Title: Creating plant molecular factories for industrial and nutritional isoprenoid production.

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Abstract

Chemical refining is a highly efficient process that has driven industrialisation and globalisation. However, dwindling fuel reserves and climatic fluctuation are now imposing key societal and economic challenges to health and welfare provision, agriculture, manufacturing outputs and energy. Plants are potentially exploitable "green" chemical factories, with vast chemical diversity that can be used for the discovery and production of food, feed, medicines and biomaterials. Despite notable advances, plant based production under real-life scenarios remains, in most cases, economically uncompetitive when compared to inherently non-sustainable petrochemical based processes. In the present review the strategies available and those emerging will be described. Furthermore, how can the new evolving molecular tools such as genome editing be utilised to create a new paradigm of plant based production? To illustrate the present *status quo*, we have chosen the isoprenoids as the class of natural products. These compounds display vast chemical diversity and have been used across multiple industrial sectors as medicines, supplements in food and feedstuffs, colorants and fragrances.

Introduction

Isoprenoids, also known as terpenoids represent the largest and oldest class of natural productsdocumented, consisting of >40,000 different molecules [1]. Biosynthetically all isoprenoids are related via a common five carbon building block (isopentenyl pyrophosphate; IPP). Commercially isoprenoids are used in cosmetics and fragrances, and as colorants and nutritional supplements in foods and feeds. Additionally, numerous isoprenoids have important medicinal properties, such as the anti-cancer agents Taxol and the isoprenoid-derived indole alkaloid vincristine and vinblastine [2]. Their industrial relevance also means they are compounds of high value with global markets in the range of $ 1 billion per annum (www.researchandmarkets.com). A contributing factor to the high cost of these molecules resides in the fact that they are mainly produced in low yields by slow growing plant species that are not readily amenable to agricultural production. It is therefore not surprising that total or semi-synthetic chemical synthesis is presently the method of choice for obtaining many of these isoprenoid molecules. However, their structural complexity makes chemical synthesis expensive, difficult and prone to the formation of non-biological stereoisomers and

contamination with reaction intermediates. There is also a significant environmental impact as the precursors used are typically derived from the chemical refining of fossil fuels [3].
In addition to their value as speciality or bulk chemicals, isoprenoids such as carotenoids (provitamin A) and tocopherols (vitamin E) are essential components of the human diet. Moreover, nutritionally enhanced foods are advocated by most national governments [4]. Therefore, enhancement in crop plants such as rice, maize and tomato to confer improved nutritional and consumer quality traits is an area of intense research and development.
The formation of isoprenoids is a complex process in plants, that is compartmentalised and under developmental regulation. The biosynthetic pathway(s) can be divided into (i) the formation of the isopentenyl pyrophosphate (IPP); (ii) the formation of prenyl phosphates and (iii) biosynthesis of isoprenoid subgroups. Since the discovery of the mevalonic acid (MVA) pathway in the 1950s, it was assumed that IPP was synthesised from acetyl-CoA via MVA. However, experimental data consistently indicated that the MVA pathway could not account exclusively for the formation of plastid-derived isoprenoids. In the early 1990s, retro-biosynthetic labelling conclusively established the presence of an alternative MVA-independent pathway for the formation of IPP and dimethylallyl pyrophosphate (DMAPP), termed the methyl *D*-erythritol phosphate pathway (MEP) [5].
Prenyl phosphates represent the central backbone of the isoprenoid pathway from which all isoprenoid sub-groups are derived. The five carbon substrates used are IPP and DMAPP. These molecules lead to the formation of longer chain prenyl lipids such as geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}), geranylgeranyl pyrophosphate (GGPP, C_{20}), octaprenyl pyrophosphate (C_{40}) and solanesyl pyrophosphate (C_{45}). GPP, FPP and GGPP are the main products synthesised in plants. The presence and action of GPP, FPP and GGPP synthases is key to the prenyl lipids produced and thus the formation of independent branches of isoprenoid biosynthesis that utilise these prenyl precursors (Figure 1). A vast array of isoprenoid subgroups exist, these include monoterpenes (C_{10} derived) such as geraniol, sesquiterpenes (C_{15} derived), which includes artemisinin, diterpenes (C_{20} derived) such as taxanes, triterpenes (C_{30} derived) e.g. phytosterols and carotenoids (C_{40} derived) such as β-carotene.
Following the elucidation of rudimental knowledge of isoprenoid formation, the last decades have seen an explosion in our ability to implement genetic intervention (metabolic engineering and Marker Assisted Selection, MAS) to alter isoprenoid levels in plants. The following sections will provide notable examples of rational strategies that have been used to achieve successful production of certain isoprenoids or which provide exploitable fundamental knowledge that could lead to future advances. An attempt will be made to judge the levels of socio-economic impetus required to fully adopt these new biosources as foods and production platforms.

Strategies used for the metabolic engineering of isoprenoid production in plants

Metabolic engineering strategies are summarised in Figure 2, for convenience we have used the plastid compartment to illustrate the approaches used. Initial efforts focused on the modulation of single key enzymatic steps in a pathway. These enzymes typically had the highest flux coefficient among the pathways components and thus, their modulation increased metabolic flux through the targeted pathway. Alternatively, the first committed step in a pathway was targeted in order to divert precursors into the pathway. In the case of endogenous biosynthetic pathways, this approach often resulted in pleiotropic effects. Not only co-

suppression due the constitutive overexpression of endogenous genes, as observed with the carotenogenic enzyme *PHYTOENE SYNTHASE* (*PSY*1) in tomato fruit [6], but also unintended biochemical effects such as dwarfism due to increased flux into carotenoids at the expense of precursors into the essential isoprenoid derived phytohormones gibberellins [6,7] or loss of vigour and senescence phenotypes due to a reduction in the levels of the phytohormone cytokinin resulting from the depletion of the IPP/DMAPP pool [8]. In order to overcome the pleiotropic effects and issues with co-suppression and allosteric regulation, alternative gene homologues were utilised and/or tissue specific promoters exploited. [9]. Also highlighted by these studies was the potential of sink organs such as tomato fruit as favourable chassis, overcoming detrimental effects when production levels in vegetative tissues reached economic viable levels. In addition, natural variation can be exploited for better performing alleles. For example, a single nucleotide polymorphism (SNP) in the coding region of the *DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE (DXS)* gene, encoding the first biosynthetic enzyme of the MEP pathway from *Vitis vinifera*, has been linked to high levels of monoterpenes (geraniol, linalool and nerol) [10].

The well characterised introgression populations [11] now available represent an untapped resource to identify intermediary and isoprenoid biosynthetic enzymes with superior catalytic activity or altered regulation that can influence isoprenoid contents.

Engineering single steps in the isoprenoid pathway cannot be sufficient to achieve the desired chemotype and a multi-gene approach is needed. Notably, it is now straightforward and cost effective to synthesise codon optimised genes [12] and test their activity in high-throughput systems [13•], for subsequent heterologous expression across diverse plant chassis. The construction of mini-pathways with heterologous enzymes is exemplified by Golden Rice where the synthesis of β-carotene in rice endosperm was achieved by expressing both a *PSY* from daffodil and a bacterial desaturase *Crt*I [12,14]. A similar approach has been used in potato tubers to produce β-carotene [15]. However, in the case of mini-pathway engineering, having each gene driven by its own promoter can result in a lack of coordinated expression and, with multiple genes, a range of promoters are needed to reduce the incidence of homology-dependent gene silencing. One way of overcoming these problems is to exploit viral systems for polycistronic expression as in the case of the foot and mouth disease virus (FMDV) 2A peptide sequence [16], a cis-acting hydrolase element (CHYSEL) which self cleaves through ribosome-skipping during translation [17]. The FMDV 2A sequence has been used successfully in the metabolic engineering of the carotenoid pathway [18,19].

Exploitation of transcription factors that simultaneously govern expression of several genes encoding biosynthetic isoprenoid enzymes and related branches has also been an area of study in order to improve metabolic engineering outcomes. For instance, several transcription factors (e.g. ORCA, MYC, BIS, WRKY) involved in the phytohormone jasmonic acid response have been characterised in *Catharanthus roseus* as regulators of terpenoid indole alkaloid biosynthesis [20]. Recent work by Paul *et al* [21] provides a detailed characterisation of the transcriptional and post-transcriptional regulation of the ORCA cluster in *C. roseus*, demonstrating a >40-fold increase in terpenoid indole alkaloid accumulation. In addition, gene products capable of enhancing isoprenoid production through a post-transcriptional mechanism have been identified. For example, the Orange (OR) gene product is a plastid localised protein, which was first described in cauliflower as a mutant allele responsible for high levels of β-carotene in the curd and stem tissue [22]. Initially this accumulation was postulated to be as a result of OR induced chromoplast differentiation, however it has since been demonstrated that

the wild type isoform of OR has a stabilising effect on PSY, enhancing its activity [23,24]. Most engineering strategies described above have been performed using nuclear transformation in order to generate stable transgenic lines. Alternatively, plastid transformation protocols have been developed for several plant species. The advantages of plastid transformation systems and their present limitations are described in [25]. A further development to improve the practical utility of plastid transformation systems is the horizontal transfer of whole chloroplast genomes between grafted species [26,27]. This horizontal transfer of plastomes between sexually incompatible species could be exploited in order to genetically engineer species not amenable to other transformation methods. The potential for nuclear genomes to be transferred via grafting has also been demonstrated [28••]. More recently a new approach, combinatorial super transformation of transplastomic recipient lines (COSTREL) has been established which combines both nuclear and plastomic transformation to introduce multiple genes and facilitate the engineering of complex processes, in this case artemisinin formation in tobacco has been achieved [29••]. It has become evident that in addition to biosynthesis, sequestration mechanisms are as important to increase or alter the isoprenoid content in plants. One solution addressing this aspect has been the modulation of specific organelles. For example, the fruit-specific downregulation of the *DE-ETIOLATED1* gene in tomato, which is a component of the light signal transduction pathway, increased the number of plastids and their size. Such changes at the cellular level delivered a metabolic sink and boosted intermediary metabolism. The resulting chemotype displayed an increase in all isoprenoid related secondary metabolites tested [30]. Similar results have been observed in the high pigment tomato mutants [31,32]. Another strategy to increase the metabolite sink is to alter structural components such as fibrillin [33]. It is also important to note that the increase in metabolites can, at a certain level, trigger the formation or differentiation of specific storage structures [34-37].

The modification of newly synthesised or elevated isoprenoid levels by esterification or glycosylation processes can facilitate improved storage and possibly protect against degradation [38,39]. The elimination of catabolic processes such as oxidation by increasing the levels of antioxidants [40•] or limiting the enzymatic degradation of the molecules by suppressing the expression of Carotenoid Cleavage Dioxygenase (CCD) genes [41] have been shown to be effective strategies to increase the levels of the isoprenoid of interest. In the case of vitamin E in soybean seed, a mutation in *HOMOGENTISATE DIOXYGENASE* (HGO) gene increased 2-fold the tocopherol/tocotrienol content. Effectively, this is an illustration of intermediary metabolism affecting end-product isoprenoids [42].

The future exploitation of isoprenoid formation in plants

The present molecular resources and tools now available have enabled the development of excellent new prototypes for the production of useful isoprenoids. However, the field is on the horizon of new paradigm changing opportunities for crop improvement using targeted genome engineering [43]. Particularly, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system [44] which is emerging as a transformative tool, due to its ease of engineering, robustness, and multiplexed target recognition [45,46]. It can efficiently generate precise modification of crop genomes via the: (i) knock-out of open reading frames (ORF) or promoters to produce loss-of-function mutants; (ii) highly precise modification of a gene sequence promoting allele replacement; (iii) targeted introduction of cis- or transgenes leading to stacking of multiple genes at a specific location, or the introduction

of regulatory sequences to modulate gene expression [47,48]. While highly efficient non homologous end joining (NHEJ) repair is involved in random insertions/deletions, the more challenging homologous recombination (HDR) drives targeted modifications involving a donor DNA template.

A significant potential advantage of using genome editing mediated by CRISPR/Cas and other site-specific nucleases over traditional genetic engineering approaches is the possibility of new crop variety production without any foreign genes. This may accelerate the regulatory approval process and thus bring products faster to the market [49-51]. Some examples of utilising CRISPR/Cas gene editing in crop plants so far include; disease and herbicide resistant crops [50-53] and output traits such as improved shelf life fruits [54•].

The potential of gene editing for introducing new alleles, promoter replacement, entire novel pathways or even multiplexing is of great interest, not only for creating plant-based systems expressing bioactive molecules but also for stacking traits linked to disease resistance, abiotic stress tolerance (e.g. heat, drought), in order to enhance crop performance.

Dramatic advances have been made in the engineering of isoprenoids in plants and unprecedented future potential exists. However, despite these developments critical, fundamental aspects of their formation and sequestration await elucidation. For example, how the cell/tissue knows when and how to switch between isoprenoid classes during development and environmental stresses is not fully known. The holistic regulation of the pathway, including potentially new epigenetic mechanisms, requires further input. At a cellular level; how the transport of isoprenoids between organelles and sub-organelle structures occurs is not understood; the topology of the biosynthetic enzymes and their organisation into metabolons awaits elucidation and also how abiotic and biotic stress conditions affect isoprenoid levels is an emerging area that needs attention with the advent of climatic fluctuations.

Conclusions and perspectives

This review highlights the plethora of strategies and emerging technologies available for the production and enhancement of nutritional and industrially useful isoprenoids in plants. It can be concluded that, in most cases, it is now possible to rapidly progress through proof of concept studies and deliver genotypes with high titres of valuable isoprenoids or enhanced levels of nutritional related isoprenoids, for direct nutritional intervention. So the question remains; why these notable advances have not been adopted and entered the market place more rapidly to deliver better products with improved environmental credentials? In the case of GM foodstuffs, consumer perception is a key issue, although the consensus from unprecedented testing is that the technology *per se* poses no adverse effects to human/animal health [55]. Instead, from a translational perspective, it would now appear the major limiting factors are the costs associated with regulatory approval. It is also surprising that Golden Rice has still not been released and it is legitimate to ask why? [56]. As besides its direct humanitarian benefits, it is an exemplar for GM output traits that could potentially stimulate or rekindle a whole industrial sector and funding stream that would contribute to the development of the bioeconomy. Modern molecular breeding approaches, such as MAS and TILLING do provide non-GM alternative approaches for the production or enhancement of isoprenoids. However, these approaches are limited to compatible sources of natural variation and, to date, it appears greater production titres are achievable through genetically modified crops. Improving nutritional quality is still a clear feasible target for modern molecular breeding. The limiting factors in this instance have been attributed to the costly European Food Safety Agency (EFSA) and Food and Drink Agency (FDA; US) practices required to substantiate health claims [57], without the

prospect of market advantage, which prevents most commercial enterprises pursuing such developments. In addition, the lack of precision in the technology, means that yield and biotic and abiotic resistance of the elite lines can be lost during the stacking of consumer traits. In this case, the development of the genome editing technologies for rapid allele transfer offer a potential solution to these problems.

For those isoprenoids used in speciality or bulk chemical markets, GM based production does not pose such a decisive influence, as the compounds of interest can be extracted and purified. The main determinant for economic viability being yield per hectare [58]. Our survey of strategies does highlight the promotion of "non-food" crops. In most cases competitive levels will have detrimental pleotropic effects when produced in vegetative or seed tissues at economically viable levels. In contrast, crops with sink tissues (e.g. tomato) offer an attractively alternative platform. Additionally, they are Generally Regarded As Safe (GRAS) materials, where minimal low–energy bioprocessing can be used or even the direct use of admixes. The levels that can be achieved in these tissues also circumvents the "food versus land" debate and emerging management practices such as vertical farming and aquaponics could be utilised in this case. There are excellent examples where derived isoprenoid (carotenoid) products fruit have reached commercial maturity (www.lycored.com/lyc-o-mato; www.in-cosmetics.com). Although many proof of concept studies exist, there is a shortage of feasibility studies performed on an industrial scale. This restricts informative Life Cycle Analysis (LCA) and the formulation of business models. To improve this aspect, it would help if funding agencies and scientific journals were more receptive to these studies. The examples highlighted in this article have often produced the target isoprenoids at levels and a purity that warrant economic viability. However, it would appear that dramatic elevation of levels are required to persuade manufacturers to adopt different production infrastructures. This step-change is however now possible with the emerging molecular techniques associated with genome editing. Alternatively, socio-economic polices similar to those subsidising biofuels could be used to drive and promote renewable production and nutritional enhancement.

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Figure Legends.

Figure 1: Overview of isoprenoid biosynthesis and compartmentalisation in plant cell.
Black arrows indicate either a single or multiple enzymatic steps. Red arrows correspond to cross-membrane transport. HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; MVA, mevalonate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-*D*-xylulose.

Figure 2: Diagrammatic representation of the potential strategies for metabolic engineering of isoprenoid compounds.

1 Heterologous expression (sole or mini-pathways) of genes encoding biosynthetic enzymes; 2, improved transcription and/or allele replacement; 3, enhanced translation; 4, improved transcription and translation; 5 enhanced transcription via regulation of transcription factor(s); 6, increased activity by controlling post-translational mechanisms; 7, plastid transformation; 8, enhancement of precursor availability by restricting its diversion; 9, interaction with intermediary metabolism to enrich precursors and other plant processes; 10, minimising non-enzymatic degradation and the diversion of target products into competing pathways; 11, metabolite modification; 12, sequestration of metabolites; 13, controlling of transport

mechanisms; 14; attenuation of the diversion into other pathways; 15 – 17; changes in cellular structure. For simplicity, only plastid metabolic pathway operating in plastid is shown