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Metabolic engineering of terpenoid biosynthesis in plants

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Abstract

Metabolic engineering of terpenoids in plants is a fascinating research topic from two main perspectives. On the one hand, the various biological activities of these compounds make their engineering a new tool for improving a considerable number of traits in crops. These include for example enhanced disease resistance, weed control by producing allelopathic compounds, better pest management, production of medicinal compounds, increased value of ornamentals and fruit and improved pollination. On the other hand, the same plants altered in the profile of terpenoids and their precursor pools make a most important contribution to fundamental studies on terpenoid biosynthesis and its regulation. In this review we describe our recent results with terpenoid engineering, focusing on two terpenoid classes the monoterpenoids and sesquiterpenoids. The emerging picture is that engineering of these compounds and their derivatives in plant cells is feasible, although with some requirements and limitations. For example, in terpenoid engineering experiments crucial factors are the subcellular localisation of both the precursor pool and the introduced enzymes, the activity of endogenous plant enzymes which modify the introduced terpenoid skeleton, the costs of engineering in terms of effects on other pathways sharing the same precursor pool and the phytotoxicity of the introduced terpenoids. Finally, we will show that transgenic plants altered in their terpenoid profile exert novel biological activities on their environment, for example influencing insect behaviour.

Abbreviations: DMADP – dimethylallyl diphosphate; FDP – farnesyl diphosphate; GDP – geranyl diphosphate; GGDP – geranylgeranyl diphosphate; IDP – isopentenyl diphosphate; MEP – methylerythritol 4-phosphate; TPSs – terpene synthases.

Introduction

Terpenoids are the most structurally varied class of plant natural products. They are commercially important due to their wide application in a vast number of industrial products such as flavouring agents, pharmaceuticals, perfumes, insecticides and anti-microbial agents (Martin et al., 2003). In nature, they play significant roles in plant–environment interactions, plant–plant communication and plant–insect and plant–animal interactions (Pichersky and Gershenzon, 2002). Although

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many of them are associated with primary metabolism (e.g. the phytol side chain of chlorophyll, carotenoid pigments, and the plant hormone gibberellin) others are typical plant secondary metabolites.

All terpenoids are synthesized through the condensation of isopentenyl diphosphate (IDP) and its allylic isomer dimethyl allyl diphosphate (DMADP) (Carretero-Paulet et al., 2002). The sequential head-to-tail addition of IDP units to DMADP yields the prenyl diphosphates geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP) (Figure 1). These three components serve as precursors for the monoterpenes, sesquiterpenes and diterpenes, respectively. Terpenoid synthases or cyclases catalyze the reactions in which the primary terpene skeletons are formed from these substrates. The parent skeletal type of mono-, sesqui- and diterpenes is normally further modified by the activity of an array of different enzymes (e.g. hydroxylases, dehydrogenases, reductases and glycosyl, methyl and acyl transferases) which together generate the many thousands of different terpenoid structures (Lucker et al., 2001). Terpenoid biosynthesis occurs in the cytosol and the plastids (Figure 1). IDP and DMADP are synthesized through the 2-methylerythritol 4-phosphate pathway (MEP) *via* deoxy-D-xylulose 5-phosphate in plastids. However, IDP is also synthesized in the cytosol *via* the mevalonate pathway (Bick and Lange, 2003). It is generally accepted that GDP and GGDP in the plastids are used as substrate for monoterpene and diterpene biosynthesis, respectively whereas FDP in the cytosol is used for sesquiterpene biosynthesis (Figure 1).

Why would we like to engineer terpenoid production in plants? Primarily, plants engineered for their terpenoid profile could serve as a tool for improving a large number of traits in different crop species. Examples for such traits are enhanced disease resistance, weed control by producing allelopathic compounds, improved pest control, increased value of ornamentals and fruit (fragrance and flavour) and improved pollination by altering scent profiles. In addition, large-scale production of terpenoids in plants, either for medicinal uses or for other industries such as

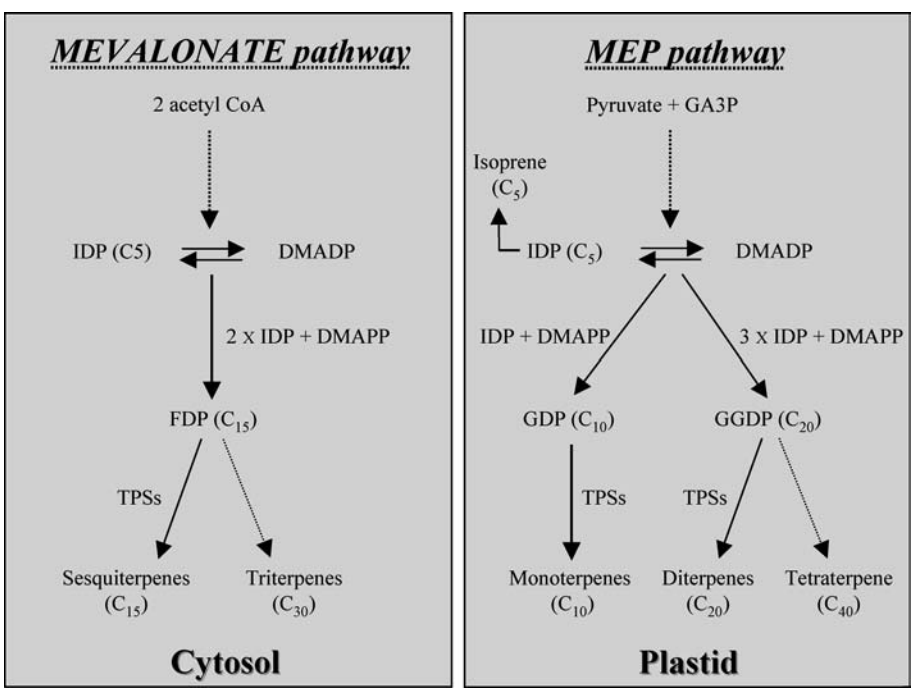

Figure 1. The mevalonate and MEP pathways producing different terpenoid classes in the cytosol and plastids, respectively. GA3P, D-glyceraldehyde-3-phosphate; TPSs, terpene synthases; IDP, isopentenyl diphosphate; DMADP, dimethylallyl diphosphate; FDP, farnesyl diphosphate; MEP, methylerythritol 4-phosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate. Broken arrows represent multiple enzymatic steps.

cosmetics and food would be attractive. A second, but not less important reason is that plants altered in the profile of terpenoids (and pool of precursors) make an important contribution to fundamental studies on their biosynthesis and regulation. For example, metabolic engineering experiments often reveal undiscovered branches to an already known metabolic pathway or point to feedback loops within a pathway or between pathways.

In recent years attempts to produce high levels of monoterpenes in transgenic plants have been successful. Several different plant species were engineered, mainly by overexpressing terpene synthases under constitutive promoters. Petunia, tomato, carnation, potato and Arabidopsis plants were generated that over-expressed genes encoding linalool synthases. Such plants produced and emitted the monoterpene linalool and its glycosylated or hydroxylated derivatives (Lewinson et al., 2001; Lucker et al., 2001; Lavy et al., 2002; Aharoni et al., 2003). Mint and tobacco plants expressing limonene, γ-terpinene and α-pinene synthases were also altered in their terpenoid profile (Diemer et al., 2001; Lucker et al., 2004b). Levels of terpenoid precursors could also be elevated by overexpressing genes encoding enzymes from various steps of the MEP pathway (*DXR* and *HDR*) (Mahmoud and Croteau, 2001; Botella-Pavia et al., 2004). In addition, genes encoding enzymes which modify monoterpene structures have been successfully over-expressed or knocked down in tobacco and mint (Mahmoud and Croteau, 2001; Wang et al., 2001; Lucker et al., 2004a; Mahmoud et al., 2004). In conclusion, in many studies it was demonstrated that it is feasible to engineer several steps of the monoterpenoid pathway. However, attempts to engineer sesquiterpenes in plants using terpene synthases resulted in only low level production (Hohn and Ohlrogge, 1991; Wallaart et al., 2001).

In this report we will describe our recent work on engineering mono- and sesquiterpenes in Arabidopsis and potato. Several issues important for this field of research will be discussed, including: (a) metabolic fate of engineered terpenoids, (b) ease of engineering monoterpenes versus sesquiterpenes, (c) availability of precursors (in different sub-cellular compartments) (d) cost of engineering terpenoids (e) biological activity of transgenic plants altered in their terpenoid profile.

The FaNES1 protein as a 'sensor' for both mono- and sesquiterpene precursors

We chose the strawberry *FaNES1* (*Fragaria ananassa Nerolidol Synthase 1*) gene for performing metabolic engineering experiments in several plant species (Aharoni et al., 2004). The recombinant FaNES1 protein was previously shown to catalyze the conversion of GDP and FDP to (*S*)-linalool and (3S)-(*E*)-nerolidol, respectively with equal efficiency. The ability of other recombinant terpene synthases to generate both sesquiterpenes and monoterpenes was already observed earlier (Crock et al., 1997; Steele et al., 1998). However, in these cases the recombinant enzyme could generate the sesquiterpene with high efficiency while a combination of monoterpenes would be formed from GDP but with low efficiency. FaNES1 could therefore serve as an excellent 'sensor' for levels of both monoterpene and sesquiterpene precursors in the cell or even, as will be described later in this report, in a specific sub-cellular compartment.

Engineering monoterpenoids in Arabidopsis plants

Recent research in Arabidopsis revealed that what at first seemed a metabolically simple plant species is in reality a reasonable producer of secondary metabolites (D'Auria and Gershenzon, 2005). For example, nearly two dozen monoterpenes and sesquiterpenes are emitted from its flowers (Aharoni et al., 2003; Chen et al., 2003; Tholl et al., 2005). Leaves of Arabidopsis on the other hand emit only traces of one monoterpene, limonene. Transgenic Arabidopsis plants were raised which expressed the *FaNES1* gene driven by the CaMV 35S promoter. The FaNES1 protein was targeted to the plastids by fusing the wild strawberry FvNES1 *(Fragaria vesca Nerolidol Synthase 1)* plastid targeting region to the N-terminal of FaNES1. Various headspace analyses methods revealed that the volatile profile of rosette leaves derived from transgenic lines was altered compared to control plants. High levels of linalool were produced by transgenic lines and levels of linalool released from the highest producing lines reached 7.2 to 13.3 μg day^{-1} plant^{-1} (Aharoni et al., 2003). Multidimensional GC-MS analysis revealed that transgenic Arabidopsis lines produced exclusively (*S*)-linalool. This result matches with the linalool enantiomer produced by ripe fruit of cultivated strawberry and the recombinant

FaNES1 enzyme *in vitro*. Interestingly, emission of the newly produced linalool showed a diurnal rhythm in the same way as other mono- and sesquiterpenes naturally emitted by flowers of wild-type plants (Aharoni et al., 2003).

Plants overexpressing *FaNES1* not only produced and emitted linalool, but also three linalool derivatives including *E*-8-hydroxy linalool, *Z*-8-hydroxy linalool and *E*-8-hydroxy-6,7-dihydrolinalool. *E*-8-hydroxy linalool and *E*-8-hydroxy-6,7-dihydrolinalool were identified as both glycosidically bound and as aglycons and *Z*-8-hydroxy linalool only as glycoside (Aharoni et al., 2003). Formation of these derivatives is most probably a result of endogenous enzyme activities mediating hydroxylation, double bond reduction, and glycosylation (e.g. cytochrome P450s, reductases and glycosyl transferases). *E*-8-hydroxy linalool and its glycoside were detected to the highest levels. This might be explained by the fact that low levels of glycosidically bound *E*-8-hydroxy linalool could also be detected in leaves of wild-type Arabidopsis, and, therefore, active endogenous enzymes apparently were present that could also act efficiently on the newly produced linalool.

Engineering monoterpenoids in potato plants

To engineer monoterpenes in potato plants we expressed the *FaNES1* gene using the same construct as the one used for transforming Arabidopsis (see above). Leaves of wild-type potato plants already emit linalool but transgenic lines emitted linalool to much higher levels (Figure 2). As described earlier (Aharoni et al., 2004), the FaNES1 recombinant protein catalyzes the biotransformation of GDP to *S*-linalool. We performed chiral analyses on linalool produced by transgenic and wild-type plants using multidimensional gas chromatography mass spectrometry

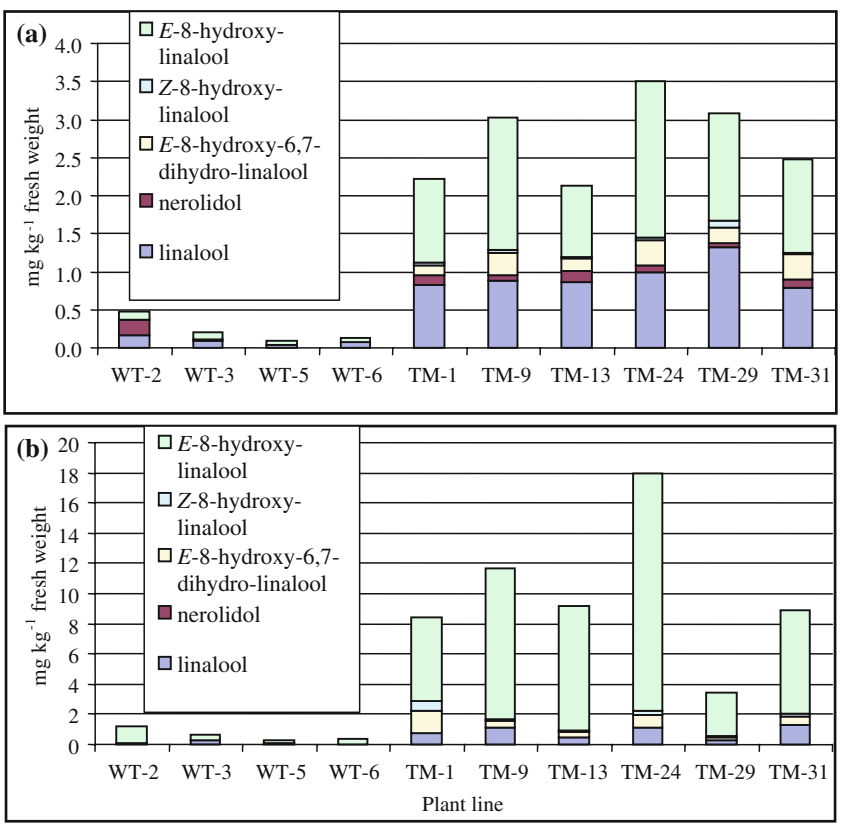

Figure 2. Concentration of linalool, nerolidol, *E*-8-hydroxy-6,7-dihydro-linalool, *Z*-8-hydroxy-linalool, and *E*-8-hydroxy-linalool in wild-type (WT) and transgenic potato (*S. tuberosum*) leaves expressing *FaNES1* with a plastidic targeting signal (TM) (a). Concentration of the metabolites released from their glycosides by enzymatic hydrolysis (b). Detection limit was 0.01 mg kg^{-1} with phenol as internal standard and assuming a response factor of 1.

(MDGC-MS). The results showed that wild-type potato leaves contain linalool with an average enantiomeric ratio of 98:2 (*R*:*S*) while in transgenic potato plants overexpressing *FaNES1* the enantiomeric ratio of linalool is completely the opposite with an average ratio of 8:92 (*R*:*S*) (Figure 3). Transgenic potato plants emitting high levels of linalool had a very distinct smell compared to wild-type plants. While leaves of wild-type plants had the typical green odor, leaves of transgenic lines had a sweet, flowery, citrus fruity impression which is characteristic of *S*-linalool.

Volatiles and glycosidically bound terpenoids produced by young potato leaf tissue were also analyzed in more detail. Wild-type leaves already produce linalool and *E*-8-hydroxylinalool (Figure 2a). However, in leaves of *FaNES1* transgenic plants, levels of linalool and *E*-8-hydroxylinalool were considerably higher than in the wild-type leaves and *Z*-8-hydroxylinalool as well as *E*-8-hydroxy-6,7-dihydrolinalool were also detected (Figure 2a). Glycosidically bound terpenoids were determined after enzymatic hydrolysis of glycosides (Figure 2b). GC-MS analysis of liberated alcohols showed that glycosylated linalool and *E*-8-hydroxylinalool were present in wild-type plants but that their levels were considerably higher in leaves of transgenic plants. Transgenic

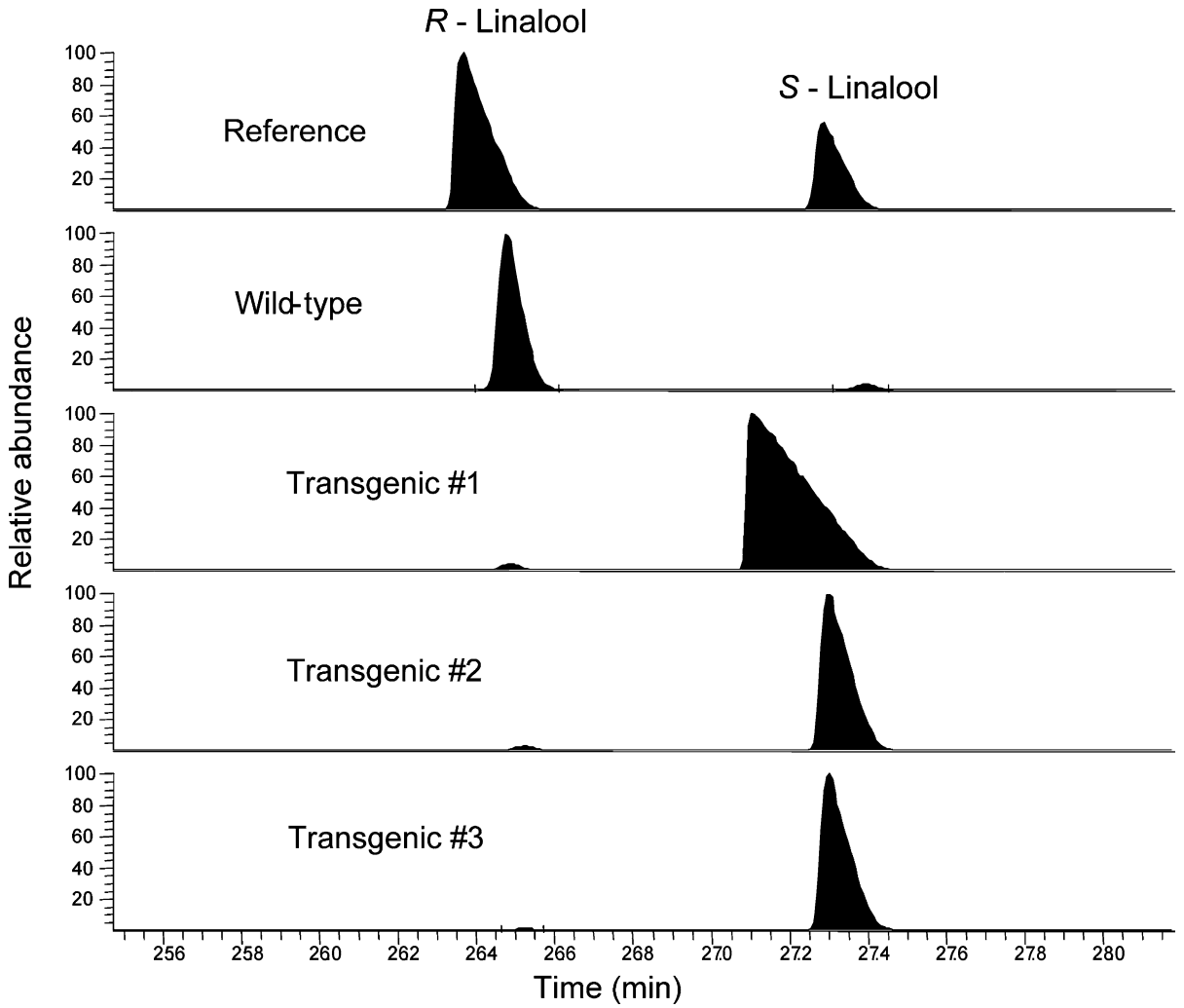

Figure 3. Enantiomeric distribution of linalool in transgenic potato plants compared to wild-type plants. Enantio-selective analysis of endogenous linalool in wild-type potato leaves and linalool extracted from leaves of transgenic potato plants expressing the *FaNES1* gene. Analysis was conducted by using Multidimensional Gas Chromatography-Mass Spectrometry (MDGC-MS; for methods see Aharoni et al., 2004).

plants also produced glycosylated *E*-8-hydroxy-6,7-dihydrolinalool and *Z*-8-hydroxylinalool, which were not detected in wild-type plants. HPLC-MS analysis confirmed the presence of linalyl-β-D-glucopyranoside in wild-type and transgenic potato leaves and we assume that the carbohydrate moiety in the glycosylated 8-hydroxy linalool derivatives are also attached to the tertiary hydroxyl group (Figure 4). Thus, the glycosylation patterns of identical linalool derivatives are different in potato and Arabidopsis.

Attempts to engineer sesquiterpenes in Arabidopsis

Transgenic Arabidopsis plants overexpressing the *FaNES1* gene emitted also the sesquiterpene nerolidol, albeit at much lower levels (levels of nerolidol emitted were 100- to 300-fold lower than of linalool) (Aharoni et al., 2003). As sesquiterpenes are known to be produced in the cytosol, and these transgenic lines produced the FaNES1 protein in the plastids, this observation indicates that FDP is also present in the plastids. In another attempt to engineer sesquiterpenes, Arabidopsis plants producing a cytosolic germacrene A synthase from chicory were generated. GC-MS analysis of volatiles emitted from transgenic vs. control plants did show the presence of the thermal rearrangement product of germacrene A, β-elemene, but only in trace amounts (Aharoni et al., 2003). Thus, engineering sesquiterpene production in plants is more difficult as compared to the engineering of monoterpenoids, most probably due to shortage in the sesquiterpene precursor pool. Future work on metabolic engineering of sesquiterpene production in plants might prove to be more successful as two very recent reports described the formation of higher levels of sesquiterpenes in transgenic Arabidopsis plants. Kappers et al. (2005) could generate higher levels of the sesquiterpene nerolidol by altering the localization of the strawberry FaNES1 protein to the mitochondria. Emission of 4,8-dimethyl-1,3(E),7-nonatriene [(E)-DMNT] the homoterpene derivative of nerolidol, which plays a crucial role in insect predator attraction in tritrophic interactions (Bouwmeester et al., 1999) was also detected in the same transgenic plants. Also in transgenic Arabidopsis, Degenhardt et al. (2005) overexpressed the maize *TPS8* and *TPS10* genes and could generate a range of sesquiterpenes *in planta*.

Cost of engineering terpenoids in plants and biological activity of transgenics

Up to now most experiments aiming at engineering terpenoids in plants utilized the constitutive CaMV35S promoter for driving the expression of a gene of interest. Although most of these plants produced and emitted the expected terpenoid (in the case of monoterpenes) it was not without paying a price in terms of plant vitality. In most cases, if transgenic plants were affected in their growth, the strength of the phenotype correlated with the production level of the new terpenoid. For example, Arabidopsis plants expressing *FaNES1* were retarded in growth compared to their wild-type counterparts (Aharoni et al., 2003). An even stronger phenotype was observed in potato plants when expressing the *FaNES1* gene under the Rubisco small subunit promoter from chrysanthemum (Figure 5). This promoter is

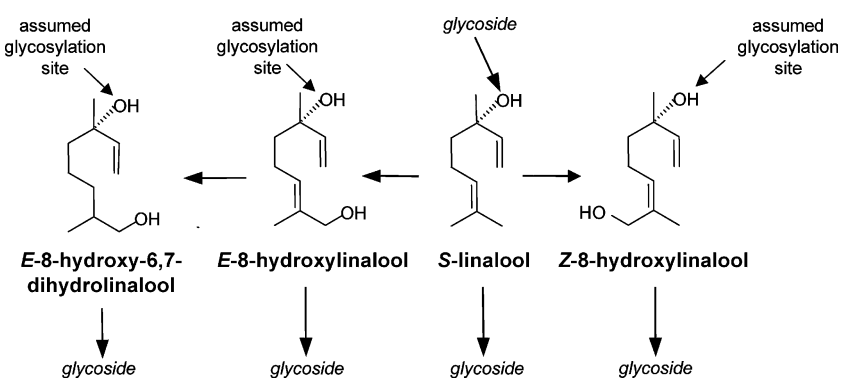

Figure 4. Structures of linalool and derivatives formed in transgenic potato plants over-expressing the strawberry *FaNES1* gene.

Figure 5. Phenotypic effect in potato plants with very strong expression of the strawberry *FaNES1* gene under the control of the chrysanthemum rubisco small subunit promoter. Upon transfer from *in vitro* to the greenhouse leaves of plants with the highest levels of expression turned white and were retarded in growth.

approximately 10-fold stronger and transgenic potato lines with such high levels of linalool production were strongly reduced in growth, but also showed heavy bleaching in leaves upon transfer from *in vitro* to the greenhouse (Figure 5). Again, plants with the highest levels of linalool showed the most severe phenotype. It is still not clear whether these effects are due to terpene toxicity or the reduction in the availability of precursors for isoprenoid primary metabolite pathways such as chlorophyll and carotenoid biosynthesis.

The biological effects and possible practical application of these compositional changes in the terpenoid profiles of engineered plants have so far hardly been tested. The first indications of the possibility of altering insect behaviour were provided by transgenic tobacco plants producing higher levels of the diterpene cembratriene-ol (Wang et al., 2001). Not only did exudates from transgenic plants have higher aphidicidal activity, *in vivo* assays with these plants showed greatly reduced aphid colonisation. Aphid behaviour was also altered in transgenic Arabidopsis plants producing linalool (Aharoni et al., 2003). In dual-choice assays with *Myzus persicae*, transgenic lines producing linalool significantly repelled the aphids. Similar results were recently obtained with transgenic chrysanthemum (*Chrysanthemum* × *grandiflorum*) and the western flower thrips, *Frankliniella occidentalis* (M.A. Jongsma, 2004. Novel genes for control and deterrence of sucking insect pests. http://www.isb.vt.edu/news/2004/Nov04.pdf). The transgenic Arabidopsis plants engineered to emit (3*S*)−*E*-nerolidol and (*E*)-DMNT, as described above, attracted carnivorous predatory mites (*Phytoseiulus persimilis*), the natural enemies of spider mites (Kappers et al., 2005).

Concluding remarks

The recent reports on metabolic engineering of terpenoids in plants have clearly shown that these type of approaches could be used successfully to generate substantial levels of terpenoids. Engineering of some classes of terpenoids is apparently more difficult than others as the pool of terpenoid precursors may not be sufficient for the production of substantial amounts of the required terpenoid. In the case of monoterpenes, the availability of GDP is apparently high in both leaf tissues (as we observed in both potato and Arabidopsis and others in additional plant species such as tobacco and mint) as well as in fruit (Lewinson et al., 2001), tuber (Aharoni and Jongsma, unpublished) and flower tissues (Lucker et al., 2004b). Newly introduced monoterpene skeletons, but most likely also those of other terpene classes, will be transformed by endogenous modifying enzymes such as cytochrome P450s, reductases and glycosyl transferases. Interestingly, in Arabidopsis and potato we detected the same 8-hydroxy derivatives (*E*-8-hydroxy linalool, *Z*-8-hydroxy linalool and *E*-8-hydroxy 6,7-dihydrolinalool) but their glycosylation pattern was different. In both plant species *E*-8-hydroxy linalool was also present at low levels in the wild-type plants, and, thus, endogenous enzymes already active in the wild-type plants could act on the introduced monoterpene and produce substantially higher levels of this particular derivative.

Engineering of sesquiterpene production in the cytosol compared to the plastidic production of monoterpenes seems more difficult. At this point it is not clear why only such a small amount of FDP, the sesquiterpene precursor is available although it was earlier speculated that this is due to tight metabolic regulation directing precursors to sterol biosynthesis (Chappell et al., 1995). In recent years several studies showed that transport of terpenoid precursors occurs between subcellular compartments (McCaskill and Croteau, 1998; Bick and Lange, 2003; Hemmerlin et al., 2003; Laule et al., 2003; Schuhr et al., 2003; Dudareva et al., 2005). We discovered, however, that in ripe strawberry fruit monoterpene synthase proteins are localized to the cytosol and not as expected to the plastids. Two independent strawberry monoterpene synthase proteins, which generate unrelated products (linalool in one case and several olefinic monoterpenes in the other), were shown to lack a plastidic targeting signal. This was identified by *in silico* analysis as well as by testing the targeting capacity of the N-termini of these proteins *in vivo* (Aharoni et al., 2004). These two proteins were active in two different strawberry species, wild (*Fragaria vesca*) and cultivated (*Fragaria* × *ananassa*). Thus, intracellular biosynthesis of the different terpene classes and the presence of their precursors is not as strict as believed earlier when the MEP pathway was discovered.

Engineering experiments to alter terpenoid biosynthesis can take advantage of these two points (intracellular transport and biosynthesis of precursors) by targeting proteins to different subcellular compartments. For example, Arabidopsis plants targeting the FaNES1 protein to the plastids unexpectedly also produced low levels of the sesquiterpene nerolidol (Aharoni et al., 2003). Targeting of the same protein to the mitochondria resulted in even higher level of nerolidol production (Kappers et al., 2005). Ohara et al. (2003) targeted the limonene synthase (*LS*) cDNA of *Perilla frutescens* to the plastid, the cytosol and the endoplasmic reticulum (ER) in transgenic tobacco. Limonene formation was detected in leaf extracts of both plastid- and cytosol-localized limonene synthase transgenic plants but not in the ER. The amount of limonene in plastid-localized LS transgenic plants was more than three times higher compared to transgenic plants with cytosol localized LS. Thus, targeting different cell compartments when engineering terpenoids might be a valuable tool for obtaining higher levels of terpenoids and producing novel ones. In addition, further modification of the introduced terpenoid might be different in each cell compartment.

Since the isoprenoid pathway in both the plastid and the cytosol supplies precursor not only for mono- and sesquiterpene biosynthesis but also for an array of other compounds (some of them crucial to plant growth and fitness such as sterols, gibberellins, carotenoids and chlorophyll), strong and constitutive expression of introduced genes could be most harmful. It is therefore highly recommended in future metabolic engineering experiments to direct gene expression to a specific tissue or organ or use an inducible system. One example for an inducible approach was recently reported for the production of the diterpene taxadiene, which was engineered using the glucocorticoid

(GR) system in transgenic Arabidopsis plants (Besumbes et al., 2004). In contrast to plants expressing taxadiene synthase using a strong/constitutive promoter (35S CaMV) which showed a growth retardation and yellowing phenotype, plants engineered with the GR induction system produced taxadiene (after induction) and had a normal appearance.

A large number of terpenoids including mono- and sesquiterpenes are highly bioactive molecules which play a significant role in the interaction of plants with other organisms. Plants producing these compounds are therefore a perfect tool for observing how they influence plant interaction with micro-organisms and insects. By improving plant disease and pest resistance they could also be used as a biotechnological product, providing improved defence properties to crops. Indeed, Arabidopsis plants producing high levels of linalool influenced aphid behaviour, deterring them compared to leaves of wild-type plants when tested in a dual choice assay. Moreover, preliminary results in our group show that high linalool producing potato show enhanced disease resistance, and deter insects when tested with both aphids and thrips in choice assays (Aharoni and Jongsma, unpublished data). It is expected that last years successes in metabolic engineering of terpenoids will boost an array of future studies on the biological activities of transgenic plants engineerined for pathways of these wonderful small molecules.

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