

## ***Title***

Engineering biosynthesis of high value compounds in photosynthetic organisms

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## ***Abstract***

The photosynthetic, autotrophic lifestyle of plants and algae position them as ideal platform organisms for sustainable production of biomolecules. However, their use in industrial biotechnology is limited in comparison to heterotrophic organisms such as bacteria and yeast. This usage gap is in part due to the challenges in generating genetically modified plants and algae and in part due to the difficulty in development of synthetic biology tools for manipulating gene expression in these systems. Plant and algal metabolism, pre-installed with multiple biosynthetic modules for precursor compounds, bypasses the requirement to install these pathways in conventional production organisms, and creates new opportunities for the industrial production of complex molecules. This review provides a broad overview of the successes, challenges and future prospects for genetic engineering in plants and algae for enhanced or *de novo* production of biomolecules. The toolbox of technologies and strategies that have been used to engineer metabolism are discussed, and the potential use of engineered plants for the industrial manufacturing of large quantities of high value compounds is explored. This review also discusses the routes that have been taken to alter the profiles of primary metabolites for increasing the nutritional quality of foods as well as production of specialised metabolites, cosmetics, pharmaceuticals and industrial chemicals. As the universe of high-value biosynthetic pathways continues to expand, and the tools to engineer these pathways continue to develop, it is likely plants and algae will become increasingly valuable for the biomanufacturing of high value compounds.

**Keywords:** Synthetic Biology; Metabolic Engineering; Genetic Modification; Plants; Algae; Natural Products; Secondary Metabolites

## ***Introduction***

Metabolic engineering is at the forefront of industrial biotechnology for production of high value compounds and reduced reliance on fossil fuels and industrial chemistry. The majority of metabolic engineering projects have used yeast or bacteria as platform microorganisms for production, exploiting their simple genetics, ease of manipulation and long history of use in food production. Pathway engineering in these organisms has focussed on specialty and fine chemicals of microbial origin, as well as production of single proteins or simple biochemicals.(1) While there are many examples of production in these microbial systems, their utility does not extend easily to all compounds. For example, many desirable compounds have intricate biosynthetic pathways and utilise complex building blocks, meaning they cannot be easily manufactured in such microorganisms. Production of these compounds often requires complex cultivation systems, expensive inputs and chemical processes with environmentally deleterious waste products. Thus there is significant incentive to develop novel production systems that mitigate these costs and effects.

Plants, algae and cyanobacteria are photosynthetic and autotrophic and thus require fewer resources than bacterial or yeast systems. This reduction in resources positions them as an attractive alternative for cheap, sustainable production of compounds on an industrial scale. In addition to this cost consideration many plants and algae contain biochemical pathways, absent from fungi and bacteria, that produce precursors from which many pharmaceuticals are manufactured. The presence of these pathways in plants and algae (such as terpenoid and alkaloid biosynthesis) has the potential to expedite metabolic engineering strategies that utilise these metabolites as building blocks, as fewer enzymes need to be introduced for product synthesis. However, the use of plants and algae for production of high value compounds has yet to realise its full potential, and most efforts to engineer plant products have reconstituted plant pathways in microbial hosts.(2) There are several reasons for this lag in utilising photosynthetic hosts. Foremost amongst these is a lack of genetically tractable plants and algae that can be grown at scale, coupled with a lack of precision tools for engineering in these species. These challenges are now being addressed by the recent development of synthetic biology tools in plants (3) and algae (4) that have potential to prime a new green revolution in industrial biotechnology.

This review summarises the range of strategies used to engineer production of compounds in plants and algae, from expression of single genes to entire pathways (for a brief overview of the technologies discussed see the Supplementary Toolbox). Although increases in biomass would also result in increased production of desired compounds, this review does not cover strategies to engineer more efficient photosynthesis.<sup>(5)</sup> Neither does it cover mutagenesis approaches to modification of metabolite production. Instead, the focus will be put on targeted metabolic engineering that has resulted in increased production and altered composition of primary metabolites (e.g. carbohydrates, lipids, vitamins and minerals). This review also covers the production of specialised metabolites for use in manufacture of cosmetics, fragrances and pharmaceuticals; either through enhanced biosynthesis in native producers or transfer of synthesis pathways to more tractable organisms. Together these success stories reveal the potential for plants and algae in industrial biotechnology and highlight a suite of challenges that remain to be solved.

### ***Modification of primary metabolites in plants***

Plants form the basis of the human diet, containing all the energy, lipids, vitamins and minerals essential for survival. Genetic engineering has potential to alter the composition and content of food crops and ultimately improve their nutritional value. The following section describes successful engineering strategies that have resulted in modification of carbohydrate, lipid, vitamin and mineral content of plants.

#### **Altering carbohydrate profiles**

Carbohydrates constitute the bulk of plant biomass, providing energy storage molecules for growth as well as structural elements such as cell walls. Whilst the former are readily turned over in plant tissues, the latter are recalcitrant to degradation. Modification of these different carbohydrate pools thus presents different challenges and opportunities.

#### ***Modification of storage carbohydrates***

Storage carbohydrates provide the majority of calories consumed by humans. They are also a bulk commodity material, with global production of starch amounting to billions of tonnes per year.<sup>(6)</sup> The ability to control production of specific classes of storage carbohydrates in plants is an attractive alternative to chemical production of these same compounds *in vitro* <sup>(7)</sup> and has been achieved in a number of ways.<sup>(8)</sup> For instance, altering expression of starch synthases, by overexpression or RNA silencing (see Supplementary Toolbox),

can be used to control the gelling properties of starch by changing the ratio of linear amylose to branched amylopectin. In potato tubers, this resulted in starch with enhanced storage stability that can also be used as a thickening agent or stabiliser.(9, 10) Similarly, a degradation resistant starch was engineered in potato tubers by using an RNAi based strategy to reduce the levels of glucan water dikinase, leading to increased shelf life of the potato.(11) It has also been possible to engineer a vaccine-displaying starch by fusing starch-binding proteins to known antigens in algal chloroplasts.(12) Modification of starch profiles of food crops has potential to radically alter their shelf life, and thus minimise wasted resources, as well as producing starches with industrially desirable properties.

Fructans are also a class of storage carbohydrates, made from fructose polymers in place of the glucose polymers that make up starch and stimulate probiotic growth. The exact makeup of fructans was modified in chicory by introducing fructosyl transferases from onion, *Allium cepa*.(13) Low molecular weight, pro-biotic fructans were produced by transferring fructosyl transferases from *Helianthus tuberosus* into sugar beet (*Beta vulgaris*) (14) and a full spectrum of globe artichoke (*Cynara scolymus*) fructans was produced in potato (*Solanum tuberosum*) by expressing two artichoke fructosyltransferases.(15) Using these techniques fructan profiles of agricultural species can be manipulated to give fructans with optimised probiotic profiles.

### ***Modification of plant cell wall composition***

A range of plants are being explored for production of lignocellulosic biofuels, but efficient conversion of structural carbohydrates to fermentable sugars is challenging. Various strategies are being explored to engineer more digestible plant materials, including increasing cellulose content, altering the proportion of other polysaccharides that make up cell walls, and modification of chemical linkages.(16) Most of this work has focused on mutagenesis and overexpression of cell wall enzymes, which often leads to pleiotropic effects on plant growth. However there has been some progress in targeted approaches for cell wall engineering through altering enzyme expression.(17)

The heterologous expression of an *Arabidopsis* small GTPase, which acts as a positive regulator for xylem development, in poplar (*Populus trichocarpa*) resulted in an increase in more readily digestible glucan content without an increase in other cell wall constituents, allowing easier extraction of the sugars.(18)

Overexpression of the transcription factor controlling secondary cell wall biosynthesis (NST1) in fibre cells of an *Arabidopsis* line previously engineered to have low lignin content, increased the density of the cell wall, raised the total carbohydrate content and improved the release of sugars.(19) The application of these changes to crop species could result in an easier extraction of sugars from left over agricultural biomass.

It is also possible to increase the processability of plant cell walls by expressing cell wall polymer degrading enzymes. For example overexpression of a native cellulase in poplar reduced the crystallinity of the cellulose making it more amenable to degradation.(20) Similarly, expression of a thermostable bacterial endoxylanase in *Arabidopsis* had no effect on growth at ambient temperatures, but upon incubation at 85 °C the molecular weights of cell wall xylans were decreased facilitating extraction of sugars.(21)

Lignin cross links the secondary cell walls and prohibits the extraction of the sugars for biofuel production. Aside from mutations in lignin biosynthesis, which often affect the growth of the plant, modification of the nature of lignin has been achieved through genetic engineering. For example, production of monolignol ferulate esters in poplar, by expressing a feruloyl-CoA monolignol transferase from *Angelica sinensis*, using a xylem specific promoter, increased the release of sugars.(22) Similarly, sugar yield was improved in fescue (*Festuca arundinacea*) by expression of fungal ferulic acid esterase (from *Aspergillus niger*), which hydrolyses lignin from the cell wall carbohydrates. This activity was further enhanced when the gene product was targeted to the Golgi.(23) Whilst there are opportunities to enhance the release of sugars from plant biomass using metabolic engineering, there are significant challenges in balancing enhanced digestibility without compromising plant growth or resilience to physical and biological challenges.(24)

### **Enhancing production and altering the composition of lipids**

Short chain fatty acids are of use as feedstock for the production of biodiesel and it is now well established that long chain poly-unsaturated fatty acids (PUFAs) are essential constituents of human nutrition, with many health benefits.(25) Fatty acids are made by repeated extension of plastid synthesised fatty acids by addition of two-carbon units attached to a carrier protein and desaturated fatty acids are formed by specific desaturation of particular bonds in the carbon chain (Figure 1). A variety of strategies are discussed below

that have been used to engineering production of specific valuable lipids, such as biofuels in cyanobacteria (26) and alga,(27) and PUFAs in plants (28) and microbes.(29)

### ***Increasing total lipid content***

Lipids can act as an energy storage molecule and their production is often increased when there is an excess supply of carbon. Overexpression of a diacylglycerol acyltransferase (DGAT) in the diatom *Phaeodactylum tricornutum* stimulated a 35% increase in lipid content, with more oil bodies and an increase in the proportion of polyunsaturated fatty acids, whilst the growth rate remained similar (30). A fourfold increase in the total lipid content was achieved in a different diatom, *Thalassiosira pseudonana*, by the anti-sense knock down of a degradative lipase.(31) Similarly in cyanobacteria, overexpression of the native acetyl-CoA carboxylase (ACC) subunits, and medium chain thioesterases from plants (*Cuphea hookeriana*, *Cinnamomum camphorum* and *Umbellularia californica*), whilst knocking out divergent pathways and fatty acid degradation, increased secreted lipids to 13% of the biomass.(32) These results suggest that modification of lipid biosynthesis for increased yield in algae can be achieved in multiple ways in the absence of deleterious effects on growth. Thus this a potential area where application of engineering principles may result in substantial further yield increases. Moreover there is substantial variability in lipid content between algal species and comparative omics approaches may provide clues to help guide these engineering efforts.

Plants do not only produce lipids in storage tissues, but they are also synthesised in vegetative tissues(33) and overexpression or down-regulation of a range of genes has been reported to increase levels of these lipids.(34) These have included: increasing synthesis, by overexpressing DGAT in tobacco leaves resulting in an accumulation of fatty acids up to 6% of dry weight;(35) reducing competing pathways, for example by RNAi of ADP-glucose pyrophosphorylase, resulting in a 1.4 fold increase in triacyl glycerols (TAG);(36) enhancing storage, by overexpressing the oil droplet stabilising protein oleosin leading to an 7 fold increase in TAG content in *Arabidopsis* leaves;(37) and transcription factor engineering, by expressing *Arabidopsis* LEC2 in tobacco leaves resulting in increased fatty acids to 6.8% of dry weight.(35) Combining these strategies, Vanhercke et al. achieved 17.7% total lipid content by stably expressing DGAT, oleosin and the transcription factor WRL1 in tobacco leaves, representing a 24 fold increase over wild-type levels.(38) These

efforts to enhance lipid accumulation in oil crops have clear applications in the production of lipid-based biofuels, although yields would need to increase further for production costs to rival that of fossil fuels.

### ***Producing short chain lipids***

Short chain lipids are of use in the biofuel industry, as they give better oxidative stability and physical properties than their longer counterparts, and their production can be engineered by early release of the growing chain from the acyl-carrier protein (ACP). Expression in *Arabidopsis* of the California bay laurel tree (*Umbellularia californica*) thioesterase (TE), which hydrolyses medium chain lipids from the ACP, changed the ratio of the seed oil lipids such that the predominant fatty acid was laurate, absent in the wild type *Arabidopsis* seeds,(39) and a common ingredient in cosmetics and soaps. In the diatom *Phaeodactylum tricornutum*, heterologous expression of two TEs from trees (*Cinnamomum camphorum* and *Umbellularia californica*), which catalyse the cleavage of medium chain fatty acids from the ACP, caused increased total lipid accumulation and production of 12 and 14 carbon fatty acids, which can be potentially used as diesel alternatives.(40) Reengineering of the profile of lipids produced may reduce the downstream processing necessary to produce fuels from these lipids and make plant based production more cost effective.

### ***Engineering long chain poly-unsaturated fatty acids (PUFAs)***

Eukaryotic microalga are the primary producers of PUFAs, which then accumulate through the food chain and are a key component of the human diet. These compounds have attracted attention due to their relevance to human nutrition and thus several attempts have been made to engineer their production. PUFAs can be engineered by desaturating endogenous fatty acids, but producing the long chain fatty acids also requires elongation of the carbon chain. Some plants can produce specific types of PUFAs, such as the Ahi flower (*Buglossoides arvensis*) and work is ongoing to increase PUFA content through conventional breeding approaches.(41) Although many cyanobacteria do not produce PUFAs, overexpression of endogenous or fungal genes encoding fatty acid desaturases in the cyanobacterium *Synechocystis* caused an accumulation of  $\alpha$ -linolenic acid (18:3) and stearidonic acid (18:4) at the expense of their saturated analogues.(42) Expression of both an elongase (Elo) and a desaturase (FAD) from the green alga *Osterococcus tauri* in the diatom *Phaeodactylum tricornutum* caused an increase in the PUFAs regarded as most beneficial for human

health.(43) Further development of this system could result in a viable source of PUFAs that could be used in both the human diet and animal feed.

One strategy to achieve industrial scale production of PUFAs is to engineer production in a conventional crop plant. However, levels of accumulation are low and significant improvements need to be made to render production commercially viable.(44) The challenges to production centre on the growing fatty acid, which is the substrate for the elongases and desaturases (Figure 1). These are typically held on different carrier molecules (45) and enzymes to transfer between these carriers must be engineered into the plant.(46) The assembly of PUFAs into storage glycerides also requires the transfer between these different carriers.(47) By expressing seven enzymes from diverse sources, including algae, moss and protists, PUFA levels comparable to those found in microalga were produced in a commercial oil-seed crop *Camelina sativa*.(48) Production of PUFAs in plants could provide a sustainable source of these compounds for supplementing the human diet without relying on marine resources.

#### ***Exploiting precursor lipids for the biosynthesis of other compounds***

Aside from their use as storage molecules and structural components of cells, lipids also provide the precursors from which several plant and animal hormones are built. Given the importance of these hormones to agriculture, human and veterinary health their biosynthesis pathways represent attractive targets for engineering in plants. One successful example of hormone engineering in plants is the biosynthesis of acetylated fatty alcohols, which act as insect pheromones. In order to produce these compounds in plants a series of enzymes were transiently expressed in tobacco (*Nicotiana tabacum*).(49) These include a specific plant thioesterase targeted to the plastid, a desaturase and a reductase from moths targeted to the endoplasmic reticulum and finally a plant enzyme to acetylate these alcohols. The final compounds displayed activity matching conventionally produced pheromones.(49) This may represent an efficient way to produce commercial quantities of these valuable compounds, or alternatively this pathway could be introduced into crop plants to disrupt the behaviour and development of insect pests, providing novel crop protection strategies.



## Enhancing production and accumulation of vitamins and minerals

Plants are the major source of many vitamins, minerals and essential amino acids in the human diet. Various strategies have been attempted for increasing the content of these compounds in crop plants, including expression of biosynthetic enzymes, enhancing transport to storage organs and improving storage of the compounds.

### **Folic acid**

Folic acid is an essential cofactor for many enzymes and the majority of folate in the human diet is derived from plants.(50) Folic acid is biosynthesised from pteridine, derived from GTP, and *p*-aminobenzoate (PABA), derived from chorismate (Figure 2A). Expressing the first enzyme in pteridine biosynthesis, GTP cyclohydrolase I (GTPCH1) from mouse, in tomato (*Solanum lycopersicum*) fruit lead to a modest (twofold) increase in folate but a large increase (3 to 140 fold) in pteridine.(51) Expression of an *Arabidopsis* aminodeoxychorismate (ACS) synthase in tomato fruit lead to a 19 fold increase in PABA concentration, and when these two lines were bred together a new strain was generated that accumulated 25 fold higher levels of folate (840 µg per 100 g).(52) Thus though biosynthetic pathways can often contain several steps, this example showed that increasing expression of the first enzymes in a pathway can sometimes lead to an increase in the level of end products.

### **Vitamin A**

β-Carotene is an essential component of the human diet as the precursor to Vitamin A, necessary for development and vision. Many staple food crops including rice, cassava and potato contain low levels of β-Carotene and thus there is significant impetus to increase the content of this compound in these plants. β-Carotene is synthesised from geranylgeranyl pyrophosphate (Figure 2B) and is essential for photosynthesis. Lycopene β-cyclase genes from various carotenoid-synthesizing source organisms were tested as transgenes in transplastomic (see Supplementary Toolbox) tomato plants, producing up to 1 mg/g dry weight of β-carotene as well as a 50% increase in total carotenoids.(53) To produce enhanced levels of β-carotene in rice (*Oryza sativa*) endosperm, from which it is normally absent, phytoene synthase (PSY), from daffodil (*Narcissus*), and carotene desaturase (CRTI), from a bacteria (*Erwinia*), were expressed and targeted to the rice endosperm to produce Golden Rice varieties.(54) Further enhanced levels of carotene (31 µg/g) in rice

endosperm was subsequently achieved by replacing the daffodil enzyme with a maize (*Zea mays*) homologue.(55) The same paradigm has been used to generate a golden potato using all three *Erwinia* genes (PSY, CRTI and CRTY), specifically expressed in the tuber.(56) Given the simplicity of the pathway, and the success in transferring it to crop plants, genetic engineering to increase the production of  $\beta$ -carotene in other staple crops is proposed as a strategy to ameliorate Vitamin A deficiency in developing countries with far reaching health benefits.

### **Vitamin E**

Vitamin E is an essential part of the human diet. It includes tocopherols and tocotrienols, fat soluble antioxidants synthesised from tyrosine and phytol in photosynthetic cells (Figure 2C).(57) The level of  $\alpha$ -tocopherol was increased by >80-fold in *Arabidopsis* by overexpressing the native methyltransferase in seeds.(58) By generating transplastomic tomato and tobacco plants expressing three key enzymes specific to tocopherol biosynthesis, homogentisate phytyltransferase (HPT), tocopherol cyclase (VET1), and  $\gamma$ -tocopherol methyltransferase (TMT) from *Synechocystis*, a sixfold enhancement of Vitamin E accumulation was achieved.(59) In contrast to folate biosynthesis above, these results suggest that over expression of later steps in a biosynthetic pathway can also increase flux through earlier enzymes to increase the accumulation of end product.

### **Vitamin C**

Ascorbic acid is also an essential component of the human diet, and is the most important antioxidant in photosynthesising organisms, reducing reactive oxygen species formed during photosynthesis.(60) Expression of ascorbate biosynthesis enzymes in leaves of model plants has had some success in increasing the levels of Vitamin C, such as expression of the rat L-gulonolactone oxidase in lettuce (sevenfold increase) (61) and D-galacturonic acid reductase in *Arabidopsis* (two to threefold increase).(62) Similarly, constitutive expression of GDP-L-galactose phosphorylase from kiwi (*Actinidia chinensis*) enhanced Vitamin C production in strawberry and tomato (two and three to sixfold respectively), and expression of the potato or *Arabidopsis* enzyme gave up to a threefold increase in potato tubers.(63) Thus there have been several parallel success

stories in modulating levels of vitamin C accumulation in multiple plant species, indicating that bioaccumulation of this important nutrient should be readily achievable in crop species.

Combining these strategies to increase vitamin content in the same plant has the potential to generate a new generation of high nutrition value crops. This potential has already been realised in maize where expression of native phytoene synthase and *Pantoea ananatis* phytoene desaturase genes for carotenoid biosynthesis, the rice dehydroascorbate reductase gene for ascorbate recycling, and the *E. coli* folE gene (encoding GTP-cyclohydrolase) for folate synthesis, increased levels of  $\beta$ -carotene, ascorbate and folate by 169-, six- and twofold respectively.(64) Thus it is possible, with relatively small numbers of genes, to produce agriculturally relevant food crops with enhanced nutritional content that could (if permitted) ameliorate nutrient deficiencies that affect large numbers of people around the world. It is likely that the major barriers to the development of these crops will not be due to technological or scientific issues.

### **Minerals**

Minerals are not biosynthesised, but instead are obtained by plants from their environment. In order to boost the mineral content of crops, attempts have focussed on increasing capacity for uptake and storage. To increase the iron storage capacity of seeds ferretin, an iron storage molecule, has been expressed in rice, also giving an increase in zinc content, raising levels of iron from 15.7 to 34.7  $\mu\text{g/g}$  and zinc from 33.6 to 55.5  $\mu\text{g/g}$ .(65) Over expression of the endogenous ferretins in wheat (*Triticum*) endosperm also leads to a 50-85% increase in the iron content of the grain.(66) Combining expression of a bean (*Phaseolus vulgaris*) ferretin with a thermotolerant fungal phytase (from *Aspergillus fumigatus*), which degrades an inhibitor of iron uptake, and over-expressing the endogenous cysteine-rich metallothionein-like protein, which enhances absorption, produced rice with double the content of bioavailable iron.(67) In contrast to this, overexpression of an iron transporter lead to only small increases in iron (13%) and zinc (12%) in rice (68) presumably through limited capacity to store transported minerals. However, combining these two strategies together by expressing enzymes for synthesis of the natural metal chelator nicotianamine and mugineic acid together with a transporter (OsYSL2) produced 4.4 fold increase in rice iron content in the field.(69) This strategy could

be useful to fortify staple foods in regions of the world that suffer from health issues caused by iron deficiency.

### ***Engineering production of specialised metabolites***

Specialised metabolites are generally not considered necessary for normal plant growth. They include many antifeedants (herbivory prevention), attractants (to attract pollinators or symbionts), and antimicrobials (to protect against pathogens). Many of these compounds find use as dyes, fragrances and pharmaceuticals. The pathways are often complicated and limited to certain non-model species, thus making their engineering somewhat challenging. Efforts to engineer biosynthesis of these compounds has focused on increasing yield or altering product profiles in native producers, or transferring the entire pathway into alternative heterologous hosts.

### **Glucosinolates and cyanogenic glucosides**

Glucosinolate are defence compounds that are generally produced upon damage to the plant.(70) Though they are defence chemicals, consumption of glucosinolates is thought to have health benefit in humans, prompting interest in boosting their production in food crops. Glucosinolates are primarily found in Brassicales species and production can be enhanced or altered by transferring parts of the pathway between species.(71) The core pathway evolved by combining four enzymatic modules allowing the production of new chemical backbones.(72) This may allow the artificial shuffling of these modules to generate novel glucosinolates. In order to produce a cultivar of broccoli with enhanced health benefits, marker assisted breeding was used to transfer genes from wild relatives which have higher levels of glucoraphanin, leading to production of commercial broccoli with up to 30  $\mu\text{mol/g}$  dry weight glucosinolates.(73)

Novel glucosinolates can also be generated by introduction of precursors into species that don't naturally produce them. For example, expression of two *Sorghum* cytochrome P450 enzymes (CYPs), which play a role in dhurrin biosynthesis (Figure 3), in *Arabidopsis* and tobacco resulted in production of a range of cyanogenic compounds structurally related to glucosinolates, including some that were stabilised by endogenous glucosylation such as *p*-hydroxybenzylglucosinolate.(74) Dhurrin was also produced in tobacco plastids by expressing these two enzymes, together with a glucosyltransferase, directly coupling to the photosynthetic reducing power.(75) Similarly, the pathway for glucoraphanin was introduced into non-glucosinolate

producing tobacco.(76) This required five genes for biosynthesis of the chain elongated methionine starting material and six further genes for biosynthesis of the glucosinolate. Transfer of these genes into crop plants outside the Brassicaceae could be used to produce a range of foods with enhanced health benefits and potential pathogen resistance.

### **Podophyllotoxin**

Etoposide is a semisynthetic anticancer agent, derived from the natural product podophyllotoxin which is produced by *Podophyllum hexandrum* (mayapple).(77) Whilst podophyllotoxin targets tubulin polymerisation,(78) when glycosylated to form etoposide this activity is completely altered to target topoisomerase.(79) The entire pathway (Figure 4) has recently been transiently expressed in *N. benthamiana* (see Supplementary Toolbox). The first four enzymes were known (80) and pluviatolide was successfully produced by transiently expressing three of these in tobacco. The next enzymes were discovered by expressing candidates in leaves engineered to produce pluviatolide and the full pathway elucidated, allowing production of desmethyl-epipodophyllotoxin, the precursor of etoposide, when all enzymes are co-expressed.(81) The yield was enhanced to 10.3 ng per mg of plant dry weight by coinfiltration with pinoresinol, suggesting there is a bottleneck early in the pathway. This represents the most comprehensive pathway engineering achieved to date *in planta*, and, although it only utilised transient expression, it shows the potential of metabolic engineering to introduce complex multistep pathways for bioproduction of pharmaceuticals.

### **Terpenoids**

Terpenoids, one of the largest and most diverse classes of metabolites in plants, are formed from sequential addition of five carbon isoprene units, followed by cyclisation and decoration with a variety of moieties. They include carotenoids, chlorophyll pigments, hormones (including gibberellin and abscisic acid), and various attractant and protectant molecules. Photosynthetic organisms are ideal for industrial scale production of terpenoids as they contain pathways for terpenoid production, and carotenoids in particular are required at high levels during photosynthesis. For example, high concentrations of the algal carotenoid astaxanthin, which is used as a food colouring, can be produced from  $\beta$ -carotene by expressing an oxygenase in

*Synechococcus* (2.7% of total carotenoids) (82) or tomato (16.1 mg/g in fruits).(83) This is approaching the levels found in *Haematococcus pluvialis*, the alga which is used as the main production system, of 40 mg/g.

There are two pathways for biosynthesis of terpenoid precursors in plants (Figure 5A), with only the mevalonic acid (MVA) pathway producing sterols and the methyl erythritol phosphate (MEP) pathway producing carotenoids, amongst other compounds. In order to achieve high levels of terpenoids these precursor pathways often need to be engineered. For example, in order to engineer efficient production of artemisinic acid in yeast two genes from the MVA pathway had to be overexpressed in an mutant background that already had high MVA and a repressed squalene synthase.(84) Engineering terpenoid pathways into other species is complicated – for example introduction of a MVA pathway in *E. coli* inhibited cell growth.(85) Since plants have functional MVA (in the cytosol) and MEP pathways (in the plastid), extensive engineering may be unnecessary to generate sufficient precursor.

Alteration of the biosynthesis of precursor pathways can be used to increase production in plants. Stable expression of snapdragon (*Antirrhinum majus*) geranyl diphosphate synthase (GPPS) in tobacco plants, under a petal specific promoter, increased monoterpene emission from leaves and flowers by 12 fold, but reduced levels of sesquiterpene emission by up to 5 fold.(86) The over-expression of the native HMGR in *Salvia miltiorrhiza* enhances production of tanshinones (2.5 fold) and squalene (3 fold) in cultured hairy roots,(87) while similarly, over-expression of GGPPS, HMGR and DXS, individually and in combination increases tanshinone production by almost 5 fold over wild type.(88) Thus, by increase expression of enzymes for the biosynthesis of precursors, higher levels of complex terpenes can be achieved in native producers. Together, these highlight the advantage of using plant systems for production of plant derived biomolecules.

### **Monoterpenes**

Monoterpenes are made from geranyl diphosphate (Figure 5B) and are typically highly volatile and fragrant compounds widely used in cosmetics, food and pharmaceuticals. Example monoterpenes include the scent molecules of lemon, mint and pine. Engineering of monoterpenes can be relatively straightforward with expression of a suitable monoterpene cyclase typically producing the desired compound.

For example, stable tobacco transformants expressing  $\beta$ -pinene, limonene, and  $\gamma$ -terpinene synthases produced the desired target compounds. When the three transgenes were stacked in one transgenic line by breeding, the tobacco plants produced more terpenoids and produced the expected suite of terpenoids.(89) Similarly, to enhance the flavour and scent of tomatoes the *S*-linalool synthase (LSO) from *Clarkia breweri* was expressed to generate the desired monoterpene.(90) However, when this same enzyme was expressed in *Petunia*, linalool could not be detected, but the non-volatile glucoside was identified, modified by endogenous enzymes,(91) indicating that in certain instances straightforward engineering of monoterpene synthesis doesn't always work. In order to produce geraniol, a monoterpene with a rose like scent commonly used in perfumes, the specific geraniol synthase (GOS) has been expressed in a range of plants. Expressing the GOS from *Valeriana officinalis* in tobacco hairy root culture allowed the synthesis and accumulation of significant amounts of geraniol, as well as the glycoside.(92) Tomatoes expressing the *Ocimum basilicum* GOS gene under the control of a fruit ripening-specific promoter accumulate monoterpenes at the expense of lycopene accumulation, leading to improvement in aroma and flavour.(93)

Monoterpenes in plants can also provide protection against herbivory and fungal infection. For example phellandrene is an insecticide against the herbivorous caterpillar *Helicoverpa zea*, and is an antifungal agent against *Botrytis cinerea*. Phellandrene synthase (PHS) can use geranyl pyrophosphate to make myrcene and ocimene, and expression of this enzyme in tomato fruit produces of a small amount of both.(94) However, in order to make phellandrene neryl (*cis*-geranyl) pyrophosphate is needed as a substrate (95) and overexpression of native neryl diphosphate synthase in tomato fruit lead to a reduction in carotenoids with a small increase in various terpenoids.(94) Crossing these two lines restored carotenoid levels and produced  $\beta$ -phellandrene, affecting caterpillar feeding behaviour and acting as an antifungal. Thus engineering production of this compound in crop plants may provide a novel multi-faceted crop protection strategy.

### **Sesquiterpenes**

Sesquiterpenes are made from farnesyl diphosphate and thus have a 15 carbon backbone (Figure 5C). They can be synthesised by a single enzyme and thus can potentially be transferred between organisms. For example expression of the  $\beta$ -caryophyllene synthase (BCS) gene from *Artemisia annua* in *Synechocystis*

produced the bicyclic sesquiterpene  $\beta$ -caryophyllene.(96) Production of sesquiterpenes can also be used to modulate insect behaviour for crop protection. Examples include, expression of the strawberry nerolidol synthase (NES) in *Arabidopsis*.(97) These plants attracted carnivorous predatory mites (*Phytoseiulus persimilis*) that aid the plants' defence mechanisms. On the other hand, tobacco hornworm was repelled from tobacco by production of patchoulol. This was achieved by expressing patchoulol synthase (PTS) from Patchouli (*Pogostemon cablin*), fused to chicken (*Gallus gallus*) farnesyl pyrophosphate synthase (FPPS), to enhance production of the precursors.(98)

To further elaborate the sesquiterpene backbones for production of modified terpenes other enzymes are needed. Three enzymes are required for the synthesis of costunolide and these were coinfiltrated into tobacco: germacrene A synthase (GAS) from feverfew (*Tanacetum parthenium*); chicory germacrene A oxidase (GAO); and costunolide synthase (COS), a cytochrome P450, also from chicory.(99) Similarly, coinfiltration of *N. benthamiana* with the cDNA encoding amorphadiene synthase (ADS) and the appropriate cytochrome P450 (CYP71) from *Artemisia annua* leads to production of artemisinic acid, which is then glucosylated by endogenous enzymes.(100) Currently artemisinic acid is obtained from the native producer, *Artemisia annua*, and yeast engineered to synthesise this compound are not commercially viable, despite significant investment by Sanofi.(101) It will be interesting to see whether metabolic engineering could increase the production within *Artemisia* or whether introducing the pathway into another crop plant could lead to further price decreases and supply stability.

### **Diterpenes**

Diterpenes are synthesised from geranylgeranyl pyrophosphate by a variety of cyclisation reactions, giving them a 20 carbon backbone (Figure 5D) and expressing the specific cyclases has been used to produce a range of valuable diterpenes. A range of diterpenes were engineered into tobacco by transiently expressing specific diterpene cyclases, including *Nicotiana glauca* cembratrienol synthase (CBS), *Ricinus communis* casbene cyclase (CBC) and *Ginkgo biloba* levopimaradiene synthase (LVS).(102) Almost 3 mg per gram dry weight was obtained of the perfume precursor sclareol in the moss *Physcomitrella patens* by removing the native



kaurene synthase and expressing labda-13-en-8-ol diphosphate synthase (LPPS) and sclareol synthase (SS).(103)

Alterations in the relative abundance of diterpenes can be achieved by altering the modification enzymes. For example, knock-downs of a specific cytochrome P450 (1-31A) in tobacco reduced the cembratrienediols and increased the cembratrieneols, resulting in a decrease in aphid colonisation.(104) To begin engineering production of a precursor to forskolin, a pharmaceutically active diterpenoid from *Coleus forskohlii*, *Synechocystis* was stably transformed with chromosomally integrated TPS2 and TPS3, to produce manoyl oxide, and maximum yields were achieved by co-expression of the plant DXS and GGPPS for the biosynthesis of the precursor.(105) Diterpenes can be produced readily by heterologous expression of the simple cyclases, but these then need to be further modified to achieve the desired compounds, increasing the complexity of the problem.

### ***Triterpenes***

Triterpenes are made from a 30 carbon backbone, which is itself derived from the joining of two molecules of farnesyl pyrophosphate by squalene synthase, followed by oxidation and cyclisation to form different ring system skeletons (Figure 5E). They are further elaborated by oxidation, glycosylation, acetylation and methylation.(106)

Over expression of early steps in the pathway can be used to enhance production of a whole suite of native triterpenes. For example, production of the simplest triterpene, squalene, was increased 150 fold by expression of FPPS and squalene synthase (SQS) from either the nuclear or plastid genomes, although the latter had 4000 fold higher expression level, suggesting metabolic limitations.(107) To increase the expression of one class of triterpenes a  $\beta$ -amyrin synthase (BAS) from *Aster sedifolius* was stably transformed into *Medicago truncatula*, resulting in an increase in derivatives of this compound in both leaves and roots, which caused an enhancement of nodulation.(108) This strategy was also used to identify the compounds made by a *Lotus japonicas* cyclase, AMY2. This enzyme was transiently expressed in tobacco, producing the novel scaffold, dihydrolupeol.(109) Co-expression of a genomically co-localised cytochrome, CYP71D353,

resulted in the production of 20-hydroxybetulinic acid through oxidation at two positions, though it cannot be certain that endogenous enzymes do not carry out the second oxidation.

Avenacin is an antifungal saponin found in oat roots, with a core  $\beta$ -amyrin scaffold decorated with sugars and an anthralinine moiety, transferred from *N*-methylantranilate glucoside.(110) It is synthesised from anthralinic acid by a methyl transferase (Sad9) and a glucosyl transferase (Sad10), which were transiently co-expressed in tobacco leaves, generating appreciable levels of product.(111) Transient expression of oat  $\beta$ -amyrin synthase (Sad1) lead to production of the expected scaffold, and co-infiltration with a cytochrome P450 (Sad2) produced the epoxy-hydroxy-amyrin.(112) This is then further oxidised by uncharacterised cytochromes, further glycosylated by uncharacterised glycosyl transferases and then acetylated by Sad7 (See Figure 5E). Once these steps have been elucidated then the entire Avenacin biosynthetic pathway can be reconstituted in other plants to protect against fungal pathogens.

### **Anthocyanins**

Anthocyanins are water-soluble natural pigments that give rise to the red, purple and blue colours of many flowers and fruit. They are synthesised by extension and cyclisation of a phenolic acid derived from phenylalanine (Figure 6). Although simple forms have been produced in microbial hosts, for example by expressing the core pathway of four genes in *E. coli*,(113) they are not as decorated as in the originating plants and much work has gone into engineering them in plants.(114) Altering the anthocyanin biosynthetic pathways can be used to increase the concentration of these health promoting compounds and enhance or alter the colour of specific plants, generating new commercially attractive cultivars.

#### ***Increasing total anthocyanins***

Overexpressing enzymes early in the pathway has been used to increase general production. For example, expressing the petunia gene for chalcone isomerase (CHI) in tomatoes gave a 78-fold increase in the flavonoid content of tomato peel and a corresponding increase in tomato paste.(115) Combining expression of chalcone synthase (CHS) and flavonol synthase (FLS) gave a further ~50% increase flavonol levels in the flesh of tomato fruits.(116)

In addition to overexpressing metabolic enzymes, transcription factor engineering has also been used to increase expression of whole pathways. During an activation tagging experiment in *Arabidopsis*, in which a strong promoter is randomly inserted into the genome, a line with strong purple pigmentation was identified. In this mutant line, the insertion of the promoter caused upregulation of a specific MYB transcription factor, which in turn caused activation of phenylpropanoid biosynthetic genes and enhanced accumulation of purple anthocyanins.(117) Similarly, the transient expression of an apple MYB in tobacco formed pigmented leaf patches and when it was stably transformed into apple, highly pigmented anthocyanin-rich fruits were generated.(118)

Expression of a MYB transcription factor from *Arabidopsis* constitutively throughout tomatoes lead to accumulation of anthocyanins in all tissue types.(119) Engineering tomatoes by expression of two transcription factors (*Del* and *Ros1*) from *Antirrhinum majus* under the control of a fruit specific promoter enhanced expression of most of the genes for anthocyanin biosynthesis and decoration, and genes related to the transport of these molecules into the vacuole.(120) This lead to accumulation of anthocyanins to 2.8 mg of anthocyanins per g fresh weight, producing a deep purple colour. These tomatoes had an increased shelf life and resistance to *Botrytis cinerea*.(121) In addition, cancer-prone rodents fed on a diet supplemented with purple tomato powder lived significantly longer than control animals.(120) To date, transcription factor engineering has been the most effective strategy for enhancing total anthocyanin production. To maximise yield and minimise negative effects (122) on plant growth careful targeting to specific tissue types and developmental stages will be necessary.

### ***Altering anthocyanin class***

The expression of specific enzymes from different branches of the flavonoid biosynthesis pathway can be used to alter the class of compound produced. For example by expressing the rose alcohol acetyltransferase in petunia, the early anthocyanin pathway intermediates phenylethyl alcohol and benzyl alcohol were successfully acetylated and released as novel volatile compounds.(97) Both free and glycosylated resveratrol were produced at up to 53 µg/g fresh weight in tomato fruits of plants expressing the grape stilbene synthase, similar to levels found in grapes.(123)

Various strategies have been utilised to alter flower colours.(124) Down regulation of the undesired pathway removes unwanted colours and often leads to an increase in desired pigments through an increase in the common intermediate. For example, a shift in colour from blue to red has been achieved by silencing F3'5'H in gentians (see Figure 6).(125) White flowers were also produced by silencing the chalcone synthase and thus derailing the entire pathway at an early stage.(125) Novel, red coloured seeds of soybean have been produced by down regulating two anthocyanidin reductases, which allows red cyanidin-based anthocyanins to accumulate in the seed coat.(126)

Heterologously expressing genes for a different pathway can be used to alter flower colour. Expression of dihydroflavonol 4-reductase (DFR) from maize in petunia can increase the conversion of aromadendrin to produce red pelargonidin-type anthocyanins.(127) Forsythia flowers are pigmented by carotenoids and are deficient in anthocyanins. By constitutively expressing *Antirrhinum majus* DFR and *Matthiola incana* anthocyanidin synthase (ANS) pink flowers were generated, overlaying red cyaniding pigments on the native yellow carotenoids.(128) By combining overexpression of genes for a desired pathway and silencing of genes for the native pathway novel pigments can be generated. In order to engineer a yellow pigment into *Torenia hybrida* the C4'GT and AS1 genes from *Antirrhinum majus* were constitutively expressed, leading to accumulation of aureusidin 6-O-glucoside in transgenic flowers.(129) To reveal the colour the DFR was silenced to prevent synthesis of the native purple delphinidins, resulting in brilliant yellow flowers.

These strategies have been exploited to generate a range of blue pigmented flowers in species for which there are no delphinidin anthocyanins and thus no violet-, purple- or blue-coloured cultivars. For example the overexpression of Campanula F3'5'H in Chrysanthemums allowed the production of delphinidins, generating blue flowers.(130) Carnations (*Dianthus caryophyllus*) naturally accumulate pelargonidin or cyanidin type anthocyanins, but expression of F3'5'H in a white flower produced a range of purple cultivars, now commercially available.(131) The anthocyanins in these transgenic flowers are delphinidin derivatives with the same glycosylation pattern as natural carnation anthocyanins, indicating that native anthocyanin decorating enzymes are able to modify delphinidin.(132) By replacing the endogenous DFR gene with the Iris homologue and introducing the viola F3'5'H gene in a rose cultivar, the flowers accumulated delphinidins

giving rise to a novel commercial strain with a mauve colour.(133) Anthocyanin class can be altered in a variety of plants to give rise to commercial cultivars with colours that are unobtainable by conventional breeding.

## **Alkaloids**

Alkaloids include a number of important medicinal compounds, most of which are still derived from plant materials. They are a structurally unrelated group of nitrogen-containing compounds produced from a range of precursors through different biosynthetic pathways.(134)

### ***Purine alkaloids***

Purine alkaloids are derivatives of purine nucleotides with caffeine being one of the most ubiquitous drugs used by humans. The biosynthesis pathway of caffeine is relatively straightforward, with the sequential transfer of methyl groups onto nitrogens of the purine core (Figure 7A).(135) A 70% reduction of caffeine content was achieved in coffee (*Coffea canephora*) by downregulating the second *N*-methyltransferases transferase via RNA silencing (see Supplementary Toolbox).(136) A more pest resistant tobacco cultivar has also been engineered by expressing three xanthosine *N*-methyltransferases from coffee to produce caffeine at up to 5 µg/g fresh weight.(137) Using genetic engineering it is thus possible to raise or lower the caffeine content of plants as desired. Production of caffeine free varieties of coffee and tea are clear commercially viable targets for these genetic engineering approaches that would save substantial costs in post-harvest processing.

### ***Tropane alkaloids***

Tropane alkaloids are a range of medicinally useful plant alkaloids, including such drugs as cocaine and atropine, found amongst the *Solanaceae*.(138) They are made from the joining of the fused bicyclic alkaloid tropane and phenyllactate (Figure 7B).

Conversion of hyoscyamine to the much more valuable scopolamine was achieved by the overexpression of hyoscyamine-6β-hydroxylase (H6H) in *Hyoscyamus muticus* (henbane) hairy root cultures.(139) By expressing this gene in whole plants of *Atropa belladonna* (deadly nightshade) complete conversion of the hyoscyamine was achieved, producing up to 1.2% dry weight scopolamine compared to 0.3% hyoscyamine in the wild

type.(140) Fluorinated littorine and hyoscyamine analogues could be produced by simply feeding fluorinated phenyllactic acid analogs to *Datura stramonium* root cultures, transformed with wild type *Agrobacterium*.(141) By combining these strategies it may be possible to produce commercially viable quantities of novel analogues of pharmaceuticals.

### ***Benzylisoquinoline alkaloids***

The benzylisoquinoline alkaloids are a structurally diverse family of alkaloids, derived from tyrosine (Figure 7C), with a wide range of pharmacological activities including the potent analgesics morphine and codeine. Overexpression of codeinone reductase (CER), the final enzyme in morphine biosynthesis, in transgenic opium poppy (*Papaver somniferum*), moderately increased morphine and codeine content by 15-30%.(142) Overexpression of scoulerine methyltransferase (SMT) resulted in a moderate (15%) increase in the amount of berberine and columbamine in a *Coptis japonica* cell culture.(143) Columbamine was also produced in the non-native producer *Eschscholzia californica* (Californian poppies) when this enzyme was expressed.(143) The overexpression of an early step in the pathway (MCH) in an opium poppy resulted in an up to 450% increase of total alkaloids without altering the product distribution, suggesting this is a key regulation step in morphine biosynthesis.(144) The cultivar used as the background for this experiment had undergone extensive selective breeding, suggesting that metabolic engineering offers opportunities unavailable through conventional strategies.

### ***Monoterpene indole alkaloids***

Monoterpene indole alkaloids are derived from tryptophan and the terpene secologanin (Figure 7D).(145) A number of metabolic engineering efforts have attempted to improve production and produce novel analogues.(146) As discussed previously, overexpressing enzymes in the early part of the pathway is an effective way to increase production of the final products, as has been shown in *Cinchona officinalis*, a tree from which the antimalarial quinine is derived. Hairy root cultures expressing tryptophan decarboxylase (TDC) and strictosidine synthase (STR) from *Catharanthus roseus*, produced large quantities of tryptamine and strictosidine, together with a 3-5 fold increase in the downstream products quinine and its stereoisomer quinidine.(147)

The Madagascan periwinkle, *Catharanthus roseus*, has been highly studied as it produces a range of monoterpene indole alkaloids, including the anticancer agent vinblastine.(148) This has been the focus of some of the most comprehensive metabolic engineering studies carried out in plants, despite many steps in the pathway remaining uncharacterised. To increase the overall level of alkaloids overexpression of strictosidine synthase in hairy root cell lines (see Supplementary Toolbox) improved the levels of ajmalicine, serpentine, catharanthine and tabersonine, although highly productive lines were not stable.(149) Expression in hairy roots of both subunits of the feedback-insensitive anthranilate synthase from *Arabidopsis* and the native tryptophan decarboxylase lead to a 14 fold increase in tryptamine and a significant increase in downstream alkaloids.(150)

In order to make unnatural derivatives, synthetic tryptamine analogues containing halogen atoms were successfully fed to cell cultures of *C. roseus* and incorporated into alkaloid products – for example fluorinated serpentine and akuammicine analogous were produce upon feeding fluorinated tryptamine.(151) Mutants of strictosidine synthases were identified which allowed more artificially fed tryptamine analogous to be incorporated in hairy root culture.(152) Rather than directly feeding halogenated tryptamine analogous to the cells it has been possible to express bacterial halogenases alongside the mutant strictosidine synthase to produce a variety of downstream halogen-containing alkaloids, including chlorinated or brominated ajmalicine.(153) These were then chemically cross coupled, giving rise to new derivatives.(154) The biosynthetic engineering of chemical handles into specific parts of these complex molecules opens up a whole new suite of analogues and a viable route to their synthesis.

## ***Industrial biomanufacturing in plants***

### **Biomanufacturing of industrial chemicals**

With the aim of reducing the use of hazardous materials in the production of industrial products, attention has been directed at biomanufacturing to supply either building blocks or even final products for the chemical industry. Proof of concept pathways have been constructed for the production of more than 20 industrial chemicals in cyanobacteria.(155) These include biofuels, such as butanol, feedstocks, such as sugars, and chemical building blocks, such as lactate.

When 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase were constitutively expressed and targeted to the chloroplast in *Arabidopsis*, the plants accumulated the biodegradable plastic polyhydroxybutyrate (PHB) at up to 4% of their fresh weight.(156) Addition of threonine deaminase led to the accumulation of a more useful copolymer.(157) PHB could also be produced at high levels in the agricultural species alfalfa (1.8% dry weight) (158) and sugarcane (4.8% dry weight).(159) Expressing these genes in the chloroplast of tobacco lead to an accumulation of up to 18.8% dry weight of PHB in leaf tissue.(160) These engineered plants are now being explored for the commercial production of these plastics.

### **Hydrogen production in algae**

Hydrogen is a promising renewable energy source, with particular emphasis in fermentation by anaerobic bacteria. However these organisms are oxygen sensitive and the hydrogenases are only active under anaerobic conditions.

Algae may be able to overcome these drawbacks by directly coupling hydrogen production to photosynthesis. For hydrogen production in *Synechocystis* the partial uncoupling of luminal proton efflux with ATP synthesis resulted in an increase in linear electron transport and a drastically reduced  $\Delta pH$  across the thylakoid membrane.(161) This concomitantly caused a reduction in CO<sub>2</sub> fixation, although this is an unnecessary step for production of hydrogen. The bacterial [FeFe] hydrogenase could be transferred in the cyanobacterial host successfully (algal hydrogenases are [NiFe]), retaining activity.(162) The hydrogenases are oxygen sensitive but it is possible to generate hydrogen without co-production of oxygen in *Synechocystis* mutants lacking all three respiratory terminal oxidases.(163) These algae are now being explored as alternatives to anaerobic bacteria for the bioproduction of hydrogen.

### ***in planta* protein production**

Transient expression (see Supplementary Toolbox) has allowed the production of a wide range of proteins and has facilitated a number of the projects described in this review.(164) This allowed, for example, production of amorpho-4,11-diene synthase and epi-cedrol synthase from *Artemisia annua* for *in vitro* assays (165). Plant derived virus like particles have been successfully produced in plants and these have gone on to be evaluated in clinic trials.(166) The surfaces of viruses expressed in plants can be modified to express antibodies for potential use in treatments.(167) Eukaryotic microalga have been explored as a



biomanufacturing host for a range of recombinant proteins, including antibodies and immunotoxins, hormones, industrial enzymes, an orally-active colostral protein for gastrointestinal health, and subunit vaccines.(168) The chloroplast has proven a valuable production platform for a range of proteins, such as a malarial vaccine candidate.(169) and camelid single chain antibodies against botulinum neurotoxin.(170) These were all achieved in *Chlamydomonas*, but other algae have been successfully used to produce proteins, such as active rabbit neutrophil peptide-1 produced in *Chlorella* (171) and an effective viral subunit vaccine which can be fed directly to shrimp in the producing alga, *Dunaliella*.(172)

For some proteins nuclear expression is required to achieve the correct post-translational modification, as was the case for production of extracellular erythropoietin in *Chlamydomonas*.(173) Recombinant human gastric lipase, produced by transient expression in tobacco was suitably glycosylated to retain activity. (174) Production of antibodies with human-like *N*-glycans was achieved in plants by coexpressing a human galactosyltransferase by coinfiltration the expression constructs in tobacco.(175) Six mammalian proteins comprising the sialic acid pathway were co-expressed together with a monoclonal antibody (mAb) in *N. benthamiana* to produce a sialylated human monoclonal antibody.(176) Production of proteins in plants has advantages over other systems in that they have complex protein glycosylation, unlike *E. coli* or yeast, and do not have the difficulty of culturing or contamination issues suffered with animal cell cultures. Plants therefore represent a flexible eukaryotic system for the production of large amounts of protein and this has allowed their use in commercial production of pharmaceutical proteins.

### ***Future outlooks***

Whilst there are many examples of plant metabolic engineering producing high value compounds *in planta*, few of these systems are yet being commercially exploited. The production and sale of blue roses and carnations, unavailable through conventional breeding but achieved through genetic engineering, (124) is an elegant example. The manufacture of vaccines in tobacco has also found commercial application.(166) Although these examples raise the profile and public acceptance of these technologies there are still a number of hurdles to overcome to realise the full potential of plant metabolic engineering.

A great deal of the work in metabolic engineering of plants for the production of high value compounds has focussed on increasing biosynthesis in native producers. As yet, there has been no work on the transfer of bacterial pathways into plants. Whilst progress can be made by conventional breeding for the production of high value compounds, as has been successful for PUFA production in the Ahi flower,(41) metabolic engineering can be used to enhance production even in high yielding cultivars, such as alkaloid production in poppies.(144) Transformation of non-model plants can also be challenging and may represent a major bottle neck for this approach. The alternative strategy is to transfer biosynthetic pathways into organisms which have already been adapted for agricultural exploitation, such as the production of PUFAs in *Camelina*.(48)

Though the technology to exploit plants and algae is rapidly advancing there are several factors that will need to be overcome. For example, there are many high-value plant products for which the biosynthesis pathway has yet to be elucidated and thus the potential for improvement, transfer between production systems and exploitation is limited. Although many of the successful examples of metabolic engineering have taken the approach of ubiquitous over-expression of enzymes, it is clear that tight regulation of the subcellular localisation and timing of expression of the enzymes will be necessary to maximise production in plants.(177) Plants that produce high levels of PHB are stunted,(156) but if this was targeted to specific organs then this growth defect may be ameliorated. One obvious route is to target pathways to the seeds, exemplified by seed specific expression of appropriate enzymes for PUFA production.(48) Similarly, by using the fruit ripening specific promoter to drive transcription factor expression in tomatoes a healthier plant with higher levels of anthocyanin was produced.(120) While engineering production in seeds is an attractive target there are a number of factors to consider. Altering seed metabolism may affect germination, and depending on the compound being stored, engineered seeds may not be suitable for consumption. Thus co-expression of easily identifiable markers, such as altered seed colour, may be necessary to prevent accidental consumption. Furthermore, many precursor pathways are only active in growing tissues and not in mature leaves or seeds. Thus activation of these pathways in seeds may be a prerequisite for efficient production.

In addition, whilst it may seem straightforward to insert the pathway to a desired product, there are often undesirable reactions catalysed by native enzymes, such as the glucosylation of linalool in *Petunia*.(91)

Additionally, interventions in one pathway can have unexpected effects in others, such as the overexpression of neryl diphosphate synthase in tomato fruit causing a reduction in carotenoids (94) and the overexpression of FPPS and SQS in tobacco lead to alterations in up to 120 unrelated metabolites and profound changes in the global transcriptome.(107)

As algae are more efficient at converting sunlight into biomass and do not have the complications of multicellularity, these may prove valuable production platforms for multiple classes of compounds. Eukaryotic algae represent an extremely diverse group of photosynthetic organisms, distinct from the prokaryotic cyanobacteria. Eukaryotic algae include primary endosymbionts, which gained their plastid from establishing a cyanobacterial endosymbiont (the red, green and golden algae), and secondary endosymbionts, who gained their plastid from establishing an endosymbiotic relationship with a primary or other secondary endosymbiont (including the diatoms, haptophytes, cryptophytes and glaucophytes to name a few).(178) The complex evolutionary history of these algae has given them unusual enzyme complements and architectures (179) and these may give them a unique advantage for the biosynthesis of high value compounds. As new algal genome sequences become available it is clear that many encode complicated natural product machinery reminiscent of modular bacterial megasynthases (180) and there is a great deal of potential for the discovery of new compounds in these organisms. As these alga already make complex natural products from minimal nutritional input they represent a promising platform system in which to start heterologous engineering of these complex bacterial pathways.(181)

Although the development of synthetic biology tools has progressed in plants,(3) it is not currently sufficiently advanced to allow the reliable construction of multi gene constructs for the high level expression of entire pathways in stable hosts. Advances in engineering complex bacterial systems, such as the refactoring of the nitrogenosome (182) and its optimisation through the application of engineering principles,(183) show that complex cellular systems can be engineered once the components have been fully characterised and understood. Thus a major block in the utilisation of plants will be the availability of reliable promoters to drive temporally controlled tissue specific expression, more detailed knowledge of the biochemical pathways

being manipulated, controlled accumulation of undesirable end products and advanced techniques for pathway construction and engineering.

Although, the major factor that will determine the most suitable platform organism for industrial scale production will be the practicality of engineering the target pathway in that organism, other factors will also contribute to this decision. Foremost among these factors are: end use (food, fuel, pharmaceuticals); required scale (milligrams to megatons); and potential hazard (health and environmental). For example, production of nutritional supplements directly in the crop plant, if feasible, will incur a lower cost than production of those compounds in yeast or bacterial systems and adding them to food. In these instances, the scale of required biosynthesis is extremely large and potential health hazard of production or effects of escape into the environment is negligible compared to the potential health benefits. Thus it is likely that many such examples of engineering will translate to industrial scale production in plants. Similarly, biosynthesis of bulk chemicals and biofuels in plants will also likely see multiple examples of translation to industrial production due to the ease of scaling offered by agriculture and reduced requirements for input resources. In contrast, production of pharmaceuticals and controlled substances, where accidental consumption could lead to adverse health or environmental effects, will need careful consideration of choice of platform plant to minimise the potential for compounds to enter human or animal food chains. In these cases legislation concerning production and access to material may mean that industrial production is easier and cheaper in yeast or bacterial systems which are more readily controlled. Finally, variability in product yield or quality due to differences in environment may also limit the potential for biosynthesis in plants.

Finally, there is substantial natural diversity in accumulation of desirable biomolecules in plants that has yet to be exploited. Comparative omics approaches that make use of these natural resources have the potential to provide new insights into the mechanisms of biosynthesis as well as guide engineering strategies for production in stable hosts.

## ***Conclusions***

Great progress has been made in engineering plants to produce high value compounds, either by augmenting the biosynthetic capacity of the native producers or by transferring genes and pathways into alternative

hosts. The majority of success stories have utilised simple technology, using random insertion into the genome and nonspecific viral promoters, and have achieved altered production of metabolites from the manipulation of a single gene. Though there has been multiple examples of success in manipulating various aspects of plant metabolism, plants and algae remain a largely untapped resource for industrial biotechnology. As the genetic manipulation technologies, and libraries of components are refined for use in more plant species, and as more information about the biosynthetic pathways becomes available, the industrial synthesis of high value compounds in plants will come to fruition.

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### ***Declaration of interest***

The authors declare no conflicts of interest.

## Figure legends

**Figure 1: Fatty acid and triacylglycerol synthesis.** In plants *de novo* synthesis of fatty acids take place in the chloroplast, extending the growing alkyl chain immobilised on an acyl carrier protein (ACP) via a CoA cofactor. FabF=3-oxoacyl-ACP synthase. FabG=3-oxoacyl-ACP reductase. FabA= $\beta$ -hydroxyacyl-ACP dehydratase. FabI=enoyl-ACP reductase. TE=Thioesterase. ACC=Acetyl-CoA carboxylase. LACS=long chain acyl-CoA synthetase. Elo=elongase. PC=phosphatidyl choline. LPCAT=lysophosphatidylcholine acetyl transferase. FAD=fatty acid desaturase. G3P=glycerol phosphate. LPA=lysophosphatidic acid. PA=phosphatidic acid. DAG=diacyl glycerol. GPAT=acyl-CoA:G3P acyltransferase. IPAAT=acyl-CoA:LPA acyltransferase. PPA=PA phosphatase. CPT=CDP-choline:DAG cholinephosphotransferase. DGAT=acyl-CoA:DAG acyltransferase. TAG=triacyl glycerol. Enzymes in red have been targeted in metabolic engineering projects in plants.

**Figure 2: Vitamin biosynthetic pathways: A.** Folate is synthesised by the joining of aminobenzoate and dihydropterine, derived from chorismate and guanine respectively. GTPCH1=GTP-cyclohydrolase I DHPP=dihydropterin phosphatase. DHNA=dihydroneopterin aldolase (DHNA) HPPK=7,8-Dihydro-6-hydroxymethylpterin-pyrophosphokinase. ACS=aminodeoxychorismate synthase. ACL=aminodeoxychorismate lyase. DHPS=dihydropteroate synthase. FPGS=Folylpolyglutamate synthase. DHFR=dihydrofolate reductase. **B.** Carotene is synthesised from phytoene, made from the head-to-head joining of two molecules of geranylgeranyl pyrophosphate, followed by desaturation and cyclisation. PSY=phytoene synthase. CRTI=carotene desaturase. CRTY=lycopene cyclase. **C.** Tocopherols are formed from tyrosine and phytane. TAT=tyrosine aminotransferase. PDS=Hydroxyphenylpyruvate dioxygenase. HPT=homogentisate phytyl transferase. VET1= tocopherol cyclase. TMT= $\gamma$ -tocopherol methyltransferase. Enzymes in red have been targeted in metabolic engineering projects in plants.

**Figure 3: Biosynthesis of cyanogenic glucosides and glucosinolates.** Glucosinolates are synthesised from an amino acid, tyrosine in this example. They are then oxidised and glucosylated, which may be through a sulphur derived from cysteine, as in *p*-hydroxybenzylglucosinolate. The starting amino acid can be modified by extension or oxidation, as is the case for glucoraphanin. CYP=Cytochrome P450. UGT=Glucosyltransferase. SUR1=Cysteine-sulphur lyase. ST5a=PAPS-desulfo-glucosinolate transferase. Enzymes in red have been targeted in metabolic engineering projects in plants.

**Figure 4: The heterologous podophyllotoxin biosynthetic pathway.** 10 genes for the biosynthesis of podophyllotoxin were transferred into tobacco from mayapple. DIR=Dirigent protein. PLR=pinoresinol-lariciresinol reductase. SDH=secoisolariciresinol dehydrogenase. CYP719A23=pluviatolide synthase. OMT3=O-methyltransferase 3. CYP71CU1=hydroxylase. OMT1=O-methyltransferase 1. 2-ODD=2-oxoglutarate/Fe(II)-dependent dioxygenase. CYP71BE54=deoxypodophyllotoxin demethylase. CYP82D61=desmethyl-deoxypodophyllotoxin hydroxylase.

**Figure 5: Terpene biosynthetic pathways.** **A.** Isoprene biosynthetic pathway. There are two pathways for the biosynthesis of the isoprene starter units: the cytosolic mevalonic acid pathway, possessed by animals, and the bacterial methyl erythritol phosphate pathway, located in the plastid. DXS=1-deoxy-D-xylulose-5-phosphate synthase. DXR=1-deoxy-D-xylulose-5-phosphate reductoisomerase. CMS=2-C-methyl-D-erythritol 4-phosphate cytidyltransferase. CMK=4-diphosphocytidyl-2-C-methyl-D-erythritol kinase. MCS=2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase. HDS=4-hydroxy-3-methylbut-2-enyl-diphosphate synthase. HDR=4-hydroxy-3-methylbut-2-enyl diphosphate reductase. ACAT=acetyl-CoA acetyltransferase. HMGS=hydroxymethylglutaryl-CoA synthase. HMGR=hydroxymethylglutaryl-CoA reductase. MVK=mevalonate kinase. PMK=phosphomevalonate kinase. MDD=mevalonate diphosphate decarboxylase. IDI=isopentenyl diphosphate isomerase. GPPS=geranyl diphosphate synthase. FPPS =farnesyl diphosphate synthase. GGPPS=geranylgeranyl diphosphate synthase. **B.** Monoterpenes are made from geranyl diphosphate phosphate hydrolysis or by cyclisation, followed by decoration. PNS= $\beta$ -pinene synthase. LMS=limonene. TPS= $\gamma$ -terpinene. LSO=S-linalool synthase. GT=glycosyl transferase. GOS=geraniol synthase. PHS=Phellandrene synthase. **C.** Sesquiterpenes are made from farnesyl diphosphate by cyclisation, followed by decoration. BCS= $\beta$ -caryophyllene synthase. PTS=patchoulol synthase. NES nerolidol synthase. DMNT=dimethylnonatriene. ADS=amorphadiene synthase. CYP71=amorphadiene oxidase. GAS=germacrene A synthase. GAO=germacrene A oxidase. COS=costunolide synthase. **D.** Diterpenes are made by the cyclisation of geranylgeranyl pyrophosphate followed by decoration. TPS2=labdaenol synthase. TPS3=manoyl oxide synthase. CBS=cembratrienol synthase. CYP1-31A=cembratrieneol hydroxylase. CBC=casbene cyclase. LVS=levopimaradiene synthase. LPPS=labda-13-en-8-ol diphosphate synthase. SS=sclareol synthase. **E.** Tripterenes are made by the head to head joining of two molecules of farnesyl

pyrophosphate to make squalene. This is then cyclised to give a range of ring structures which are further decorated. SQS=Squalene synthase. SQM=squalene monooxygenase. AMY2=dihydrolupeol synthase. CYP71D353=dihydrolupeolhydroxylase. BAS= $\beta$ -amyrin synthase. Sad2= $\beta$ -amyrin oxidase. Sad9=anthranilic acid methyl transferase. Sad10=*N*-methyl anthranilic acid glucosyl transferase. Sad7=avenacin acetyl transferase. All annotated enzymes have been targeted in metabolic engineering projects in plants.

**Figure 6: Flavonoid biosynthetic pathway.** Flavanoids are synthesised from phenylalanine and three equivalents of malonyl CoA, followed by specific cyclisation and oxidation to generate compounds with vastly different colours (matching those in the diagram). ANS=anthocyanidin synthase. AS=aureusidin synthase. C4'GT=C4'glycosyltransferase. C4H=cinnamate-4-hydroxylase. CHI=chalcone isomerase. CHS=chalcone synthase. 4CL=4-coumarate:CoA ligase. DFR=dihydroflavonol 4-reductase. F3H=flavanone 3-hydroxylase. F3'H=flavonoid 3'-hydroxylase. F3'5'H=flavonoid 3',5'-hydroxylase. FLS=flavonol synthase. FNR=flavanone 4-reductase. FNS=flavone synthase. PAL=phenylalanine ammonia-lyase. Enzymes in red have been targeted in metabolic engineering projects in plants.

**Figure 7: Alkaloid biosynthetic pathways. A.** Caffeine is synthesised from xanthosine by sequential methylation. XMT=xanthosine methyltransferase. PNP purine nucleoside phosphorylase. MXMT=methylxanthine methyltransferase. DMXMT=dimethyl xanthine methyltransferase (DMXMT). **B.** Tropine, derived from ornithine, and phenyllactate, derived from phenylalanine, are condensed to make littorine, the core tropane alkaloid. H6H=hyoscyamine-6 $\beta$ -hydroxylase. **C.** The core of benzyloisoquinoline alkaloids, noroclaurine is formed by the condensation of two derivatives of tyrosine, dopamine and 4-hydroxyphenylacetaldehyde, by noroclaurine synthase, which is then derivative to give rise to codeine, berberine and scoulerine. NCS=Noroclaurine synthase. CMT=Coclaurine methyl transferase. MCH=methylcoclaurine hydroxylase. SMT=scoulerine methyltransferase. CER=codeinone reductase. **D.** Monoterpene indole alkaloids are biosynthesised by the condensation of tryptamine, made by the tryptophan decarboxylase catalysed decarboxylation of tryptophan, and secologanin, derived from geranyl pyrophosphate, to make strictosidine. This core is then modified to give rise to the range of alkaloid products through diverse pathways that have not yet been fully characterised. GOS=geraniol synthase.



TDC=tryptophan decarboxylase. STR=Strictosidine synthase. Enzymes in red have been targeted in metabolic engineering projects in plants.

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Figures

Figure 1

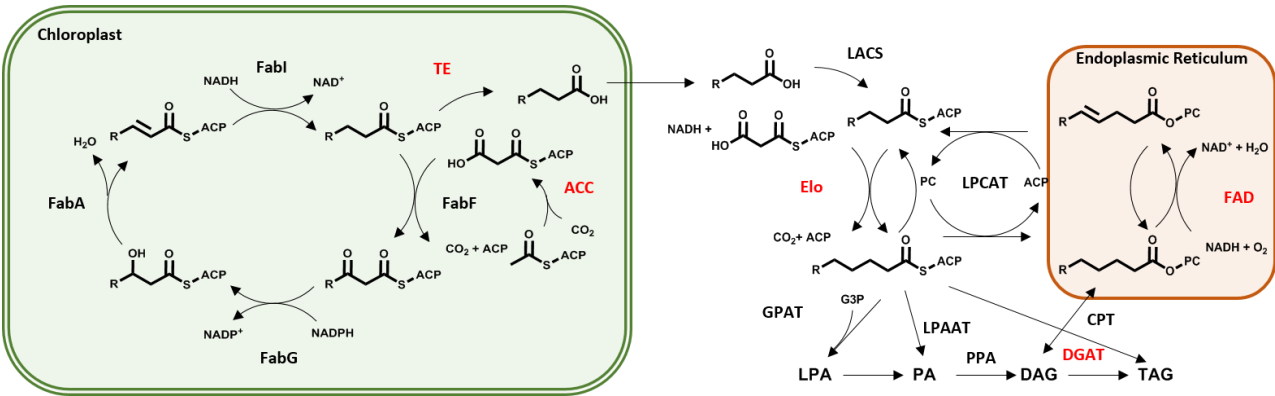


Figure 2

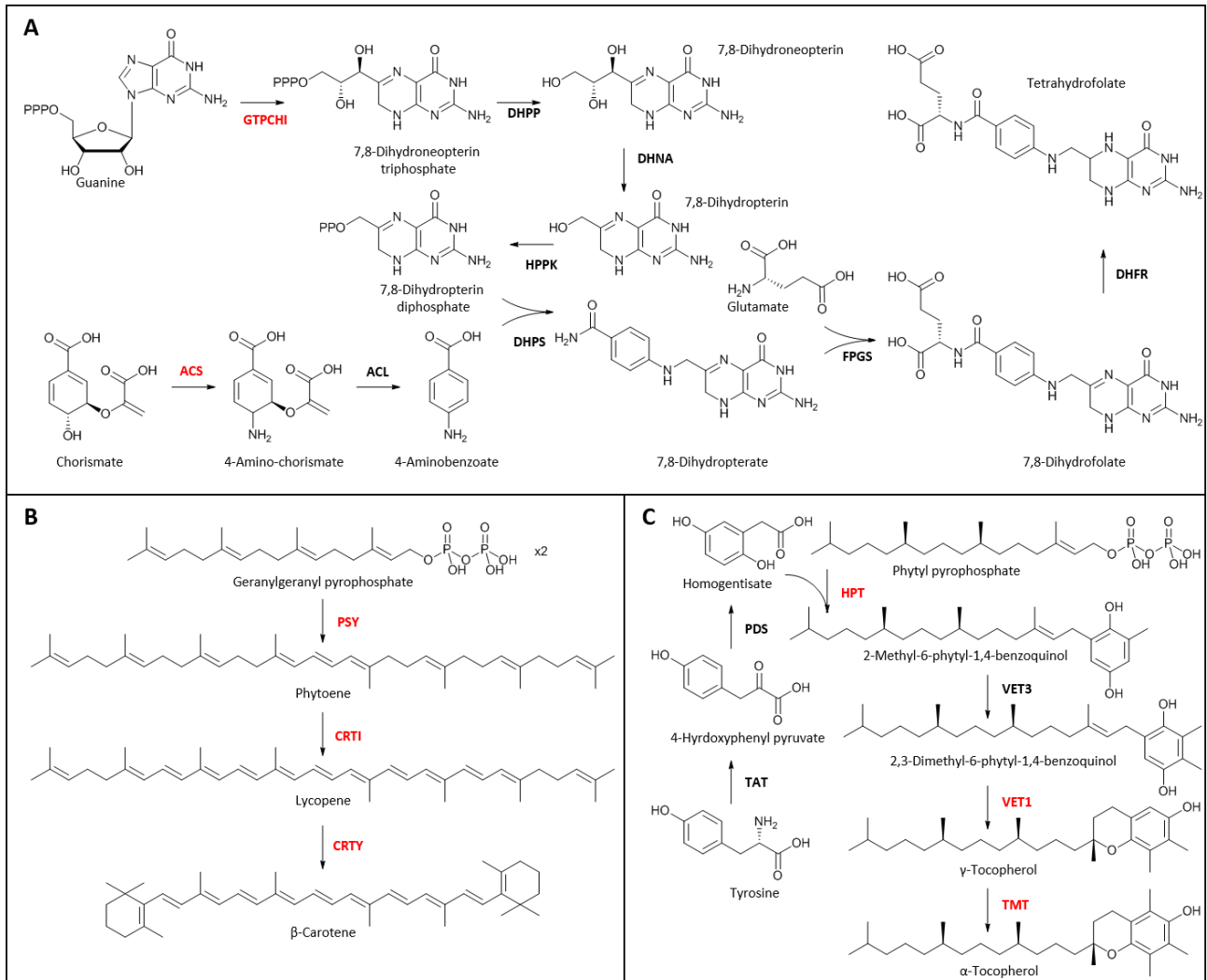


Figure 3

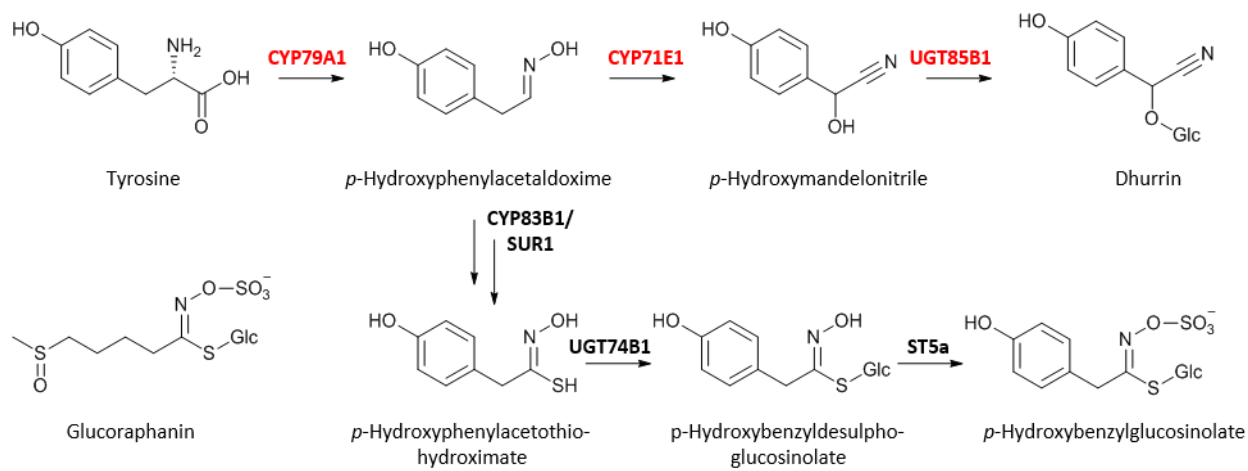


Figure 4

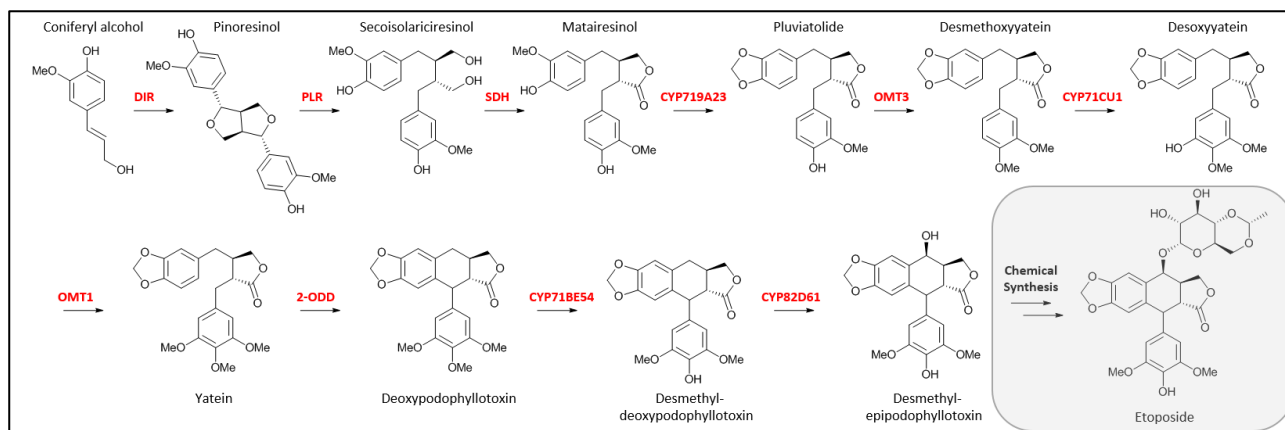


Figure 5

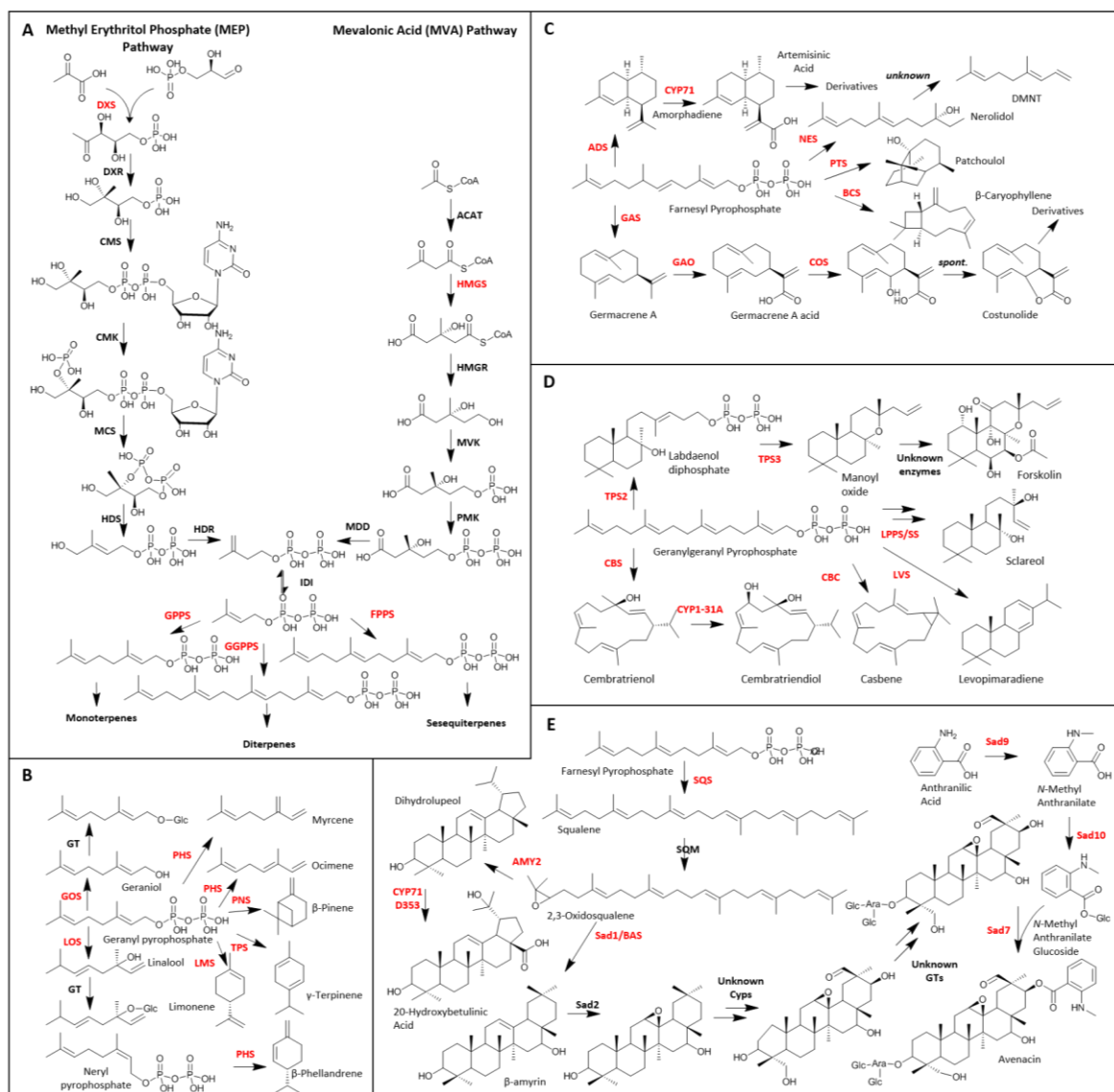




Figure 6

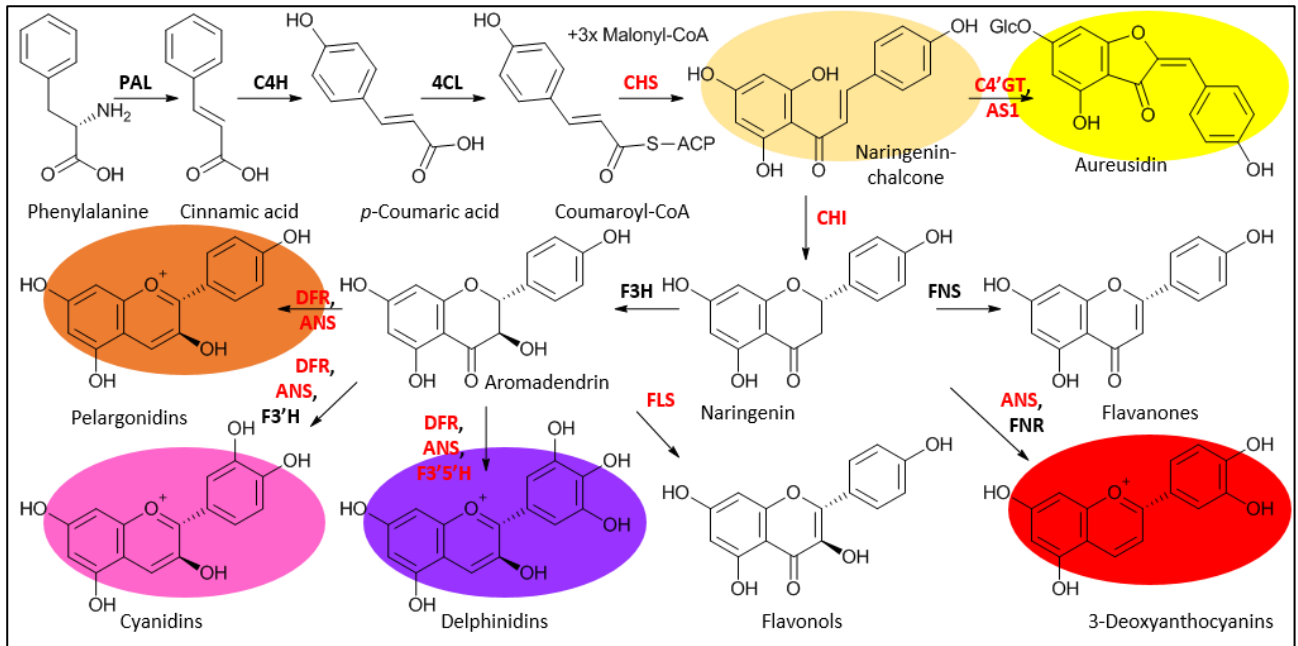


Figure 7

