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SI SYNTHETIC BIOLOGY

Synthetic biology for production of natural and new-to-nature terpenoids in photosynthetic organisms

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SUMMARY

With tens of thousands of characterized members, terpenoids constitute the largest class of natural compounds that are synthesized by all living organisms. Several terpenoids play primary roles in the maintenance of cell membrane fluidity, as pigments or as phytohormones, but most of them function as specialized metabolites that are involved in plant resistance to herbivores or plant–environment interactions. Terpenoids are an essential component of human nutrition, and many are economically important pharmaceuticals, aromatics and potential next-generation biofuels. Because of the often low abundance in their natural source, as well as the demand for novel terpenoid structures with new or improved bioactivities, terpenoid biosynthesis has become a prime target for metabolic engineering and synthetic biology projects. In this review we focus on the creation of new-to-nature or tailor-made plant-derived terpenoids in photosynthetic organisms, in particular by means of combinatorial biosynthesis and the activation of silent metabolism. We reflect on the characteristics of different potential photosynthetic host organisms and recent advances in synthetic biology and discuss their utility for the (heterologous) production of (novel) terpenoids.

Keywords: combinatorial biosynthesis, heterologous biosynthesis, metabolic engineering, genome editing, conjugates, specialized metabolism.

INTRODUCTION

Due to their sessile nature, plants rely on a vast array of natural products to defend themselves against pests and predators. Many of these natural products have valuable commercial applications or therapeutic potential, and plants that accumulate specific natural products have been used for thousands of years as herbal medicines, for example sweet wormwood (*Artemisia annua*) that was applied for the treatment of malaria in traditional Chinese medicine (Qinghaosu Antimalaria Coordinating Research Group, 1979). Recently, Tu Youyou was awarded the 2015 Nobel Prize in Medicine for her discovery of the anti-malarial drug artemisinin, a sesquiterpenoid that accumulates in *A. annua* (Miller and Su, 2011), underscoring the vast metabolic potential of plants (Lau *et al.*, 2014) synthesizing these metabolites *de novo* from just water and atmospheric CO_2 using sunlight as an energy source. Despite the enormous metabolic potential and cheap energy and nutrient requirements of plants, the commercialization of natural products from plant sources is often hampered by the low *in planta* production levels and/or the occurrence of the metabolites in rare or slow-growing plant species, leading to high market prices or even insufficient quantities of the compounds. Furthermore, due to the discovery of new drug targets, the emergence of new diseases and the growing microbial drug tolerance, the pharmaceutical industry is in constant need of novel metabolites (Pollier *et al.*, 2011; Moses *et al.*, 2013). To address these issues, metabolic engineering could be applied to generate plants

with increased metabolite productivity, or entire biosynthetic pathways could even be transferred to heterologous, fast-growing or easy-to-cultivate plants or microbes (Ajikumar *et al.*, 2008). Finally, by generating novel enzyme–substrate combinations through combinatorial biosynthesis, the production of new-to-nature metabolites could be rationally designed (Pollier *et al.*, 2011; Moses *et al.*, 2013).

In this review, we will focus on one specific class of plant natural products, i.e. terpenoids or isoprenoids, of which at least 40 000 compounds have been identified in plants (Roberts, 2007; Tholl, 2015). *In planta* these metabolites fulfil primary roles as pigments and phytosterols in basic functions such as photosynthesis or as signalling molecules in the regulation of plant growth and development (Bohlmann and Keeling, 2008; Vranová *et al.*, 2012). The vast majority of terpenoids, however, are specialized metabolites crucial for the interaction of plants with their environment (Tholl, 2015). Commercially important terpenoids include the anti-cancer and anti-malarial drugs paclitaxel and artemisinin, respectively, flavours and fragrances such as menthol, linalool, camphor and limonene, and natural rubber. We will discuss the current status of metabolic engineering of photosynthetic organisms for the production of terpenoids and will particularly explore the various possibilities for creating new enzyme–substrate combinations that may finally lead to the production of 'new-to-nature' terpenoids.

TERPENOID BIOSYNTHESIS

Synthesis of metabolic precursors

The structurally diverse terpenoids are all derived from the universal C_5 building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can be generated via two distinct metabolic routes, the mevalonate (MVA) and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Figure 1). The MVA pathway is localized in the cytosol, peroxisome and endoplasmic reticulum (ER) and starts with acetyl-CoA and ends with the formation of IPP, which is interconverted to its allylic isomer DMAPP by IPP isomerase (IDI). A key intermediate in the MVA pathway is the eponymous mevalonic acid which is generated from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the ER-localized HMG-CoA reductase (HMGR). Being the target of multi-level regulation, HMGR is considered to be the central enzyme of the entire MVA pathway (McGarvey and Croteau, 1995; Hemmerlin, 2013; Pollier *et al.*, 2013; Vranová *et al.*, 2013).

Whereas the MVA pathway is believed to have evolved in archaea, the alternative, plastidial MEP pathway is of prokaryotic origin (Koga and Morii, 2007; Hemmerlin *et al.*, 2012). Unlike the MVA pathway, the MEP pathway is fed by pyruvate and glyceraldehyde-3-phosphate (GAP) originating from the Calvin cycle (Rohmer *et al.*, 1996; Lichtenthaler, 1999). Both molecules are condensed to

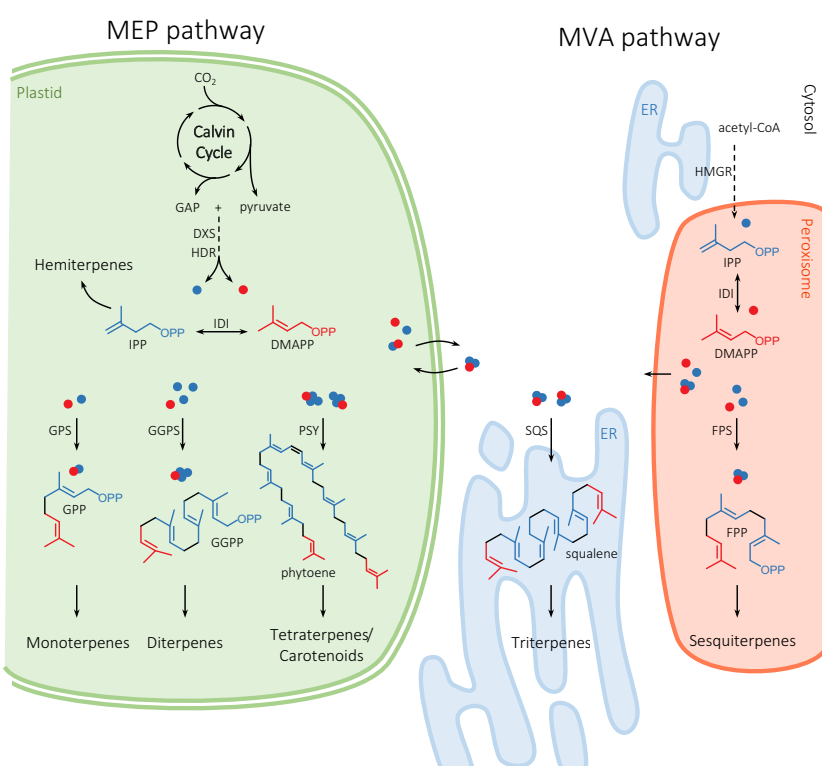

Figure 1. Biosynthesis of terpene building blocks in plants.
Both the plastidial methyl-D-erythritol 4-phosphate (MEP) pathway and the cytosolic/ER/peroxisomal mevalonic acid (MVA) pathway generate the C_5 precursors isopentenyl diphosphate (IPP, blue) and dimethylallyl diphosphate (DMAPP, red). While the plastidial 4-hydroxy-3-methylbut-2-enyldiphosphate reductase (HDR) generates both monoprenyl diphosphates, the MVA pathway leads to the formation of only IPP, which is isomerized to DMAPP by IPP isomerase (IDI). Key enzymes in the individual pathways are mentioned, dashed arrows indicate multiple enzymatic reactions. To simplify the chemical structures, phosphates are represented by 'P'.
DXS, 1-deoxy-D-xylulose 5-phosphate synthase; ER, endoplasmic reticulum; FPP, farnesyl diphosphate; FPS, FPP synthase; GAP, glyceraldehyde-3-phosphate; GGPP, geranyl geranyl diphosphate; GGPS, GGPP synthase; GPP, geranyl diphosphate; GPS, GPP synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; PSY, phytoene synthase; SQS, squalene synthase.

1-deoxy-D-xylulose 5-phosphate (DXP) by DXP synthase (DXS). Analogous to HMGR in the MVA pathway, DXS often functions as the gatekeeper and catalyses the limiting step in the MEP pathway (Lois *et al.*, 2000; Estévez *et al.*, 2001). In contrast to the MVA pathway, the final products of the MEP pathway, i.e. IPP and DMAPP, are both simultaneously synthesized from their common precursor 4-hydroxy-3-methylbut-2-enyldiphosphate (HMBPP) by HMBPP reductase (HDR); IDI converts excess IPP to DMAPP (Figure 1; Hemmerlin *et al.*, 2012).

Whereas animals, fungi, archaea and some Gram-positive bacteria exclusively rely on the MVA pathway, and most bacteria (including photosynthetic cyanobacteria) use the MEP metabolic route for terpene synthesis, plants use both due to the acquisition of the MEP pathway through cyanobacterial endosymbiosis (Lichtenthaler, 1999; Gould *et al.*, 2008; Hemmerlin *et al.*, 2012; Vranová *et al.*, 2013). Algae have a unique position: green algae (including unicellular organisms, such as the model green alga *Chlamydomonas reinhardtii*) do not possess the genes for the MVA pathway, while others, such as red or golden algae, use both biosynthetic routes (Disch *et al.*, 1998; Lichtenthaler, 1999; Vranová *et al.*, 2013).

From metabolic precursors to functional terpenes

The further biosynthetic fate of terpenes is of a modular nature and their nomenclature is based on their biosynthetic origin and the number of carbon atoms they have incorporated (Figure 1). IPP or DMAPP are the direct C_5 precursors of the hemiterpenes, the class of terpenes that are derived from a single isoprene unit. Geranyl diphosphate (GPP), the C_{10} precursor of the monoterpenes, results from the condensation of IPP and DMAPP by GPP synthase. The C_{15} sesquiterpene precursor farnesyl diphosphate (FPP) is synthesized by FPP synthase either directly through two successive condensations of IPP with DMAPP or through condensation of IPP with GPP. Likewise, the C_{20} diterpene precursor geranylgeranyl diphosphate (GGPP) is synthesized by GGPP synthase through condensation of (multiple) IPP units to DMAPP, GPP or FPP substrates (Figure 1). Unlike these all-*trans*-prenyl diphosphates, *cis*-prenyltransferases synthesize substrates such as neryl diphosphate (NPP, C_{10}), *Z,Z*-FPP (C_{15}), or nerylneryl diphosphate (NNPP, C_{20}), thereby adding to the diversity of terpene biosynthesis (Akhtar *et al.*, 2013). Furthermore, irregular or non-head-to-tail linkage of **DMAPP** leads to the synthesis of prenyl diphosphates with distinct conformations, such as lavandulyl and chrysanthemyl diphosphate (Rivera *et al.*, 2001; Demissie *et al.*, 2013). Longer prenyl diphosphates are generated for the biosynthesis of ubiquinones, phylloquinones or dolichol.

The next step is the conversion of the different prenyl diphosphate precursors by terpene synthases (TPSs) that eliminate the diphosphate group, generating carbocations that lead to complex rearrangements of the carbon skeleton. Numerous terpenes therefore have sophisticated structures arranged in multiple rings. Many plant genomes harbour dozens of different TPS-encoding genes, and because many TPSs have relaxed product specificities a myriad of different terpene structures are known (Tholl, 2006; Chen *et al.*, 2011).

Tri- and tetraterpenes do not originate from the synthesis of a direct diphosphate precursor. For the biosynthesis of the C_{30} triterpenes, two molecules of FPP are condensed to squalene by squalene synthase (SQS; Figure 1). The resulting squalene is subsequently epoxidized by squalene epoxidase (SQE) to 2,3-oxidosqualene. Protonation of the epoxide by oxidosqualene cyclases (OSCs) generates a carbocation whose rearrangement triggers cyclization of 2,3-oxidosqualene into various sterol or triterpene scaffolds (Phillips *et al.*, 2006; Augustin *et al.*, 2011; Moses *et al.*, 2013; Thimmappa *et al.*, 2014). Similarly, phytoene synthase (PSY) condenses two molecules of GGPP to produce the C_{40} tetraterpene precursor phytoene. Unlike squalene for the synthesis of triterpenoids or the prenyl diphosphate precursors for that of hemi-, mono-, sesqui- or diterpenes, the C_{40} tetraterpene precursor does not undergo carbocation rearrangements and remains mostly linear. Other, rarer classes of terpenes are the C_{25} sesterterpenes and the C_{35} sesquarterpenes which have been reviewed elsewhere and will therefore not be discussed here (Sato, 2013; Wang *et al.*, 2013). Finally, terpene moieties can also be coupled to moieties originating from other biosynthetic pathways, giving rise to the meroterpenoids.

The biosynthesis of most terpenes does not end there, because the backbone may be subject to various modifications that give rise to the tremendous diversity of terpenoids. The skeletons can be modified by methylation or de-methylation, thereby yielding terpenoids with different numbers of carbon atoms, as is the case for sterols (Weete, 1973; Fujioka and Yokota, 2003; Nes, 2011), or can be decorated with hydroxyl or carbonyl moieties by the action of cytochrome P450 mono-oxygenases (P450s), contributing significantly to their structural diversity (Hamberger and Bak, 2013; Weitzel and Simonsen, 2015). Further modifications include (de)saturation, esterification, glycosylation and many others (Aharoni *et al.*, 2003; Nes, 2011; Sawai and Saito, 2011).

The synthesis of prenyl diphosphate precursors, and as such of terpenes, is highly compartmentalized in plants (Figure 1). GPP and GGPP are generally synthesized in the plastids, hence, mono-, di- and tetraterpenes are of plastidial origin. Conversely, FPP, the precursor of sesqui- and triterpenes, is generated in the cytosol of plants. Exceptions are for instance the *cis*-prenyl diphosphate synthases of the Solanaceae family, which mostly are plastid-localized (Akhtar *et al.*, 2013). Furthermore, green algae synthesize cytosolic terpenes, such as sterols, via the MEP

pathway (Disch *et al.*, 1998). Despite the compartmentalized biosynthesis, low levels of GPP and FPP have been found in the cytosol and plastids, respectively, pointing towards a cross-flow between the two (Lichtenthaler, 1999, 2007; Vranová *et al.*, 2013).

COMBINATORIAL BIOSYNTHESIS, THE PRINCIPLE FOR 'NEW-TO-NATURE' TERPENE PRODUCTION

Despite the numerous challenges for metabolic engineering of plants and other photosynthetic organisms for the production of terpenes, there are several success stories that have been covered extensively in recent reviews (Farhi *et al.*, 2011; Lange and Ahkami, 2013; Moses *et al.*, 2013; Giuliano, 2014; Vickers *et al.*, 2014; Angermayr *et al.*, 2015; Davies *et al.*, 2015; Kempinski *et al.*, 2015; Pattanaik and Lindberg, 2015). Here we will give and discuss examples of heterologous terpene production in photosynthetic organisms, and specifically for the production of 'new-to-nature' terpenes. It should be noted that only a fraction of the photosynthetic organisms has been chemically characterized, for instance less than 10% of the approximately 400 000 species of flowering plants, implicating that thousands of plant-derived chemicals and their biological purposes probably still need to be catalogued (Martin, 2013). Hence, with the term 'new-to-nature' we here mean compounds that either do truly not exist in nature or, alternatively, have not been detected in nature and reported to date.

Creation of new-to-nature terpenes can be achieved through combinatorial biosynthesis, an engineering strategy that allows the generation of novel, structurally similar but distinct compounds, which is achieved by combining biosynthetic genes of related metabolic pathways from different organisms in a single host (Julsing *et al.*, 2006; Pollier *et al.*, 2011). This requires the use of enzymes that have some kind of relaxed specificity and thus are able to incorporate derivatives of their natural substrates. Because terpenoids are attractive compounds with various pharmacological activities, the use of combinatorial biosynthesis for the generation of novel terpenoids with new bioactivities is of great interest. Apart from pioneering success stories for the synthesis of novel carotenoids in non-photosynthetic *Escherichia coli* (Umeno and Arnold, 2003), there are only a few reports on successful targeted combinatorial biosynthesis of (novel) terpenoids in plants, the first being about the biosynthesis of monoterpenoid indole alkaloids (MIAs) in the Madagascar periwinkle (*Catharanthus roseus*). Expression of two different bacterial halogenases led to the halogenation of tryptophan, which then was incorporated in the regular MIA pathway, yielding new-to-nature halogenated derivatives (Runguphan *et al.*, 2010). In a recent study, biosynthetic genes of five different plants were combined in yeast to produce a monoglycosylated triterpene saponin that does not occur in any of the parent plant species (Moses *et al.*, 2014b).

COMBINATORIAL BIOSYNTHESIS OF CAROTENOIDS, PIONEERING SUCCESS STORIES IN NON-PHOTOSYNTHETIC MICROBES

Combinatorial biosynthesis was first successfully applied for the generation of novel carotenoids (Figure S1 in the Supporting Information). These most commonly occur as C_{40} tetraterpenes or as C_{25} and C_{30} apocarotenoids that are cleavage products of oxidized C_{40} carotenoids. However, organisms such as *Staphylococcus* and *Streptococcus* synthesize 'genuine' C_{30} carotenoids from dehydrosqualene, a carotenoid precursor resulting from the condensation of two FPP molecules. In *Staphylococcus aureus* this condensation is catalysed by the C30 carotene synthase CrtM and the resulting dehydrosqualene is further converted to staphyloxanthin (Lin *et al.*, 2010), the carotenoid pigment that is responsible for the characteristic golden colour of the bacterium. C_{50} carotenoids are found in some halophiles and a few Gram-positive and Gram-negative bacteria, and result from the diprenylation of regular tetraterpenes (Heider *et al.*, 2014).

Using combinatorial biosynthesis, novel carotenoids with uncommon sizes have been generated in *E. coli*. In a pioneering study, Umeno and Arnold (2003) combined the *Erwinia* gene *crtW*, encoding GGPPS, and the *S. aureus* gene *crtM*, encoding C_{30} carotenoid synthase, ultimately resulting in a CrtM with the unnatural substrate GGPP and leading to the heterocondensation of FPP and GGPP, thereby yielding the asymmetric C_{35} carotenoid 4-apophytoene. This novel carotene skeleton was accepted as a substrate by enzymes of the regular C_{40} and C_{30} pathways. PSY fully desaturated the novel backbone, and α- and ε-cyclases could asymmetrically cyclize some of the intermediates to yield at least four new-to-nature carotenoids (Umeno and Arnold, 2003). Later, CrtM was engineered to accept the C_{25} farnesyl geranyl diphosphate (FGPP) as a substrate, thus generating novel C_{45} and C_{50} carotenoids as well as an asymmetrical C_{40} backbone (Umeno and Arnold, 2004; Tobias and Arnold, 2006). Combining this engineered CrtM with phytoene desaturase resulted in the synthesis of a whole set of new-to-nature carotenoids (Tobias and Arnold, 2006).

Combinatorial biosynthesis was also used to produce novel oxygenated carotenoids in *E. coli*. In a first study, three different carotenoid desaturases were co-expressed with a carotenoid hydratase, a cyclase and a hydroxylase. The engineered strain accumulated 12 oxygenated carotenoids normally not found in *E. coli*, of which four were new to nature (Albrecht *et al.*, 2000). Similarly, expression of the carotenoid oxygenase (*crtOx*) gene from *S. aureus*, which normally oxidizes linear carotenoids to their di-acid forms, in an *E. coli* strain engineered for lycopene production, yielded a structurally novel, fully saturated tetraterpene di-aldehyde (Mijts *et al.*, 2005). Further successful

pathway engineering was achieved by combining a hydroxylase, a desaturase, a ketolase and a carotene glucosylase in an *E. coli* strain with a C_{40} carotenoid background. As such, a whole set of novel, highly oxygenated carotene glucosides was produced (Lee *et al.*, 2003).

ENGINEERING OF (NOVEL) TETRATERPENOIDS IN PHOTOSYNTHETIC ORGANISMS

In plants, carotenoids appear as regular C_{40} tetraterpenoids that are synthesized using eight C_5 isopentenyl building blocks from the plastidial MEP pathway (Rodríguez-Concepción, 2010). Carotenoids and their oxygenated derivatives, i.e. xanthophylls, are biologically important metabolites that are found in all photosynthetic plant tissues; they are associated with photoperception and photoprotection, but are also precursors of breakdown products that act as aromatic volatiles and signalling molecules (strigolactones and abscisic acid; Auldridge *et al.*, 2006). Since animals are not able to synthesize carotenoids, they depend on their uptake as vitamin A precursors and nutritional antioxidants. Besides carotenoids, xanthophylls have high anti-oxidative activities and are used as food supplements and feed additives in fisheries (Giuliano, 2014). To meet the nutritional and economic demand for carotenoids and xanthophylls, a range of photosynthetic organisms have been engineered for their production, ranging from different cyanobacteria (Harker and Hirschberg, 1997; Lagarde *et al.*, 2000; Albrecht *et al.*, 2001) and micro-algae (Varela *et al.*, 2015) to staple crops such as rice, maize, (sweet) potato, carrot, melon, tomato and canola (for reviews see Giuliano *et al.*, 2008; Giuliano, 2014).

One of the earliest efforts at carotenoid engineering in plants was the development of golden rice. Because natural rice does not produce carotenoids such as β-carotene, the precursor of vitamin A, in its endosperm, engineering of rice to produce β-carotene in its endosperm may provide an invaluable tool for alleviating vitamin A deficiency in developing countries (Lim *et al.*, 2012). Upon expression of bacterial *PSY* and phytoene desaturase (*crtI*) genes, transgenic rice was generated of which the seeds were yellow (golden) due to the accumulation of β-carotene and xanthophylls in the endosperm. It turned out that all genes encoding the enzymes necessary for the conversion of lycopene into carotenoids (carotene *cis-trans*-isomerase, α/β-lycopene cyclase, β-carotene hydroxylase) were constitutively active in rice endosperm, whereas *PSY* was probably lost during rice domestication. Complementation of the missing step and overcoming the metabolic bottleneck of phytoene desaturation was sufficient for the production of α- and β-carotene, as well as different hydroxylated derivatives, as a consequence of rice endogenous silent metabolism (Ye *et al.*, 2000; Schaub *et al.*, 2005).

A newer approach for the generation of crops with a new or increased carotenoid composition is combinatorial nuclear transformation through which embryonic plants are transformed using DNA-covered metal particles. In an initial study, five carotenogenic genes and a selectable marker driven by different endosperm-specific promoters were used to transform a white maize variety that lacks PSY activity (Zhu *et al.*, 2008). Thirteen per cent of the resulting population contained all five carotenoid genes and a total of 12 different gene combinations were identified. Using this approach, six new maize varieties were generated that differed considerably in carotenoid composition and abundance (Zhu *et al.*, 2008). The same approach was used to generate a multivitamin maize variety with increased levels of carotenoids, ascorbate and folate (Naqvi *et al.*, 2009) and carotenoid-accumulating rice (Bai *et al.*, 2014, 2016).

Novel carotenoids have been produced in plants as by-products of heterologous xanthophyll production. In an attempt to generate plants that produce ketocarotenoids, Shindo *et al.* (2008) expressed bacterial β-carotene ketolase (*crtW*) and β-carotene hydroxylase (*crtZ*) from *Brevundimonas* sp. in plastids of *Nicotiana tabacum*. Besides substantial amounts of astaxanthin, an array of other β-carotene-derived ketocarotenoids and several precursors, a novel compound was detected, which was identified as 4-ketoantheraxanthin and presumably resulted from the combinatorial conversion of β-carotene to zeaxanthin by CrtZ and the joined action of the endogenous tobacco zeaxanthin epoxidase and CrtW (Figure 2). The novel ketocarotenoid was shown to possess considerable anti-oxidative activity *in vitro* (Hasunuma *et al.*, 2008; Shindo *et al.*, 2008).

In a similar approach, *crtW* was introduced into an endosperm-derived rice callus that had previously been engineered for carotenoid production. Besides astaxanthin and precursors thereof, the novel 4-keto-α-carotene was detected in transgenic calli, whereas 4-ketoantheraxanthin was not detected (Figure 2). The generation of the new reaction product was attributed to the high concentration of α-carotene in the callus and the relaxed substrate specificity of *Brevundimonas* CrtW, which seems to oxidize the C-4 position of β-ionone rings regardless of the carotenoid (Breitenbach *et al.*, 2014). Both 4-keto-α-carotene and 4-ketoantheraxanthin were later shown to accumulate in transgenic *E. coli* expressing *Brevundimonas crtW* along with other carotenogenic genes (Takemura *et al.*, 2015).

ENGINEERING OF (NOVEL) DITERPENOIDS IN PHOTOSYNTHETIC ORGANISMS

As tetraterpenoids, the diterpenoids are derived from the plastidial MEP pathway. Diterpenoids are built from four isopentenyl building blocks and include the ubiquitous gibberellin phytohormones and specialized metabolites that are only found in certain plant species or families (Zerbe and Bohlmann, 2015). The most renowned diterpenoid is

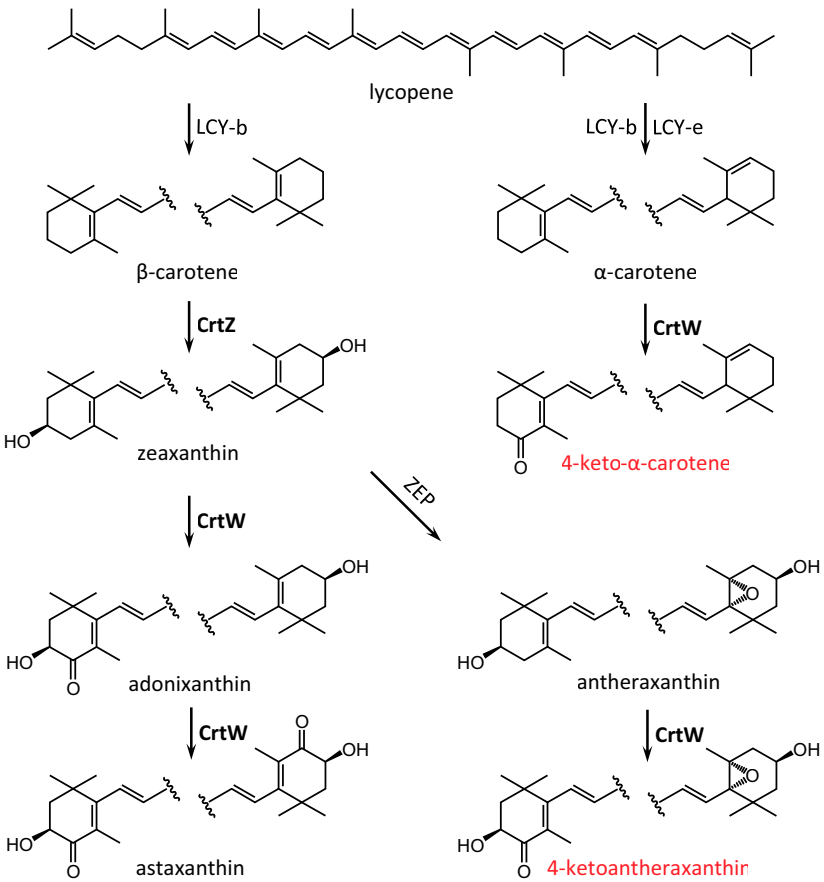

Figure 2. Generation of novel ketocarotenoids as byproducts of astaxanthin production in transgenic tobacco and rice.
Heterologous enzymes are in bold type and novel carotenoids are indicated in red. CrtW, β-carotene ketolase from *Brevundimonas* sp.; CrtZ, β-carotene hydroxylase from *Brevundimonas* sp.; LCY-b, endogenous lycopene beta cyclase; LCY-e, endogenous lycopene epsilon cyclase; ZEP, endogenous zeaxanthin epoxidase.

the highly functionalized paclitaxel that is used as an efficient drug for the treatment of several types of cancer (Cusido *et al.*, 2014). As paclitaxel is found only in minute amounts in the bark of the Pacific yew tree (*Taxus brevifolia*), much effort has been expended in recent years to produce taxol precursors in heterologous microbial hosts such as yeast (Engels *et al.*, 2008) and *E. coli* (Ajikumar *et al.*, 2010) and in plants such as *Arabidopsis thaliana* (Besumbes *et al.*, 2004), tomato (Kovacs *et al.*, 2007), *A. annua* (Li *et al.*, 2015), ginseng (Cha *et al.*, 2012), tobacco (Rontein *et al.*, 2008; Hasan *et al.*, 2014) and the moss *Physcomitrella patens* (Anterola *et al.*, 2009).

Diterpenes are synthesized in a modular fashion, starting with the carbocation-driven cyclization of the skeleton, followed by P450-mediated decoration of the backbone and further chemical modification and conjugation. In contrast to the other terpene classes, the enzymatic generation of diterpene backbones itself often happens in a modular way, which grants the diterpene synthases (diTPSs) a unique place among TPSs. First, class II diTPSs cyclize GPP into bicyclic prenyl diphosphates that may differ in stereochemistry and oxygenation. Then, these class II diTPS products are further converted through the action of class I diTPSs via the ionization of the diphosphate group. As such, further cyclization, oxygenation or rearrangement of the carbon skeleton is achieved. In contrast to this, bifunctional class I/II diTPSs contain the active sites of both classes and prenyl diphosphate intermediates may diffuse freely between enzyme domains. Whereas, for instance, the biosynthesis of labdane-type diterpenoids is carried out by functional pairs of class I and II diTPSs in angiosperms, the mechanistically identical reaction is carried out by bifunctional class I/II diTPSs in various gymnosperms. However, some class I diTPSs may directly convert GGPP or NNPP to linear or macrocyclic structures without the need for prior action of class II diTPSs. Examples of the latter can be found widely spread across the plant kingdom, including taxadiene synthase (TXS) for paclitaxel biosynthesis in *Taxus* spp. or casbene-type macrocyclases in Euphorbiaceae (Peters, 2010; Zerbe and Bohlmann, 2015).

The modularity of diterpene biosynthesis holds great potential for the combinatorial biosynthesis of (new) diterpenes. Class II diTPSs are generally regio- and stereo-specific and can be combined with class I diTPSs that can utilize stereoisomers generated by different class II enzymes (Figure 3a; Morrone *et al.*, 2011; Zhou *et al.*, 2012; Zerbe and Bohlmann, 2015). Furthermore, diTPSs have great functional plasticity. Product specificity of diTPSs often relies on just one amino acid in the active site of the enzyme. Replacing this residue with another amino

acid has led to altered product specificities of several enzymes (recently reviewed in Zerbe and Bohlmann, 2015). In a recently published study (Potter *et al.*, 2014), either of the two active site residues, histidine or asparagine, of the *A. thaliana* *ent*-copalyl-PP (*ent*-CPP) synthase were mutated to alanine. Rather than abolishing the enzymatic conversion, the engineered enzymes both produced an epimeric pair of 8-hydroxylated *ent*-CPPs by the incorporation of water (Figure 3d). Whereas 8α-hydroxy-CPP is the naturally occurring precursor of diterpenes such as manoyl oxide or sclareol (Figure 3a), 8-hydroxy-*ent*-CPP is a class II diTPS product that has not been described in nature. Combination of this engineered 8-hydroxy-*ent*-CPP synthase with the respective class I diTPS activities is likely to result in the formation of novel stereoisomers of manoyl oxide, sclareol or abienol (Potter *et al.*, 2014).

Applying means of rational enzyme design to diTPSs and exploiting the modular nature of diterpene biosynthesis by combining class I/II enzymes from different plant species with activities that naturally do not occur together, may lead to enzymes with tailor-made product spectra. In fact, a modular yeast platform for the combinatorial biosynthesis of several rare and new-to-nature labdane-type diterpenes has recently been established: combination of two class II diTPSs with three promiscuous class I diTPSs led to the production of six different diterpene scaffolds in yeast, which furthermore could be modified by different engineered P450 variants (Ignea *et al.*, 2015).

In a similar approach, eleven class II and nine class I diTPSs were combined through *N. benthamiana* agroinfiltration, creating 51 functional gene combinations, 41 of which do not occur in nature, and generating more than 50 different diterpenoid skeletons (Andersen-Ranberg *et al.*, 2016). It was also shown that the new-to-nature combination of diTPSs can challenge production levels of compounds such as (13*R*)-(+)-manoyl oxide by the native combinations of enzymes. Furthermore, it was illustrated how the stereochemistry of diterpenoid products can be controlled by the use of different class II diTPSs. For instance, three manool derivatives were generated by pairing the class I diTPS SsSCS with diTPSs forming *ent*-CPP, (+)-CPP, or *syn*-CPP (Andersen-Ranberg *et al.*, 2016). Notably, this study also reports the production of diterpenes by the mere expression of class II diTPS, probably through the action of endogenous phosphatase activity in *N. benthamiana*. This phosphatase activity was also observed in other studies, when class II diTPSs were expressed in the absence of their class I counterpart in *N. benthamiana*: infiltration with different class II diTPSs led to the production of copalol, *ent*-copalol and labda-7,13*E*-dien-15-ol instead of their corresponding diphosphates. Co-expression with the class I genes, however, outcompeted the dephosphorylation and yielded the regular diterpene skeletons (Figure 3c; Brückner *et al.*, 2014; Zerbe *et al.*, 2015). Next to phosphatase activity, other actions of silent metabolism were also observed when the structurally distinct casbene, cembratrienol, and levopimaradiene were produced by the heterologous expression of diterpene synthases in *N. benthamiana*. Interestingly, the synthesis of levopimaradiene was accompanied by the accumulation of side products. Next to abietatriene (a dehydration product) and a sandaracopimaradiene- or a pimaradiene-like compound, a putative hydroxy-levopimaradiene and two different hydroxy-abietatrienes were detected that were probably formed by the action of endogenous tobacco hydroxylases. Notably, metabolic turnover was only detected for levopimaradiene and not for cembratrienol or casbene, indicating that the endogenous metabolization of diterpenoids in tobacco is not a random process. Rather, levopimaradiene may induce the expression of oxidases that are associated with detoxification processes or such oxidases are to some extent substrate specific (Brückner and Tissier, 2013). Similar effects were observed when the *Rosmarinus officinalis* CPP synthase (RoCPS1) and two different miltiradiene synthases were co-expressed in *N. benthamiana* leaves. Next to miltiradiene and abietatriene (a spontaneous oxidation product), monohydroxylated derivatives thereof were observed to accumulate (Figure 3b; Brückner *et al.*, 2014). Interestingly, none of these derivatives were reported in a more recent study, in which CPP synthase and miltiradiene synthase from *Salvia fruticosa* were combined with different P450s to produce ferruginol from miltiradiene, indicating that an additional biosynthetic step may outcompete the endogenous conversion (Božić *et al.*, 2015).

SILENT METABOLISM DRIVES PRODUCTION OF NOVEL COMPOUNDS IN OTHER TERPENOID CLASSES

The latter examples of the generation of novel diterpenoids in infiltrated *N. benthamiana* leaves can all be considered as consequences of the activation of 'silent metabolism'. Indeed, in plants, many biosynthetic pathways are not fully active or detectable, and many enzymes without any apparent endogenous substrate are annotated in the genomes, pointing towards the existence of 'occult' metabolic capabilities that can be 'awakened' by providing the appropriate substrate to the existing enzymes (Lewinsohn and Gijzen, 2009; Pollier *et al.*, 2011), thus generating numerous opportunities to obtain novel plant-derived compounds. As with the heterologous production of diterpenoids, the engineering of other terpene classes in plants was often accompanied by the activation of silent metabolism, as discussed below.

Monoterpenoids

The first dedicated step in monoterpenoid biosynthesis in plants is catalysed by monoterpene synthases that convert the C_{10} GPP into simple linear, mono- or bicyclic struc-

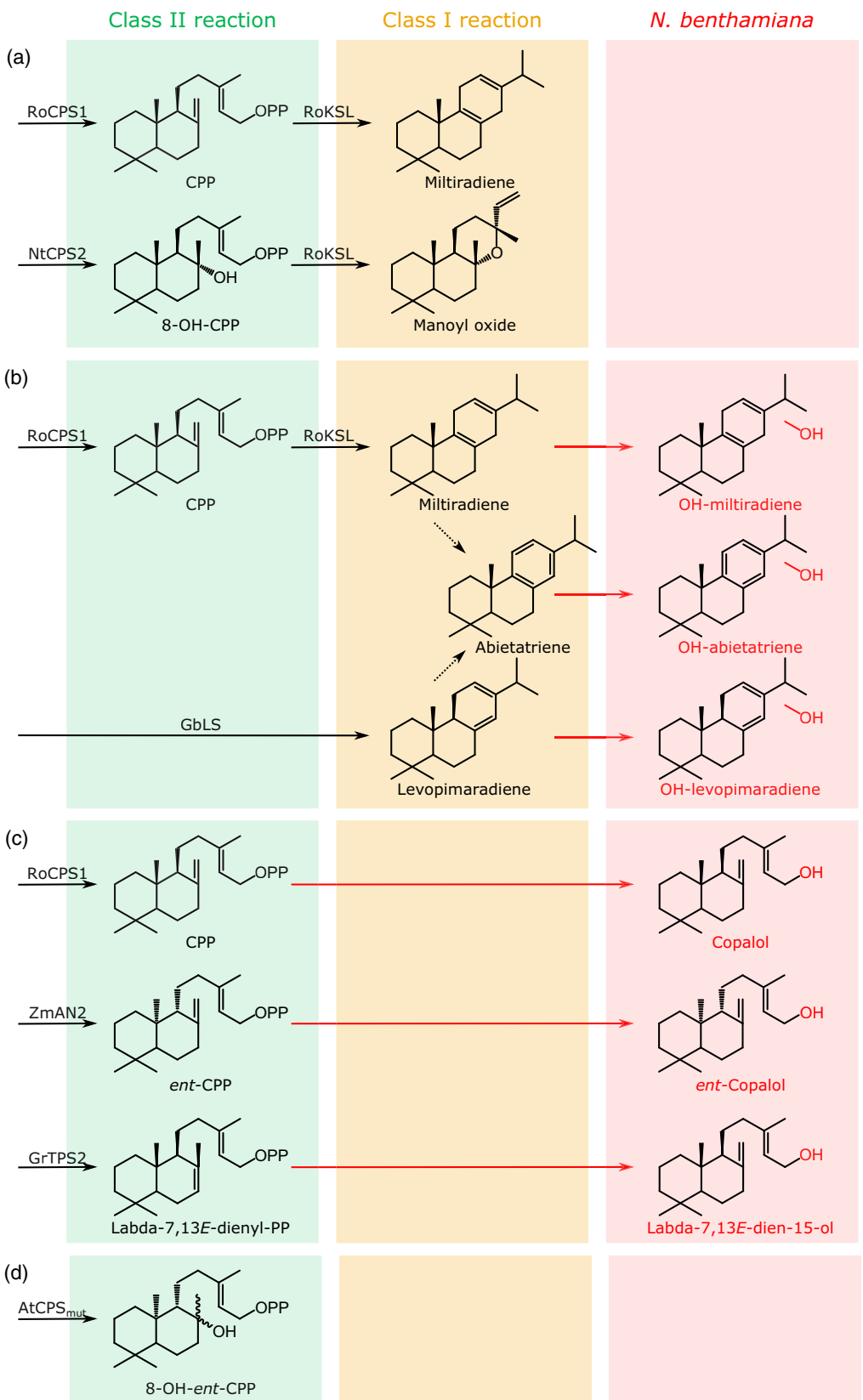

Figure 3. Modular fashion of diterpene biosynthesis and metabolism in *Nicotiana benthamiana*.
(a) Relaxed substrate specificity of class I diterpene synthases (diTPSs). Miltiradiene synthases from *Rosmarinus officinalis* accept both copalyl diphosphate (CPP) and 8-hydroxy-CPP as a substrate, giving rise to two distinct diterpenoids.
(b) Hydroxylation of class I diTPS reaction products in *N. benthamiana.*
(c) Dephosphorylation of different class II reaction products in *N. benthamiana.*
(d) Mutagenized *ent*-CPP synthase from *Arabidopsis thaliana* produces a racemic mixture of 8-hydroxy-*ent*-CPP.
Class II diTPSs generate bicyclic prenyl diphosphates from GGPP, which is not shown to keep the figure simple. Hypothetically spontaneous reactions are indicated with a dotted arrow. Modifications carried out by endogenous enzymes and their reaction products are shown in red. To simplify the chemical structures, phosphates are represented by 'P'.
$AtCPS_{mut}$, *A. thaliana* CPP synthase; GbLS. *Gingko biloba* levopimaradiene synthase; GrTPS2, *Grindelia robusta* labda-7,13*E*-dienyl diphosphate synthase; NtCPS2, *Nicotiana tabacum* 8-hydroxy CPP synthase; RoCPS1: *R. officinalis* CPP synthase; RoKSL, *R. officinalis* kaurene synthase-like; ZmAN2, *Zea mays ent*-CPP synthase.

tures. Monoterpenoids are small, volatile terpenoids that often have aromatic properties and are major components of floral scents and essential oils. Certain representatives act as attractants for pollinators, while others function in herbivore repellence. Prominent monoterpenoids, such as geraniol, limonene or menthol are used as flavours and fragrances for food and cosmetics or as pharmaceuticals (Yu and Utsumi, 2009). Recently, there has been growing interest in the production of monoterpenoids for use as biofuels (Withers and Keasling, 2007; Fortman *et al.*, 2008; Zhang *et al.*, 2011a; Yang *et al.*, 2013a; Wang *et al.*, 2015b), and several success stories of the production of monoterpenoids in classical hosts such as *E. coli* or yeast (Herrero *et al.*, 2008; Zhang *et al.*, 2011a; Hong and Nielsen, 2012; Alonso-Gutierrez *et al.*, 2013; Yang *et al.*, 2013a), but also in photosynthetic micro-organisms such as microalgae (Wang *et al.*, 2015b) and cyanobacteria (Davies *et al.*, 2014; Formighieri and Melis, 2014; Halfmann *et al.*, 2014; Kiyota *et al.*, 2014), have been published.

A monoterpenoid that has received great attention recently is geraniol, as it is the starting point in the biosynthesis of the seco-iridoid part of the MIAs vinblastine and vincristine. These anti-cancer drugs are produced by *C. roseus* at extremely low levels and consequently have a restricted availability and a resulting high market price (Miettinen *et al.*, 2014). Therefore, different geraniol synthases (Dong *et al.*, 2013), production hosts (Dong *et al.*, 2013; Fischer *et al.*, 2013) and plant organs and cultures (Vasilev *et al.*, 2014) have been analysed for their biosynthetic production capacities (see Table S1 for expression hosts and yields). By the combined expression of *CYP76B6* for the oxidation of geraniol to 8-oxogeraniol (Höfer *et al.*, 2013) and the remaining nine genes of the *C. roseus* seco-iridoid pathway, strictosidine could be produced in *N. benthamiana* leaves (Miettinen *et al.*, 2014). Notably, this study, as well as virtually all other studies on the production of geraniol and further downstream products, reports on the accumulation of derivatives through action of silent metabolism. For instance, hydroxylated and reduced derivatives of geraniol, as well as their corresponding aldehydes and acids, were observed, with the number and type of derivatives varying between different plant hosts (Figure 4, Table S1). While in some cases, geraniol and its derivatives occur in their free form, substantial amounts of the monoterpenoids accumulate as complex sugar conjugates or as acetylated or malonylated derivatives (Davidovich-Rikanati *et al.*, 2007; Yang *et al.*, 2011; Dong *et al.*, 2013; Fischer *et al.*, 2013; Höfer *et al.*, 2013; Ritala *et al.*, 2014; Vasilev *et al.*, 2014). For instance, in tobacco plants stably transformed with geraniol synthase from *Valeriana officinalis*, a total of 19 compounds were produced, identified as mono-, di- or tri-glycosides and malonyl or acetyl conjugates of geraniol, hydroxy-geraniol, geranic acid, hydroxy-geranic acid and hydroxy-dihydrogeranic acid (Dong *et al.*, 2013). The engineering of the downstream MIA pathway in *N. benthamiana* (Miettinen *et al.*, 2014) was accompanied by similar modifications (Table S1).

Another well-studied monoterpenoid is linalool, which is synthesized by linalool synthase (LIS) and has been heterologously produced in carnation (Lavy *et al.*, 2002), petunia (Lücker *et al.*, 2001), tomato (Lewinsohn *et al.*, 2001), potato (Aharoni *et al.*, 2006), *A. thaliana* (Aharoni *et al.*, 2003), *Chrysanthemum morifolium* (Yang *et al.*, 2013b) and the cyanobacterium *Anabaena* sp. PCC 7120 (Zhou and Gibbons, 2015). As with the production of geraniol, the activation of silent metabolism was observed upon *LIS* expression and a range of hydroxylated and saturated compounds as well as different conjugates thereof were detected in various hosts (Figure 4b, Table S1; Lewinsohn *et al.*, 2001; Lücker *et al.*, 2001; Lavy *et al.*, 2002; Aharoni *et al.*, 2003, 2006; Yang *et al.*, 2013b).

Another intriguing example for the activation of silent metabolism was observed with the plastid-targeted expression of monoterpene synthases in ripening tomato fruits. Besides geraniol, *GES*-expressing tomatoes also produced substantial amounts of nerol (*trans–cis* isomerization), citronellol (double-bond reduction) and oxidized forms, as well as acetyl esters thereof. Furthermore, other monoterpenes that were not direct geraniol derivatives became abundant, most notably myrcene, limonene and different ocimene species, whereas these were virtually absent in wild-type fruits (Davidovich-Rikanati *et al.*, 2007). A similar effect was observed when linalool was produced in ripening tomatoes by expression of *FaNES1* (Lewinsohn *et al.*, 2001). Further examples for monoterpenoid production in

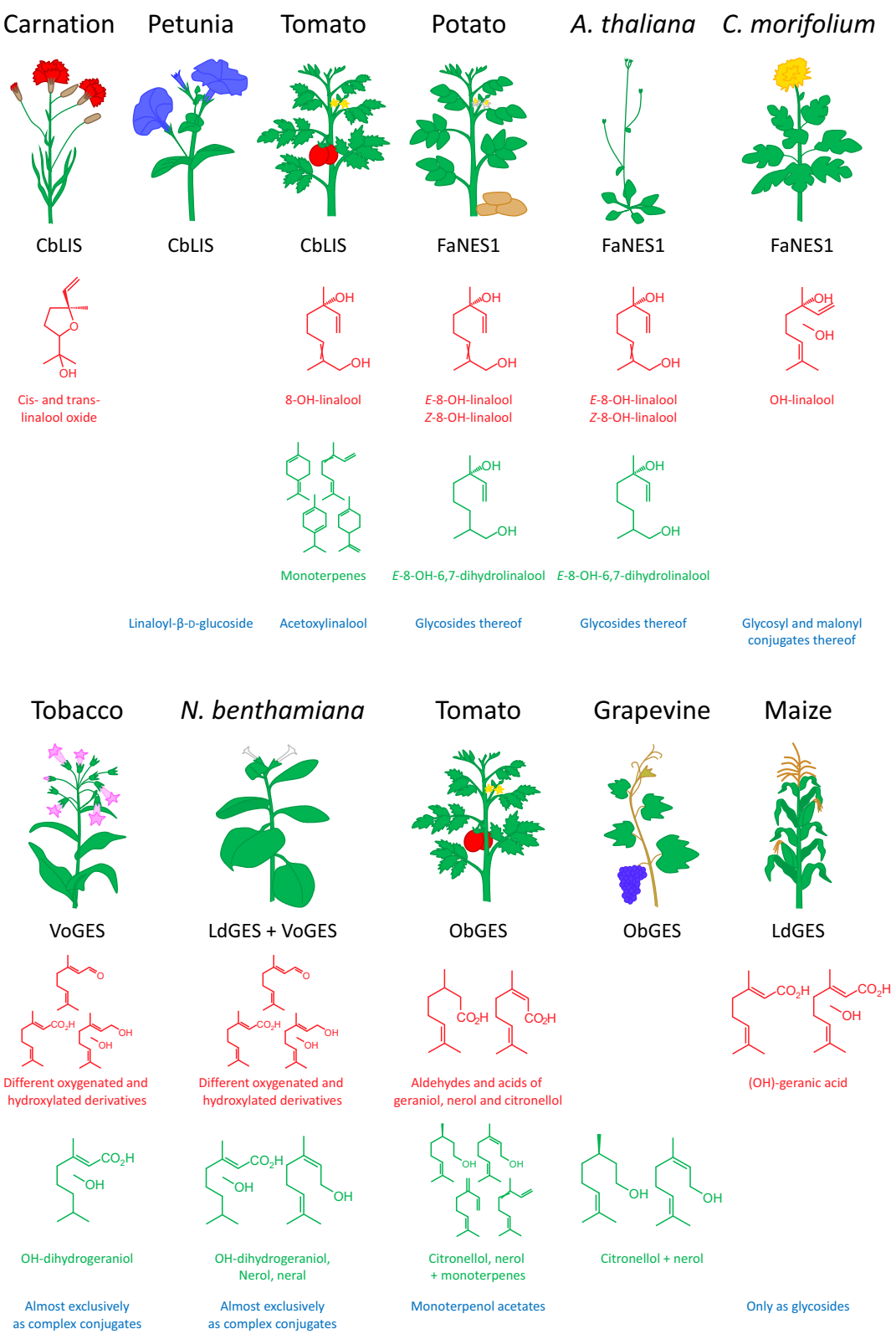

Figure 4. Metabolism of linalool and geraniol in different heterologous plant hosts.
Hydroxylation and oxygenation products are shown in red, double bond reduction and other products in green and conjugates in blue.
CbLIS, *Clarkia breweri* linalool synthase; FaNES1, bifunctional linalool/nerolidol synthase from *Fragaria* × *ananassa*; LdGES, *Lippia dulcis* geraniol synthase; ObGES, *Ocimum basilicum* geraniol synthase; VoGES, *Valeriana officinalis* geraniol synthase. Supporting information is provided in Table S1.

plants and the activation of silent metabolism are summarized in Table S1.

Sesquiterpenoids

Heterologous sesquiterpene production has also coped with unintended combinatorial effects. The direct precursor of all sesquiterpenoids, i.e. FPP, is synthesized in the cytosol by FPP synthase. Sesquiterpene synthases will eliminate the phosphate group of FPP, thereby creating a carbocation that can be neutralized to relatively simple, linear structures, such as farnesol. The carbocation can also undergo rearrangements leading to complex di- and tricyclic structures. As monoterpenoids, sesquiterpenoids are constituents of plant essential oils and resins, and are valued for their aromatic properties and their potential use as biofuels. Sesquiterpenoids have been engineered in various photosynthetic hosts, including cyanobacteria (Reinsvold *et al.*, 2011; Davies *et al.*, 2014; Halfmann *et al.*, 2014), moss (Zhan *et al.*, 2014) and a number of plant species.

Probably the best-known sesquiterpenoid is the antimalarial drug artemisinin, which is recommended by the World Health Organization for the treatment of uncomplicated malaria caused by the protozoan parasite *Plasmodium falciparum*. The natural source of artemisinin is *A. annua*, which produces the artemisinin precursor dihydroartemisinic acid from FPP via a three-step enzymatic pathway; this is subsequently converted to artemisinin via a photochemical reaction that is stimulated by sunlight. Considerable effort has been made to establish *Saccharomyces cerevisiae* (Ro *et al.*, 2006; Paddon *et al.*, 2013) and *E. coli* (Tsuruta *et al.*, 2009) as microbial platforms for the biosynthesis of artemisinin, leading to a challenging yeast-based semi-synthetic production pipeline (Peplow, 2013). Recently, the complete artemisinin biosynthetic pathway was also re-established in tobacco by expressing four *A. annua* genes [amorphadiene synthase (*ADS*), cytochrome P450 *CYP71AV1*, artemisinic aldehyde reductase (*DBR2*) and cytochrome P450 reductase (*CPR*); Figure 5] in combination with a truncated and deregulated yeast HMGR (Farhi *et al.*, 2011). However, awakening of silent metabolism was also observed for the production of artemisinin precursors in tobacco. For instance, transient expression of *ADS* together with *HMGR* and *FPPS* in *N. benthamiana* leaves resulted in the production of amorpha-4,11-diene. However, a second compound, probably an amorphadiene derivative, was more abundant than free amorphadiene in the infiltrated leaves. Furthermore, additional expression of *CYP71AV1* did not result in the accumulation of artemisinic acid, but rather of its diglucoside, indicating that the produced artemisinic acid was further metabolized by *N. benthamiana* glycosyltransferases (van Herpen *et al.*, 2010). In a similar attempt to produce artemisinic acid in *N. tabacum*, only artemisinic alcohol was detected. Additional co-expression of *DBR2* led to the accumulation of dihydroartemisinic alcohol (Figure 5; Zhang *et al.*, 2011b). As DBR2 is only active towards the aldehyde but not the alcohol (Zhang *et al.*, 2008), it was hypothesized that the aldehyde formed by CYP71AV1 is converted back to dihydroartemisinic alcohol by an endogenous alcohol dehydrogenase (Zhang *et al.*, 2011b).

Activation of silent metabolism was also observed when a three-gene pathway for the sesquiterpene lactone costunolide was reconstituted by agroinfiltration of *N. benthamiana*. While the cyclized sesquiterpene germacrene A was detected when expressing germacrene A synthase (*GAS*), co-infiltration with the next two genes in the pathway did not lead to the accumulation of free costunolide as shown for this gene combination in yeast. Instead, glutathione and cysteine conjugates were detected, generated either spontaneous or through the action of glutathione *S*-transferase (Liu *et al.*, 2011; Eljounaidi *et al.*, 2014).

Triterpenoids

Triterpenoids comprise primary metabolites such as phytosterols and brassinosteroid hormones, and specialized metabolites, such as saponins. Triterpenoids and their biosynthetic intermediates are bioactive compounds with applications in the pharmaceutical, cosmetic and food industries. The various triterpenoid backbones are produced from 2,3-oxidosqualene by cyclization of the linear precursor by OSCs, and can then be further decorated by cytochrome P450s, UDP-dependent glycosyltransferases and tailoring enzymes such as acyltransferases (Moses *et al.*, 2013, 2014a; Thimmappa *et al.*, 2014; Seki *et al.*, 2015). The heterologous production of glycosylated plant triterpenoids has been reported in yeast (Moses *et al.*, 2014b; Yan *et al.*, 2014; Wang *et al.*, 2015a), using recombinant glycosyltransferases *in vitro* (Achnine *et al.*, 2005; Meesapyodsuk *et al.*, 2006; Naoumkina *et al.*, 2010; Augustin *et al.*, 2012) and in the plant host *N. benthamiana* (Khakimov *et al.*, 2015).

Compared with the other terpenoid classes, the engineering of triterpenoids in photosynthetic hosts is still in its infancy. OSCs have been expressed in several heterologous hosts. For instance, expression of the *Panax ginseng* dammarenediol-II synthase gene in rice led to the production of dammarane-type sapogenins in the transgenic plants (Huang *et al.*, 2015b). Interestingly, the heterologously produced dammarenediol-II was recognized by endogenous rice enzymes that converted it to the ginsenoside aglycones protopanaxadiol and protopanaxatriol (Figure 6). Likewise, expression of β-amyrin synthase in rice led to the accumulation of oleanolic acid (Huang *et al.*, 2015a), an oxidation product of the β-amyrin produced by the introduced β-amyrin synthase (Figure 6). In plants, the oxidation of dammarenediol-II and the C-28 oxidation of β-amyrin is carried out by CYP716 family members (Carelli

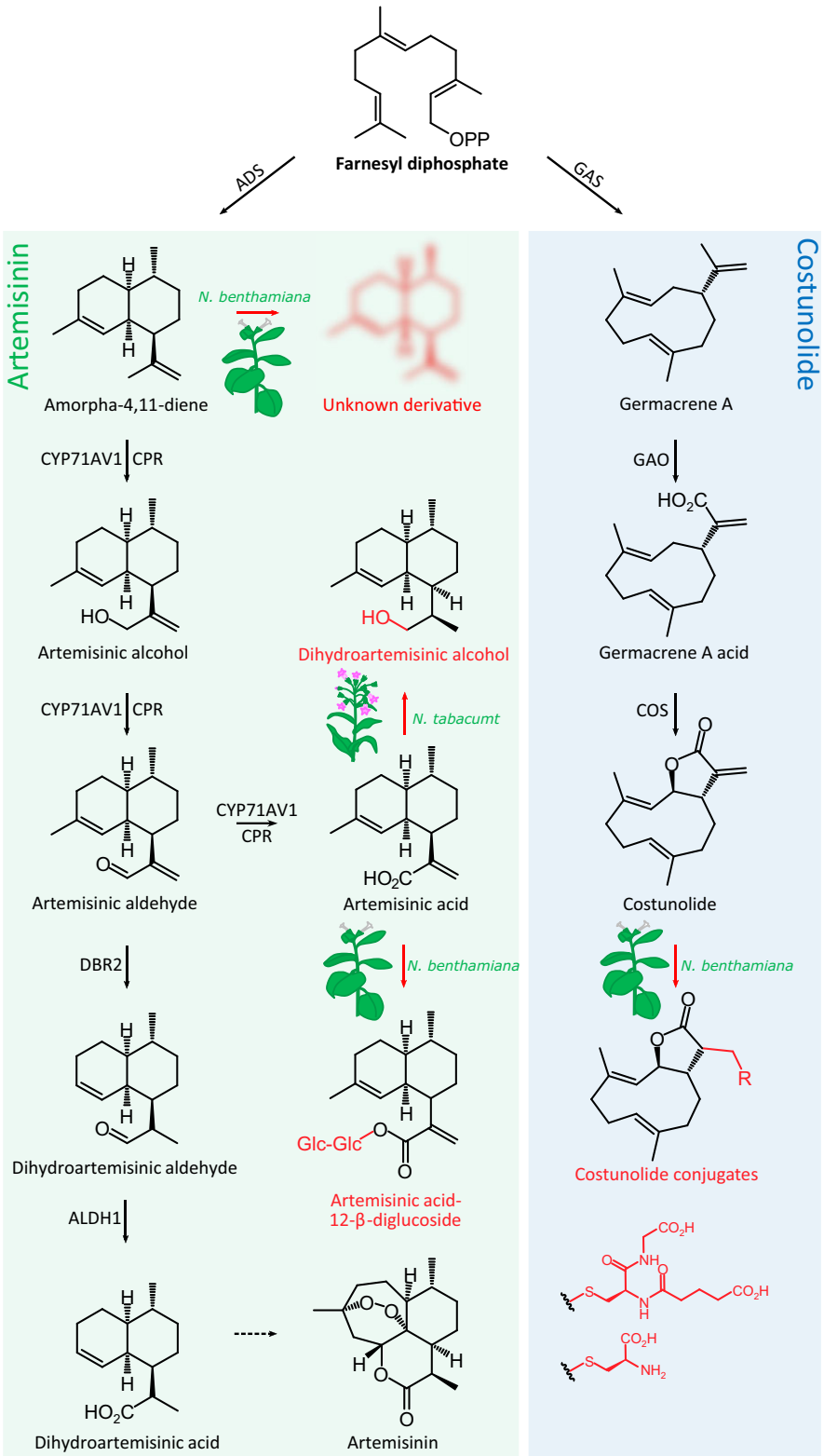

Figure 5. Sesquiterpenoid metabolism in different heterologous plant hosts.
Multiple enzymatic conversions are indicated with a dashed arrow. Modifications carried out by endogenous enzymes are shown in red.
ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; COS, costunolide synthase (from *Cynara cardunculus* or *Cichorium intybus*); CPR, cytochrome P450 reductase from *Artemisia annua*; DBR2, artemisinic aldehyde Δ11(13) reductase; CYP71AV1, amorpha-4,11-diene 12-hydroxylase; GAO, germacrene A oxidase (from *C. cardunculus* or *C. intybus*); GAS, germacrene A synthase (from *C. cardunculus* or *Tanacetum parthenium*).

et al., 2011; Fukushima *et al.*, 2011; Han *et al.*, 2011, 2012, 2013; Huang *et al.*, 2012; Khakimov *et al.*, 2015; Moses *et al.*, 2015a), a family of P450s that is not present in monocots such as rice (Nelson, 2009), indicating that hitherto unknown rice enzymes may be able to catalyse the oxidation of the produced triterpenes.

The triterpene backbones produced by the various OSCs are further decorated in plants to yield oxidized triterpenoids and saponins. Initial plant engineering efforts focused mainly on the reconstitution of triterpenoid biosynthetic pathways, with *N. benthamiana* being the preferred plant host. For instance, the two-step pathway leading to the compendium of specialized triterpenoids that accumulate on the leaf surface of *A. annua* was reconstituted in *N. benthamiana*. Expression of the multifunctional *A. annua OSC2* in *N. benthamiana* led to the accumulation of α-amyrin, β-amyrin and δ-amyrin that were further converted to their corresponding amyrones by co-infiltration of *A. annua CYP716A14v2* (Figure 6; Moses *et al.*, 2015b). Similarly, co-infiltration in *N. benthamiana* of the three-step pathway leading to 3-*O*-Glc-oleanolic acid led to the heterologous production of the *Barbarea vulgaris* insect-deterring metabolite (Figure 6; Khakimov *et al.*, 2015). As for other types of terpenoids, the heterologously produced triterpenoids were further metabolized by endogenous *N. benthamiana* enzymes. Next to 3-*O*-Glc-oleanolic acid, *N. benthamiana* accumulated more than 20 other saponins, with up to six hexoses and multiple glycosylations

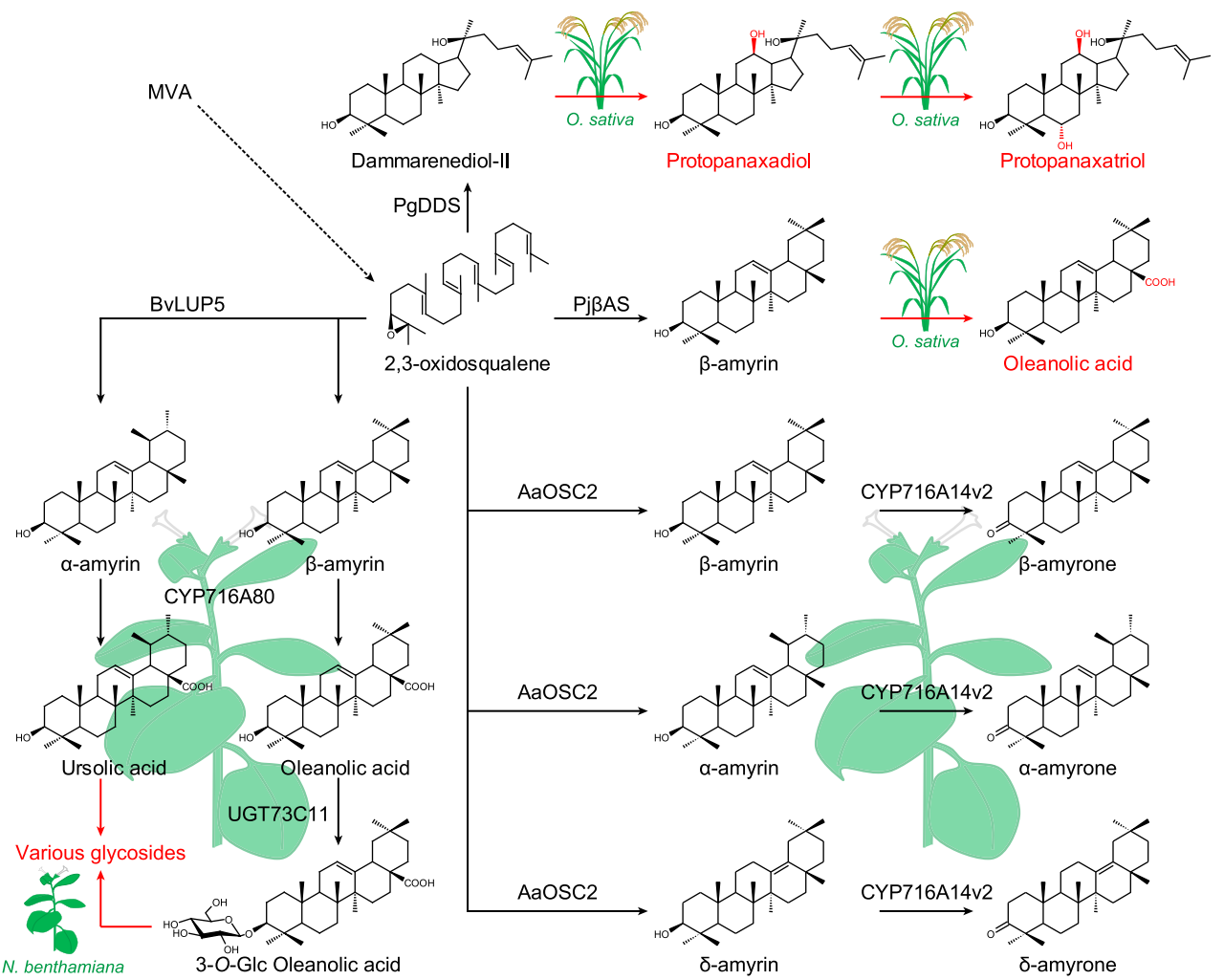

Figure 6. Biosynthesis of (novel) triterpenoids in heterologous plant hosts.
Modifications carried out by endogenous enzymes are shown in red. Expression of *Panax ginseng* dammarenediol-II synthase (PgDDS) and *Panax japonicus* β-amyrin synthase (PjβAS) in rice leads to the accumulation of dammarenediol-II and β-amyrin, respectively, that are further metabolized by endogenous rice enzymes. Expression in *Nicotiana benthamiana* of *Artemisia annua* oxidosqualene cyclase 2 (AaOSC2) and CYP716A14v2 leads to the production of various amyrins and amyrones. Expression in *N. benthamiana* of *Barbarea vulgaris* β-amyrin and α-amyrin synthase (BvLUP5), CYP716A80 and UGT73C11 leads to the accumulation of 3-*O*-Glc oleanolic acid and a set of diverse triterpene glycosides derived from conversion of the produced metabolites by endogenous enzymes.
CYP, cytochrome P450; MVA, mevalonate pathway; UGT, UDP glycosyltransferase.

occurring on both the C-3 and C-28 positions (Khakimov *et al.*, 2015).

PERSPECTIVES FOR TERPENOID PRODUCTION PLATFORMS

In this section we will discuss different photosynthetic hosts and plant organs for the production of terpenoids. We will reflect on recent advances in engineering and synthetic biology to convert photosynthetic organisms into well-performing terpenoid production platforms and to exploit their endogenous metabolism for the synthesis of novel compounds.

Possible photosynthetic hosts for synthetic biology programmes

The vast and diverse plant kingdom offers a wide range of possible biosynthetic hosts for the (heterologous) production of (novel) terpenoids (Table 1). Despite this enormous potential, to date only a small number of heterologous photosynthetic hosts have been used. For lab-scale experiments, currently the most widely used host plant is *N. benthamiana*, the leaves of which can be easily infiltrated with multiple *Agrobacterium* strains, leading to transient expression of the infiltrated genes. Agroinfiltration of *N. benthamiana* allows rapid analysis of the terpenoid production potential of multiple enzymes or enzyme combinations, and can already be scaled up to industrially relevant levels for protein production (Sainsbury and Lomonossoff, 2014; Sack *et al.*, 2015; Walwyn *et al.*, 2015), bringing commercial biosynthesis of high-value terpenoids within reach (Table 1).

However, as infiltration requires large volumes of expensive *Agrobacterium* cultures, transient expression in *N. benthamiana* may not be the best choice for a commercial photosynthetic terpenoid production platform. Hence, once a biosynthesis pathway for the heterologous synthesis of terpenoids has been established, the entire pathway could be expressed in a stably transformed plant. Currently, several plant species have been engineered to produce terpenoids. The use of staple crops such as rice, maize and potato (Giuliano *et al.*, 2008; Giuliano, 2014) offers the advantage that these are domesticated crops for which modern agricultural tools are available, enabling cheap and abundant production. Furthermore, the produced compounds do not necessarily need to be purified and can be directly taken up by humans and animals through food and feed. For instance, the β-carotene present in engineered golden rice has been shown to be an effective source of vitamin A in humans (Tang *et al.*, 2009). Also more specialized, high-value compounds can be produced in crop species. Expression of two algal genes in tomato led to the accumulation of high amounts (16.1 mg g^{-1}) of esterified astaxanthin in the tomato fruits, opening up the way for the commercial production of natural astaxanthin in engineered plants (Huang *et al.*, 2013).

Table 1 Comparison of different photosynthetic and microbial hosts for the (heterologous) production of terpenoids

Host	Advantages	Disadvantages
Escherichia coli	Scalable in bioreactors Fast growth Simple genetic makeup Easy transformation	Requires sugar source Prokaryote, absence of ER may impair P450 activity Incorrect post-translational modifications and protein folding
Yeast	Scalable in bioreactors Fast growth Simple genetic makeup Easy transformation	Requires sugar source Incorrect post-translational modifications and protein folding
Cyanobacteria	Scalable in bioreactors Fast growth Simple genetic makeup Photoautotrophic No need for arable land	Lack of engineering tools Prokaryote, absence of ER may impair P450 activity
Algae	Scalable in bioreactors Fast growth Simple genetic makeup Photoautotrophic No need for arable land	Lack of engineering tools
Mosses	Scalable in bioreactors Fast growth Simple genetic makeup Photoautotrophic Stress tolerant Homologous recombination	Lack of engineering tools
Transient plant expression	Rapid analysis of multiple genes Photoautotrophic	Requires large volumes of expensive *Agrobacterium* cultures
Plant cell cultures	Scalable in bioreactors	Requires sugar source Expensive cultivation media Complex genetic makeup and genetic instability
Transgenic plants	Photoautotrophic Modern agricultural tools available	Complex genetic makeup and metabolic background Slow growth Possible competition with food production

ER, endoplasmic reticulum.

As non-vascular plants, mosses constitute another potential plant host for the heterologous production of terpenoids (Table 1). In a pioneering study, the model moss *P. patens* was used for the heterologous production of taxa-4(5),11(12)-diene by expression of the taxadiene synthase gene from *T. brevifolia* (Anterola *et al.*, 2009). Likewise, the sesquiterpenoids patchoulol and α/β-santalene

(Zhan *et al.*, 2014) and the diterpenoid sclareol (Pan *et al.*, 2015) were also successfully produced in *P. patens*. A major advantage of *P. patens* over other plant species is its ability to undergo homologous recombination (Schaefer and Zrÿd, 1997), which may allow targeted integration of the transgenes. More importantly, however, the potential use of homology-directed repair during genome editing of *P. patens* using the CRISPR/Cas9 system may enable more precise genome editing and more elaborate engineering of the endogenous metabolism to optimize (heterologous) terpenoid production.

Algae may be another attractive host for the heterologous production of specialized, high-value terpenoids (Table 1). In recent years, a lot of effort has been put into the engineering of algae for the production of biofuels (Georgianna and Mayfield, 2012; Chisti, 2013; Gimpel *et al.*, 2013) and high-value ketocarotenoids such as astaxanthin (Guedes *et al.*, 2011). Algae have the advantage that they are fast-growing and easily scalable, and that they can be cultivated using low-quality water sources such as waste or salt water on non-arable land, thereby not interfering with food production (Georgianna and Mayfield, 2012). With the constant progress being made in algal engineering, the production of any desired terpenoid, either endogenous or heterologous, natural or new to nature, in algal cell factories could be achievable within the foreseeable future.

Lastly, cyanobacteria could also be considered as photosynthetic hosts (Table 1). Cyanobacteria are a group of photosynthetic bacteria, prokaryotes, that may also serve as hosts for the large-scale photosynthetic production of various terpenoids. Several plant-specific terpenoids have already been produced in different cyanobacterial strains (Angermayr *et al.*, 2015; Pattanaik and Lindberg, 2015). For instance, expression of the *Schizonepeta tenuifolia* limonene synthase gene in the model cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*) led to the production of the volatile monoterpene limonene by the recombinant strains (Kiyota *et al.*, 2014).

Although some terpenoids have already been heterologously produced in cyanobacteria, many challenges remain. For instance, cyanobacteria only have the MEP pathway for the production of terpenoids and hence the maximum terpenoid production level is determined by the rate-limiting MEP pathway. Attempts to overcome this metabolic bottleneck have been undertaken but have so far only been moderately successful. Further engineering will be necessary for the commercial production of terpenoids in cyanobacterial hosts (Pattanaik and Lindberg, 2015).

Synthetic biology in plant or microbial hosts?

Besides the above-describe photosynthetic hosts, microbial hosts such as yeast and *E. coli* are extensively used for the production of terpenoids. Other than plants, microbes such as *E. coli* and yeast are fast growing, are independent of weather conditions and are easy to genetically modify. However, the choice of the heterologous host for the production of a certain metabolite is specific for each desired compound, and each system has its own advantages and disadvantages (Table 1).

Traditionally, plants are often disregarded as potential hosts for synthetic biology programmes due to their slow growth, inefficient transformation protocols and complex metabolic network and metabolite composition. However, a major advantage of photosynthetic hosts compared with microbial hosts such as yeast and *E. coli* is that they synthesize the desired metabolites using sunlight, water and carbon dioxide. In contrast, yeast and *E. coli* require fermentable sugars as an energy source. These sugars may be obtained from plant-derived lignocellulose from agricultural waste. However, the sugars in this material are protected by cross-linked polymers such as lignin that first need to be broken down using energy-intensive processes (Wang *et al.*, 2012) or represent a complex mix of hexoses and pentoses that cannot be readily utilized by wild-type yeast (Kim *et al.*, 2013; Sànchez Nogué and Karhumaa, 2015). Furthermore, microbes may be unable to carry out the post-translational modifications that are necessary for correct protein folding and enzyme functionality (Ikram *et al.*, 2015).

Like yeast and *E. coli*, cyanobacteria and algae grow faster than vascular plants and have a simple genetic makeup that should be relatively easy to manipulate. These hosts also have the advantage over vascular plants that they can be cultivated in large bioreactors in controlled and axenic conditions. Mosses and plant cell cultures such as callus cultures and hairy roots can also be grown in bioreactors. Mosses offer the advantage that they can be cultivated as genetically stable protonema (Simonsen *et al.*, 2009), whereas plant cell cultures that consist of undifferentiated callus cells have a high level of somaclonal variation. In addition, mosses grow photoautotrophically in simple inorganic media, whereas plant cell cultures need a carbon source, vitamins and phytohormones for their cultivation. Finally, the ability of mosses such as *P. patens* to undergo homologous recombination (Schaefer and Zrÿd, 1997) is another major advantage over vascular plant hosts.

Another consideration may be the need for arable land. Algae and cyanobacteria, for instance, are cultivated submerged in water and as such do not require arable land for their cultivation, and hence do not compete with food production. In addition, they can be cultivated in abundantly available seawater or in nutrient-laden agricultural wastewater (Dismukes *et al.*, 2008). Furthermore, water allows for higher carbon dioxide concentrations than the atmosphere and could consequently enable the use of concentrated carbon dioxide from industrial emissions (Nozzi *et al.*, 2013). The need for arable land can also be compensated

by producing desired metabolites directly in food or feed crops for which modern agricultural tools are available. This allows for a very cheap and abundant production of compounds that can be directly taken up as food or feed without the need for metabolite purification. Because heterologous terpenoid production could potentially increase resistance to herbivores, pathogens or pests, it could also enhance the overall fitness of the crop species (Ikram *et al.*, 2015). However, using crops for the production of terpenoids that are not directly used as food or feed, such as biofuels, may lead to competition with food production and result in higher food prices. Furthermore, proper separation of the downstream processing pipelines from the food chain might become another critical parameter to be addressed.

In contrast to microbial systems, mosses and plant cell cultures, plants have different organs that may have different (heterologous) terpenoid production capacities. Some plants have dedicated organs such as trichomes, glands, laticifers or secretory ducts for the production of large quantities of metabolites. Targeting the terpenoid production to these organs may dramatically improve the production capacity and fitness of the transgenic plants. However, not only specialized organs can be targeted. Tomato fruits or carrot roots, for instance, are rich in carotenoids and could hence be targeted for the heterologous production of specific carotenoids. Similarly, triterpenoids are especially abundant in the cuticular wax of leaves. Furthermore, organs or tissues that are rich in a certain metabolite could also be targeted for the production of metabolites that share the same biochemical precursor. For instance, like carotenoids, mono- and diterpenoids originate from the MEP pathway and hence tomato fruits or carrot roots could be targeted for mono- or diterpenoid engineering.

Challenging microbial production platforms

To date, genetic toolboxes for the design and engineering of microbial production platforms are better established than those for plant hosts, in particular when dealing with multigenic traits such as complex biosynthetic pathways. Traditionally, multigene pathways have been engineered in plants by sequential transformation of transgenic lines with additional genes or by crossing of homozygous transgenic lines. Both techniques are slow and labour-intensive and may lead to segregation of the transgenes in later generations (Naqvi *et al.*, 2010; Pollier *et al.*, 2011; Zorrilla-López *et al.*, 2013; Farré *et al.*, 2014).

To express multiple genes from a single promoter, viral mechanisms for pseudo-polycistronic gene expression based on internal ribosomal entry sites (IRESs) or, more recently, 2A peptides have emerged as a tool in metabolic engineering (recently reviewed by Farré *et al.*, 2014). Like this, the number of necessary transformations for the expression of entire metabolic pathways can be minimized. The 2A system has now been employed in various studies on terpene engineering of plants, ranging from the production of amorphadiene in *N. benthamiana* (van Herpen *et al.*, 2010) via the expression of sclareol synthases in the moss *P. patens* (Pan *et al.*, 2015) to carotenoid production in tobacco, tomato, rice and soy (Ralley *et al.*, 2004; Ha *et al.*, 2010; Kim *et al.*, 2012).

Recently, cloning systems such as Golden Gate MoClo (Engler *et al.*, 2008) or GoldenBraid (Sarrion-Perdigones *et al.*, 2013) have been gaining attention for plant metabolic engineering. Based on type IIS restriction enzymes, these systems allow seamless cloning of transcriptional units made from BioBricks, which comprise promoters, untranslated regions (UTRs), coding sequences (CDSs), terminators and many more (Knight, 2003). Using modular vector sets, these transcriptional units can then be combined to large multigene constructs. Recently, a standard syntax of 12 fusion sites for the assembly of BioBricks to eukaryotic transcriptional units was suggested by members of the international plant science and synthetic biology communities (Patron *et al.*, 2015). Because they are so fast and easy to use, it is to be expected that these techniques will become the standard for multigene expression in plants. In combination with binary bacterial artificial chromosomes (BIBACs), which allow *Agrobacterium*-mediated transformation of plants with fragments up to 150 kb (Hamilton *et al.*, 1996), entire metabolic pathways may easily be introduced into plants in the future, thereby challenging microbial platforms for the production of heterologous terpenoids.

Genome editing

Until recently, higher plants did not allow for the targeted knockout of genes, because they do not undergo homologous recombination, unlike microbes and mosses. This, however, may be desired in metabolic engineering programmes when competing side branches of metabolic pathways or negative feedback loops are to be shut down. However, thanks to recent technological developments, this may now be addressed through genome editing techniques, in which highly specific zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPRs) are used to introduce double-strand DNA breaks at almost every genomic position, after which the endogenous DNA repair mechanism will try to mend the loose DNA ends. In this way random deletions or insertions may be introduced that often lead to a loss of function of the target gene (Hsu *et al.*, 2014). Because of the high efficiency of these techniques, even polyploid crops can be targeted (Li *et al.*, 2012; Wendt *et al.*, 2013; Sawai *et al.*, 2014) and the simultaneous knock-out of up to eight genes in plants has been reported (Shan *et al.*, 2013). Genome editing techniques have already been

employed to successfully modify the metabolic profile of plants. Using TALENs in tetraploid potatoes, the sterol side chain reductase 2 (SSR2) was knocked out, thereby generating plants that contained significantly lower amounts of cholesterol and steroidal glycoalkaloids (Sawai *et al.*, 2014). Furthermore, techniques such as CRISPR interference for transcriptional gene repression (CRISPRi; Larson *et al.*, 2013) and synthetic, TALEN-derived transcription factors (Perez-Pinera *et al.*, 2013) may well be considered for plant metabolic engineering programmes in the future. Latest advances in (plant) genome editing have recently been reviewed in more detail elsewhere (Puchta and Fauser, 2014; Baltes and Voytas, 2015).

Expanding the metabolic potential

Plants possess an enormous potential for combinatorial biosynthesis because they are outstanding chemists with exceptional metabolic powers. The staggering diversity of plant metabolites – thousands of different metabolites can be produced by a single plant – can be attributed to several factors. First, a substantial number of plant genes encode enzymes that are involved in metabolism. In *A. thaliana* for example, about 22% of the protein-encoding genes are currently annotated as enzymes [5995 out of 27 206 genes; TAIR10 Genome (https://www.arabidopsis.org/) and AraCyc 13.0 (http://www.plantcyc.org/)]. Alternative splicing of pre-mRNAs may lead to structurally and functionally distinct enzymes that originate from the same gene, thereby expanding the number of possible enzymatic reactions. According to a recent study, at least 42% of intron-containing genes are alternatively spliced in *A. thaliana*, thus probably also contributing to the diversity at protein level (Filichkin *et al.*, 2010). Furthermore, many plant enzymes have a broad spectrum of reaction products, and others are promiscuous, meaning they accept similar, but distinct, compounds as substrates. As such, a combinatorial number of compounds may be generated from only a few precursor molecules and enzymes. Another aspect of the chemical versatility of plants is their powerful detoxification mechanisms. Due to their immobility, plants are regularly challenged with xenobiotics that may be toxic to them and which have to be efficiently detoxified. This may be achieved by P450-mediated oxygenation and conjugation with hydrophilic molecules such as glutathione, malonate or different sugars (Coleman *et al.*, 1997).

Such modifications also underlie the effects caused by the activation of silent metabolism and were observed when terpenoids were heterologously produced in various plant hosts (see the above sections). The nature of the observed modifications often matches those generated through biotransformation, a process in which (plant) cells are exogenously fed with a compound which may then be chemically transformed by the endogenous plant metabolism (Ishihara *et al.*, 2003; Pollier *et al.*, 2011). For instance, cell suspension cultures of *C. roseus* oxidized geraniol and nerol to their corresponding allyl alcohols (Hamada *et al.*, 1997), events which were also almost always observed when geraniol was heterologously produced in various plant hosts (Figure 4, Table S1).

While such modifications are the actual targets in biotransformation strategies, they are primarily regarded as unwanted, flux-disturbing effects in metabolic engineering programmes. However, such effects may be circumvented by careful choice of the production host, tissue type and culture condition. For instance, when different plant hosts for heterologous geraniol production were compared, grapevine and *N. benthamiana* produced nerol or citronellol next to geraniol, while in *A. thaliana*, none of these derivatives were detected (Fischer *et al.*, 2013).

Also the tissue, organ and culture type used for gene expression are important variables. While, for instance, the geraniol derivatives detected in tobacco hairy roots did not vary too much from those obtained after agroinfiltration (Dong *et al.*, 2013; Ritala *et al.*, 2014), tissue-specific differences in the distribution of geraniol derivatives were noticed in stably transformed tobacco plants. Besides differences in the glycosylation profile, acetylated and malonylated derivatives were more abundant in mature leaves, indicating that this enzymatic conversion is more active in the later plant developmental stages (Dong *et al.*, 2013). Conversely, a particular organ may react distinctly to different terpenes. Tomato fruits responded to the heterologous expression of monoterpene synthases with the upregulation of endogenous monoterpenoid metabolism (Lewinsohn *et al.*, 2001; Davidovich-Rikanati *et al.*, 2007), but this effect was not observed during heterologous tetraterpenoid production in tomato fruits (Ralley *et al.*, 2004; Huang *et al.*, 2013).

On the other hand, the endogenous metabolic activity of different plant hosts may also be exploited for the production of novel terpenoids. Stably transformed plants may, for instance, readily produce glycosylated terpenoid drugs with higher bioavailability and would thus outcompete expensive cell suspension cultures. However, data on heterologous terpene production in photosynthetic organisms are not comprehensive enough yet. Expression systems such as *N. benthamiana* are only starting to boom and engineering tools in photosynthetic organisms are not substantial enough yet and/or are not performing equally well as in microbial hosts. Still, sequence resources of photosynthetic organisms are growing explosively (Goossens, 2015), hence, in the future, the metabolic capacities of plants may be well predictable and the choice of photosynthetic host species or a certain tissue for the heterologous production of terpenes may just become as much a part of the metabolic engineering toolbox as the mere use of different promoters or genes. Continuous exploitation of the

metabolic capacities of plants and other photosynthetic organisms may therefore enable sustainable production of new-to-nature and tailor-made terpenoids as efficiently as in microbial hosts.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Combinatorial biosynthesis for the production of novel carotenoids in *Escherichia coli*.

Table S1. Metabolic engineering of terpenoids in plants and algae.

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