Terpenoid biosynthesis in plants

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Introduction

Plants produce a wide range of structurally diverse natural products. These natural products play key roles in the interaction of plants with organisms in their environment. They, for example, act as defence compounds against herbivores and pathogens, or as attractants of insects for pollination. They also provide a natural resource for humans and are used as medicine (e.g. artemisinin and parthenolide), pigments (e.g. β-carotene and lycopene), fragrance (e.g. limonene and linalool) and flavours (e.g. vanillin and menthol).

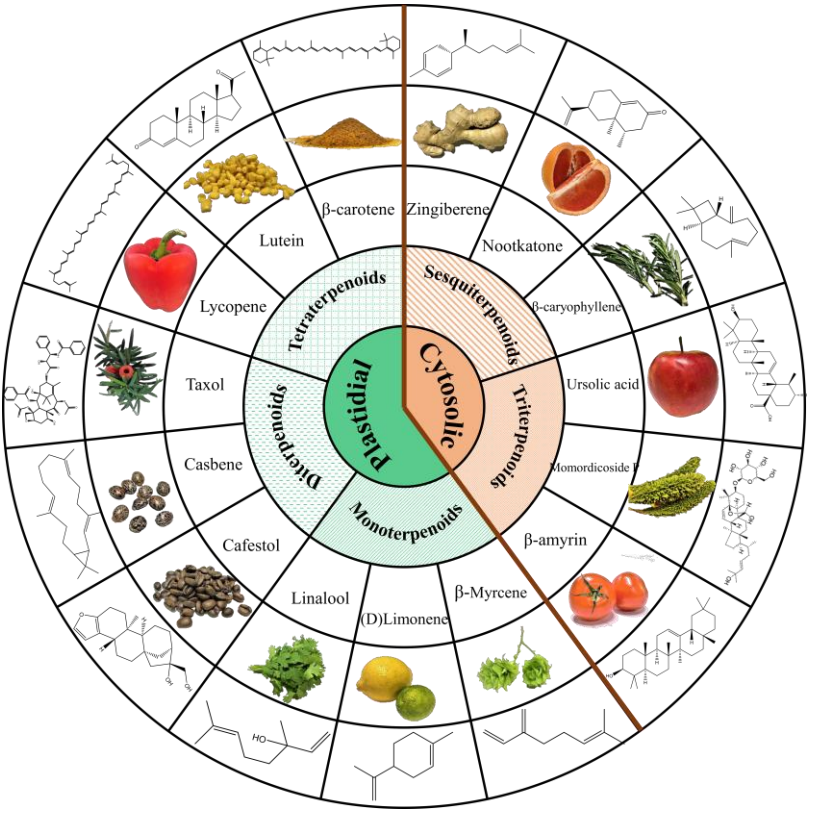

Figure 1: Schematic representation of plastidial and cytosolic terpenoids. Examples of different plant derived terpenoids and plant organs (seed, fruit, leaf or root) containing these terpenoids are shown.

Plant natural products can be divided into several classes such as nitrogen-containing (e.g. alkaloids, glucosinolates and cyanogenic glucosides) and nitrogen-free metabolites such as terpenoids, phenolics and flavonoids [1]. Among these, the terpenoids are the

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most diverse class, constituting almost 12% of all known plant metabolites [2] and possessing many different functions in plants. Low molecular weight volatile terpenoids are involved in plant protection mechanisms during biotic and abiotic stresses [3,4] and when emitted from flowers can act as pollinator attracting signals [5]. Terpenoids can be antifeedant compounds that protect the plant against insects, such as for example, geranyllinalool in *Nicotiana obtusifolia* [6] and can be an activator signal for systemic acquired resistance [7]. Several terpenoids, such as gibberellins, abscisic acid and strigolactones are plant hormones and act as signalling molecules in physiological processes. Strigolactones, for example, are a regulator of plant axillary bud outgrowth and thus branching [8]. In addition to their role as plant hormone, strigolactones are also secreted into the soil surrounding the plant's roots where they recruit the symbiotic arbuscular mycorrhizal fungi.

Biosynthesis of Terpenoids

Biosynthesis of the basic building blocks of terpenoids

Terpenoids are produced from the universal building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are synthesized through two different biosynthetic pathways. One of these occurs in the plastids and supplies mostly the substrates for the production of monoterpenoids (often present in essential oils), diterpenoids and tetraterpenoids (carotenoids) (Figure 2). The other pathway, known as the mevalonate (MVA) pathway takes place in the cytosol. The IPP and DMAPP derived from this pathway are mostly used as substrates in the production of sesquiterpenoids and triterpenoids.

The condensation of the IPP and DMAPP building blocks produced by the MEP and MVA pathways provides the prenyl diphosphate substrates such as C10 (geranyl diphosphate, GPP), formed by condensation of IPP and DMAPP through enzymatic activity of geranyl diphosphate synthase (GPS) or C15 (farnesyl diphosphate, FPP), formed from condensation of GPP and IPP by farnesyl diphosphate synthase (FPS) (Figure 2). GPP and FPP are the universal precursors of monterpenoids (C10) and sesquiterpenoids (C15), respectively. Geranylgeranyl diphosphate synthase (GGPS) catalyses the condensation of FPP with IPP, which results in the formation of the C20 precursor of the diterpenes, geranylgeranyl diphosphate (GGPP), while dimerization of two FPP molecules and removal of the diphosphate groups through the activity of squalene synthase (SQS) results in biosynthesis of squalene (C30) [9]. Squalene monooxygenase or epoxidase adds an oxygen group to the squalene, resulting in production of 2,3-oxidosqualene, the precursor of triterpenoids (C30) as well as sterols and steroids in plants. Dimerization of two GGPP molecules and elimination of the two diphosphate groups by phytoene synthase (PS) results in the formation of a C40 compound, phytoene, the precursor of the tetraterpenoids or carotenoids (Figure 2).

Biosynthesis of monoterpenoids (C10)

Monoterpenoids are C10 compounds derived from GPP. Monoterpenoids are well-known for their biological activity, but also for their strong odour and aromatic properties [10]. These compounds are used for various applications such as fragrances, drinks, food additives, perfumes and cosmetics [11]. Geraniol (isolated from rose flowers) and linalool (from coriander; Figure 1) are two of most important monoterpenoids used in the flavour industry which is reaching to annual consumption of 5000 tons/year [12]. Monoterpenoids in plants are often induced upon biotic and/or abiotic stress conditions and they are supposed to possess properties to enable plants to deal with these stresses

[13]. 1,8-Cineole, for example, is toxic to certain insects and is produced by *Artemisia annua* upon infestation by the root feeding insect, *Diuraphis noxia* [14]. GPP synthase (GPS), the enzyme responsible for synthesis of the monoterpenoid precursor GPP, was first characterised from the essential oil glands of sage [15]. Formation of monoterpenoids takes place through the activity of enzymes called monoterpene synthases (sometimes also called monoterpene cyclase if catalysing the formation of a cyclic monoterpene).

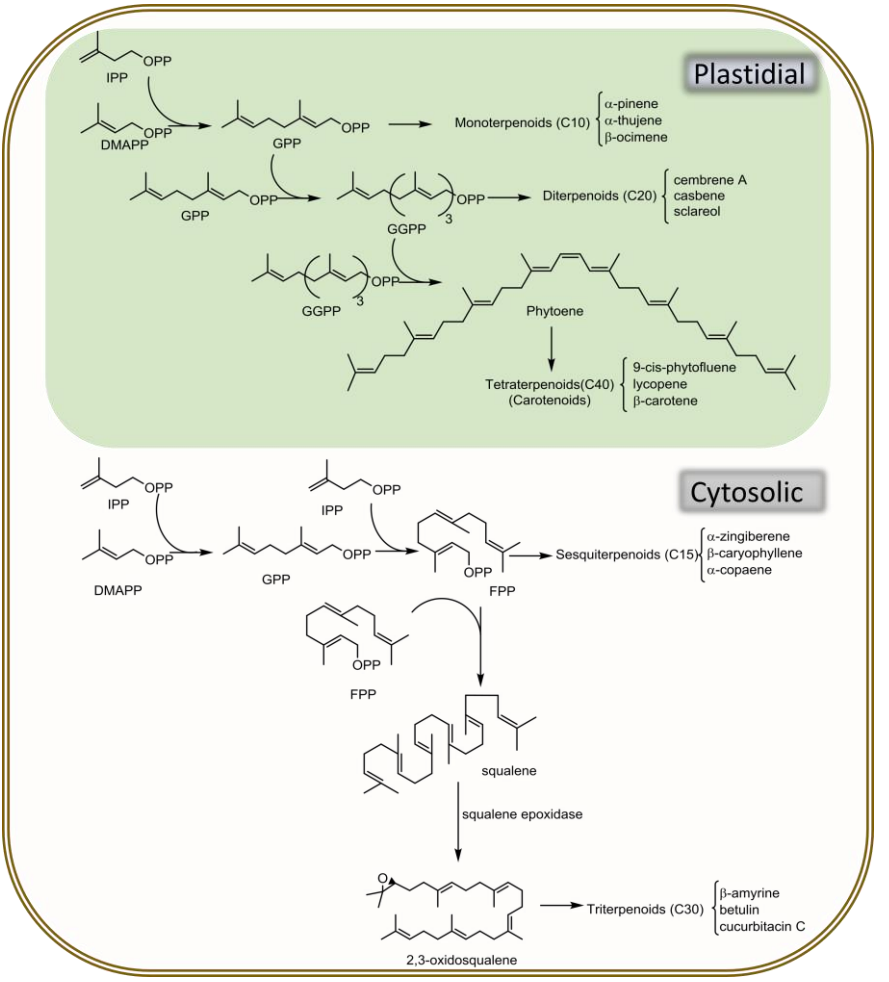

Figure 2: Terpenoid biosynthesis pathways. Biosynthesis of monoterpenoids (C10), diterpenoids (C20) and teteraterpenoids (C40) takes place in the plastids (in green) while the biosynthesis of sesquiterpenoids (C15) and triterpenoids (C30) is localized in the cytosol (in white).

The DDxxD motif for Mg^{2+} cation binding is conserved among all terpene synthases, which allows their identification [16]. A single monoterpene synthase often catalyses formation of several monoterpenoids from GPP. For example, a promiscuous monoterpene synthase enzyme, *Cs*TPS2FN, isolated from *Cannabis sativa* encodes the formation of (+)-α-pinene, (+)-β-pinene, myrcene, (-)-limonene and β-phellandrene [17]. As detailed above, it is generally assumed that the plastids are the major organelle for production of monoterpenoids and their substrate, GPP. Intriguingly, however, exchange

of mitochondrial produced GDP to the plastids for production of monoterpenoids has been demonstrated [18].

Biosynthesis of sesquiterpenoids (C15)

Sesquiterpenoids are produced from FPP, again through the catalytic activity of terpene synthases, in this case called sesquiterpene synthases. Sesquiterpenoids are often aromatic, and constituents of plant essential oils. The sesquiterpene β-caryophyllene (Figure 1) has been reported to be present in many plant species; it is the major essential oil component of basil (*Ocimum spp*.), oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis*) [19] (Figure 1) and, together with humulene, is the main sesquiterpene obtained from cannabis plants and responsible for its odour [20]. β-caryophyllene is widely used in frozen dairy, chewing gums and beverages [21]. Zingiberene, a sesquiterpene present in ginger (*Zingiber officinale*) is a spider mite repellent (Figure 1) [22]. In chamomile (*Matricaria chamomila*) it was shown that sesquiterpene biosynthesis starts in the plastids with GPP that is exported to the cytosol where IPP is added [23]. Interestingly, results from transient expression of a sesquiterpene synthase from feverfew (*Tancetum parthenium*) show that addition of mitochondrial targeting to a sesquiterpene synthase will result in higher sesquiterpene biosynthesis, presumably because mitochondrial FPP is accessed [24]. Indeed, localization of one of the Arabidopsis FPP synthases in the mitochondria has been demonstrated [25]. Protein localization studies using GFP fusions of *cis*-FPS, and santalene and bergamotene synthase (SBS) suggest that biosynthesis of these unusual sesquiterpenoids take place in the plastids using IPP and DMAPP from the MEP pathway [26].

Sesquiterpene lactones are a sub-class of the sesquiterpenoids with over 4000 different known structures. Sesquiterpene lactones are mainly colourless, bitter, compounds found mainly in plant species in the *Asteracea* [27]. Their biological properties such as antibacterial (e.g. vernolide [28]), antifungal (8α-hydroxy-4-epi-sonchucarpolide [29]), anticancer (e.g. parthenolide [30]) make them of interest for medical use. Sesquiterpene lactones are classified in six bicyclic or tricyclic classes named guianolides, pseudoguaianolides, xanthanolides, eremophilanolides, eudesmanolides and germacranolides [31]. Costunolide may then serve as the precursor of the other germacranolides (e.g parthenolide) and/or guaianolides (e.g. the main constituents of bitter compounds in chicory and endive). Further modification of sesquiterpene lactones is carried out by double bond reductases and glycosyl transferases [32,33].

Biosynthesis of diterpenoids (C20)

With more than 10,000 different natural plant derived structures, the diterpenoids are one of the most diverse classes of plant secondary metabolites [34]. They are also part of plant primary metabolism as plant growth regulators such as the gibberellins are diterpenes [35]. Many diterpenoids have medicinal properties, such as taxol (Figure 1), which is isolated from the Pacific yew (*Taxus brevifolia*) [36], and is used for the treatment of ovarian and breast cancer [37]. Cafestol (Figure 1) and the structurally related kahweol are two diterpenes from *Coffea arabica* that induce apoptosis in malignant pleural mesothelioma (MPM) cancer cells [38]. A valuable compound for the fragrance industry is *cis*-abienol which is an aromatic diterpene isolated from fir trees (*Abies balsamea*). *Cis*-abienol is an important oxygen containing diterpenoid serving as the precursor of Ambrox® in perfume formulations [39] and the major labdane type diterpenoid responsible for the fragrance of tobacco leaves. Biosynthesis of *cis*-abienol proceeds in two sequential steps. First a diterpene synthase converts GGPP to 8-hydroxy-copalyl

diphosphate and then a kaurene synthase like enzyme converts the latter into *cis*-abienol by removing the diphosphate group [40].

Biosynthesis of triterpenoids (C30)

This class of specialized metabolites constitutes more than 20,000 identified plant compounds so far [41]. Triterpenoids show a lot of diversity in plant families. Saponins, glycosylated triterpenoids, are, for example, found in *Quillaja saponaria* (a native Chilean tree) and *Camellia oleifera*. Saponins are used in detergents, shampoos and emulsifiers due to their foaming properties [42,43]. Many plants produce saponin type triterpenoids during normal growth (e.g. apple fruit peel, producing ursolic acid [44]), however their saponin levels strongly depends on plant species, organs and developmental stage [45]. Butelin, isolated from the bark of *Butela spp*., is another natural triterpenoid which is used in cosmetic products such as hair conditioners [46]. Many triterpenoids are used to cure major diseases such as cancer and HIV. Celastrol, a triterpenoids isolated from *Tripterygium wilfordii* exhibits Tat inhibitory action [47]. 'Tat' is a virus encoded protein which is required for HIV genome transcription. Triterpene synthases convert 2,3-oxidosqualene through a Chair-Boat-Chair (CBC) or the Chair-Chair-Chair (CCC) conformation into the different triterpene skeletons. An example of a triterpene synthase is β-amyrin synthase [48] responsible, for example, for β-amyrin biosynthesis in tomato (Figure 1). P450s and glycosyl transferases play an important role in further decoration of triterpenoids, for example, for the production of the triterpene glycoside glycyrrhizin.

Biosynthesis of tetraterpenoids (C40)

The tetraterpenoids contain 750 different reported structures [49]. The carotenoids [50](tetraterpenoids) are the most common natural pigments and also possess antioxidant properties. Carotenoids are industrially used as dyes and colorants, in the food industry (e.g. β-carotene), as nutraceuticals and in the pharmaceutical industry, as well as in cosmetics [51] (Figure 1). They are mostly present in photosynthetic organisms [50] and often are responsible for red, orange and yellow colours [52]. Carotenoids are essential and play a vital role in photosynthesis. Carotenoid biosynthesis starts with the activity of phytoene synthase making pre-phytoene diphosphate [53]. Phytoene synthase then converts pre-phytoene diphosphate to 15-*cis*-phytoene. Several other enzymes namely a desaturase and an isomerase are involved to produce *trans*-lycopene. Cyclisation is the next step; activity of an α-cyclase results in α-carotene biosynthesis, while a β-cyclase can convert *trans*-lycopene to β-carotene. Another class of naturally occurring carotenoid-derived terpenoid type molecules are the strigolactones. Their biosynthesis starts with isomerization of β-carotene by D27 [54]. Then a carotenoid cleavage (CCD7) cleaves the resulting 9-*cis*-β-carotene which then results in production of 9-*cis*-β-apo-10-carotenal and β-ionone [55]. Then, another carotenoid cleavage enzyme, CCD8, converts 9-*cis*-β-apo-10-carotenal into carlactone [54]. This ubiquitous strigolactone precursor will be oxidised by a cytochrome P450, the MAX1 homologs, which results in the formation of carlactonoic acid or *ent*-2-*epi*-5-deoxystrigol [56,57].

Heterologous production of terpenoids in plants and micro-organisms

As explained above, the terpenoids are very important compounds from medicinal, nutraceutical and nutritional point of view. However, commercialization of these compounds is often restricted due to their low concentrations in the plant and their high structural complexity which makes chemical synthesis approaches too costly [58]. In addition, some of the plant species that produce attractive molecules grow slowly, may

have a low yield, are threatened by extinction, or are susceptible to environmental conditions. Several approaches have been followed in the last decades to overcome these limitations. In an approach called metabolic engineering, scientists use alternative organisms (expression platforms) to optimize production of these metabolites. Terpenoid production in microbial systems, for example, is an appealing approach. Rapid growth and regeneration (e.g. 1 to 3 days for *Escherichia coli* and *Saccharomyces cerevisiae*, respectively) and well established tools for transformation make them suitable organisms for metabolic engineering purposes. However, ectopic expression of plant derived genes (enzymes) in these microbial platforms comprise some limitations which needs to be solved for a successful engineering strategy. For example, neither *E. coli* nor *S. cerevisiae* contain plastids. Hence in order to prevent possible miss-folding of the enzymes in these platforms, removal of a possible plastid targeting signal is suggested [59]. The subcellular targeting strategy used by plants makes expressing cytochrome P450s in micro-organisms even more challenging. S. cerevisiae, however is a suitable expression platform for cytochrome P450s as it is a eukaryotic microorganism containing endoplasmic reticulum, the maturation and activity site of cytochrome P450s. Another advantage of yeast is the ability of in vivo recombination of DNA fragments, such that several DNA fragments (harbouring homologous flanking regions) can be recombined upon transformation into yeast in a so called transformation associated recombination (TAR) [60]. Almost all required precursors for the biosynthesis of the different terpenoids are produced in yeast. Carotenoid and diterpenoid production in yeast is achieved often by overexpression of a GGPP synthase as yeast produces GGPP in small quantities. Carotenoids biosynthetic pathway genes have been successfully expressed in yeast [61]. Overexpression of genes such as HMGR, the rate limiting enzyme in the MVA pathway, has been shown to enhance the pool of precursor for the biosynthesis of, for example, sesquiterpenoids and triterpenoids. Alternatively, down regulation of competing pathways like sterol biosynthesis through down regulation of *ERG9* (squalene synthase) [62] are molecular strategies which are implemented for successful engineering programs. Successful in-vivo production of artemisinic acid and costunolide in WAT11 yeast (optimal yeast strain for expression of recombinant cytochrome P450s) is reported by introduction of sesquiterpene synthases (amorphadiene synthase and germacrene A synthase) and P450s (amorphadiene oxidase (for artemisininc acid).

Metabolic engineering can also be pursued in the plant species that is already making the attractive product by overexpression of biosynthetic pathway genes or downregulation of competing pathways. However, this homologous engineering - optimization and boosting of metabolic pathways in the original plant species - is sometimes difficult and time consuming. Hence, other *in planta* expression systems have been explored for heterologous expression of genes involved in the biosynthesis of secondary metabolites. Here we discuss a number of examples of such hosts that have been used for metabolic engineering and reconstruction of terpenoid biosynthesis pathways. Overexpression of taxadiene synthase, which converts GGDP to taxadiene - a precursor of the anti-cancer molecule taxol (Figure 1) - has been studied in *Nicotiana benthamiana*. Taxadiene was produced to an astonishing yield of 27 µg/g dry weight [63]. An example of successful reconstruction of a full biosynthetic pathway is the biosynthesis of parthenolide in *N. benthamiana*. The transient co-expression of germacrene A synthase (*GAS*), germacrene A oxidase (*GAO*), costunolide synthase (*COS*) and parthenolide synthase (*PTS*) yielded 1.4 mg/g fresh weight parthenolide in the leaves [30]. Artemisinin was also successfully synthesized in *N. benthamiana*, by transient expression of five biosynthestic pathway genes, amorphadiene synthase (*ADS*), amorphadiene oxidase (*ADO*), alcohol

dehydrogenase 1 (*ALDH1*), artemisinic aldehyde double-bond reductase (*DBR*) and aldehyde dehydrogenase 1 (*ALDH1*). *Physcomytrella patens* is another plant expression platform which recently has raised a lot of interest for metabolic engineering of valuable terpenoids. Novel and relatively easy transformation technology [64] has made this platform a suitable putative heterologous system for bulk production of terpenoids. Successful artemisinin production in *P. patens* was shown recently with a yield of 0.21 mg/g dry weight [65]. This yield was obtained upon co-expression of the same five biosynthesis pathway genes mentioned above, *ADS*, *ADO*, *ALDH1*, *DBR* and *ALDH1*. Yield in these heterologous production platforms is still quite low. A better knowledge of the natural site of biosynthesis and accumulation, the chemical properties of the terpenoids produced, and the mechanisms involved in their transport (from the biosynthesis site to the accumulation site) will provide novel solutions to be implemented in metabolic engineering programs [66,67].

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