltransferase (Hou *et al*., 2004; Won *et al*., 2007), therefore the hypothesis was tested that their addition could improve vase life. In this experiment addition of exogenous xanthine, oleic acid, and linoleic did not improve vase life in any dahlia cultivar assessed (Fig. 44, 46A, 47A). This may be because U-glucosyltransferase is not an enzyme involved in dahlia floral senescence. Alternatively, previously these chemicals were applied differently to plants, either as sprays on leaf discs in the case of xanthine

(Hou *et al*., 2004) or to an extract of ground leaves in the case of oleic and linoleic acid (Won *et al*., 2007) so their effects upon plant tissue as a solution were not known. In this study as a continuous solution they were ineffective, however this was also found with BA treatment and future work using pulses or sprays may find these different application methods to be successful.

As cytokinins delay senescence, endogenous cytokinin levels fall during leaf senescence (van Staden, 1973; Singh *et al*., 1992), and upregulation of *IPT* delays flower senescence (Chang *et al*., 2003; Fig. 22), it would be expected for *IPT* to be downregulated during flower senescence. The downregulation of the expression of the *DvIPT3* gene in on-plant flowers between 1 and 4 days after cutting stage suggests that cytokinins are being downregulated days before visible wilting in on-plant flowers (Fig. 56, 57). This may because *IPT3* is implicated in nutrient signalling, and this may be less important in less senescent on-plant flowers where nutrient remobilisation from senescing to developing tissues is not yet crucial (Masclaux *et al*., 2000; Sakakibara, 2006; Kieber & Schaller, 2014). The downregulation of *DvIPT3* was found in both cultivars assessed, 'Sylvia' and 'Onesta' suggesting this effect is consistent between cultivars, however more samples, perhaps of more time points and more cultivars, would need to be examined for this to be verified.

Type-B ARR's are genes implicated in the cytokinin signal transduction pathway which positively regulate the downstream activity caused by cytokinin reception, for example in sink activity and maintenance of cellular integrity (Argyros *et al*., 2008; Kieber & Schaller, 2014; Hwang & Sheen, 2001; Sakai *et al*., 2001; Fig. 23). The RT-qPCR showed that *DvARR2* did not significantly change in expression between control and BA treated dahlias, or between day 1 and day 4 dahlias treated with BA (Fig. 56, 57). This suggests that exogenous BA has no effect on the signal transduction of dahlia, however it may be that other genes in the pathway were affected by the BA.

In the future, more research needs to be performed on cut dahlia with stem lengths comparable to industry standards to ensure BA pulses are still effective at stem lengths of up to 50 cm, as in this study a 5 cm stem length was used to maximise replication for multiple experiments in a short growing season. More research could also include performing RNA-sequencing on cut dahlia treated with exogenous BA to examine its effects on gene expression or performing further RT-qPCR on genes implicated in cytokinin signalling, breakdown, or the positive effects of cytokinin, e.g. those involved in cell membrane maintenance. A greater number of time points could be used in future

experiments to determine changes in individual florets over time, and experiments across multiple seasons to check that the results are consistent from year to year which will be vital if the dahlia is to become a commercially viable cut flower. Finally, future work could also focus on investigating the effects of cytokinins in conjunction with other phytohormones, such as ethylene, ABA and gibberellic acid, to decipher the complex interactions between them during the flower senescence process.

7. General discussion and conclusion

The aim of this study was to investigate the dahlia floral senescence process and find ways to delay it, so that dahlia vase life could be extended, thus making dahlias commercially viable as cut flowers. The work involved examining how dahlias senesce when left attached to their parent plant compared with when they were cut. Postharvest treatments relating to the phytohormones ethylene and cytokinins were also used in conjunction with RT-qPCR and RNA-sequencing to investigate the role these play in dahlia floral senescence. RNA sequencing was also used to examine the expression of genes involved in ABA biosynthesis or signalling and of potentially senescence-associated enzymes during the dahlia floral senescence process.

7.1. Comparison of senescence in cut *Dahlia* flowers vs. on-plant *Dahlia* flowers

In this study the membrane permeability and floret mass of several dahlia cultivars was measured, and these physiological measurements were then compared between dahlias left on the plant, and those cut and placed in dH2O, or treated with a 100 µM spray of BA or a 1 h pulse of 4 mM STS. The results showed that average percentage conductivity, as a measure of membrane permeability, in on-plant flowers of cultivars 'Karma Prospero', 'Onesta', and 'Sylvia' remained below 5% 7 days after cutting stage (Fig. 25B-D). This compares to average conductivity of 28.1, 30.5, and 45.5% respectively for the cut flowers of each of those cultivars at the same time point. This shows that once cut, dahlia inflorescences age very quickly compared to those left attached (Fig. 25B-D). In cv. 'Gerrie Hoek' there was no significant difference in conductivity between cut or on-plant flowers after 7 days and average conductivity of controls (dH2O) was just 8.4% (Fig. 25A). This suggests that measurements more than 7 days after cutting need to be taken in 'Gerrie Hoek' to see when or if there is a divergence between cut and on-plant flowers, however it does also suggest they do perform well as cut flowers. In the search for post-harvest treatments that prolong vase-life, an aim might therefore be to identify a postharvest treatment which would result in conductivity, and thus membrane integrity, as close as possible to the on-plant flowers. The effects of an STS pulse or BA spray varied between cultivars. In STS treated florets conductivity only remained below 5% in cv. 'Sylvia', on a par with on-plant florets (Fig. 37). Conductivity remained lower compared to controls (dH2O) in all other cultivars, though in 'Gerrie Hoek' this difference was not significant in part because control (dH2O) conductivity was very low

compared to other cultivars anyway (Fig. 37A). This suggests that STS is improving membrane integrity but not to the level that would make cut flowers comparable to on-plant flowers and that, ageing of florets is occurring even with treatment. This therefore indicates that STS is slowing down senescence but not enough to make cut flowers equivalent to uncut flowers. Treatment with BA was not as effective as STS in delaying increases in conductivity, with all cultivars showing more significant increases in conductivity after 7 days in BA sprayed florets compared with STS pulsed florets (Fig. 45). This could be due to multiple factors, for example it may be that ethylene is more involved in membrane breakdown during senescence than the presence of cytokinins is in maintaining them, hence inhibition of ethylene action had a greater effect on cell membrane integrity. There is evidence that ethylene is involved in upregulation of lipases which break down cell membranes and it has been hypothesized that ethylene may stimulate degradative phospholipid activity (Fan *et al*., 1997; Li & Huang, 2011; Griffiths *et al*., 1999; Sheng *et al*., 2000). There is also evidence that addition of exogenous cytokinins can upregulate enzymes which protect cellular membrane integrity (Trivellini *et al*., 2015; Ehneß & Roitsch, 1997), though research comparing expression of genes following STS or BA treatment would be needed to examine whether suppression of ethylene upregulated lipases by ethylene action inhibition was greater than upregulation of protective enzymes through exogenous BA treatment. However, BA sprays resulted in longer vase lives than STS pulses, suggesting that the visible wilting seen was not as strongly linked to membrane breakdown as expected, and that although conductivity was higher in BA sprayed florets, their floret tissues appeared visibly more intact. Nevertheless, as different trials were used for vase life and conductivity measurements, it is possible that variation in weather over the season led to differing results, and in future more trials need to be carried out. These need to be not only over multiple seasons, but at multiple points over the season, and trials of the same treatment measuring different parameters need to be carried out on flowers cut on the same day. Growing plants indoors under constant conditions could also mitigate this issue. The floret mass measurements also showed similar results, with STS and BA performing much better than controls (dH2O), but still falling short of the mass of on-plant florets (Fig. 26, 27, 46). However, BA treatment was better at maintaining floret mass in all cultivars except ‘Gerrie Hoek’ compared to STS treatment, though again neither treatment was enough to make florets comparable to on-plant florets (Fig. 26, 27, 46).

Ethylene is known to affect the activity of some lipases, suggesting that STS is helping to maintain membrane integrity by preventing ethylene reception and indirectly causing the upregulation of lipases implicated in membrane breakdown during senescence. This has been found in flowers from the ornamental species carnation (Hong *et al*., 2000) and *Gladiolus* (Peary & Prince, 1990). In this study the phospholipase PLDP2 was found to be upregulated in group 3 florets (Table 8), it is also known to be highly upregulated in senescent leaves in *A. thaliana* (Fig. 18A; Table 9). In *A. thaliana* suppression of the lipase PLD, implicated in degradation of membrane lipids (Thompson *et al*., 1998) resulted in a delay of both ethylene and ABA-promoted senescence (Fan *et al*., 1997). The cv. 'Sylvia' upon which the RNA-sequencing was performed was a cultivar which showed an improve vase life after STS treatment (Fig. 29) which suggest ethylene does have a role in senescence in this cultivar. However, as PLD has also been found to have a role in ABA promoted senescence it may be that this enzyme is not specific to ethylene dependent flower senescence (Fan *et al*., 1997). Nevertheless, as ABA and ethylene may positively regulate each other's biosynthesis it is possible that endogenous increase in either may stimulate PLD activity (Li & Huang, 2011). Lipoxygenase 2 was also found to be upregulated in this study, though not as strongly as *PLDP2* (Table 8), however in *A. thaliana LOX2* is downregulated between mature and senescent leaves (Table 9; Fig. 18B). *LOX2* is also highly expressed in floral tissues in *A. thaliana*, though its upregulation in this study was quite weak (Table 8, 9, Fig. 18B), suggesting that the upregulation seen in dahlia may be because of the floral specificity of this gene, and the limitations of comparing to senescing leaves, or because the downregulation of this gene in senescing tissue occurred outside of the stages examined in this experiment. Increasing lipoxygenase activity has been reported in *Gladiolus* during senescence (Peary & Prince, 1990) and lipoxygenase pathway metabolites, including jasmonic acid, have also been implicated in stimulating ethylene production in tomato fruits, hastening ripening (Sheng *et al*., 2000). Treating fruit with lipoxygenase inhibitors inhibited ethylene production. Furthermore, it has been found in tomato that ethylene upregulates some *LOX* genes (Griffiths *et al*., 1999). This suggests that *LOX* could be involved both in promoting ethylene biosynthesis and are themselves upregulated by ethylene indirectly, however more research would need to be performed to examine if this is the case in dahlia. The results here suggest that ethylene is involved in activation of phospholipases and lipoxygenases implicated in cell membrane breakdown or in ethylene biosynthesis, therefore the treatment with STS and inhibition of ethylene reception that leads to inhibited membrane breakdown in dahlias treated with STS may be due to an inhibition

of lipase action. This also implicates ethylene in the dahlia floral senescence process, at least in cultivars with improved vase life following STS treatment, but more work would be needed to confirm that the lipases found to be upregulated in thus study are being regulated by ethylene. The upregulation of *PLDP2* and *LOX2* in group 3 florets compared with the inner florets of groups 1 and 2 (Table 8) suggests that as the flower ages there is an upregulation of these particular enzymes implicated in cell membrane breakdown and that this upregulation is occurring in advance of visible wilting as group 3 florets show no wilting (Fig. 5a). However, more sequencing data from later stage florets or RT-qPCR investigating such genes would be needed to confirm this.

The best performing BA postharvest treatments were pulses of BA for 3h ('Gerrie Hoek'; Fig. 42C; 'Karma Prospero'; Fig. 43C), 6h ('Onesta'; Fig. 45B), or 18 h ('Sylvia'; Fig. 47B) in length depending on the cultivar. In the future conductivity and mass measurements of florets from BA pulse treated inflorescences would be useful in examining whether they can match on-plant flowers in physiological conditions 7 days after cutting, as in this study only BA sprayed flowers had their mass and conductivity measured. The improvements seen in BA and STS treated florets compared with controls (dH2O) shows that ethylene inhibition and increased cytokinin delay the senescence which occurs when flowers are cut. Based on data from other systems, a possible mechanism for this effect would be through repression of ethylene biosynthesis by cytokinin or inhibition of ethylene action by STS. This would then result in downregulation or delay in upregulation of lipases and proteases, and upregulation of genes involved in cell membrane maintenance, nutrition supply, and protection against ROS (Hong *et al*., 2000; Jones *et al*., 2005; Trivellini *et al*., 2015; Schnablová *et al*., 2006; Siranidou *et al*., 2002; Ehneß & Roitsch, 1997). These treatments delay senescence compared to controls (dH2O) but are not enough to stop cell breakdown induced following cutting completely as otherwise treated florets would have performed equally as well as on-plant florets. Hence either these pathways are only being partially blocked or other pathways are involved that are not affected by the inhibition of ethylene perception or increase in cytokinin availability in the florets. Other ornamentals are known to have ethylene independent pathways of flower senescence, including gladiolus (Kumar *et al*., 2014), iris (Woltering & van Doorn, 1988), *Lilium* (Woltering & van Doorn, 1988) and daylily (van Doorn & Woltering, 2008).

ABA has been implicated in flower senescence in gladiolus (Kumar *et al*., 2014), iris (Zhong & Ciafré, 2011), and daylily (Panavas *et al*., 1998) suggesting it may have a role

in marginally ethylene sensitive dahlia. However, in this study the expression patterns suggested overall downregulation of ABA biosynthesis from group 1 (inner Stage III) to group 3 florets (outer Stage IV), suggesting that ABA does not have a major role in dahlia flower senescence (Fig. 13, 14). Further research using RT-qPCR to investigate ABA related genes over a longer time period, using treatments of exogenous ABA, ABA biosynthesis inhibitors such as fluridone, or endogenous measurements of ABA in dahlia florets would give a better idea of whether it is a major regulator of dahlia floral senescence.

In cut flowers of cv.'s 'Karma Prospero' and 'Onesta', *DvIPT3* expression did not change significantly between the samples measured, however on-plant flowers showed a significant decrease in *DvIPT3* expression between 1 and 4 days after cutting (Fig. 56B, 57B), this is consistent with the results in the differential expression analysis which found downregulation of *IPT* (Fig. 20). *IPT3* has been found to be upregulated in senescing leaves of *A. thaliana* (Winter *et al*., 2007; Schmid *et al*., 2005). It has also been implicated in nutrient signalling, suggesting involvement in nutrient transport and remobilisation of nutrients (Masclaux *et al*., 2000; Sakakibara, 2006). The downregulation of *DvIPT3* in on-plant flowers 4 days after cutting stage, and no significant change in cut flowers, suggests that nutrient signalling is not important at this stage in on-plant flower senescence. In fact, in this study it was found that wilting was more advanced 7 days after cutting stage in controls (dH2O) compared to on-plant flowers in cv. 'Onesta' and 'Karma Prospero' (Fig. 24B, C). However, the lack of change in expression of this gene in cut flowers suggests a role for *IPT3* even in this early stage of senescence that could be involved with nutrient signalling and possibly nutrient remobilisation.

Overall on-plant senescence occurs at a slower rate than senescence in cut dahlias placed in dH2O. This shows that dahlias do last longer on the parent plant and if vase life can be extended to such a length they could be suitable as commercial cut flowers. Additionally, treatments with a 100 µM spray of BA or a 1 h pulse of 4 mM STS improve conductivity and mass in florets, reaching values closer to those left on the plant. This shows that postharvest treatments can be effective in delaying senescence in dahlia to some extent but do not completely stop the ageing which occurs once dahlias are cut.

7.2. Interaction of phytohormones during *Dahlia* floral senescence

The relationship amongst all phytohormones during senescence remains unclear though they usually fall into two categories, those that induce or promote senescence, such as ABA and ethylene, and those that delay it, such as cytokinins or gibberellins (Tripathi & Tuteja, 2007). In dahlia there are relatively little data on the floral senescence process compared with other species (Woltering & van Doorn, 1988; Dole *et al*., 2009; Shimizu-Yumoto & Ichimura, 2013). Work has been performed in this study investigating the role of ethylene, the hormone most commonly implicated in plant senescence, and cytokinins, which have been found to delay senescence (Tripathi & Tuteja, 2007).

It has been found in past studies that cytokinins can inhibit endogenous ethylene biosynthesis (Guo *et al*., 2003). The upregulation in ageing dahlia florets on the plant of *ACS*, the ethylene biosynthetic gene known to catalyse the rate limiting step of ethylene biosynthesis, (Fig. 17) along with the effectiveness of treatments with an inhibitor of ethylene reception, STS, in multiple dahlia cultivars, suggests that ethylene does have a role in dahlia floral senescence (Fig. 29). Moreover, another study found that ethylene production one day after cutting was relatively high in the ray florets, ovaries and receptacle of dahlia flowers, however this decreased up to 5 days after harvesting, and ethylene production in the calyx remained low for the whole experimental period (cv. 'Kokucho) (Shimizu-Yumoto & Ichimura, 2013), further suggesting that there is a role for ethylene in dahlia flower senescence at least in some floral organs. However, it is worth noting that levels of ethylene production from the ray florets was low compared with receptacle and ovary tissue in that study, and it is senescence in ray florets which matters most in assessing postharvest value.

Exogenous BA treatments may have extended vase life through suppression of ethylene biosynthesis, delaying the activation of senescence specific genes activated by ERF's (Breeze *et al*., 2011; Buchanan-Wollaston *et al*., 2003). In cv. 'Sylvia' flowers ageing on the plant it was found through differential expression analysis, that cytokinin biosynthetic genes were downregulated, whilst cytokinin catabolic genes were upregulated (Fig. 20), suggesting that the amount of endogenous cytokinins would begin to decrease. Without exogenous BA treatment, the reduction in cytokinin may then result in the repressive effect on ethylene being dampened.

The role of other phytohormones in flower senescence is less clear and in dahlias remains unknown. In other flowers, GA has been found to delay senescence and ABA to accelerate it (Saks *et al*., 1992; Kumar *et al*., 2014). GA has been found to repress rises in ethylene in carnation (Saks *et al*., 1992) and it is possible they may have a similar function in dahlia, however this would require further work. ABA has been implicated in ethylene-independent senescence but also in mediating ethylene responses, as it has been found that the transcription factor EIN3, which mediates ethylene responses, responds to ABA treatment in rose and *Dianthus* (Waki *et al*., 2001; Müller *et al*., 2000). In this study the results from differential expression analysis suggested that there was an overall downregulation of ABA biosynthesis as florets matured (Fig. 14). Moreover, it was found that *ABF* genes implicated in the transcriptional regulation of the ABA (Yoshida *et al*., 2010) response showed no significant change in expression as florets matured (Fig. 13). This suggests that ABA does not have a major role in dahlia flower senescence, however it has been found that ABA can promote ethylene biosynthesis, suggesting that ABA may trigger senescence indirectly through upregulation of ethylene biosynthesis (Ronen & Mayak, 1981) and that ABA's upregulation in dahlia may have occurred in floret tissue of a stage not sampled in this study. However, RNA-sequencing on samples of different stages would be needed to confirm this and shed more light on ABA's role in dahlia floral senescence and its potential interactions with ethylene.

7.3. Inter-cultivar comparison

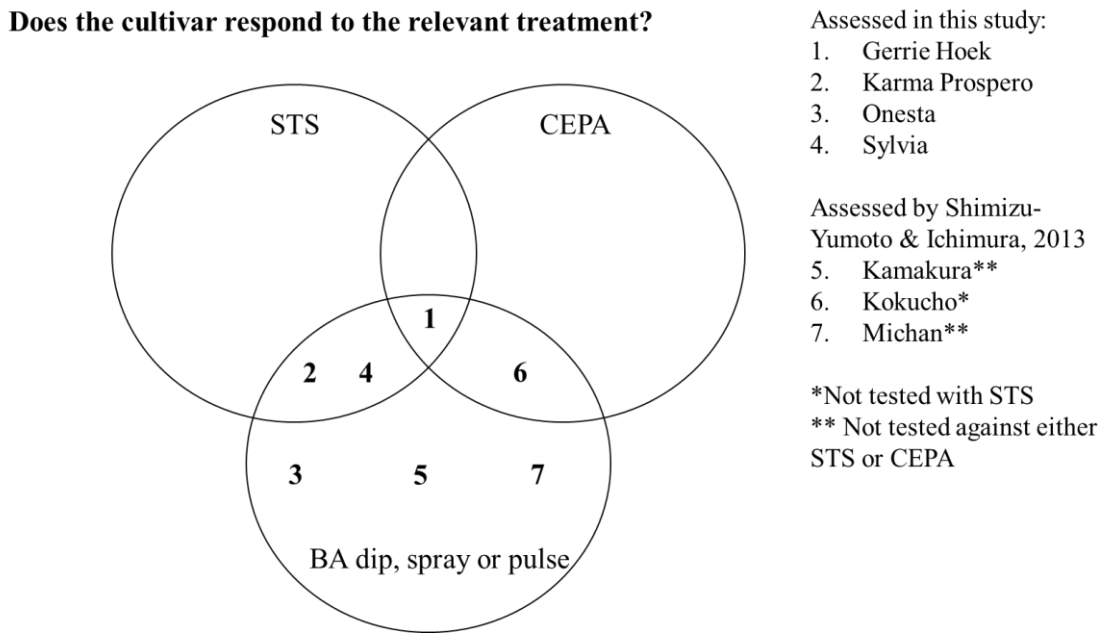
Sensitivity to cytokinins and ethylene has been found to vary in the dahlias assessed in this study, this has also been found in previous studies (Shimizu-Yumoto & Ichimura, 2013; Woltering & van Doorn, 1988; Dole *et al*., 2009; Fig. 58). There are tens of thousands of dahlia cultivars (RHS, 2014), therefore the variation in response to postharvest treatments between them is of interest as greater variability between cultivars means screening cultivars will be of greater importance. This presents dahlia researchers with questions which include:

1. Are the dahlia cultivars in this study varying in their responses in a similar range to dahlias in other studies?
2. Is there a link between morphology of dahlia and their floral senescence process?
3. Is a cultivar's sensitivity to exogenous cytokinins linked to sensitivity to ethylene?

These questions are important for dahlia researchers and horticulturalists because learning more about the variation in response to postharvest treatments amongst the few cultivars

of dahlia that have been studied, out of the tens of thousands of dahlia cultivars that exist (RHS, 2014), and whether that variation is linked to morphology or the sensitivity to a different hormone, can help us target which dahlia cultivars to focus on in the future. As dahlias need to be repotted each year and only have a relatively short growing season in the UK it is important to be as informed as possible when choosing dahlia cultivars to investigate for commercial development as cut flowers.

Firstly, the variation between dahlia cultivars in this study is not exceptional compared to dahlias in other studies, however there are limited examples for comparison, as studies in the past have focused on one cultivar (Dole *et al.*, 2009). In another study it was found that all dahlias assessed were equally sensitive to ethylene, though the vase lives were not listed, and ethylene sensitivity was found to be low (Woltering & van Doorn, 1988). In another recent study it was found that when treated with a 100 µM BA spray the cultivar 'Michan' had an average vase life of 8.4 d compared to 6.7 d in cv. 'Kamakura', a difference of 1.7 d (Shimizu-Yumoto & Ichimura, 2013; Fig. 58). In this study variation was greater with cv. 'Karma Prospero' showing a vase life of 12.2 d after a 100 µM BA spray and cv. 'Onesta' a vase life of 8.5, a difference of 3.7 d. This suggests that the cultivars in this study were more variable in their response to exogenous BA compared to other dahlia studies, but far more research needs to be carried out to assess if this is exceptional in dahlia. Additionally, treatment with 0.2 mM STS for 24 h had no effect on cv. 'Kokucho' vase life (Shimizu-Yumoto & Ichimura, 2013), similarly a 1 h pulse of 4 mM STS had no effect on 'Onesta' vase life in this study (Fig. 29, 20) however the treatments are slightly different. This suggests that with regards to STS treatments cultivars do show variation, with some not reacting to treatment at all. Furthermore, comparisons may be difficult to make as different studies have different methods of assessing vase life and the qualitative nature of vase life measurement can lead to variation in results.



Dahlia Cultivar	Vase life increase or decrease compared to control in relevant treatment				
	STS (1h, 4 mM)	**CEPA (20 µM)**	**BA pulse (3h, 100 µM)**	**BA spray (100 µM)**	**BA solution (100 µM)**
Gerrie Hoek	+	-	+	+	No change
Karma Prospero	+	No change	+	+	No change
Onesta	+	No change	+	+	-
Sylvia	+	No change	+	+	No change
Kamakura				+	
Kokucho		- (*10 µM)		+	No change
Michan				+	

Figure 58: Summary of the effects of different postharvest treatments on different dahlia cultivars. No change means the change was not significant (Shimizu-Yumoto & Ichimura, 2013).

Secondly, the results of this study suggest there is no link between sensitivity to certain postharvest treatments and dahlia floral morphology. In this study water lily type dahlia such as 'Gerrie Hoek' and 'Karma Prospero' reacted positively to STS treatments but the water lily type 'Onesta' did not (Fig. 29). In ball type cultivars both 'Sylvia' and 'Boom Boom Yellow' reacted to STS, however more than two cultivars would be needed to be establish whether this is the case in all ball types (Fig. 29). In another study, it has been found that the decorative type dahlia 'Kamakura' and the pompom type dahlia 'Michan' reacted positively to BA treatments, as did all cultivars in this study, suggesting that BA sprays are beneficial across at least three morphological types, ball/ pompom, decorative,

and water lily (Shimizu-Yumoto & Ichimura, 2013). This also suggests that cytokinins are more reliable than ethylene inhibitory treatments in extending dahlia vase life.

Lastly, is there a link between ethylene and cytokinin sensitivity? It is possible that a cultivar which shows relatively high ethylene sensitivity would also respond well to cytokinins, assuming cytokinins repress ethylene biosynthesis in dahlias as they have been suggested to do in chrysanthemum, as the exogenous BA would be expected to repress the plant's endogenous biosynthesis of ethylene (Fig. 2) (Guo *et al*., 2003). The reverse would be found in less ethylene sensitive species and cultivars where BA would have less of an effect due to reduced importance of ethylene. Measuring this is complicated as there is no single measurement for sensitivity. In this study mass, conductivity and vase life all showed varying effects of the same postharvest treatment on a cultivar. For example, in the cultivar 'Karma Prospero' STS had a significant effect on conductivity but not on floret mass 7 days after cutting (Fig. 37B, 27B). STS had no significant effect on 'Onesta' vase life and 'Onesta' also performed the worst in vase life trials of any cultivar after its optimum pulse or spray treatment of BA, despite controls (dH2O) of this cultivar performing similarly to controls (dH2O) of other cultivars (Fig. 29, 20, 37). This suggests it has poor sensitivity to both exogenous cytokinins and to ethylene inhibition. However, this hypothesis is complicated by the observation that the dahlia homolog of *ACS6* was upregulated in STS treated 'Onesta' flowers 4 days after treatment (Fig. 41B), whereas controls (dH2O) showed no upregulation. This suggests that inhibition of ethylene signalling resulted in 'Onesta' upregulating ethylene biosynthesis to compensate. This might explain why despite treatment with STS it still showed wilting comparable to controls (dH2O) (Fig. 35). This would suggest that rather than being ethylene insensitive 'Onesta' is better at synthesising endogenous ethylene compared to other cultivars or has a more sensitive signalling mechanism to activate ethylene biosynthesis. A summary of the reaction to postharvest treatments by dahlias in this study and of those used by Shimizu-Yumoto & Ichimura (2013) can be found in Fig. 58. Overall, dahlia floral morphology does not correlate with reaction to postharvest treatments, and sensitivity to exogenous cytokinins does not necessarily predict sensitivity to ethylene. The variation in this study was greater thasn in other studies, but far more work needs to be performed to assess what the variability between dahlia cultivars is.

7.4. A brief summary of *Dahlia* flower senescence: from closed bud to senesced florets

Dahlias develop sequentially, with the outermost florets opening first, this appears as whorls but is a spiral pattern with florets circling inwards becoming less mature towards the centre of the inflorescence (personal communication, Tony Stead). In water lily type dahlia, the florets open outwards to angle 90 degrees to the pedicel and only curve slightly beyond this in mature inflorescences. In contrast the tightly packed florets of ball type dahlias unfurl and bend backwards. In the outer florets of mature ball type inflorescences this angle is 180 degrees to the pedicel so that the outer florets are curled underneath the pedicel, obscuring it and forming the distinctive ball shape.

The exact point at which senescence is triggered in unpollinated flowers, if such a point exists at all, or how it is triggered is unknown. Moreover, in composite inflorescences like dahlia where each floret is a reduced flower, it is not known if senescence is triggered floret by floret or if there is a pan-floral signal, i.e. a signal which triggers senescence across the entire capitulum, rather than floret by floret. Based on evidence in this study it is likely that ethylene is playing some role in regulating senescence, and it has been found that ethylene production is relatively high in dahlias after harvesting, though this decreases from 1 day after cutting (Shimizu-Yumoto & Ichimura, 2013), though whether it is the trigger for senescence as it is in pollinator induced senescence (Jones, 2008) is not known. As less cytokinins are synthesized and their breakdown increases (Fig. 20) their repressive effect on ethylene biosynthesis will weaken (Fig. 2) (Guo *et al*., 2003). The expression of *EBF*'s, found to be upregulated in ageing florets in this study (Fig. 16), can regulate the ethylene response to ensure it does not occur too quickly (Merchante *et al*., 2013). It is also possible that ABA is influencing ethylene biosynthesis as it has been found in *Lupinus luteus* that treatment with exogenous ABA accelerated transcriptional activity of ethylene biosynthesis genes *ACS* and *ACO*, and that ABA accumulation on its own was not enough for petal abscission to occur, suggesting both ABA and ethylene were required (Wilmowicz *et al*., 2016). Ethylene has also been found to positively regulate ABA biosynthesis in *A. thaliana* (Li & Huang, 2011) suggesting they positively regulate one another.

The upregulation of ethylene biosynthesis-related genes in florets still days from showing signs of visible wilting (Fig. 4, 32) suggests that senescence is being induced in relatively young florets, this may allow the transcriptional cascade of upregulated ERF's to activate

senescence associated genes involved in PCD, such as proteases, lipases, DNases and RNases (Tripathi *et al*., 2009; Xu & Hanson, 2000; Hong *et al*., 2000). The induction of these genes will then undertake breakdown of tissues and allow nutrients to be remobilised to other tissues (Chapin & Jones, 2009; Hew *et al*., 1989).

In composite flowers outer florets senesce before inner florets, this is similar to species with flower spikes, such as gladiolus, where the florets at the bottom of the spike senescence before those nearer the top of the spike (Serek *et al*., 1994). The upregulation of senescence associated genes is likely to occur floret by floret and this perhaps occurs by a gradient of ethylene or ABA, rather than senescing as one single inflorescence, it has been suggested that the degeneration of petal margins before the centre is due to a gradient of a signal or receptor, though whether this is the case across complex composite flowers is not known (Tripathi & Tuteja, 2007).

This pattern may develop through a feedback cycle between ethylene and (Fig. 2) cytokinins or gibberellins whereby as cytokinins or gibberellins are downregulated, repression of ethylene is lifted as it has been found that cytokinins can inhibit ethylene biosynthesis (Guo *et al*., 2003), reduced GA content can enhance ethylene mediated flower senescence (Yin *et al*., 2015) and ethylene can repress GA biosynthesis (Wang *et al*., 2013a). In the future a factorial experiment using varying doses of exogenous phytohormones, including ethylene, cytokinins, GA or ABA to assess the effects they have on cut dahlia, could be performed to shed more light on their interactions with each other during the floral senescence process. During this study, the limitations of the length of the flower season and the number of quality flowers produced in that period meant that experiments were focused on ethylene and cytokinins, to build on the existing knowledge we have in dahlia (Woltering & van Doorn, 1988; Dole *et al*., 2009; Shimizu-Yumoto & Ichimura, 2013), which is less well studied compared to some other ornamentals. Flowers will begin to wilt and discolour and may eventually abscise. Frequency of abscission was not measured in this study, but it was observed to be low, and it is likely that dahlias fall into the group of flowers which lose a large percentage of fresh mass before abscising, like daylilies (Bieleski, 1995), if they abscise at all. In other composite flowers, including sunflower and gerbera, it has been found that abscission does occur and that this affects postharvest value of flowers (Tata & Wien, 2014; Emongor, 2004). In dahlia it may be that abscission is not identified as an issue as flowers wilt too soon before abscission for it to be viewed as an issue. However, in this study most observations were of cut flowers in control conditions; florets from uncut flowers may abscise eventually, however it is

not uncommon to see dahlia inflorescences fully wilted on the plant with a large portion of florets still attached. Senescence is fully over when all florets have either abscised or wilted.

7.5. Recommendations for improving *Dahlia* vase life

To improve dahlia vase life, it is likely that treatment with a 1 h pulse of 4 mM STS in conjunction with a pulse or spray of 100 µM BA would be most beneficial for 'Gerrie Hoek', 'Karma Prospero' and 'Onesta' (Fig. 42, 43, 45), whereas a BA pulse of 18 h would be most beneficial to 'Sylvia' (Fig. 47). Plants could be grown in the same manner as in this study as it produced healthy floriferous plants, however growing plants indoors could be an option to lengthen the flowering season and the constant conditions provided in a glasshouse environment could improve the consistency of flower quality over the flowering period. More studies should be carried out using stem lengths used in industry to assess what is most effective. Flowers should be cut at Stage III (Fig. 4). Open florets can be easily damaged though this could be mitigated with mesh sleeves already in use in some sunflowers to allow dahlias inflorescences to develop and then be transported without damage (personal communication, Paula Edgington).

It is likely that certain cultivars are also better than others with regards to vase life. However, in this study, cultivars 'Boom Boom Yellow', 'Gerrie Hoek', 'Karma Prospero', 'Onesta' and 'Sylvia' all showed a vase life of more than 10 days in at least one postharvest treatment. This suggests that these cultivars could perform well in future trials. Transport and storage trials and longer stem lengths (50 cm) as used in industry would also need to be researched to assess how these cultivars would fare in the supply chain which entails harvesting, then transportation from growers to retailers before a retail phase whilst till guaranteeing 5 days of vase life. It has been found that dahlias stored at 2 ^{o}C either wet or dry showed significant drop in vase life compared to unstored controls, suggesting that cold storage would not be beneficial in dahlia, however this study assessed just one cultivar (Dole *et al*., 2009).

7.6. Future work

There is great scope for future work on dahlia flower senescence. This includes further work studying phytohormones, examination of proteases or lipases and their inhibitors, experiments varying pre-harvest conditions to examine effects on vase life, and trials mimicking the supply chain, for example to examine how dahlias react to different storage

temperatures. Examining postharvest treatments could also include more fundamental work using RNA-sequencing and RT-qPCR to further investigate the gene expression patterns underlying the composite floral senescence in dahlia, and applied work using postharvest treatments to examine the physiological basis of findings from molecular work.

Pre-harvest conditions could be varied to assess if they affect vase-life. In this study planting dahlias outside in peat compost with a handful of bone meal and later treating them with a balanced fertiliser of 20% N, 20% P, and 20% K with micro nutrients including Mn and trace elements was sufficient to maintain healthy plants, and flowers cut from these plants gave vase lives greater than 10 days if treated with BA pulses or sprays.

Nutrition is another pre-harvest factor that must be considered. For example, cut *Anthurium* flower vase-life differed when growing plants were subjected to a combination of different fertilizers. A vase-life variation of 13% and 17% was accounted for by levels of nitrogen and potassium, respectively (Paul *et al*., 1992). The use of fertilisers, in proportions of 10% nitrogen, 10% phosphate, and 10% potassium, on dahlia plants has been recommended (Armitage & Laushman, 2003), whilst other research has suggested only 5% nitrogen (Romer & Nelson, 2003) for healthy plants. Furthermore, application of a combination of urea, P_2O_5 sourced from diammonium phosphate (DAP), and manure was found to have a significant positive effect compared to controls (dH2O) on early flowering as well as the number of flowers per plant in dahlia (Ahmed *et al*., 2004). The size of flowers was also significantly increased by the application of the combination of the three fertilisers, or the application of DAP alone, or in combination with urea compared with controls (dH2O). This suggests that pre-harvest conditions can affect productivity of the plants, even if it does not affect the vase life of flowers. Additionally, the effects of adding specific growth regulators pre-harvest has rarely been investigated however, flower yield of the rose cultivar ‘Poison’ was improved by the application of GA (Hashemabadi *et al*., 2009), whilst salicylic acid (SA) improved vase-life (Hashemabadi & Zarchini, 2010). Moreover, pre-harvest application of GA to chrysanthemum also resulted in higher vase life (Sajid *et al*., 2018), though salicylic acid has only been found to improve vase life postharvest in chrysanthemum (Selahvarzi *et al*., 2012). However, the data in these studies suggests that pre-harvest application of SA and GA may therefore be of interest in dahlia to extend vase life and yield quality.

Photoperiod has been found to affect flowering in dahlia, with long days inducing flowering and also causing faster flowering (Armitage & Laushman, 2003). A short-day length of eight hours inhibits flowering (Armitage & Laushman, 2003), whilst an optimum day length for flowering has been found to be 13-15 hours (Konishi & Inaba, 1964). During the transportation and storage stage, which is unavoidable once flowers reach a commercial supply chain, lack of light can accelerate the yellowing of leaves in dahlias (Nowak & Rudnicki, 1990). It would be interesting in the future to investigate the effects of light in dahlias both pre and post-harvest, to examine how day length effects the cultivars in this study, or if lighting conditions postharvest affect vase life, either due to flower senescence, or possibly dahlia leaf senescence, either of which could affect commercial value.

The stem length (5 cm) used in this study was useful in maximising replication in a limited growing season, as dahlias often produce many flowers off small stems, thus in order to focus on single flowers and get a stem length of 50 cm, many other flowers would have had to be removed. However, in industry a stem length of 50 cm is more commonly used when flowers are first cut. Therefore, trials will need to be carried out using postharvest treatments, especially pulses, to ensure they are still effective at longer stem lengths and that STS or BA are still being taken up and transported to floral tissue. Moreover, research in sunflowers suggested that stem length was positively correlated with vase life (Mensuali-Sodi & Ferrante, 2005). Sunflowers with stem lengths of 70 cm had an average vase life of 9 days, whereas those with stem lengths of 50 cm had vase lives of just 5 days. This may be because there are extra nutrients for plants to draw on in vascular tissue when more of the stem is left, or it may be that longer stems with thicker bases have a better ability to draw up nutrients and fluid, however more research would be needed to establish this. This also suggests that longer stem lengths in dahlia could be beneficial and could further improve vase life.

It would also be helpful to look at on-plant flower senescence over a longer timeframe in the future, for example 10 or more days rather than 7. Examining more cultivars would also allow us to better establish a link between how long flowers of the same cultivar last on the parent plant compared to when they are cut and whether or not the timing of on-plant senescence is a good indicator of whether a cultivar has potential as a good commercial cut flower. Performing post-harvest treatments on flowers across multiple seasons and at different times across the same season would be needed to ensure that the effects seen in this study are consistent across seasons and therefore commercially viable.

Further investigation of phytohormones such as ethylene, cytokinins or ABA could improve our knowledge of dahlia flower senescence. This could include RT-qPCR on a wider range of genes involved in biosynthesis, signal transduction or catabolism, or RNA-sequencing on tissue treated with exogenous BA, ethylene or ABA, as this has already been done on petunias treated with exogenous BA (Trivellini *et al*., 2015). The ethylene inhibitor 1-MCP could also be used to examine if it is as effective as STS (Serek *et al*., 1995). Treatment with exogenous ABA or with ABA inhibitors such as fluridone would also shed light on the role of ABA in dahlia and whether it has a greater role in dahlias which show less ethylene sensitivity, as it has been found that ABA plays a role in senescence in gladiolus (Kumar *et al*., 2014), daylily (Panavas & Rubinstein, 1998), carnation (Drory *et al*., 1995), and daffodil (Hunter *et al*., 2004a). Exogenous GA could be tested as a postharvest treatment in dahlia as it has been found to improve vase life in a range of other ornamental species including carnation (Saks *et al*., 1992) and gladiolus (Singh *et al*., 2008), and also in the composite species gerbera (Emongor, 2004).

Treatments with other typical postharvest solutions such as citric acid could also extend vase life as this has been found in sunflower (Mensuali-Sodi & Ferrante, 2005). Treating dahlias with citric acid in conjunction with the BA sprays, pulses, or STS pulses shown to be effective in this study could allow us to examine if these treatments can have an additive effect to extend vase life further. The use of the same cultivars in future studies would be helpful in establishing a comprehensive database of a few cultivars of dahlia rather than a little knowledge on many cultivars. However, examination of more dahlia cultivars from a wider range of dahlia morphologies, for example semi-cactus type dahlias which were not assessed in this study, would be give us a better view of the ranges of sensitivity to ethylene and exogenous BA in dahlia. Such studies could also give commercial interests more options when searching for cultivars to further develop commercially.

Finally, RT-qPCR and RNA sequencing can be used to understand the molecular basis of dahlia flower senescence. The differential expression analysis of the sequencing performed in this study has yielded a database of thousands of genes up and downregulated between different stages of floral tissue (Table 4, 5, Fig. 11). This will be a useful dataset for future researchers, however it could be made more comprehensive by sampling cv. ‘Sylvia’ florets from both later and earlier stages to give a wider picture of how genes are changing in expression throughout the senescence process. In the future further analysis of pathways could be done to further our knowledge of this topic. RNA-

sequencing using samples from outer florets from Stages I-V would give a better overview of expression in florets from bud stage to senescing stage and may be more easily comparable than florets from inner and outer parts of the flower, more stages would also give us an idea of when senescence is triggered. Other cultivars which show differences in reaction to postharvest treatments e.g. STS pulses or BA sprays, could also be used to see if expression of genes or pathways of interest is significantly different between cultivars, and RNA-sequencing on treated samples could be performed to examine differences in expression between control and treated inflorescences.

Future work would help to extend vase life further and give us a better understanding of the gene expression changes involved in composite floral senescence which could be applicable across members of the Asteraceae family, including in the more commercially important chrysanthemum and sunflower.

7.7. Conclusion

In conclusion, the data in this study showed that uncut flowers do not senesce as quickly as cut flowers showing that they do have potential as cut flowers if treatments can be found to delay cut flower senescence. The data also suggests that ethylene may play a role in dahlia flower senescence, however inhibition of ethylene action is unlikely to lengthen dahlia vase life enough to make its use a way to make cut dahlia flowers commercially viable. However, treatment with a spray or pulse of exogenous BA can improve dahlia vase life to within a range of commercial viability, which was a major aim of this study. RNA-sequencing showed that ethylene biosynthetic genes are upregulated in dahlia florets as they mature whilst at the same stage cytokinin biosynthetic genes are downregulated and cytokinin catabolic enzymes are upregulated in expression. Moreover, based on RNA-sequencing data, genes implicated in ABA biosynthesis were also downregulated as florets matured. The performance of RNA-sequencing provides a large database for future research into dahlia floral senescence.

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Appendix I. Weather Data

Weather data was taken from 'World Weather Online' (2018). The data presented shows the growing season when plants were outside, young plants were grown in a polytunnel before being planted outside from May and their tubers dug up in October. Thus, the data here shows temperature and precipitation from the months May to October over the three growing seasons covered by the project (2015-2017). Season 1 data are from Wisley, Surrey, Season 2 and 3 data are from Egham, Surrey, this corresponds with the locations plants were grown in the different years.

Season 1 (2015)

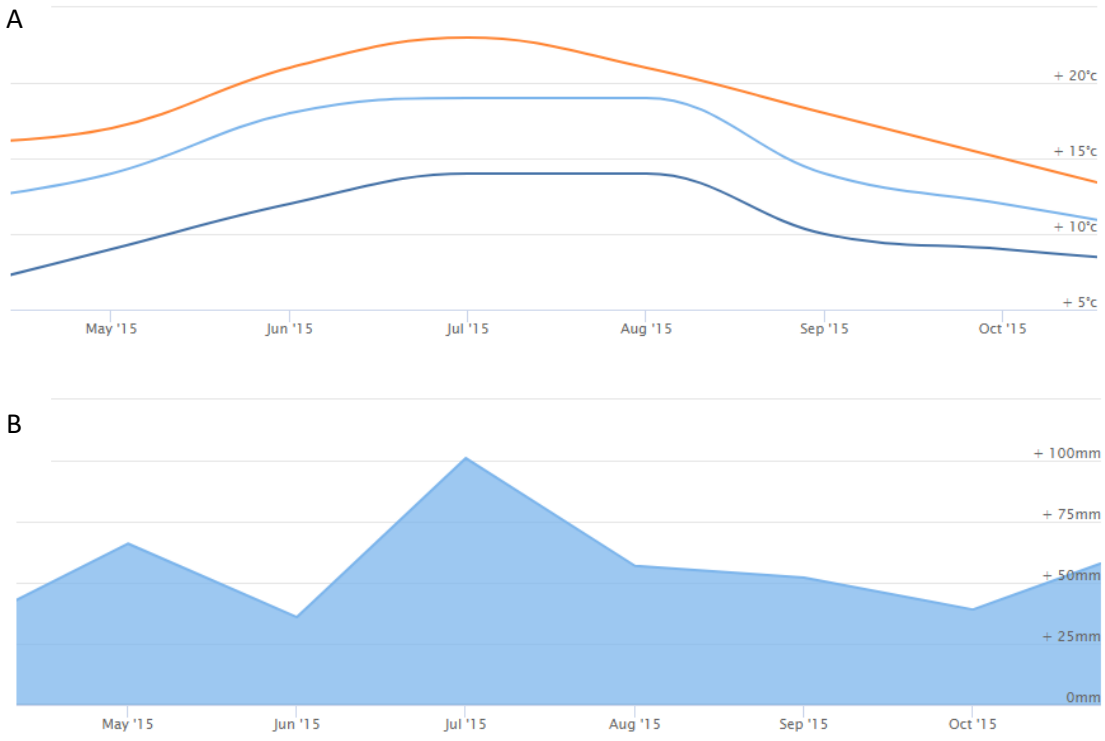

Figure 59: A) Average temperature, maximum temperature, and minimum temperature (°C) from May to October 2015 in Wisley, Surrey.

B) Precipitation (mm) from May to October 2015 in Wisley, Surrey.

Season 2 (2016)

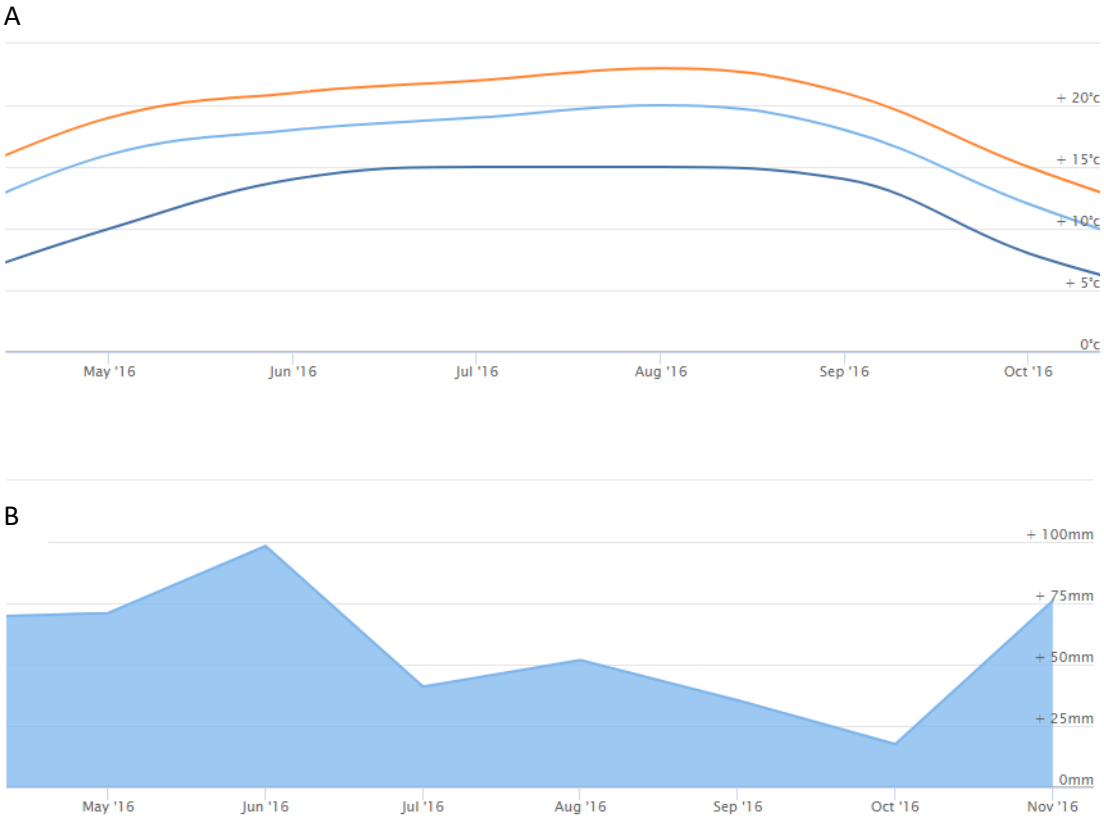

Figure 60: A) Average temperature, maximum temperature, and minimum temperature (°C) from May to October 2016 in Egham, Surrey.

B) Precipitation (mm) from May to October 2016 in Egham, Surrey.

Season 3 (2017)

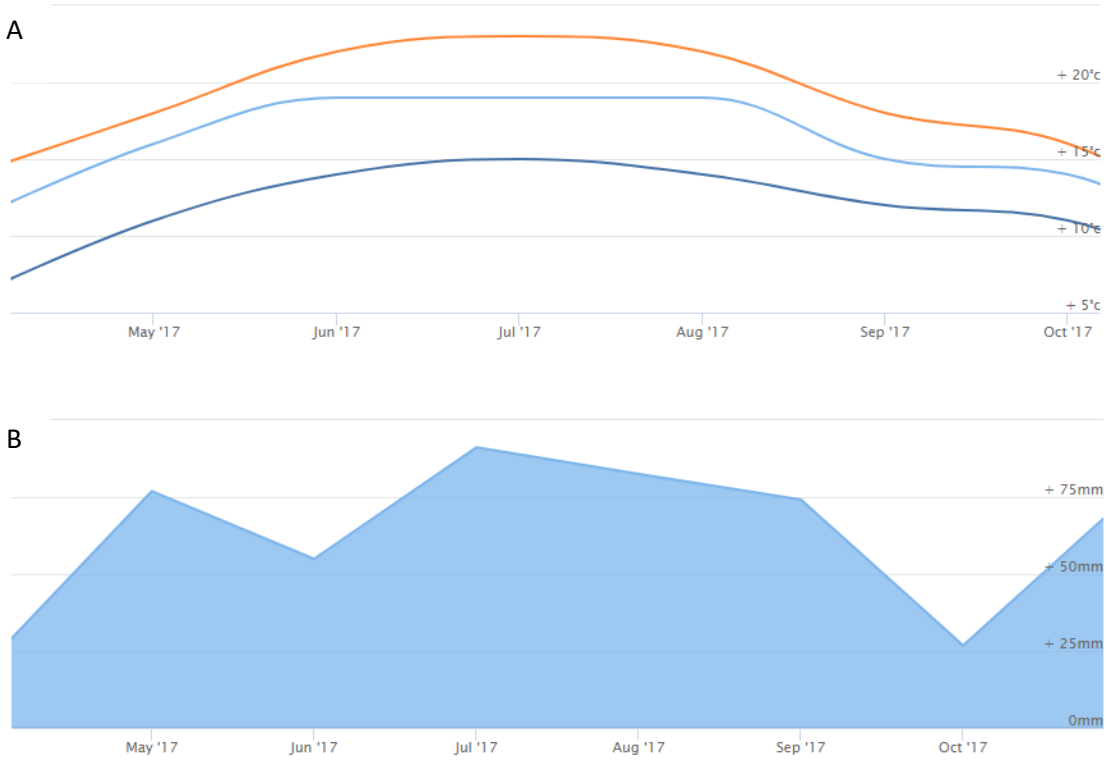

Figure 61: A) Average temperature, maximum temperature, and minimum temperature (°C) from May to October 2017 in Egham, Surrey.

B) Precipitation (mm) from May to October 2017 in Egham, Surrey.

Appendix II. Primers

ARR2

Product:

TGGGTTGTCATTATTGAGGGAGAATAAGAGTGGGTTTGATGTTGTTTTAAGT GATGTTCATATGCCGGATATGGATGGATTCAAGCTTCTTGAATACATTGGCC TTGAGATGGACCTTCCTGTT

Product Size: 124

Table 10 – Summary of ARR2 primers

	Forward	*Reverse*
Primer	TGGGTTGTCATTATTGAGGG	AACAGGAAGGTCCATCTC
Length (bp)	20	18
GC Content (%)	45%	50%
Tm (^{o}c)	58	54

Table 11 – Homology of ARR2 primer product

	Blastn Homolog (General)	*Blastn Homolog (*A. thaliana*)*
Homolog	PREDICTED: *Helianthus annuus* two-component response regulator ARR1-like (LOC110897868), mRNA NCBI Reference Sequence: XM_022144600.1	Arabidopsis thaliana response regulator 2 (RR2), mRNA NCBI Reference Sequence: NM_117704.6
Query Cover (%)	100	86
Identity (%)	90	81
e-value	3e-30	2e-21

Alignment of product to general blastn ARR2 homolog:

```
Query  1    TGGGTTGTCATTATTGAGGGAGAATAAGAGTGGGTTTGATGTTGTTTTAAGTGATGTTCA  60
            ||| ||||| || |||||||||||||| |||||||||||| | ||||| |||||||| ||
Sbjct  362  TGGTTTGTCGTTGTTGAGGGAGAATAAAAGTGGGTTTGATATAGTTTTGAGTGATGTGCA  421

Query  61   TATGCCGGATATGGATGGATTCAAGCTTCTTGAATACATTGGCCTTGAGATGGACCTTCC  120
            |||||||||||||||||||||||||||||| | ||||| ||||| |||||||| ||
Sbjct  422  TATGCCGGATATGGATGGATTCAAGCTTCTTGAACATATTGGGCTTGAAATGGACCTCCC  481

Query  121  TGTT  124
            ||||
Sbjct  482  TGTT  485
```

IPT3

Product:

TCGGCATTCTTCTTCGGCTATTTTATTGGTAAGGATATCTAATCCATCGTACA
ATTGAATCTTGTCTGAGTTGATTACCTCCCCCGAAAAGCGGGTGGCGATATC
GATCGCAAGTCTTGACTTTCCGGTGCCGGTGGCACCTATCACGAACACT

Product Size: 154

Table 12 – Summary of IPT3 primers

	Forward	*Reverse*
Primer	TCGGCATTCTTCTTCGGCTA	AGTGTTCGTGATAGGTGCCA
Length (bp)	20	20
GC Content (%)	10	10
Tm (^{o}c)	60	60

Table 13 – Homology of IPT3 primer product

	Blastn Homolog (General)	*Blastn Homolog (*A. thaliana*)*
Homolog	PREDICTED: *Helianthus annuus* adenylate isopentenyltransferase 3, chloroplastic-like (LOC110927578), mRNA NCBI Reference Sequence: XM_022171128.1	*Arabidopsis thaliana* isopentenyltransferase 3 (IPT3), mRNA NCBI Reference Sequence: NM_116176.3
Query Cover (%)	99	87
Identity (%)	79	73
e-value	2e-27	1e-12

Alignment of product to general blastn IPT3 homolog:

```
Query  2    CGGCATTCTTCTTCGGCTATTTTATTGGTAAGGATATCTAATCCATCGTACAATTGAATC  61
            || || |||||||| |  | |||||||||   ||||||||| || || ||||  ||||||
Sbjct  320  CGACACTCTTCTTCAGTGACTTTATTGGTGGCGATATCTAACCCTTCATACATCTGAATC  261

Query  62   TTGTCTGAGTTGATTACCTCCCCCGAAAAGCGGGTGGCGATATCGATCGCAAGTCTTGAC  121
            || |||||||| |||| |||  ||||||| |||||||| |  ||||||| ||| |||||
Sbjct  260  TTATCTGAGTTTATTATCTCTGCCGAAAAACGGGTGGCAAGGTCGATCGAAAGCCTTGAT  201

Query  122  TTTCCGGTGCCGGTGGCACCTATCACGAACACT  154
             |||||||||| || || || ||||| | ||||
Sbjct  200  TTTCCGGTGCCTGTAGCGCCCATCACAACCACT  168
```

ACO4

Product:

GAGAGAGATGTCACGATGGAGAAGATCAAAGATGCGTGCGAGAATTGGGG
ATTTTTTGAGTTGGTGAATCATGGGATTGCTCATGAATTACTTGACAAAGTG
GAGAAAATGACTAAGGATCATTACAAGAAGTGTATGGAGCAGAGG

Product Size: 147

Table 14 – Summary of ACO4 primers

	Forward	*Reverse*
Primer	GAGAGAGATGTCACGATGGA	CCTCTGCTCCATACACTTCT
Length (bp)	20	20
GC Content (%)	50	50
Tm (^{o}c)	60	60

Table 15 – Homology of ACO4 primer product

	Blastn Homolog (General)	*Blastn Homolog (*A. thaliana*)*
Homolog	PREDICTED: *Helianthus annuus* 1-aminocyclopropane-1-carboxylate oxidase 3 (LOC110910493), mRNA NCBI Reference Sequence: XM_022155132.1	*Arabidopsis thaliana* ethylene-forming enzyme (EFE, ACO4, EAT1), mRNA NCBI Reference Sequence: NM_100380.4)
Query Cover (%)	100	99
Identity (%)	89	80
e-value	6e-46	6e-30

Alignment of product to general blastn ACO4 homolog:

```
Query  1    GAGAGAGATGTCACGATGGAGAAGATCAAAGATGCGTGCGAGAATTGGGGATTTTTTGAG  60
            ||||||| ||| || |||||||||||||| ||||| || || || |||||||| ||||||
Sbjct  149  GAGAGAGGTGTTACCATGGAGAAGATCAATGATGCATGTGAAAACTGGGGATTCTTTGAG  208

Query  61   TTGGTGAATCATGGGATTGCTCATGAATTACTTGACAAAGTGGAGAAAATGACTAAGGAT  120
            |||||||| ||||||||| ||||||| |||||||||||||| || || ||||| ||||||
Sbjct  209  TTGGTGAACCATGGGATTCCTCATGATTTACTTGACAAAGTCGAAAAGATGACAAAGGAT  268

Query  121  CATTACAAGAAGTGTATGGAGCAGAGG  147
            |||||||||||||||||||||||||||
Sbjct  269  CATTACAAGAAGTGTATGGAGCAGAGG  295
```

ACS6

Product:

CATGAACCAAGTACGAGGAAACCGCGTGAAGTTTGACCCTGACCGGATTGT
GATGAGCGGTGGCGCCACCGGAGCTCATGAAACGCTGGCTTTCTGCTTAGC
AAATCCCGGAGAAGCATTTTTAGTCCCCACCCCTTATTATCCAGCGTTCGAT
CGAGATTTGAGATGGCGAACTGGAGT

Product Size: 180

Table 16 – Summary of ACS6 primers

	Forward	*Reverse*
Primer	CATGAACCAAGTACGAGGAA	ACTCCAGTTCGCCATCTCAA
Length (bp)	20	20
GC Content (%)	45	50
Tm (°c)	58	60

Table 17 – Homology of ACS6 primer product

	Blastn Homolog (General)	*Blastn Homolog (*A. thaliana*)*
Homolog	PREDICTED: *Lactuca sativa* 1-aminocyclopropane-1-carboxylate synthase-like (LOC111886468), mRNA NCBI Reference Sequence: XM_023882709.1	*Arabidopsis thaliana* 1-aminocyclopropane-1-carboxylic acid (acc) synthase 6 (ACS6), mRNA NCBI Reference Sequence: NM_117199.2
Query Cover (%)	100	86
Identity (%)	93	85
e-value	4e-69	1e-41

Alignment of product to general blastn ACS6 homolog:

```
Query  1    CATGAACCAAGTACGAGGAAACCGCGTGAAGTTTGACCCTGACCGGATTGTGATGAGCGG  60
            |||||||| ||| |||||||||| ||||  |||||||||||||| ||||||||||||||
Sbjct  415  CATGAACCGAGTTCGAGGAAACCGTGTGACATTTGACCCTGACCGCATTGTGATGAGCGG  474

Query  61   TGGCGCCACCGGAGCTCATGAAACGCTGGCTTTCTGCTTAGCAAATCCCGGAGAAGCATT  120
            |||||||||||||||||||||||||||||||||| |||||||||||||| ||||||||
Sbjct  475  TGGCGCCACCGGAGCTCATGAAACGCTGGCTTTCTGTTTAGCAAATCCCGGCGAAGCATT  534

Query  121  TTTAGTCCCCACCCCTTATTATCCAGCGTTCGATCGAGATTTGAGATGGCGAACTGGAGT  180
             ||||| |||||||||||||||||| |||||||||||||| ||||||||||||||||
Sbjct  535  CTTAGTTCCCACCCCTTATTATCCAGGGTTCGATCGAGATTTGGGATGGCGAACTGGAGT  594
```

Beta-Tubulin

GAGTCTCCGGTGTACTTGCCGGTGACATCAATACCGTGTTCGGCGCAGACG ACCTCCCAGAACTTGGCTCCGATCTGGTTGCCGCATTGGCCGCCCTGGATGT GAAGGATT

Product Size: 111

Table 18 – Summary of beta-tubulin primers

	Forward	*Reverse*
Primer	GAGTCTCCGGTGTACTTGCC	CGCCCTGGATGTGAAGGATT
Length (bp)	20	20
GC Content (%)	60	55
Tm (^{o}c)	64	62

Table 19 – Homology of beta tubulin primer product

	Blastn Homolog (General)	*Blastn Homolog (*A. thaliana*)*
Homolog	PREDICTED: *Helianthus annuus* tubulin beta chain	*Arabidopsis thaliana* tubulin beta chain 2 (TUB2)
Query Cover (%)	96	99
Identity (%)	94	83
e-value	2e-141	1e-94

Alignment of product to general blastn beta-tubulin homolog:

```
Query  1    AGCGCCTTCCGTATAATGTCCTTTGGCCCAATTATTACCAGCACCGCTCTGGCCAAAGAC  60
            ||||||||| || || || ||||||||||||||||||||||||| |||||||||| ||
Sbjct  410  AGCGCCTTCAGTGTAGTGACCTTTGGCCCAATTATTACCAGCACCACTCTGGCCAAAAAC  351

Query  61   GAAGTTATCAGGCCTGAAGATTTGACCGTATGCGCCAGATCTGAGGCTGTCCATAGTCCC  120
            |||||||||||||| |||||||||||||| |||||||||||||| |||||||||| ||
Sbjct  350  GAAGTTATCAGGCCGGAAGATTTGACCGTAGGCGCCAGATCTGAGACTGTCCATAGTTCC  291

Query  121  TGGCTCCAGATCCATGAGCACGGCGCGTGGAACGAACCTTCCGCCGCTAGCTTCGTTGTA  180
             ||||| |||||||||| |||||||||||||| |||||||||||||||||||||||||
Sbjct  290  AGGCTCGAGATCCATGAGGACGGCGCGTGGAACGTACCTTCCGCCGCTAGCTTCGTTGTA  231

Query  181  ATAGACGTTGATCCTCTCGAGCTGGAGCTCGGAGTCTCCGGTGTACTTGCCGGTGACATC  240
            |||||||||||| |||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  230  ATAGACGTTGATTCTCTCGAGCTGGAGCTCGGAGTCTCCGGTGTACTTTCCGGTGACATC  171

Query  241  AATACCGTGTTCGGCGCAGACGACCTCCCAGAACTTGGCTCCGATCTGGTTGCCGCATTG  300
             || |||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  170  GATGCCGTGTTCGGCGCATACGACCTCCCAGAACTTGGCTCCGATCTGGTTGCCGCATTG  111

Query  301  GCCGCCCTGGATGTGAAGGATTTCACGCATTTTC  334
            ||||||||| |||||||||||||||||||||||||
Sbjct  110  GCCGCCCTGAATGTGAAGGATTTCACGCATTTTC  77
```

Table 20 – Summary of Eurofins sequences and their Blastn results

Primer used	PCR product sequence as determined by Eurofins (unclipped)	Blastn result: Asteraceae
ACS6	GGAGGAGGTCGATGCTGATCGGGTTGGGGGAGAGGTGGCGCTCCCGG GGATCTGGAAACGCTGGCTTTCTGCTTCATGAT CCCGGGTGGCCCCTCTGATCCCCCACCCCTTATTAGGGTCCGTTTTCTCGC GGTTTGCTCGGGCTTGGTGGAGTAAGTGG TTGCTGGCCTTTTCTGATGCTTACTTCCATAACTTATTTATTTCTGTTGGCG GTAATTGACCTCTATGGTGGTTCATGTT TAGGTTTGTTCGATCTTGTTTTGGGAGATTCGGGGGAGACATGGTGGCT GGGATACTCTGATTCCTTTATTTAAACCGGG ACAAACTGTGCTAATATAATGAACCCTACCTGAGATGACGTCTTCTTCCTA AGTTGTACCGGCATCTTTGACTGTGGGCG GATAGGCAGCACCTCCTTTACTGACAGCCTCCTCTCCCACAGAGGCGGG GGGCAGAGAGGGCGTCGACGGTGCTGTACCG GAGTACAATCGATAACGTTAACTGCTGAGCTGCTGGCTGATATTGATTCT CGAGGACTTCCGACATTGTCACACGAAATT TTTGTTTGGATAGCTCGCTTCCATTTTACCGGATTATTATACAAAATTCAT TTATACTAAACTTGTCAAACTAATTTATT CTTTCGCGATATTTACTTCCGCAGGACCCGGCCTTTCTCTCCCTCAGATTG GAACTCTTTCTCGTCTTCCTGCTGTCATT GACTCCCGCCTACCCTTCATCCAAGCTAAT	Predicted *Lactuca sativa* ACS Query Cover: 11% e-value: 4e-04 Identity: 70%
ARR2	GTAAGGCGACGGCAATGTTGTTTTATGTGATGTTCATATGCCGGATATG GATGGATTCTCGCTTCTTGTCTTATTCGCCC TCGATATGGACCTTCCAAGTTATAACTTAGAGGCTGACTGTATCCCGCCT GCTATCCGTGAATAACTTTCGAATAATTTG GGGTGTCAGGATTCGAAGTCATAAGCTGCCTGCAAGGTCGTGAAATAAA AAATCAAACCCGGCCAATACGAGACACAACG GACTCCCACACAACGGGAACGAAGCATCTGCTTTACATCCGAAATATTCA TGACCACCCCCTCAATACATGGGGTGTAAA CTGCGTGCCTCAATGCACAGGACATTGCTGGATCCTTGTGGCTCGGTTGG GCCGAGTCGTTTTACTCTTCTACCCTACTA ACTCCGCTGTCCACCGAGATGGAATTGCAGTGTGGAGCCCAAGATCGTA GACGTCGCCGCTGAGATATTGAATGAAACCA AAATGATAACTATGTCCCGGCTCCCGTTTCTGTGCTTACGGACTGAAACC ACTGGAGGGTCATACAGTTTAGCCCAATCT TGCGGGCTATGCCTTGGGATGCCTGATTTGTTATGCAATAAGGGACACTA TAGTAGTTTTGCTCAATAAACGGTTCGACG AGTCAC	PREDICTED: *Cynara cardunculus var. scolymus* two-component response regulator ARR2-like Query Cover: 10% e-value: 9e-11 Identity: 85%

ACO4	CAGCGCGTGTGAGATCGGGGGTTTTTGAGTTGTGGGATCTCGGGTGAGT CAATGAATTACTGGACCAGAGTAGAAAATGA GAAGGAGAATTACAAGAAGGGGATGTGAGCAGTCCAATTATGGAAGTG GGGGTCTTTGATGTTTTAGTAGGGGACGGAGC TGACGAGTCCGCTTTGTATAGTCTTGGAAAGGGCTTTACATTCGTATGAC AATCGTAAATTTTTTCCAGACAGCGGTATC ATCACGAAACAACGGATCTGTGGTTTGCTTCGAAACGAACTGGAATTAC GGGTACGGTCGAGAAGATTGCTACCAAATAA ATACTGCTTCTAACTCAGAAGTGTATGAACGCATGATTCTTTATCTTTACA GGGAACACCTGCATTATTCGGACCTGCTT CCGGCGTTCCTCACACTTATCGCTGTTGTTTATTTTCTGCCAACACCGGAT CCAGACCAAGTTTGCGTGCGTATAAAAAA ACCGCCATAAAGGCTCAGTGGGACTCCTTCGCCATTTTTGATGTTGAAAT ACAATCGTCGATAGTTACTGAAATTAAACT TAGGATTTTCCCCACCCTATTAAATGAGCATTCATACATTTCAAACGTTTT AGAAGAGGGGAGTGCAGACCTGTTAACCA ATGTGCGTTCATCAGAAGAGCGCAGCTGCTACCACCTTATTGCAGTTCAT CCCACCCTCAGGTATTCCGTGCTGCGGGCG CATATTCCTGCAGTCTCA	PREDICTED: *Lactuca sativa* 1-aminocyclopropane-1-carboxylate oxidase 3 Query Cover: 14% e-value: 3e-07 Identity: 76%
IPT3	GGGGTGGGAGAACTAGCCTCGTCTCATGACTCGTGTCTGAGTTGAGGCC TCCCCCGAAACCGGGTGGCGAGATCGATGGC TTATCTTGACTTTCCGGTGCCGGTGGCACCTATCACGAACAAAAAGTATG AATGGTATAGTGTAATTTGGGCCGTGCTTC AGTTCGGCCTCTTTCGAATACATGCTGTTGTTACAAGGACTTTAGCGGTA GGTTTTACCCGCCTGATGATCGTGTCTAAA AACAACGGCGCCTGACAAGAACAATATTC	PREDICTED: *Cynara cardunculus var. scolymus* isopentenyltransferase 3 Query Cover: 20% e-value: 9e-08 Identity: 84%

Appendix III. ANOVA tables

Table 21 - Results of two-way ANOVAs on mass (mg) of florets from uncut or cut flowers, 1, 4 or 7 days after cutting stage. Treatment refers to cut or uncut flowers.

Gerrie Hoek	**d.f**	**F**	**Sig**
Day	2	5.063	P<0.05
Treatment	1	0.062	P>0.05
Day* Treatment	2	7.804	P<0.05
Karma Prospero			
Day	2	1.498	P>0.05
Treatment	1	38.019	P<0.05
Day* Treatment	2	14.538	P<0.05
Onesta			
Day	2	17.23	P<0.05
Treatment	1	29.63	P<0.05
Day* Treatment	2	11.88	P<0.05
Sylvia			
Day	2	6.956	P<0.05
Treatment	1	150.559	P<0.05
Day* Treatment	2	19.794	P<0.05

Table 22 - Results of two-way ANOVAs on membrane permeability of florets from uncut or cut flowers, 1, 4 or 7 days after cutting stage. Treatment refers to cut or uncut flowers.

Gerrie Hoek	**d.f**	**F**	**sig**
Day	2	9.788	P<0.05
Treatment	1	0.594	P>0.05
Day* Treatment	2	1.230	P>0.05
Karma Prospero			
Day	2	20.37	P<0.05
Treatment	1	32.11	P<0.05
Day* Treatment	2	21.36	P<0.05
Onesta			
Day	2	103.3	P<0.05
Treatment	1	153.1	P<0.05
Day* Treatment	2	133.7	P<0.05
Sylvia			
Day	2	135.5	P<0.05
Treatment	1	182.4	P<0.05
Day* Treatment	2	167	P<0.05

Table 23 - Results of two-way ANOVAs on protein content (µg/ g FW) of florets from uncut or cut flowers, 1, 4 or 7 days after cutting stage. Treatment refers to cut or uncut flowers.

Karma Prospero	**d.f**	**F**	**sig**
Day	2	17.844	P<0.05
Treatment	1	1.096	P>0.05
Day* Treatment	2	0.074	P>0.05
Onesta			
Day	2	16.781	P<0.05
Treatment	1	5.021	P<0.05
Day* Treatment	2	4.447	P<0.05

Table 24 - Results of two-way ANOVAs on mass (mg) of florets from control (dH_2O), STS (1h pulse at 4 mM) or CEPA (20 μM) treated flowers, 1, 4 or 7 days after cutting.

Gerrie Hoek	**d.f**	**F**	**sig**
Day	2	29.383	P<0.05
Treatment	2	6.951	P<0.05
Day* Treatment	4	1.884	P>0.05
Karma Prospero			
Day	2	6.041	P<0.05
Treatment	2	1.837	P>0.05
Day* Treatment	4	8.217	P<0.05
Onesta			
Day	2	32.23	P<0.05
Treatment	2	12.48	P<0.05
Day* Treatment	4	6.034	P<0.05
Sylvia			
Day	2	21.669	P<0.05
Treatment	2	22.298	P<0.05
Day* Treatment	4	4.269	P<0.05

Table 25 - Results of two-way ANOVAs on membrane permeability of florets from control (dH_2O), STS (1h pulse at 4 mM) or CEPA (20 μM) treated flowers, 1, 4 or 7 days after cutting.

Gerrie Hoek	**d.f**	**F**	**sig**
Day	2	15.359	P<0.05
Treatment	2	6.424	P<0.05
Day* Treatment	4	3.504	P<0.05
Karma Prospero			
Day	2	81.685	P<0.05
Treatment	2	5.458	P<0.05
Day* Treatment	4	6.687	P<0.05
Onesta			
Day	2	89.74	P<0.05
Treatment	2	24.94	P<0.05
Day* Treatment	4	13.97	P<0.05
Sylvia			
Day	2	157.8	P<0.05
Treatment	2	193.7	P<0.05
Day* Treatment	4	118.6	P<0.05

Table 26 - Results of two-way ANOVAs on protein content (µg/ g FW) of florets from control (dH_2O) or STS (1h pulse at 4 mM) treated flowers, 1, 4 or 7 days after cutting.

Karma Prospero	**d.f**	**F**	**Sig**
Day	2	22.2	P<0.05
Treatment	1	0.163	P>0.05
Day* Treatment	2	0.709	P>0.05
Onesta			
Day	2	41.59	P<0.05
Treatment	1	2.01	P>0.05
Day* Treatment	2	1.18	P>0.05

Table 27 - Results of two-way ANOVAs on mass (mg) of florets from control (dH_2O) or BA (100 μM) treated flowers, 1, 4 or 7 days after cutting.

Gerrie Hoek	**d.f**	**F**	**sig**
Day	2	13.11	P<0.05
Treatment	1	1.36	P>0.05
Day* Treatment	2	1.367	P>0.05
Karma Prospero			
Day	2	7.395	P<0.05
Treatment	1	14.33	P<0.05
Day* Treatment	2	3.504	P<0.05
Onesta			
Day	2	9.467	P<0.05
Treatment	1	10.296	P<0.05
Day* Treatment	2	13.429	P<0.05
Sylvia			
Day	2	23.1	P<0.05
Treatment	1	90.37	P<0.05
Day* Treatment	2	14.65	P<0.05

Table 28 - Results of two-way ANOVAs on membrane permeability of florets from control (dH_2O) or BA (100 μM) treated flowers, 1, 4 or 7 days after cutting.

Gerrie Hoek	**d.f**	**F**	**sig**
Day	2	31.55	P<0.05
Treatment	1	14.37	P<0.05
Day* Treatment	2	17.95	P<0.05
Karma Prospero			
Day	2	43.993	P<0.05
Treatment	1	12.568	P<0.05
Day* Treatment	2	4.727	P<0.05
Onesta			
Day	2	156.35	P<0.05
Treatment	1	67.43	P<0.05
Day* Treatment	2	28.1	P<0.05
Sylvia			
Day	2	196.91	P<0.05
Treatment	1	33.27	P<0.05
Day* Treatment	2	19.55	P<0.05

Table 29 - Results of two-way ANOVAs on protein content (µg/ g FW) of florets from control (dH_2O) or BA (100 µM) treated flowers, 1, 4 or 7 days after cutting.

Karma Prospero	**d.f**	**F**	**sig**
Day	2	4.325	P<0.05
Treatment	1	1.516	P>0.05
Day* Treatment	2	0.263	P>0.05
Onesta			
Day	2	20.548	P<0.05
Treatment	1	0.329	P>0.05
Day* Treatment	2	2.814	P>0.05