Metabolic Engineering of Monoterpene Biosynthesis in Plants

Joost Lücker

Promotor:

Prof. dr. L.H.W. van der Plas, hoogleraar in de Plantenfysiologie, Wageningen Universiteit

Co-promotoren:

Dr. ir. H.A. Verhoeven, senior onderzoeker, Business Unit Celcybernetica, Plant Research International

Dr. ir. H.J. Bouwmeester, senior onderzoeker, Business Unit Celcybernetica, Plant Research International

Promotiecommissie:

Prof. dr. J. Gershenzon (Max Planck Institute for Chemical Ecology, Germany)

Prof. dr. M. Dicke (Wageningen Universiteit)

Dr. M.C.R. Franssen (Wageningen Universiteit)

Prof. dr. R. Verpoorte (Universiteit Leiden)

Joost Lücker

Metabolic Engineering of Monoterpene Biosynthesis in Plants

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 8 oktober 2002
des namiddags te vier uur in de Aula

Joost Lücker

Metabolic engineering of monoterpene biosynthesis in plants

Proefschrift Wageningen Universiteit

ISBN 90-5808-717-4

'Don't panic,

it's organic'

Francisco Goya & Jason Prior (Double or Nothing, 1993)

Cover:

Background : Setup of headspace analysis using Tenax trapping of transgenic and control tobacco flowers of different developmental stages (**Chapter 4**). The flowers are placed on wetted floral foam blocks wrapped in aluminium foil to prevent evaporation of water.

Front : Top view of tobacco flowers of stage 12 ready for headspace analysis.

Cover design: Johannes Kalma, Nienke de Bode

Layout: Joost Lücker

Uitnodiging (wave): Johannes Kalma, Antine Breimer

Printed: PrintPartners Ipskamp, Enschede

Contents

Abbreviations

ATP: Adenosine triphosphate
B93: *Citrus limon* γ-terpinene synthase
BAP: 6-Benzylaminopurine
BSA: Bovine serum albumin
C62: *Citrus limon* (+)-limonene synthase 1
cDNA: Complementary deoxyribonucleic acid
CIAP: Calf intestinal alkaline phosphatase
CID: collision-induced dissociation
Cl(-)βPINS: *Citrus limon* (-)-β-pinene synthase
Cl(+)LIMS1: *Citrus limon* (+)-limonene synthase 1
Cl(+)LIMS2: *Citrus limon* (+)-limonene synthase 2
ClγTS: *Citrus limon* γ-terpinene synthase
CTAB: cetyltrimethylammonium bromide
D85: *Citrus limon* (-)-β-pinene synthase
DMAPP: Dimethylallyldiphosphate
DNA: Deoxyribonucleic acid
DOXP: 1-Deoxyxylulose 5-phosphate
DXR: DOXP-reductoisomerase
e.e.: enantiomeric excess (| %R-%S |)
ER: Endoplasmatic reticulum
ESI: Electrospray ionisation
EST: Expressed sequence tag
FPP: Farnesyl diphosphate
FID: Flame ionisation detector
FW: Fresh weight
GA_3: Gibberellic acid
GAP: Glyceraldehyde 3-phosphate
GC-MS: Gas Chomatography-Mass Spectrometry
GPP: Geranyl diphosphate
GGPP: Geranyl geranyl diphosphate
His-tag: Histidine tag
HMG-CoA: hydroxymethyl glutaryl CoA
HPLC: High pressure liquid chromatography
i.d.: internal diameter
IPP: Isopentenyl diphosphate

IPTG: Isopropyl-1-thio-ß-D-galactopyranoside
LB broth: Luria Bertani broth
LC-MS-MS: Liquid Chromatography tandem Mass Spectrometry
lis: *S*-linalool synthase
LPP: linalyl diphosphate
M34: *Citrus limon* (+)-limonene synthase 2
MAR: matrix associated region
MDGC-MS: Multidimensional tandem GC-MS system
MEP: 2C-methyl-D-erythritol 4-phosphate
mRNA: Messenger ribonucleic acid
MS: Murashige & Skoog medium
NADPH: Nicotinamide adenine dinucleotide phosphate, reduced form
ORF: Open Reading Frame
PDMS: Polydimethylsiloxane
PVPP: Polyvinylpolypyrrolidone
SPME: Solid phase micro extraction
TDNA: Transfer DNA
TIC: Total ion count
Tps: Terpene synthase
tRNA: Transfer ribonucleic acid
UTR: Untranslated region

Chapter 1

General Introduction

Terpenoids, also called isoprenoids, constitute the largest group of natural metabolites, predominantly found in plants and currently consisting of more than 36000 compounds (Hill, 2002). They are highly variable in structure as they contain a vast amount of different carbon skeletons and many functional groups. Still, they are all derived from a single molecule, the five- carbon isoprenoid unit isopentenyldiphosphate (IPP). They are classified according to the number of isoprenoid units as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes (larger than C_{40}) such as rubber (Gershenzon and Croteau, 1993).

In plants, terpenoids are involved in important processes such as photosynthesis (e.g. chlorophyll side chains or carotenoids), electron transport (ubiquinone, menaquinone and plastoquinone), cell membrane architecture (sterols) and regulation of cellular development (gibberellins, abscisic acid, brassinosteroids) (Gershenzon and Croteau, 1993). Furthermore they can exhibit more specialised functions in defence (some are phytoalexins, insect feeding and oviposition deterrents, or competitive phytotoxins) and reproduction (as attractants of pollinators and seed dispersing animals). These functions normally occur in the C_{10}, C_{15} and C_{20} families of terpenoids (Harborne, 1991).

The monoterpenes are volatile, colourless, lipophilic substances, of which around one thousand different structures have been identified (Haudenschild and Croteau, 1998). Examples of structures of monoterpenes are shown in Figure 1.

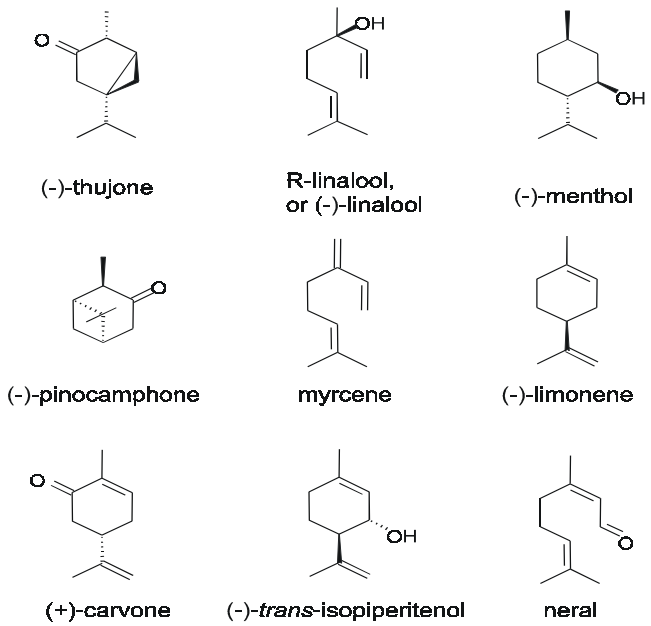


Figure 1. Diversity in structures of monoterpenes that are found in nature

Monoterpenes are emitted from flowers of many plant species (Knudsen et al., 1993), where they are among other volatiles involved in the attraction of species-specific pollinators (Raguso and Pichersky, 1999; Pichersky and Gershenzon, 2002). Monoterpenes can also act as repellents for herbivores, as was shown in a study with aphids (Hori, 1998). They can also be emitted as semiochemicals after herbivore attack to attract predators of the herbivores (Pare and Tumlinson, 1997; Weissbecker et al., 1997; Bouwmeester et al., 1999b; Arimura et al., 2000; Birkett et al., 2000; Baldwin, 2001; Kessler and Baldwin, 2001; van Poecke et al., 2001).

Apart from their ecological functions, monoterpenes are of commercial importance as they are commonly used as flavour and fragrance compounds in foods, beverages, perfumes and cosmetics, and in a broad range of cleaning products (Verlet, 1993). Monoterpenes can be applied as herbicides (Vaughn and Spencer, 1993; Romagni et al., 2000), have potent antimicrobial activity (Espinosa and Langenheim, 1991; Belaiche et al., 1995; Neirotti et al., 1996; Caccioni et al., 1998; Tsao and Zhou, 2000; Aggarwal et al., 2002) and pharmacological properties (Beckstrom Sternberg and Duke, 1996), such as anticarcinogenic effects (Crowell and Gould, 1994; Mills et al., 1995; Yu et al., 1995).

Monoterpenes at high concentrations have been reported to be detrimental to biological structures (Weidenhamer et al., 1993). This phytotoxicity could be a reason for the necessity of the development of specialised structures for the accumulation of high levels of these compounds. Monoterpenes are commonly found in essential oils that accumulate in secretory structures of common herbs and spices as well as in *Citrus* species (Fahn, 1979). In gymnosperm species such as fir and pine, turpentine oil is stored in resin ducts while the biosynthesis occurs in the epithelial cells surrounding these ducts (Fahn, 1979). In angiosperms, the glandular structures are mostly located at the surfaces of leaves or floral organs. In *Mentha* species, monoterpenes are mainly produced and accumulated in glandular trichomes on the leaves. Peltate glandular trichomes consist of a radial cluster of eight secretory cells, with an underlying stalk and basal cell and surmounted with a droplet of oil enclosed by a thick cuticular layer (Fahn, 1979; Gershenzon et al., 1989). In *Citrus*, the essential oil is accumulated in secretory cavities surrounded by schizogenous secretory cells (Fahn, 1979; Turner et al., 1998).

Ultrastructural studies revealed that the specialised secretory cells, present in secretory structures, usually contain a high amount of leucoplasts and have an extensively developed network of smooth endoplasmatic reticulum (ER) probably involved in the transport of the produced terpenoids (Schnepf, 1974; Kolalite, 1994).

In some flowers, the entire petal surface may be glandular and designed more for scent emission than for storage (Fahn, 1979). In these species the production of the volatiles can be localised in epidermal cells, such as in snapdragon and in evening primrose (Dudareva et al., 1996; Kolosova et al., 2001b). In the flowers of rose and gardenia, large parts of the

monoterpenes that are produced in the petals are conjugated as a glycoside. These aroma precursors contribute to the scent production as they can be hydrolysed after flower opening (Watanabe et al., 1994; Oka et al., 1998; Oka et al., 1999). Glycosidally bound monoterpenes are found in many plant species (Stahl Biskup et al., 1993). These glycosides can be commercially important for instance in grapes as they can contribute to the flavour upon wine making after hydrolysis (Park and Noble, 1993; Baek and Cadwallader, 1999).

Emission of volatiles from flowers often occurs in a circadian rhythm (Loughrin et al., 1991; Dudareva et al., 1996; Helsper et al., 1998; Dudareva et al., 2000; Dudareva and Pichersky, 2000; Helsper et al., 2001; Kolosova et al., 2001a). Plants have evolved emission patterns to lure specialised pollinators that are adapted to forage on these flowers (Pichersky and Gershenzon, 2002). Bumblebees are foraging during the day and also the emission pattern of volatiles from bumblebee-pollinated flowers such as snapdragon peaks during daylight, in a diurnal rhythm (Dudareva et al., 2000; Kolosova et al., 2001a). A diurnal rhythm of emission of volatiles was also observed for rose (Helsper et al., 1998; Helsper et al., 2001). In contrast, moths are active at night, and flowers of moth-pollinated species such as petunia and tobacco display a nocturnal circadian rhythmicity of volatile emission (Kolosova et al., 2001a). The expression of the genes and the activity of the resulting enzymes synthesising the volatiles are highly correlated with circadian rhythmicity of volatile emission, and show the same pattern (Dudareva et al., 1996; Dudareva et al., 2000; Kolosova et al., 2001a).

Formation of the general terpenoid precursor IPP

The classical acetate/ mevalonate pathway (Figure 2A) was the first defined biosynthetic route leading to isopentenyldiphosphate, IPP and was reported to occur in all living organisms (Gershenzon and Croteau, 1993). This pathway involves the two-step condensation of three molecules of acetyl-CoA by the enzymes acetyl-CoA acyltransferase and hydroxymethylglutaryl-CoA (HMG-CoA) synthase to produce HMG-CoA. This C_6 compound is then reduced by HMG-CoA reductase to (3*R*)-mevalonic acid (mevalonate). This conversion is considered to be the main regulatory step in the pathway. Mevalonate is then converted to the C_5 compound IPP, in three steps, involving two subsequent phosphorylation steps by mevalonate kinase and phosphomevalonate kinase, and one last decarboxylation step, by phosphomevalonate decarboxylase. The IPP isomerase catalyses the conversion of part of the IPP in a reversible reaction to dimethylallyldiphosphate (DMAPP). IPP and DMAPP are the basic building blocks of which eventually the enormous variety of terpenoids is formed. Most of the genes encoding enzymes of this pathway have now been cloned from plants (Scolnik and Bartley, 1996).

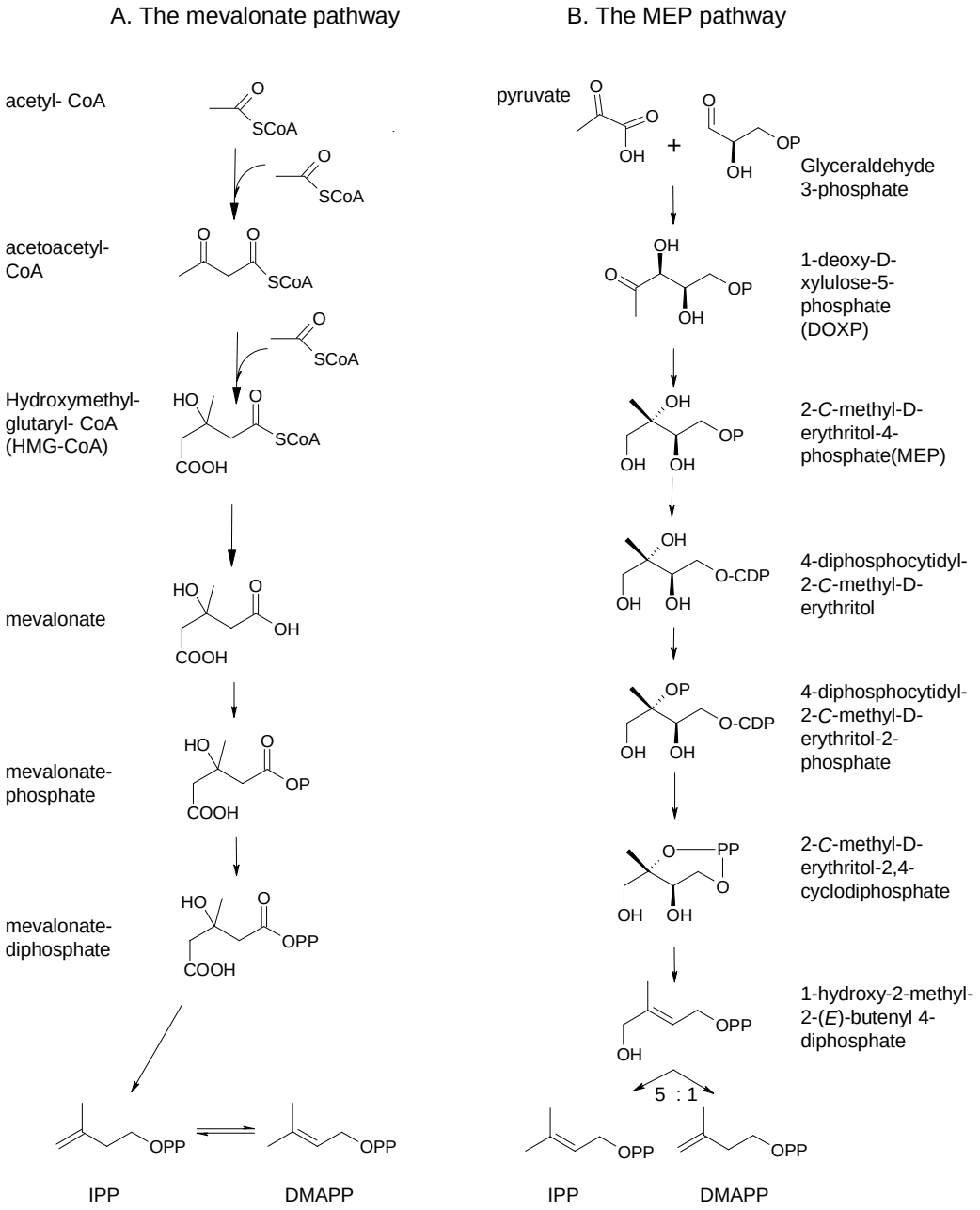


Figure 2. Two metabolic pathways leading to the general terpenoid precursors IPP and DMAPP

However, a number of results from incorporation studies with ^{14}C-labelled substrates, studies with inhibitors and feeding experiments with isolated plastids could not be explained by terpenoid synthesis via the mevalonate pathway (Lichtenthaler, 1999). Almost a decade ago, a new pathway leading to IPP was proposed, where a C_5 precursor for IPP, 1-deoxy-D-xylulose 5-phosphate (DOXP), was formed from glyceraldehyde 3-phosphate (GAP) and pyruvate (Rohmer et al., 1993; Broers, 1994; Schwarz, 1994). The pathway has been reviewed several times in the last years (Lichtenthaler, 1999; Rohmer, 1999; Eisenreich et al., 2001) and was found to occur in eubacteria, green algae and in plastids of higher plants. DOXP is formed by DOXP-synthase, a novel type of transketolase (Lois et al., 1998) and is also a biosynthetic precursor for vitamins B_1 and B_6 (Hill et al., 1972; White, 1978; Lois et al., 1998; Cane et al., 1999). The pathway has been elucidated rapidly and was named the MEP pathway (Figure 2B), after the first committed intermediate for IPP biosynthesis, 2*C*-methyl-D-erythritol 4-phosphate (MEP), formed by DOXP-reductoisomerase (DXR) from DOXP. Subsequently, MEP is converted in several steps to the last intermediate 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, which is subsequently converted into a 5:1 mixture of IPP and DMAPP (Hecht et al., 2001; Rohdich et al., 2002) (Figure 2B). The isomerase activity, which is active in the mevalonate pathway resulting in the conversion of IPP to DMAPP has been hypothesised to be insignificant in this MEP pathway (Rohdich et al., 2002). The occurrence of the proposed two product enzyme was postulated to occur in plants based on feeding experiments with deuterated substrates (Rieder et al., 2000; Rohdich et al., 2002).

Prenyltransferases

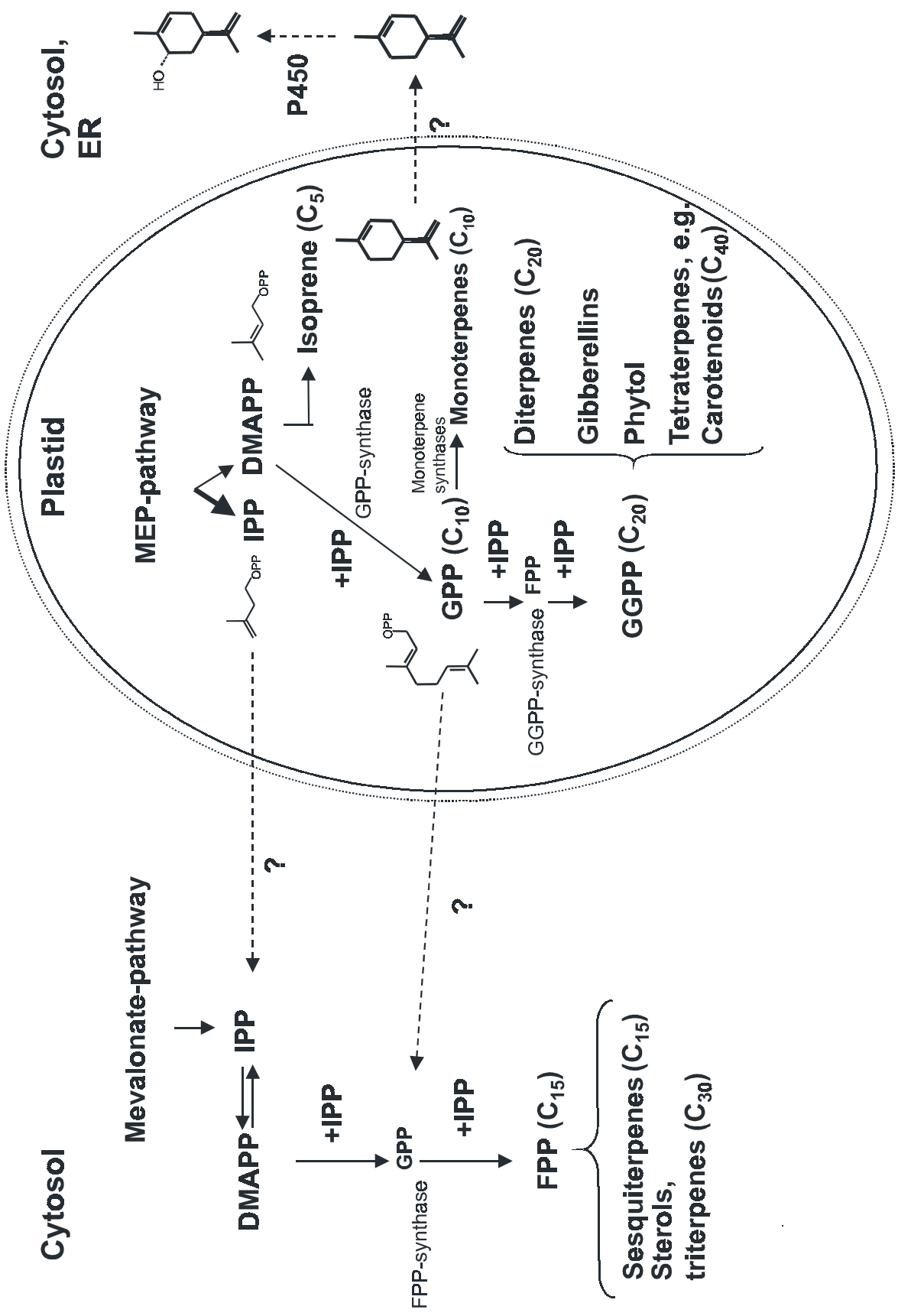
All terpenoid compounds are synthesised from IPP and DMAPP, originating from either of the two pathways outlined above. The head to tail condensation of one molecule of IPP with one molecule of DMAPP yields the C_{10} compound geranyl diphosphate (GPP), which is the immediate precursor of the monoterpenes (Figure 3). The addition of another IPP unit to GPP generates farnesyl diphosphate (FPP), the precursor of sesquiterpenes, triterpenes and sterols. The latter two groups both require the initial condensation of two units of FPP to form squalene. Addition of IPP to FPP provides geranyl geranyl diphosphate (GGPP), the precursor of diterpenes and tetraterpenes consisting of two units of GGPP formed via phytoene. Longer prenyl (allylic) diphosphates are similarly produced by subsequent additions of IPP, as for natural rubber (Poulter and Rilling, 1981). The electrophilic condensation reactions are catalysed by prenyltransferases of which the ones leading to the major terpene classes are specifically named GPP-synthase, FPP-synthase and GGPP-synthase, respectively after the corresponding end products (Gershenzon and Croteau, 1993). The reactions catalysed by these prenyltransferases are believed to be multi-step and sequential in which intermediate elongation products are not released from the enzyme surface in appreciable amounts. Plant

prenyltransferases appear to be similar in size and other properties, require only a divalent metal ion for catalysis and share common primary structural elements including an aspartate-rich motif involved in substrate binding (Ohnuma et al., 1993; Chen et al., 1994). GPP synthase was believed to be limited to plants that produce high quantities of monoterpenes in specialised secretory structures and a gene for GPP-synthase was isolated from the glandular trichomes of peppermint (Haudenschild and Croteau, 1998; Burke et al., 1999; Little and Croteau, 1999). However, a gene for this enzyme was isolated from Arabidopsis, a plant producing little or no monoterpenes at all, indicating that the GPP-synthase is more widespread than previously thought (Bouvier et al., 2000).

Compartmentalisation of terpene biosynthesis

All the enzymes of the MEP pathway leading to IPP and DMAPP that are cloned from plants have plastid targeting signals (Eisenreich et al., 2001; Querol et al., 2002), and also geranyldiphosphate synthase, producing GPP, the general precursor for all monoterpenes, has a plastid targeting signal (Burke et al., 1999; Bouvier et al., 2000). Localisation studies indicated that GPP synthase, DOXP synthase and monoterpene synthases were localised in chloroplasts of leaf parenchyma cells and in leucoplasts of secretory cells found in peltate glandular trichomes and cells surrounding resin ducts and secretory cavities (Turner et al., 1999; Bouvier et al., 2000)(Figure 3). GGPP synthase and the diterpene and tetraterpene biosynthesis derived from the GGPP precursor are localised in the plastids as well. The mevalonate pathway leading to IPP and DMAPP is localised in the cytosol, just like FPP synthase and the sesquiterpene synthases (Figure 3) (Bohlmann et al., 1998). IPP, and possibly also GPP, could be leaking out of the plastid to the cytosol and these plastidic derived precursors can then be used in the subsequent production of FPP by the FPP synthase (McCaskill and Croteau, 1995; Adam and Zapp, 1998) (Figure 3). Enzymes responsible for the hydroxylation of the monoterpenes, the monoterpene P450 hydroxylases, are localised in the ER. This implies that following synthesis in the plastids, transfer of monoterpenes to the cytosol is necessary for further modifications like hydroxylations (Figure 3).

Figure 3. Terpenoid biosynthesis and compartmentation of the different enzymes involved in the different pathways. GPP is synthesised in the plastids via the MEP pathway. Monoterpene synthases are located in the plastids, but subsequent enzymes, like the P450 hydroxylases are located on the ER in the cytosol


Cytosol,
ER
Plastid
Cytosol
Mevalonate-pathway
MEP-pathway
IPP
DMAPP
+IPP
GPP
FPP-synthase
FPP (C15)
Sesquiterpenes (C15)
Sterols,
triterpenes (C30)
GPP-synthase
GPP (C10)
Isoprene (C5)
Monoterpene
synthases
Monoterpenes (C10)
FPP
GGPP-synthase
GGPP (C20)
Diterpenes (C20)
Gibberellins
Phytol
Tetraterpenes, e.g.
Carotenoids (C40)
P450
OPP
HO
?

Terpene synthases

Terpene synthases are enzymes that use the prenyl diphospate precursors to generate the enormous diversity of carbon skeletons characteristic for terpenoids. These hydrocarbon structures can be either acyclic, cyclic or consist of multiple ring systems. The terpene synthases may be involved in the regulation of pathway flux since they function at the metabolic branch points and catalyse the first step leading to the various terpene classes (Alonso and Croteau, 1993; Gershenzon and Croteau, 1993). The terpene synthases are quite similar in properties, and operate with electrophilic reaction mechanisms just like the prenyltransferases (Poulter and Rilling, 1981).

Monoterpene synthases all use GPP as the natural substrate. GPP has a trans C2-C3 double bond, which prevents direct cyclisation of the substrate. Therefore the reaction mechanism involves both isomerisation and cyclisation steps, shown in Figure 4 (Croteau, 1987). GPP ionises with the aid of a bivalent metal ion. The resulting allylic cation-diphospate anion pair then rearranges to form the enzyme-bound tertiary allylic isomer, 3*R* or 3*S*-linalyl diphosphate (LPP), depending on the conformation of the substrate. After rotation to the cisoid conformation, the pyrophosphate group (OPP) is split off and the resulting carbocation can be deprotonated directly, resulting in the formation of myrcene or is cyclised to the corresponding 4*R*- or 4*S*-α-terpinyl cation. From this universal intermediate, the reaction may take several routes involving internal additions to the remaining double bond, hydride shifts, or rearrangements before the terminal carbocation is deprotonated to an olefin or captured by water or the diphosphate anion (Bohlmann et al., 1998). The simplest reaction however is the formation of limonene where the α-terpinyl cation is immediately deprotonated (Rajaonarivony et al., 1992). All monoterpene synthases are capable of catalysing both the isomerisation and the cyclisation reactions, and these steps occur via a series of ion pairs at the active site (Croteau, 1987). Monoterpenes synthases are known to produce multiple products (Wagschal et al., 1991; Rajaonarivony et al., 1992).

Several monoterpene synthases have been partially purified and characterised from angiosperms, gymnosperms and bryophytes (Alonso and Croteau, 1993; Savage et al., 1994; Adam et al., 1996). They have similar properties, native molecular masses of around 50 to 100 kDa (either monomers or homodimers), a requirement for a divalent metal ion, usually Mn^{2+} or Mg^{2+}, a pI value near 5.0 and a pH optimum within a unit of pH7 (Alonso and Croteau, 1993). Monoterpenes are operationally soluble, but they are associated with plastids *in vivo* (Colby et al., 1993; Gershenzon and Croteau, 1993; Bohlmann et al., 1997).

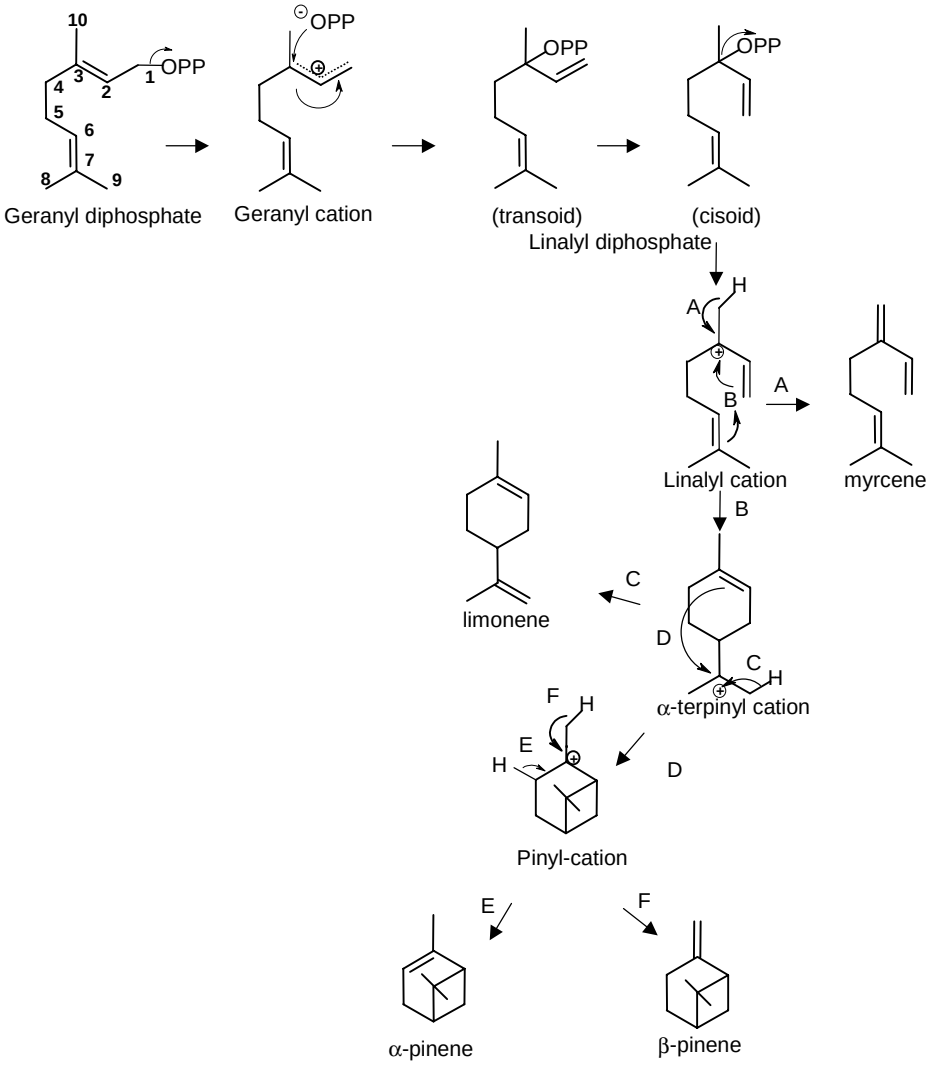


Figure 4. Conversion by monoterpene synthases of GPP, via linalyl diphosphate, to some monoterpene representatives. OPP indicates the diphosphate group (modified after Little and Croteau, 1999).

The overlap in properties of the terpene synthase enzymes of the various classes is reflected in the similarity of their primary structure (Bohlmann et al., 1998). Monoterpene synthases contain an RRX_8W motif, close to the N-terminus, which is necessary for the activity of the monoterpene synthase when it is heterologously expressed in *E. coli*. This motif is thought to be involved in the diphosphate migration step accompanying formation of the intermediate linalyl diphosphate before the final cyclisation step catalysed by the monoterpene synthases

(Williams et al., 1998). The DDXXD motif, present in all terpene synthases and in prenyltransferases, is supposed to bind the bivalent metal ion cofactor, usually Mn^{2+} or Mg^{2+}, and is responsible for the ionisation of the diphosphate group of GPP (Tarshis et al., 1994; Lesburg et al., 1997; Starks et al., 1997; Bohlmann et al., 1998; Rynkiewicz et al., 2002). Terpene synthases are often isolated from cDNA libraries by PCR using degenerate primers based on previously obtained sequence information. However, the function of an isolated terpene synthase cDNA can only be elucidated upon expression in a heterologous system (Bohlmann et al., 1998). Monoterpene synthases are therefore functionally analysed by expression in *E. coli*. Monoterpene synthases are preferably heterologously expressed in a truncated form, without the plastid-targeting signal, since the targeting signal has a negative influence on the enzyme activity (Williams et al., 1998). cDNAs encoding monoterpene synthase enzymes have now been isolated and characterised from a number of gymnosperm and angiosperm species (Colby et al., 1993; Dudareva et al., 1996; Yuba et al., 1996; Bohlmann et al., 1997; Wise et al., 1998; Bohlmann et al., 1999; Jia et al., 1999; Bohlmann et al., 2000; Fischbach et al., 2001; Maruyama et al., 2001b).

The terpene synthase (*Tps*) family

Amino acid sequence relatedness of plant terpene synthases allows the subdivision of the *Tps* gene family into six subfamilies, designated *Tpsa* to *Tpsf*, distinguished by a minimum of 40% identity among the members (Bohlmann et al., 1997; Bohlmann et al., 1998).
Figure 5 shows a phylogenetic tree of the different *Tps* families including the whole family of the recently identified *Arabidopsis Tps* genes (Auburg et al., 2002). The *Tpsa* family consists of angiosperm sesquiterpene synthases. The *Tpsb* family consists of isoprene synthase and most of the angiosperm monoterpene synthases. *Tpsc* consists of copalyl diphosphate synthases. *Tpsd* consists of gymnosperm monoterpene, sesquiterpene and diterpene synthases. *Tpse* consists of kaurene and ent-kaurene synthases. The *S*-linalool synthase that was isolated from *Clarkia breweri* (Dudareva et al., 1996) only shows little similarity to the other isolated monoterpene synthases, and is considered to be of ancient origin (Bohlmann et al., 1998; Cseke et al., 1998). This gene and its *Arabidopsis* homologue are grouped in the *Tpsf* family (Auburg et al., 2002). Gymnosperm terpene synthases are more similar to each other than the gymnosperm monoterpene synthases are to angiosperm monoterpene synthases of the *Tpsb* family and are grouped into the *Tpsd* family. The fact that the similarity between mono-, sesqui- and diterpene synthases in this family is higher than the similarity with enzymes producing the same main products in other families such as *Tpsb*, is a nice example of convergent evolution (Bohlmann et al., 1997; Bohlmann et al., 1998).

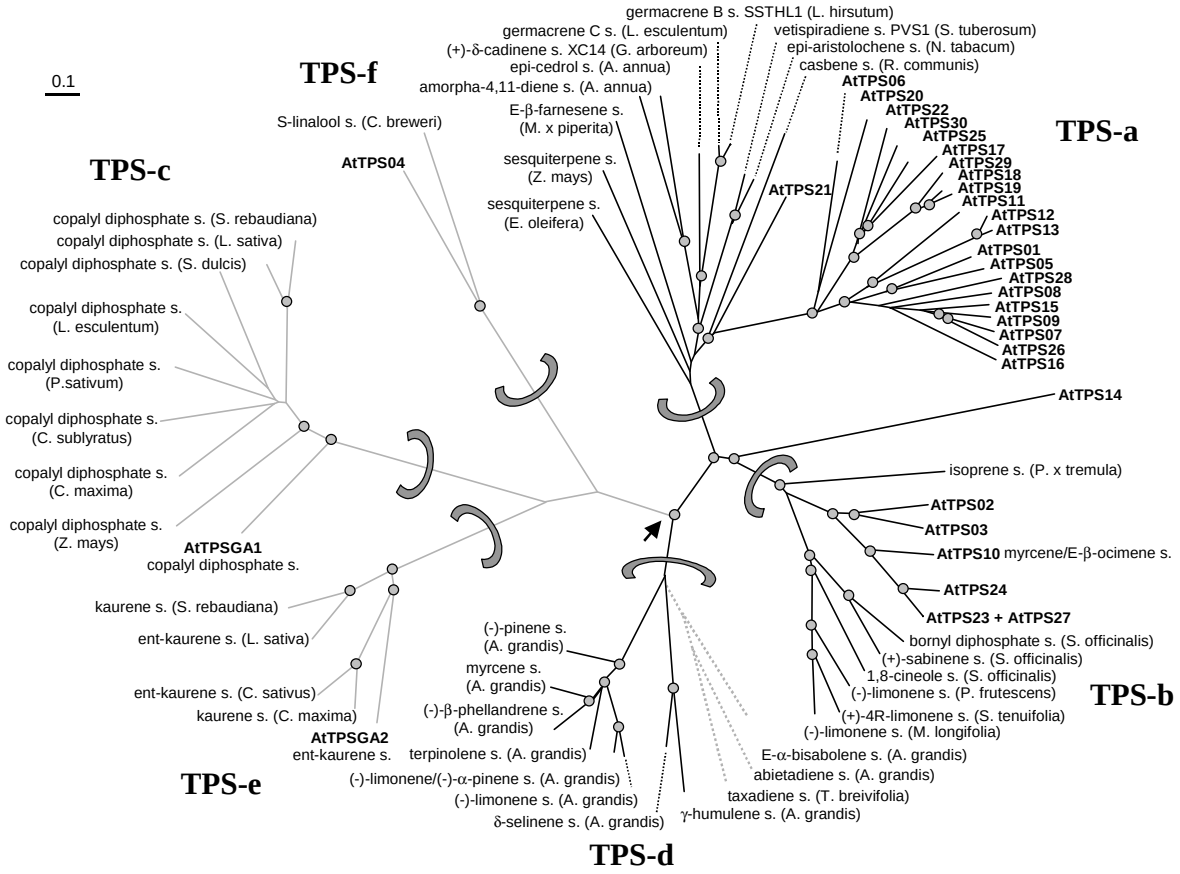


Figure 5. Phylogenetic tree of the TPS family of terpene synthases including all the TPS genes from *Arabidopsis thaliana*. With kind permission of Joerg Bohlmann (Auburg et al., 2002). s.= synthase; AtTPS = *Arabidopsis thaliana* TPS gene.

Secondary modifications of the primary monoterpene olefins

After the formation of the primary terpene skeletons from the prenyl diphosphate substrates by the terpene synthases, a variety of other enzymes can modify these molecules. An important class is formed by the cytochrome P450 monooxygenases that can initiate a whole chain of secondary modifications, mostly consisting of redox reactions and conjugations (Mihaliak et al., 1993). As a result, one parent hydrocarbon structure can lead to the formation of multiple structurally related derivatives bearing an identical oxygenation pattern initially established by the hydroxylase (Karp and Croteau, 1988; Little and Croteau, 1999) (Figure 6).

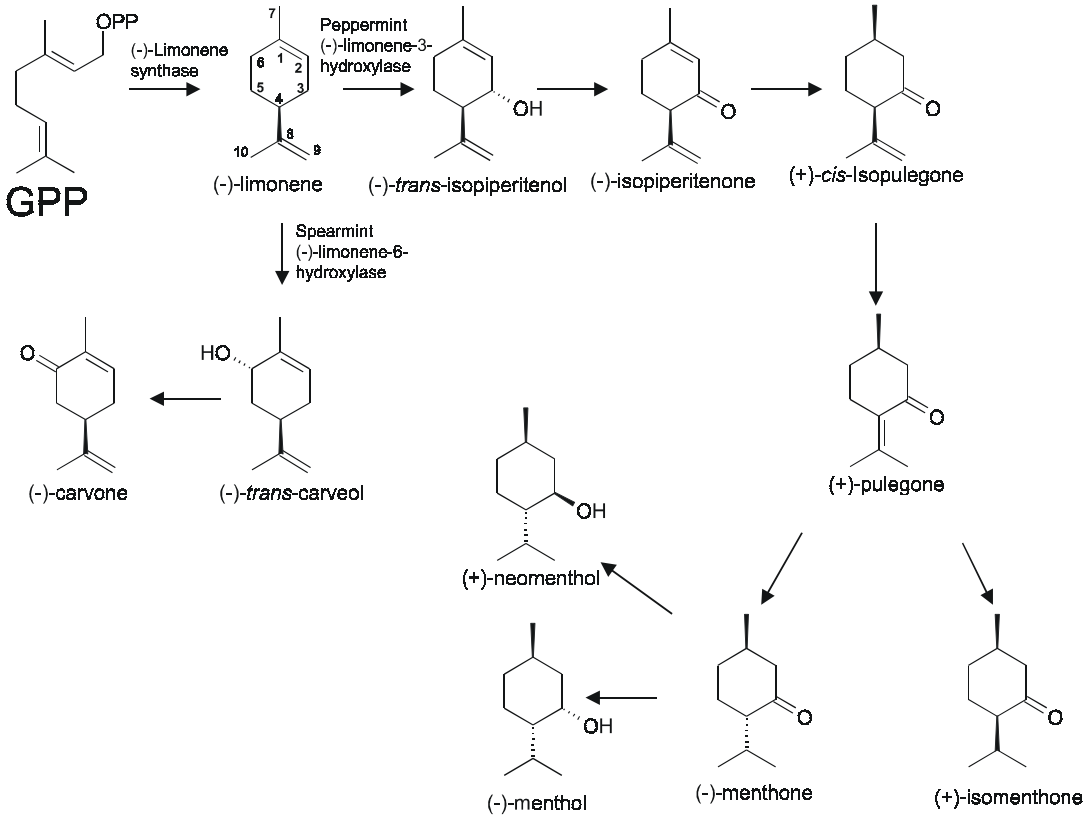


Figure 6. Secondary transformations of (-)-limonene in peppermint and spearmint initiated by regioselective cytochrome P450s.

For example, the cytochrome P450 catalysed hydroxylation at the C10 methyl group of geraniol and nerol is the first step in the biosynthesis of several indole and iridoid alkaloids and valepotiates (Mihaliak et al., 1993). Cytochome P450 hydroxylases are also involved in the hydroxylation of the cyclic monoterpene olefins sabinene (Karp et al.), limonene (Karp et al., 1990; Bouwmeester et al., 1998) (Figure 6), and α-and β-pinene (Karp and Croteau, 1988). Hydroxylation of sesquiterpenes and diterpenes has also been reported (Mihaliak et al., 1993; de Kraker et al., 2001).

Plant cytochrome P450 systems are comprised of a heme containing protein (cytochrome) and a flavoprotein, (NADPH cytochrome P450 reductase) associated with the endoplasmatic reticulum or provacuole. Reducing equivalents from NADPH, molecular oxygen and the substrate interact with the hydroxylase to form the oxygenated terpene product, water and oxidised pyridine nucleotide. During the catalytic cycle, the oxidised form of the cytochrome first binds the substrate, followed by the transfer of one electron from NADPH, via NADPH cytochrome P450 reductase, to reduce the heme iron. The substrate bound to the reduced cytochrome P450 binds a molecule of O_2 to form a reactive complex. A second electron enters the complex via the reductase and the dioxygen is cleaved, resulting in the release of one molecule of water and the hydroxylated product (Mihaliak et al., 1993).

P450 enzymes contain several conserved sequence motifs (Chapple, 1998). The (A/G)Gx(D/E)T(T/S) motif is supposed to form a threonine containing binding pocket for the oxygen molecule required in catalysis (Von Wachenfeldt and Johnson, 1995). A conserved FxxGxxxCxG motif is the supposed heme-binding region (Von Wachenfeldt and Johnson, 1995)

The proline rich region, described as the (P/I)PGPx(G/P)xP motif, probably acts as a hinge, required for optimal orientation of the enzyme in the membrane (Yamazaki et al., 1993). Microsomal P450s contain an N-terminal hydrophobic signal sequence for transfer into the ER, which is not cleaved, but anchored in the membrane. The catalytic domain of the protein is exposed on the cytosolic side of the membrane (Schuler, 1996).

Relatively few genes encoding P450 monoterpene hydroxylases have been described so far (Little and Croteau, 1999). The limonene-3-hydroxylase from peppermint and limonene-6-hydroxylase from spearmint have recently been isolated and show a remarkably high sequence similarity (Lupien et al., 1999). Other cytochrome P450 monoterpene hydroxylase cDNAs that have been isolated are the menthofuran synthase, involved in the production of the undesired monoterpene menthofuran in peppermint (Bertea et al., 2001) and the geraniol-10-hydroxylase from *Catharanthus roseus*, producing a precursor for alkaloid biosynthesis, 10-hydroxy-geraniol (Collu, 1999).

Scope of the thesis

The increasing knowledge about isoprenoid biosynthesis has lead to a number of ideas about the possibilities for genetic modification of monoterpenoid composition and production in plants. These include changes in essential oil characteristics, floral scent profiles, ecological interactions with the environment of a plant and the production of highly valuable metabolites or larger quantities of monoterpenes (McCaskill and Croteau, 1997; Bouwmeester et al., 1998; Haudenschild and Croteau, 1998; McCaskill and Croteau, 1998; Lange and Croteau, 1999).

Moreover, the engineering of secondary metabolism such as monoterpene biosynthesis can also be helpful in increasing our understanding of regulation, flux and fate of biosynthetic processes in plants. Both the commercial and the ecological importance of monoterpenes make a study of the possibilities and restrictions of metabolic engineering of monoterpene biosynthesis worthwhile.

In order to easily study alterations in volatile profiles, an approach was chosen using plants that are easily transformable and for which studies have already been carried out on the floral scent emission. During the years that the work for this thesis was achieved, other groups have concurrently worked on the metabolic engineering of monoterpene biosynthesis. Their results will be discussed in Chapter 6.

Petunia hybrida W115 was chosen as a model system to introduce monoterpene biosynthesis in a plant with no detectable monoterpene production. Transformation of petunia with *S*-linalool synthase from *Clarkia breweri* flowers was chosen as a proof of concept to investigate if GPP was present and available as a precursor for linalool production. The *S*-linalool synthase cDNA (*lis*) was introduced in the plants under regulation of the constitutive CaMV d35S promoter to achieve high expression in all tissues. This work is described in Chapter 2.

From a cDNA library made of lemon (*Citrus limon* L. Burm. f.), an important essential oil producing plant, four new cDNA encoding monoterpene synthases were isolated and heterologously expressed in *E. coli*. The isolation, characterisation and the identification of the product specificity of these enzymes is described in Chapter 3.

Tobacco does emit volatile monoterpenes (Andersen et al., 1988; Loughrin et al., 1990), and was therefore chosen as a model for the modification of the emission pattern of monoterpenes from a plant. An important question was whether there is, in addition to the GPP that is used for monoterpene production in the wild type, a sufficient amount of GPP available for production of new monoterpenes and if newly introduced enzymes can successfully compete with endogenous enzymes.

Three of the lemon monoterpene synthase enzymes responsible for the production of different monoterpene main products were transformed to common tobacco (*Nicotiana tabacum* 'Petit Havana' SR1). They were combined into one plant by crossing to obtain a plant with maximal usage of the GPP pool, which is described in Chapter 4.

Since secondary modifications of monoterpenes are essential to obtain the large variety of monoterpenoids in plants, we wanted to investigate whether it was feasible to add a second step to the pathway already introduced in tobacco. To achieve this, the pathway that was introduced into the plastids of tobacco was extended into the cytosol using a cytochrome P450 to hydroxylate the monoterpenes formed in the primary transgenic tobacco plant possessing the three additional monoterpene synthases. This research is described in Chapter 5.

Finally, in Chapter 6, the general discussion describes an overview of the new insights that are obtained from our experiments. Furthermore some suggestions are given for future work.

Chapter 2

Expression of *Clarkia S*-linalool synthase in transgenic petunia plants results in the accumulation of *S*-linalyl-β-D-glucopyranoside

Joost Lücker[1], Harro J Bouwmeester[1], Wilfried Schwab[2], Jan Blaas[1], Linus H W van der Plas[3], Harrie A Verhoeven[1]

[1] Business unit Cell Cybernetics, Plant Research International, P.O. Box 16, 6700 AA, Wageningen, The Netherlands,

[2] University of Würzburg, Chair of Food Chemistry, Am Hubland, 97974, Würzburg, Germany

[3] Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD, Wageningen, The Netherlands

Published in the Plant Journal (2001) **27** (4) 315-324

Summary

Petunia hybrida W115 was transformed with a *Clarkia breweri S*-linalool synthase cDNA (*lis*). *Lis* was expressed in all tissues analyzed and linalool was detected in leaves, sepals, corolla, stem and ovary, but not in nectaries, roots, pollen and style. However, the *S*-linalool produced by the plant in the various tissues is not present as free linalool, but was efficiently converted to non-volatile *S*-linalyl-β-D-glucopyranoside, by the action of endogenous glucosyltransferase activity. The presented results demonstrate that monoterpene production can be altered by genetic modification and that the compounds produced can be converted by endogenous enzymatic activity.

Introduction

Isoprenoids are the largest family of natural compounds, and are based on varying numbers of C_5 isoprene units. In plants they play important roles: for example carotenoids are involved in photosynthesis and mono- and sesquiterpenoids are important in cell to cell interactions and in communication between organisms. Monoterpenes, the C_{10} branch of the isoprenoid family, were first investigated for their economical importance as flavor and fragrance additives in foods and cosmetics. Anti-carcinogenic effects (Crowell and Gould, 1994; Mills et al., 1995; Yu et al., 1995), antimicrobial (Belaiche et al., 1995) and antifungal properties (Vaughn and Spencer, 1991)have also been demonstrated for some monoterpenes. They have also been shown to be of ecological significance (Harborne, 1991), for instance in the interaction between plants, plants and insects and plants and microorganisms. Commonly used monoterpenes are often produced by chemical synthesis, but at present interest for naturally produced monoterpenes is increasing. Plants producing monoterpenes have been investigated in more detail and this has resulted in a better understanding of the biochemical pathways leading to the formation of monoterpenes and their derivatives.

Linalool is an acyclic monoterpene alcohol that has a peculiar creamy floral, sweet taste (Arctander, 1969). In *Clarkia breweri* linalool, among other compounds, is responsible for the attraction of pollinating moths (Raguso and Pichersky, 1995). Linalool can also act as a repellent against aphids, as shown in a study with *Myzus persicae* (Hori, 1998); it is also one of the volatile compounds released as a semiochemical after herbivore attack in some plant species (Rose et al., 1996; Pare and Tumlinson, 1997; Weissbecker et al., 1997) and as such may attract predators of the herbivores.

Many monoterpenes have been reported to be detrimental to biological structures (Weidenhamer et al., 1993). Linalool for example, softens the structure of potato tubers incubated under a continuous flow of the compound (Vaughn and Spencer, 1991). When not emitted, linalool, like other terpenols, can be detoxified in many plant species by conjugation for example as a glycoside of β-D-glucose or a disaccharide. In this form it could also be transported via the phloem to other plant tissues (Raguso and Pichersky, 1999). In a later stage, bound linalool could still be emitted by the action of glycosidase enzymes (Raguso and Pichersky, 1999).

Genes encoding enzymes catalysing the biosynthesis of the monoterpenes *S*-linalool (Dudareva et al., 1996; Cseke et al., 1998), *R*-linalool (Jia et al., 1999), (-)-(4*S*) limonene (Colby et al., 1993; Bohlmann et al., 1997), myrcene, (-)-(1*S*,5*S*)-pinene (Bohlmann et al.,

1997), myrcene and (*E*)- β-ocimene (Bohlmann et al., 2000), (+)bornyl diphosphate, 1,8-cineole, (+)-sabinene (Wise et al., 1998), (-)-β-phellandrene, (-)-camphene, terpinolene and one making (-)-limonene and (-)-α-pinene (Bohlmann et al., 1999) have been isolated in the past few years from a number of plant species. The enzymes leading to the production of precursors for the synthesis of the primary monoterpene skeletons all appear to be active in the plastids, as all genes of this pathway that have been cloned to date have plastid-targeting signals (Haudenschild and Croteau, 1998). Recently they have been located in leucoplasts of secretory cells (Turner et al., 1999) and in chloroplasts of parenchyma cells (Bouvier et al., 2000). Partitioning to other cellular compartments of the precursors formed has been shown to occur (Schwarz, 1994; Arigoni et al., 1997; Adam and Zapp, 1998).

Increasing knowledge about isoprenoid biosynthesis has lead to various ideas on the genetic modification of monoterpenoid composition and production in plants. Now there are opportunities to alter essential oil characteristics, floral scent profiles, ecological interactions with the environment of a plant and production of larger quantities of highly valuable monoterpenes (McCaskill and Croteau, 1997; Haudenschild and Croteau, 1998; McCaskill and Croteau, 1998; Lange and Croteau, 1999). Recently peppermint plants have been transformed with limonene synthase but no drastic alterations were found in the terpenic profile (Krasnyanski et al., 1999). We are still unable to predict interactions between an inserted gene and the host genome and its subsequent influence on host metabolism (Buiatti and Bogani, 1995). Therefore we investigated monoterpene production in a model system, for use as a proof of concept of metabolic modification. *Petunia hybrida* W115 is an easily transformable plant (Horsch et al., 1985) that contains virtually no monoterpenes in floral tissues or any other organs (Dr. C.H.R. de Vos, unpublished data), as determined by gas chromatography-mass spectrometry (GC-MS) studies. This means that after the introduction of genes encoding enzymes from the monoterpene biosynthetic pathway, all monoterpenes detected will be the result of the genetic modification. This paper shows that *Petunia hybrida* W115 transformed with the *Clarkia breweri S*-linalool synthase cDNA under control of a constitutive double enhanced CaMV 35S promoter produces *S*-linalool. However the plant does not store or release the *S*-linalool but converts it into a non-volatile form, namely *S*-linalyl-β-D- glucopyranoside.

Results

Introduction of linalool synthase cDNA (*lis*) into Petunia hybrida W115

Wild type petunia W115 plants were transformed with a cDNA encoding linalool synthase under control of the CaMV d35S promoter. All regenerated PCR positive plants were transferred to greenhouse conditions for further examination. Transgenic plants exhibited normal development, compared with non-transformed and empty vector control plants, which went through the same regeneration process.

Molecular and genetic analysis of transgenic plants

The number of inserts of *lis* was determined by Southern blotting. No signal was detected in the control plants. Of the *lis* transformed plants, lines 2, 3, 17, 19, 22, and 24 showed a single gene insert (data not shown).

In a seed plating experiment, after self-pollination of the primary transformed plants and the control, all seeds of the non-transformed control were sensitive to kanamycin. The seeds of the transformed plant lines 17 and 24 exhibited the expected mendelian 1:3 segregation for a single gene insertion, assuming that the introduced kanamycin resistance gene, included on the T-DNA of the binary vector used for transformation, is co-localised with the *lis* gene in the genome.

Total RNA was extracted from young leaves of two non-transformed plants, 10 plants transformed with an empty binary vector, and 21 plants transformed with *lis*. Figure 1 shows that linalool synthase expression in a subset of five transformed plants varied strongly between the various independent transformants. The presence of the *lis* transcript was also detected by Northern blotting of the progeny of the plant lines 17 and 24, showing that the gene was stably integrated (data not shown).

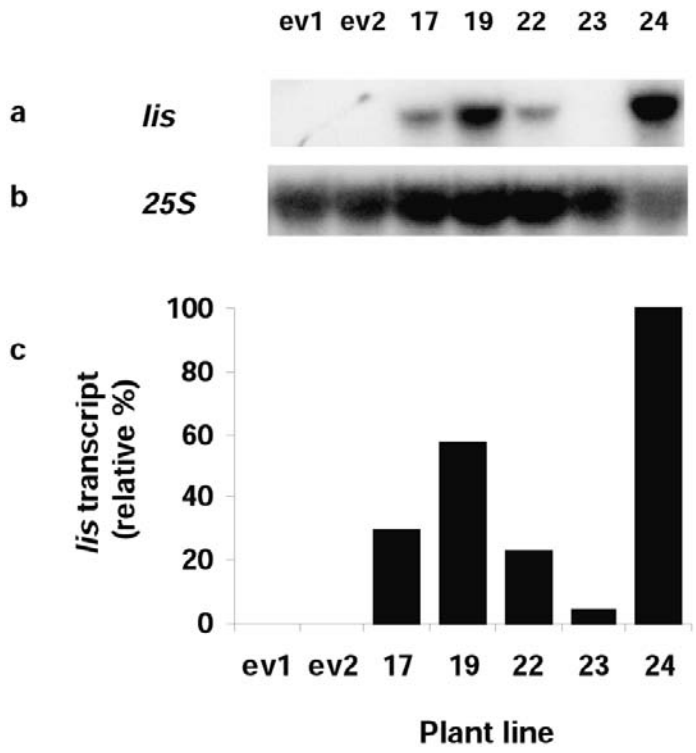


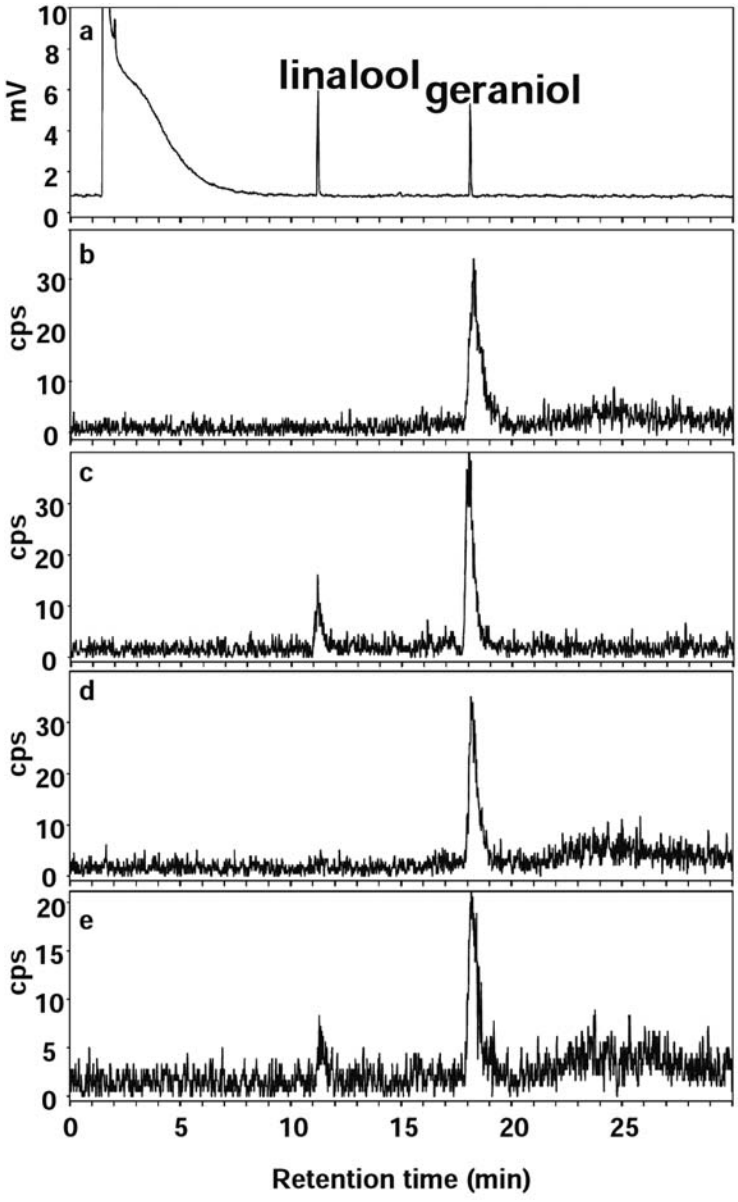
Figure 1. Northern blot analysis of linalool synthase mRNA for leaves of different transgenic plant lines: a) Hybridization with linalool synthase cDNA (*lis*). b) The same blot hybridised with ribosomal DNA (25S). c) Quantified expression levels using densitometry (standardised for ribosomal DNA). ev1, ev2: plants transformed with an empty binary vector. 17, 19, 22, 23 and 24: independent transgenic plant lines.

Enzyme activity

In order to determine whether the linalool synthase gene was properly transcribed and processed to a mature protein, crude enzyme extracts were prepared from the corolla and leaves of control and transgenic petunia plants. Linalool synthase activity was detected in corolla (1 day after anthesis) and young leaves (leaves 1-4 from the top of non-flowering plants) of transformed petunia plants, at levels of 2.3 nmol g^{-1} fresh weight h^{-1} and 4.9 nmol g^{-1} fresh weight h^{-1} respectively, while no activity was found in extracts from control plants (Figure 2). Despite the presence of the inhibitor sodium orthovanadate, considerable phosphohydrolase activity still remained, as is clear from the presence of geraniol in all enzyme assays, resulting in a non-linear assay. In a crude extract from older leaves (mature leaves on branches of a flowering plant), no linalool synthase activity could be detected in

either control or *lis* transformants. It is not clear whether this is due to a lower linalool synthase activity or the result of an even higher phosphohydrolase activity (data not shown).

Figure 2. Radio-gas chromatographic analyses of radiolabelled products formed from 10 µM [^{3}H]-geranyl diphosphate (GPP) by enzyme preparations of leaves and flowers of wild-type and transgenic petunia. a: Flame ionisation detector (FID) response to co-injected, unlabelled standards of linalool and geraniol; b-e: radio-traces showing radio-labelled products of assays with leaves of non-transgenic control (b) and transgenic plant line 17 (c) and flowers of control (d) and plant line 17 (e). cps: counts per second.



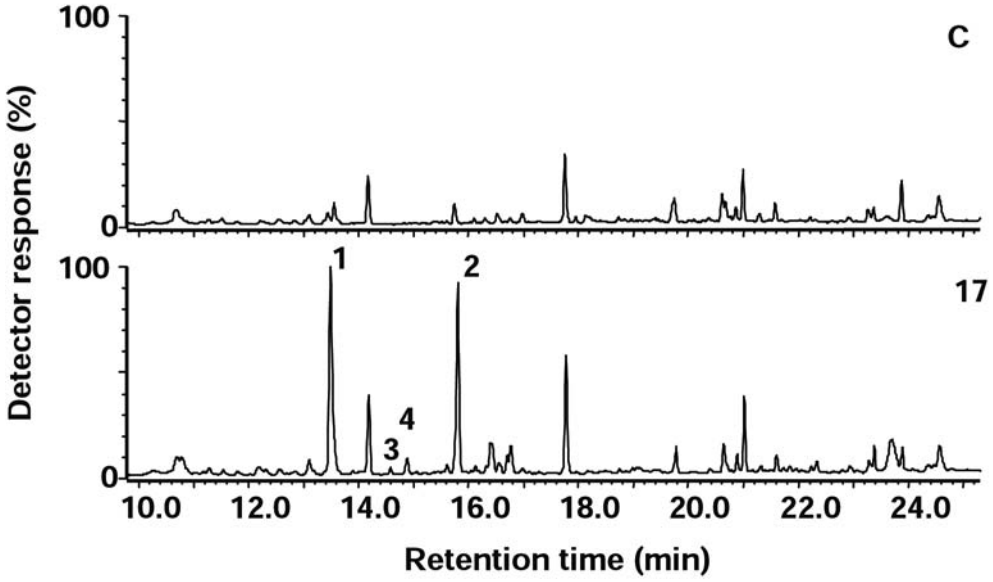


Figure 3. Typical GC-MS chromatograms obtained by headspace solid phase micro extraction (SPME) on $CaCl_2$ extracts of (C) young leaves of W115 control and (17) young leaves of transgenic plant line 17. The total ion count (TIC) spectrum is shown as a relative scale (100% scale = 5.8 e^6 ions). (1): linalool, (2): α-terpineol. Peaks 3 and 4 are unidentified trace peaks.

Expression of the *lis* gene and volatile production

Gas chromatography-mass spectrometry (GC-MS) analysis of the headspace of the flowers and leaves on intact transgenic petunia plants using solid phase micro extraction (SPME) resulted in detection of a trace level of linalool in the flowers. No linalool was detected in the leaf tissue (data not shown). However, linalool could be detected in the transgenic leaf tissue by homogenising frozen tissue with a saturated calciumchloride ($CaCl_2$) solution in water and subsequent GC-MS analysis of the headspace above the mixture using SPME. A typical chromatogram of young leaves of transgenic plant line 17 and a non-transformed control plant C, using the $CaCl_2$ extraction method is shown in Figure 3. In the control plant, only a trace of monoterpenes could be detected. In the transgenic plant 17 linalool is detected (peak 1) together with α-terpineol (peak 2), at similar levels.

From a non-transformed control plant and plant 17, roots, stem, leaf, flower bud, open flower, corolla, pollen, styles, sepals, ovary and nectaries were isolated for both RNA expression and SPME/ GC-MS analysis, as described under Experimental procedures. In the control plant no expression of *lis* and only trace amounts of monoterpene products could be detected. Figure 4 shows that in the transgenic plant line 17, *lis* was expressed in all tissues analysed, although

the level of expression strongly varied between the tissues. The monoterpenes linalool and α-terpineol were detected in stem, leaf, flower bud, open flower, corolla, sepals and ovary but not in roots, pollen, styles and nectaries.

In a similar experiment expression levels in leaves of different transformed plant lines were compared with levels of detected monoterpenes. The monoterpene levels detected in the leaves of the transgenic lines were high, between 1-5 μg g^{-1} fresh weight (8-40 μM), but showed large variation between samples from the different plants and did not seem to correlate with the expression levels (data not shown).

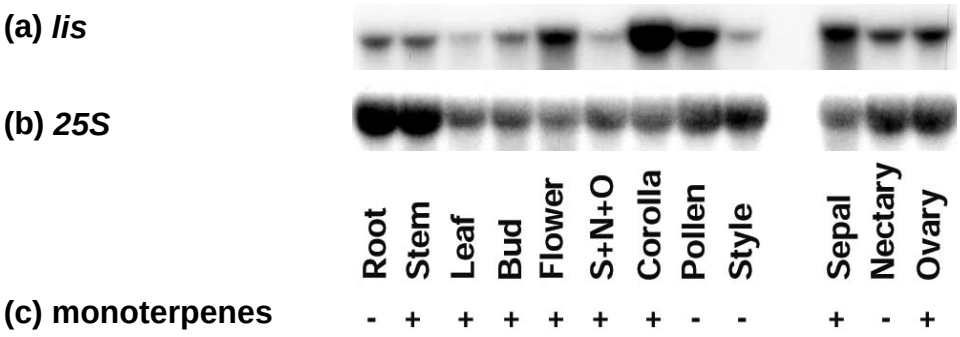


Figure 4. Northern blot analysis of tissues of transgenic plant line 17, showing expression of linalool synthase and subsequent GC-MS analysis for the presence of linalool: (a) Hybridization with linalool synthase cDNA (*lis*), (b) Hybridization with ribosomal DNA (*25S*). (c) Presence (+) or absence (-) of monoterpenes (linalool and α-terpineol) by GC-MS analysis.

Conjugation of linalool

Storage of linalool as a conjugate could explain the absence of linalool emission from the transgenic plants. To investigate the presence of conjugates, leaf tissue was incubated with several hydrolysing enzymes. Free linalool, and no α-terpineol, was detected after incubation with an almond β-glucosidase enzyme and also with Rohapect 7104, but at a level about fivefold lower. The Rohapect enzyme mixture mainly contains β-glucosidase activity, hence the results indicate the presence of linalool as a glucoside in the transgenic petunia. In control tissue, release of linalool could not be detected, with any enzyme treatment. Addition of 5M $CaCl_2$ solution to the β-glucosidase assay on the transgenic tissue resulted in additional

release of linalool but also α-terpineol. Apparently the β-glucosidase enzyme did not release all the linalool from the transgenic tissue after overnight incubation (data not shown).

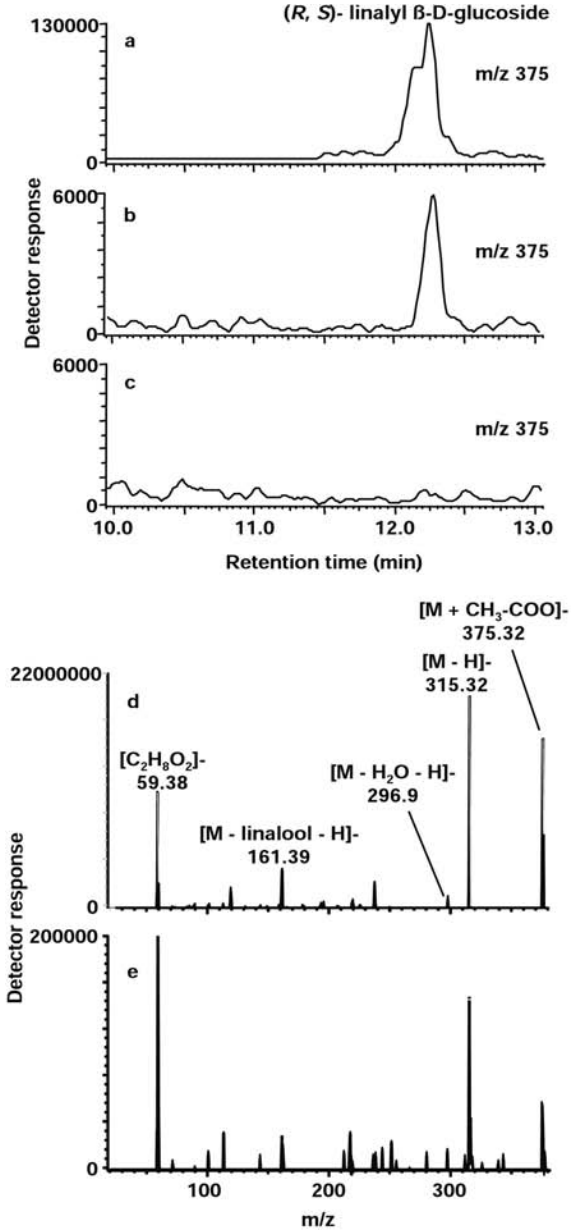
Identification of the glucoside in petunia leaf tissue

Linalyl-β-D-glucopyranoside was synthesised in order to verify the identity of the putative glycoside present in the transgenic petunia tissue. Subsequent HPLC-MS/MS analysis on control and transgenic petunia tissue as shown in Figure 5, revealed that the m/z 375 ion trace of the compound detected in the transgenic petunia tissue (Figure 5b) had the same retention time as one of the two diastereomers of the synthesised reference (*R*,*S*)-linalyl-β-D-glucoside; these diastereomers are slightly resolved in Figure 5a. Figure 5 shows that also the product ion spectrum of the synthesised reference compound (Figure 5d) is the same as the spectrum of the peak detected in the transgenic petunia tissue (Figure 5e). The control petunia tissue ion trace m/z 375 (Figure 5, c) showed only a minor peak at the retention time of the linalyl-β-D-glucoside, indicating that there might also be a trace of linalyl-β-D-glucoside in the control plants. However, this putative peak could not be identified as it was below the limit of detection of 0.5 μg g^{-1}. In the transgenic petunia leaf tissue the concentration of the linalyl-β-D-glucoside was estimated to be around 5-10 μg g^{-1} (12-27 μM).

Chiral phase multidimensional gas chromatography mass spectrometry (MDGC-MS) analysis, after enzymatic hydrolysis of the highly concentrated glycoside fraction of leaf tissue (Figure 6), revealed that the transgenic petunia leaf contains highly enriched *S*-linalyl-β-D-glucoside, shown by the vast abundance of *S*-linalool detected in the m/z 93 spectrum. The hydrolysed glycoside fraction of the control plant appeared to contain low levels of slightly more *R*-linalool as opposed to *S*-linalool.

Finally we also checked incubation of the synthesised linalyl-β-D-glucopyranoside with a 5M $CaCl_2$ solution. This resulted in the release of linalool and α-terpineol, showing that the $CaCl_2$ solution is indeed responsible for the release of α-terpineol from the linalyl-glycoside present in transgenic tissues.

Figure 5. Identification of linalyl-β-D-glucopyranoside in petunia tissue using HPLC-MS/MS. a): Ion trace m/z 375 of the synthesised (*R*,*S*)-linalyl-β-D-glucopyranoside, b): Ion trace m/z 375 of the transgenic petunia leaf tissue, c): Ion trace m/z 375 of the control petunia leaf tissue, d): Product ion spectrum of the synthesised (*R*,*S*)-linalyl-β-D-glucopyranoside and e): the compound isolated from the transgenic petunia tissue. Retention time and product ion spectrum of the synthesised (*R*,*S*)-linalyl-β-D-glucopyranoside are the same as for the compound detected in the transgenic petunia tissue.



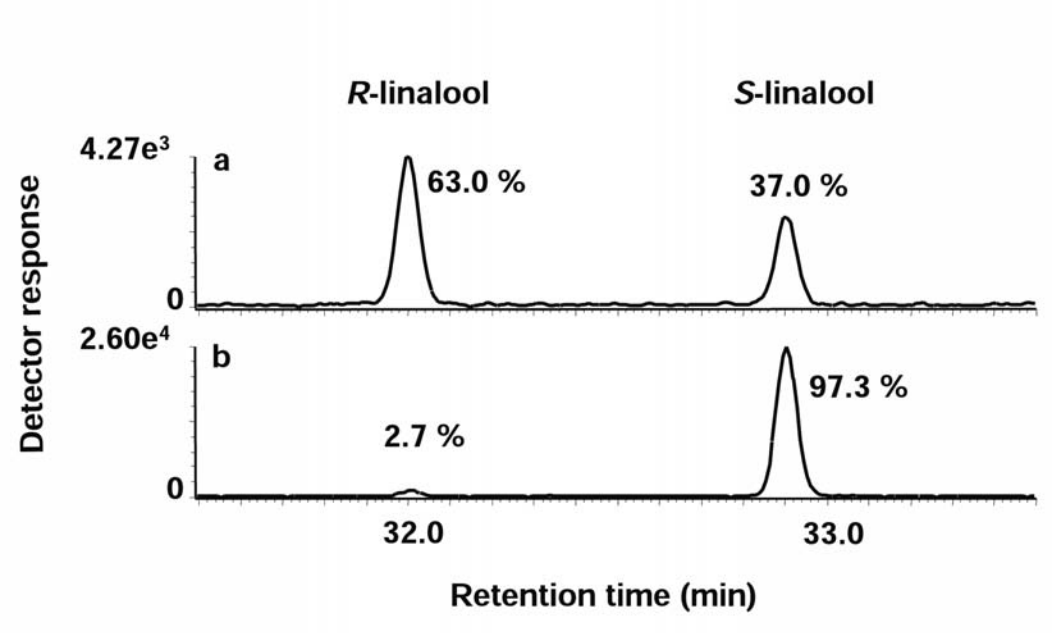


Figure 6. Determination of the enantiomeric distribution of *S*- and *R*-linalool after enzymatic hydrolysis of the concentrated glucoside fraction obtained from plant tissue using chiral phase MDGC-MS analysis. a: m/z 93 spectrum of control plant; b: m/z 93 spectrum of transgenic plant. The transgenic plant accumulates highly enriched *S*-linalyl-β-D-glucopyranoside. m/z 93 is a typical m/z value for many monoterpene mass spectra including linalool and α-terpineol.

Discussion

Genetic modification of *Petunia hybrida* W115 with the *S*-linalool synthase cDNA from *Clarkia breweri* (Dudareva et al., 1996) resulted in the expression of a functional enzyme. The linalool produced by this enzyme in the transgenic plants was verified to be the *S*-enantiomer and was predominantly glucosylated, as established by the molecular and biochemical analysis. Instead of emitting the produced volatile *S*-linalool, the plants are storing it as *S*-linalyl-β-D-glucoside. Differences between organs in the production of linalool or its glycoside (Figure 4) suggest that biosynthesis depends more on the availability of the substrate GPP than on expression of the *lis* gene. However the expression of *lis* under control of the CaMV-35S promoter can also be highly variable within a tissue of the transgenic plant, as has been shown previously for petunia plants with this promoter under control of a luciferase reporter system (van Leeuwen et al., 2000). The lack of correlation between expression of *lis* and levels of monoterpenes detected with SPME/GC-MS in leaf tissue between different plant lines might be explained in part by differences in environmental conditions, but is most probably due to the continuous production of *S*-linalool and the highly efficient conversion into the non volatile *S*-linalyl-β-D-glucoside. The level at which the

glucoside is accumulated in the leaf is around 5-10 μg g^{-1} (12-27 μM), which is more than 1000 fold higher as what was detected when a fungal sesquiterpene cyclase was expressed in transgenic tobacco (Hohn and Ohlrogge, 1991). Apart from biosynthesis of GPP and linalool synthase, the tissue content of linalool depends on emission, storage in tissues, conjugation reactions and possible degradation or even transport.

The linalool synthase gene has been described to contain a putative plastid-targeting signal (Cseke et al., 1998) that was included in the construct transformed into petunia. Although correct targeting would result in the localisation of the active linalool synthase enzyme in the plastids, which are the most likely source for monoterpene precursors in the form of GPP, mainly synthesised via 2-C-Methyl-D-erythritol-4-phosphate in the MEP-pathway (Eisenreich et al., 1997), this has not been determined in the transgenic petunia. The presence of trace amounts of linalyl-glucoside in the control petunia plant indicates that GPP is available and that there is a very low level of linalool synthase activity in the control plants (Figures 5 and 6).

The emission rate of linalool from the transgenic plants is expected to be extremely low, as only traces of linalool were detectable when the headspace of the transformed plants was analysed (data not shown). Volatilisation only occurred from the flowers and not from leaves.

Storage of linalool may occur in a hydrophobic cell compartment, as in *Mentha*, where the terpenes are secreted to the extracellular space between the cell membrane of the secretory cells and the cuticle (Gershenzon et al., 1989). Intracellular storage may occur in lipophilic vesicles, or the component may be chemically modified to become hydrophilic, e.g. by glycosylation, in order to prevent cell membrane damage by the highly reactive monoterpenes (Stahl Biskup et al., 1993). In petunia tissue the linalool might also cause cell membrane damage if it was present in a free form. As no tissue-specific promoter for expression was used, the enzyme can be formed in all plant organs and will give a product in all cells where GPP is present. By the action of an endogenous glycosyltransferase that is able to efficiently conjugate the *S*-linalool produced by the transgenic plants to *S*-linalyl-β-D-glucoside, cellular damage can be prevented. Such a highly active glycosyltransferase was also reported in transgenic kiwi fruit expressing stilbene synthase, leading to the accumulation of picied, a resveratrol-glucoside instead of resveratrol (Kobayashi et al., 2000). It is not clear whether the enzymatic activity in petunia responsible for the glycosylation of linalool represents a constitutive activity or an activity that is induced in transgenic plants by the presence of phytotoxic linalool.

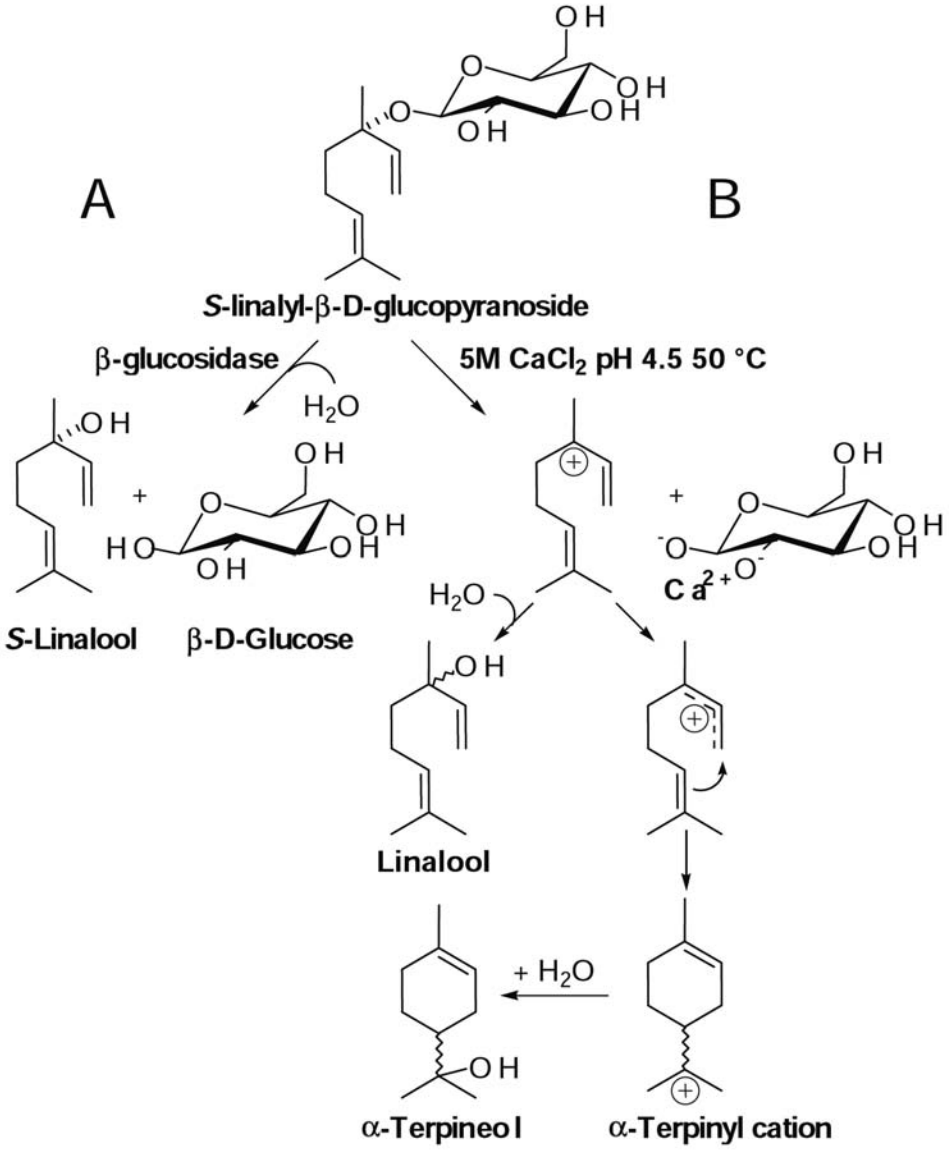


Figure 7. Reaction scheme of how *S*-linalyl-β-D-glucopyranoside is hydrolysed by (A) β-glucosidase and by (B) $CaCl_2$. Hydrolysis in a saturated $CaCl_2$ solution will result in the formation of racemic linalool and α-terpineol, via a putative carbocation, while hydrolysis by the β-glucosidase enzyme results in the formation of only the specific enantiomer.

Introduction of the linalool synthase in petunia may result in a redirection of the flux of the isoprenoid precursors dimethylallyldiphosphate and isopentenyldiphosphate towards GPP, thus competing with the formation of FPP and GGPP, which are required for the production of sesquiterpenes, sterols, diterpenes, carotenoids, gibberellins and higher terpenes such as ubiquitin. A depletion in some of these isoprenoids could have a dramatic effect on the plant phenotype, as was the case when a phytoene synthase gene was overexpressed in tomato, and depletion of the gibberellin pathway occurred, resulting in a dwarf phenotype (Fray et al.,

1995). In our plants however, no obvious phenotypic changes were visible, suggesting that the production of linalool did not result in drastic changes in the flux to other vital pathways.

In the transgenic petunia tissue samples that were measured using a saturated $CaCl_2$ solution, α-terpineol was always detected along with the expected linalool. Incubation of the synthesised linalyl-glucoside standard with a $CaCl_2$ solution also resulted in the formation of linalool and α-terpineol in a similar ratio as with the transgenic plant extracts. So the formation of α-terpineol is merely a result of hydrolysis of the linalyl-β-D-glucopyranoside in the $CaCl_2$ solution, and not caused by any other factor. Figure 7 explains how linalool and α-terpineol could be formed during incubation in $CaCl_2$ solution via a putative carbocation intermediate. Measurements using saturated $CaCl_2$ solution during volatile detection appeared to be an efficient way to release glycosidally bound volatiles.

We have shown convincingly that petunia transformed with *S*-linalool synthase produces *S*-linalool and that most of it is directly conjugated to linalyl-β-D-glucopyranoside. There are indications that this efficient glycosylation of the monoterpenol is not common to all plant species and this question is currently under investigation. The combination of endogenous glycosyltransferase activity and the introduction of the *lis*-gene leads to exciting new opportunities, not only to produce terpenoids, but also for accumulation and non-toxic storage of the components produced in suitable plants.

Experimental procedures

Plant material and transformation

The *Petunia hybrida* variety W115 was used for all experiments. Plants were grown in a greenhouse at 18°C with 18/6 h light/dark conditions, propagated using cuttings, and selfed for progeny analysis by controlled pollination.

A *Clarkia breweri* (Gray) Greene (*Onagraceae*) pBluescript cDNA clone, *lis*, encoding linalool synthase, was kindly provided by Dr. E. Pichersky and was used for all experiments. The linalool synthase *BamH*I, *Sal*I cDNA insert was ligated (Sambrook et al., 1989) with T4-DNA ligase (Life Technologies B.V., Breda, The Netherlands) between the *BamH*I site of an enhanced CaMV-35S promoter and the *Sal*I site before a Nos terminator sequence of a pFlap10 vector (kindly provided by Dr. A. Bovy, Plant Research International). The ligation product was transformed to *E. coli DH5*α competent cells and transformed colonies were grown for 16 h at 37°C and 250 rpm on a rotary shaker. The expression cassette was removed from the resulting vector by using *Pac*I and *Asc*I restriction enzymes (NEB, Hitchin,

Hertfordshire, England) and ligated into the binary vector pBinPLUS (van Engelen et al., 1995), containing a kanamycin resistance selection marker (*npt*II), after digestion with *Pac*I and *Asc*I. Colonies were checked after transformation by back-transformation to *E. coli DH5α* competent cells. Leaf cuttings of petunia W115 were transformed with *Agrobacterium tumefaciens* strain LBA4404 using a standard plant transformation protocol (Horsch et al., 1985). Regeneration of the transformants was performed as described previously (van Tunen et al., 1990).

As control, leaf cuttings were also transformed with LBA4404 containing the original pBinPLUS vector. Some non-transformed leaf cuttings were also taken through the regeneration process. Rooting plants, arising from the *lis Agrobacterium* transformation were tested with PCR for the presence of the gene. Positive plants, pBinPLUS transformed and non-transgenic plants were transferred to the greenhouse.

Northern and genetic analysis

Total RNA was isolated from petunia leaf and several other tissues as described previously (Verwoerd et al., 1989). For RNA gel blot analysis 10 µg of total RNA was denatured for 1 h at 50°C in 15 mM sodium phosphate buffer, pH 6.5, 1.7 M glyoxal (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 50% dimethylsulfoxide (DMSO) prior to electrophoresis. RNA was separated on 1.3% agarose gels in 15 mM sodium phosphate buffer, pH 6.5. The gels were blotted with 25 mM sodium phosphate buffer onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands). Labelling of cDNA was done using the RadPrime DNA labeling system (Life Technologies). RNA gel blots were hybridized with a [α-^{32}P]ATP labelled *Bam*HI-*Sal*I fragment of 2.7kb of the *lis* cDNA insert. Equal loading of RNA in the gel slots was verified by hybridization with a [α-^{32}P]ATP labelled 1.2kb fragment of a 25S ribosomal cDNA from potato (kindly provided by Dr. D. Florack, Plant Research International). Hybridisation was performed for 16 to 20 h, at 65°C in 1 M NaCl, 1% (w/v) SDS and 10% dextran sulphate. The membranes were washed two times for 30 min at 65°C in 0.1x SSC, 0.1% (w/v) SDS and exposed to an autoradiographic film (Fuji, Japan) at –80°C.

Quantification of mRNA abundance of the *lis* gene on the Northerns was done using bio-imaging plates that were developed in a FUJIX BAS2000 bio-imaging analyser (Fuji, Japan) and analyzed using TINA software (Raytest, Straubenhardt, Germany). The results were standardised using the 25S ribosomal transcript levels.

Plants were selfed and backcrossed with the wild type W115 plant. Seeds were stored dry for one month before sowing. 100 seeds of non-transgenic control plant, and two transgenic plants with one insertion of the *lis* cDNA, were plated on solid MS-medium with 10 g l^{-1} sucrose including 100 mg l^{-1} kanamycin to select for transgenics.

Isolation of linalool synthase activity and product identification

During enzyme isolation and preparation of the assays, all operations were carried out on ice or at 4°C. Frozen tissues were ground in a pre-chilled mortar and pestle: leaves (1.5 g) in 7 ml of buffer, flowers (2.4 g) in 9 ml of buffer containing 50 mM Mopso (pH 6.8), 20% (v/v) glycerol, 50 mM sodium ascorbate, 50 mM $NaHSO_3$, 10 mM $MgCl_2$ and 5 mM DTT slurried with polyvinylpolypyrrolidone (PVPP; 0.5 g g^{-1} tissue) and a spatula tip of purified sea sand. Polystyrene resin (0.5 $g.g^{-1}$ tissue, Amberlite XAD-4, Serva Electrophoresis GmbH, Heidelberg, Germany) was added and the slurry was stirred carefully for 2 min and then filtered through cheesecloth. The filtrate was centrifuged at 20,000*g* for 20 min (pellet discarded), and the supernatant centrifuged at 100,000*g* for 90 min. Then 3 ml of the 100,000*g* supernatant was desalted using a desalting column to a buffer containing 15 mM Mopso (pH 7.2), 10% (v/v) glycerol, 5 mM sodium orthovanadate, 1 mM sodium ascorbate, 1 mM $MnCl_2$ and 2 mM DTT. To 1 ml of these enzyme preparations 10 µM [1-^{3}H]GPP at 0.1 Ci $mmol^{-1}$ was added ([1-^{3}H]-GPP from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA); unlabelled GPP from Sigma after a buffer change as described for FPP (de Kraker et al., 1998). After addition of a 1 ml redistilled pentane overlay, the assays were incubated for 60 min at 30°C. After the assay, the tubes were vortexed, and the pentane layer was removed and passed over a short column of silica overlaid with anhydrous $MgSO_4$. The aqueous phase was extracted again with 1 ml of redistilled diethyl ether, which was also passed over the silica column, and the column washed with 1.5 ml of diethyl ether. The total volume of the pentane/diethyl ether extract was determined and 100 µl of the extract was removed for liquid-scintillation counting in 4.5 ml of Ultima Gold cocktail (Packard Bioscience, Groningen, The Netherlands). Before radio-GLC-analysis unlabelled reference compounds linalool and geraniol were added to the extract, which was then slowly concentrated under a stream of N_2.

Radio-GLC was performed on a Carlo-Erba 4160 Series gas chromatograph (Carlo-Erba, Milano, Italy) equipped with a RAGA-90 radioactivity detector (Raytest, Straubenhardt, Germany) essentially as described previously (Bouwmeester et al., 1999b). The GC was equipped with an EconoCap EC-WAX column (30 m x 0.32 mm i.d., 0.25 µm film thickness; Alltech Associates, Breda, The Netherlands) and the oven temperature was programmed to 70°C for 5 min, followed by a ramp of 5° min^{-1} to 210°C and a final time of 5 min.

Samples were also analysed by GC-MS using an HP 5890 series II gas chromatograph and an HP 5972A Mass Selective Detector essentially as described before (Bouwmeester et al., 1999b). The GC was equipped with an HP-5MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness) and programmed at an initial temperature of 45°C for 1 min, with a ramp of 5° min^{-1} to 120°C, a ramp of 20° min^{-1} to 270°C, and final time of 5 min.

Analysis of monoterpenes produced by the petunia plants

To measure the release of volatiles by the petunia plants, headspace of leaves and flowers was measured both on the plant in the greenhouse and the detached from the plant. A glass cylinder was used to capture the plant part to be analysed and aluminium foil was used as seal. A 100 μ Polydimethylsiloxane (PDMS) coated Solid Phase Micro Extraction (SPME) fiber (Supelco, Belfonte PA USA) was used in order to capture all volatiles released from the intact plant tissue. Measurements were done for 30 min to 24 hours and repeated several times.

The tissues to be analysed for volatile contents and gene expression were collected in the greenhouse and frozen in liquid nitrogen. After grinding, half of the sample was stored for Northern and one for GC-MS analysis. For the latter, in routine assays, 200 mg frozen material was homogenised in a mortar containing 1.5 ml 5 M calciumchloride ($CaCl_2$) solution, to inhibit enzymatic conversions, and a small amount of purified sea sand. For some tissues smaller samples were used: pollen, 30 mg; styles, 20 mg; ovaries, 25 mg and 12 single nectaries. These samples were mixed with 0.75 ml 5 M $CaCl_2$ solution. The material was ground rapidly and thoroughly with a pestle and 0.75 ml of the sample was introduced into a 1.8 ml GC vial containing a small magnetic stirrer. The vial was then closed with an aluminium cap with a PTFE/Butylrubber septum and incubated during 20 min in a water bath at 50°C under continuous stirring. The headspace was sampled during 30 min with a 100 μ PDMS SPME fiber.

For quantification of linalool and α-terpineol in transgenic petunia leaves a range of known amounts of linalool or α-terpineol were added to non-transgenic petunia tissue. The headspace was analysed as described above and the correlation between the added amounts and peak areas (m/z 93) was determined. The value m/z 93 is typical for many monoterpenes including linalool and α-terpineol (Adams, 1989).

To check if linalool was present in the petunia in a bound form, different glycosidases and phosphatases were added to the petunia tissue. From a batch of frozen and ground transgenic

leaf material 60 mg of tissue was taken to 0.5 ml buffer with an enzyme specific pH. The following enzymes were added: 52 units alkaline phosphatase to phosphate buffer (10 mM) of pH 8, 8 units acid phosphatase to citrate buffer pH 4.5, 9 units α-glucosidase to phosphate buffer pH 7, 140 units β-glucosidase, one unit α-mannosidase, 0.3 units β-mannosidase to citrate buffer pH 4.5 (all from Sigma), Rohapect D5S (10% w/v) and Rohapect 7104 (10% w/v) to citrate buffer pH 4.5 (Röhm, Darmstadt, Germany). The enzyme mixtures were incubated overnight at 25°C and the headspace was sampled by SPME for 30 min and analysed as described above. After the first measurement, 0.5 ml 5 M $CaCl_2$ solution was added to the same samples, to check for possible additional linalool in the tissue and again the headspace was sampled. The β-glucosidase enzyme was also used in a next experiment, where 100 mg of petunia leaves in triplo was used from control and transgenic plants to incubate overnight at 37°C (optimum temperature for the enzyme) at pH 5 in 1 ml of a 10 mM phosphate/citrate buffer. As a control the same tissues were also incubated overnight without the enzyme added to the assay. The next day all samples were stored at –20°C to stop enzymatic reactions. After the first measurement of the 12 samples 1 ml of 5 M $CaCl_2$ solution, pH 4.5 was added to the samples and they were measured again after 30 min incubation at 50°C. In the m/z 93 ion count profile the peak areas of linalool and α-terpineol were compared.

GC-MS analysis was performed using a Fisons (Fisons Instruments, Manchester, UK) 8060 gas chromatograph coupled to an MD 800 mass spectrophotometer (Interscience, Breda, The Netherlands). An HP-5 column (50 m x 0.32 mm, film thickness 1.05 μm) was used with He (37 kPa) as carrier gas. The GC oven temperature was programmed as follows: 2 min 80°C, ramp to 250°C at 8° min^{-1} and 5 min at 250°C. Mass spectra in the electron impact mode were generated at 70 eV. Injection was performed by thermal desorption of the SPME fiber in the injector at 250°C during 1 min using the splitless injection mode with the split valve being opened after 60 sec (Verhoeven et al., 1997). The compounds were identified by comparison of GC retention times and mass spectra with those of authentic reference compounds.

Identification of glucoside and analysis of petunia leaf samples

R,S-Linalyl β-D-glucopyranoside was synthesised from *R,S*-linalool and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide according to a modified Koenigs-Knorr synthesis (Paulsen et al., 1985).

Petunia leaves (3 to 7 g) were homogenised in 50 ml of 80% methanol and centrifuged (2000 g for 5 min). The residue was washed with 50 ml of 80% methanol and the supernatants were combined. Methanol was removed in vacuum and the remaining aqueous solution was

extracted with 2 x 20 ml diethyl ether. The aqueous extract was subjected to XAD-2 (20 cm, 1 cm inner diameter) solid phase extraction. The column was successively washed with 50 ml water and 50 ml diethyl ether. Glycosides were eluted with 80 ml methanol and the eluate was concentrated in vacuum. The residue was dissolved in 1 ml of 50% methanol in water and analyzed by high-performance-liquid-chromatography electrospray-ionisation tandem mass spectrometry (HPLC-ESI-MS-MS).

Analysis of methanol extracts was performed on a triple stage quadrupole TSQ 7000 LC-MS-MS system with an electrospray ionisation (ESI) interface (Finnigan MAT, Bremen, Germany). The temperature of the heated capillary was 240 °C. The ESI capillary voltage was set to 3.5 kV, resulting in a 3.4 µA current. Nitrogen served as both the sheath (70 psi) and auxiliary gas (10 l min^{-1}). Data acquisition and evaluation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). HPLC separations were carried out on a Eurospher 100 C-18 column (100 x 2 mm, 5 µm, Knauer, Berlin, Germany) using a linear gradient with a flow rate of 200 µl min^{-1}. Solvent A was 5 mM ammonium acetate in water, and solvent B was 5 mM ammonium acetate in methanol. The gradient program was as follows: 0-30 min 5 to 100% B. Mass spectra were acquired in the negative mode. Product ion spectra were available by collision-induced dissociation (CID) (1.5 mTorr of Argon: -20 eV).

The synthesised linalyl-β-D-glucoside had a concentration of 1 mg ml^{-1}. From the peak heights found in the HPLC-MS spectra the concentration of linalyl glycoside in the transgenic petunia leaf was estimated to be in the range of 5-10 µg g^{-1} (12-27 µM).

Multidimensional gas chromatography mass spectrometry (MDGC-MS)

The enantiomeric distribution of linalool produced by transgenic petunia was analysed using MDGC-MS. Enzymatic hydrolysis was performed by dissolving an aliquot of the methanol extract, as described above, in 2 ml of 0.2 M phosphate buffer pH 5.5. Subsequently, 200 µl of Rohapect D5L (Röhm, Darmstadt, Germany), a pectinolytic enzyme preparation exhibiting glycosidase activity, was added. After an incubation period of 24 h at 37°C, the liberated aglycons were extracted twice by 1 ml of diethyl ether. The combined organic layers were dried over Na_2SO_4 and concentrated.

MDGC-MS analyses were performed with a Fisons 8160 GC connected to a Fisons 8130 GC and a Fisons MD 800 quadrupole mass spectrometer and using Fisons MASSLAB software (Version 1.3). The first GC was equipped with a split injector (1:10, at 230°C) and a flame ionisation detector (at 250°C). This GC employed a 25 m x 0.25 mm i.d. fused silica capillary

column coated with a 0.25 µm film of DB-Wax 20 M (J & W Scientific Inc., Folsom, CA, USA) for the pre-separation of the target molecule. Separation of enantiomers was achieved with the second GC using a 25 m x 0.25 mm i.d. fused silica capillary column coated with a 0.15 µm film of 2,3-di-O-ethyl-6-O-tert-butyl dimethylsilyl-β-cyclodextrin/PS086. A multicolumn switching system (Fisons) connected the column in GC1 to the column in GC2. The retention time of the compound of interest was determined by GC separation while the column in GC1 was connected to the FID. Separation of the enantiomers was achieved in the second GC after transfer of the compound of interest from the capillary column in GC1 to the column in GC2 via the switching device. The fused silica capillary column in GC1 was maintained at 60°C then programmed to 240°C at 10°C min^{-1} with He gas flow at 3 ml min^{-1}. The fused silica capillary column in GC2 was maintained at 60°C (15 min) then programmed to 200°C at 2°C min^{-1} with He gas flow at 3 ml min^{-1}. The compound of interest was transferred from GC1 to GC2 from 9.8 min to 10.3 min. The MS operating parameters were ionisation voltage, 70 eV (electron impact ionisation); ion source and interface temperature, 230°C and 240°C, respectively.

Acknowledgements

We like to thank Dr. Eran Pichersky, for providing the *Clarkia breweri S*-linalool synthase cDNA, C. Ruff and B. Weckerle for the MDGC-MS analyses, John Franken for help with the transformation, Prof. Dr. Arjen van Tunen and Dr. Ric de Vos for helpful discussions. The support of the Joseph-Schormüller-Gedächtnisstiftung for WS is gratefully acknowledged.
Genbank accession number: *Lis*, *S*-linalool synthase, U58314

Chapter 3

Monoterpene biosynthesis in lemon (*Citrus limon*): cDNA isolation and functional analysis of four monoterpene synthases

Joost Lücker[1], Mazen K. El Tamer[1], Wilfried Schwab[2], Francel W. A. Verstappen[1], Linus H. W. van der Plas[3], Harro J. Bouwmeester[1], Harrie A. Verhoeven[1]

[1] Business unit Cell Cybernetics, Plant Research International, P.O. Box 16, 6700 AA, Wageningen, The Netherlands,

[2] University of Würzburg, Chair of Food Chemistry, Am Hubland, 97974, Würzburg, Germany and

[3] Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD, Wageningen, The Netherlands

Published in the European Journal of Biochemistry (2002) **269**, 3160-3171

Summary

Citrus limon possesses a high content and large variety of monoterpenoids, especially in the glands of the fruit flavedo. The genes responsible for the production of these monoterpenes have never been isolated. By applying a random sequencing approach to a cDNA library from mRNA isolated from the peel of young developing fruit, four monoterpene synthase cDNAs were isolated, that appear to be new members of the previously reported *tpsb* family. Based on sequence homology and phylogenetic analysis these sequences cluster in two separate groups. All four cDNAs could be functionally expressed in *Escherichia coli* after removal of their plastid targeting signals. The main products of the enzymes in assays with geranyl diphosphate as substrate were (+)-limonene (two cDNAs), (-)-β-pinene and γ-terpinene. All enzymes exhibited a pH optimum around 7; addition of Mn^{2+} as bivalent metal ion cofactor resulted in higher activity than Mg^{2+}, with an optimum concentration at 0.6 mM. K_m values ranged from 0.7 to 3.1 μM. The four enzymes account for the production of 10 out of the 17 monoterpene skeletons commonly observed in lemon peel oil, corresponding to more than 90% of the main components present.

Introduction

Lemon, *Citrus limon* (L.) Burm. f., is a member of the large Rutaceae family containing 130 genera in seven subfamilies, with many important fruit and essential oil producers. Lemon essential oil has the highest import value of all essential oils imported to the USA and is widely used as flavouring agent in bakery, as fragrance in perfumery and also for pharmaceutical applications (Weiss, 1997). The essential oil is produced from the peel or flavedo of the fruit. This layer consists of the epidermis covering the exocarp consisting of irregular parenchymatous cells, which are completely enclosing numerous glands or oil sacs. Below this green layer in maturing fruits is the albedo layer (mesocarp), a thick spongy white mass of tissue, rich in pectins, surrounding the fleshy, juicy interior of the fruit. Aldehydes, such as citral, are minor components present in the *C. limon* essential oil. However, they contribute more to the characteristic flavour than the bulk components, which are the olefinic monoterpenes (Weiss, 1997). Monoterpenes are the C_{10} branch of the terpene family and consist of two head to tail coupled isoprene units (C_5). They are beneficial for plants as they function in the defence against herbivores and plant pathogens or as attractants for pollinators. Sites for biogenesis of monoterpenes have been investigated extensively. In gymnosperms, such as grand fir, terpenes are produced in resin ducts (Fahn, 1979; Lewinsohn et al., 1991). Their biosynthesis is induced upon wounding (Lewinsohn et al., 1993; Funk et al., 1994; Steele et al., 1998), indicating their role in the defence against bark beetle infestation. For angiosperms many investigations have been carried out on Labiatae, especially on *Mentha* species, where monoterpenes are formed in the glandular trichomes, and on the umbelliferous caraway, where monoterpenes are produced in essential oil ducts of the fruits (McCaskill et al., 1992; Colby et al., 1993; Bouwmeester et al., 1998; Turner et al., 1999; Gershenzon et al., 2000; McConkey et al., 2000). In *Citrus*, the specialised structures for the storage and accumulation of large amounts of terpenes are the glands in the flavedo, the so-called secretory cavities. Research on lemon showed that these cavities develop schizogenously on most aerial plant parts (Fahn, 1979; Turner et al., 1998). The cells lining these secretory cavities are thought to be responsible for the production of the terpenoids (Turner et al., 1998). In cold pressed lemon peel oil from different origins, around 61% of the total monoterpene content consists of limonene together with lower levels of β-pinene (17%) and γ-terpinene (9%) (Weiss, 1997). Recently, the enantiomeric composition of some of the chiral terpene olefins present in the lemon oil was determined using a multidimensional tandem GC-MS system (MDGC-MS) (Mondello et al., 1999). The main chiral components of the cold pressed lemon oil were 4*R*-(+)-limonene with 96.6% enantiomeric excess (e.e.), and (1*S*, 5*S*)-(-)-β-pinene with 88% e.e. (Mondello et al., 1999).

The main monoterpenes of lemon can be obtained by heterologous expression of enzymes from several plant species that were isolated using a number of different strategies. cDNAs encoding (-)-limonene synthase were previously isolated from several *Mentha* species, *Abies grandis* and *Perilla frutescens*, using a PCR based approach, with sequence information obtained by protein sequencing of the purified enzyme (Colby et al., 1993), or by using the first cloned *Mentha spicata* cDNA as a probe (Yuba et al., 1996). For *A. grandis* homology-based cloning, degenerate PCR primers based on conserved domains of a number of terpene synthase genes were used (Bohlmann et al., 1997). So far only one cDNA encoding a (+)-limonene synthase has been isolated from *Schizonepeta tenuifolia*, a member of the Labiatae family (Maruyama et al., 2001b).

(-)-(1*S*, 5*S*)-β-pinene was the major product of a β-pinene synthase cDNA from *Artemisia annua* submitted to GenBank (accession number: AF276072), and of a (-)-(1*S*, 5*S*)-pinene synthase that was previously isolated from *Abies grandis* (Bohlmann et al., 1997). This enzyme produces 58% (-)-(1*S*, 5*S*)-β-pinene, but also 42% (-)-(1*S*, 5*S*)- α-pinene. A cDNA encoding γ-terpinene synthase as its main activity has not been reported on yet.

Although the composition of lemon essential oil has had considerable attention and enzymes responsible for the production of monoterpenes in the peel of lemon have been partially purified (Chayet et al., 1977), their corresponding cDNAs have never been isolated and characterized. So far only the cDNA of a sesquiterpene synthase producing (*E*)-β-farnesene as main product has been described from *Citrus junos* (Maruyama et al., 2001a). Here we report on the isolation of four new monoterpene synthase cDNAs by random sequencing of a flavedo derived cDNA library of *C. limon* and their characterization by functional expression in *Escherichia coli.*

Experimental procedures

Plant material, substrate, and reagents

Lemon plants (*Citrus limon* (L.) Burm. f.), obtained from a nursery in Sicily, Italy, were grown in pots in the greenhouse in peat moss/ clay mixture (50:50, v/v), under 18 h supplemental lighting provided by two 400 Watt high pressure sodium lamps (Philips, Eindhoven, The Netherlands), at 28 °/ 20 °C (day/night) temperature cycle. Plants were watered as needed and fertilised weekly with a liquid fertiliser.

[1-^{3}H]Geranyl diphosphate (GPP) and [1-^{3}H]Farnesyl diphosphate (FPP) were obtained from American Radiochemicals Inc. (St Louis, MO, USA) and Amersham Biosciences (Piscataway, NJ, USA) respectively. Unlabelled GPP and FPP were purchased from Sigma-Aldrich (Sigma-Aldrich, Chemie b.v., Zwijndrecht, The Netherlands) and were used after a buffer change as described for farnesyl diphosphate (de Kraker et al., 1998).

Unless otherwise stated reagents were obtained from Sigma-Aldrich. DNA sequences were assembled and analysed using DNASTAR software (DNASTAR, Inc., Madison, WI, USA). Sequencing primers were ordered from either Isogen Bioscience (Maarssen, The Netherlands) or Amersham Biosciences. Sequencing reagents were supplied by Perkin Elmer (Foster City, CA, USA). Restriction enzymes, enzymes and buffers used were from Gibco BRL (Invitrogen corporation, Breda, The Netherlands). DNA fragments were isolated from Agarose gel by a GFX™ PCR DNA and Gel band purification kit (Amersham Biosciences). Amino acid alignment was made using Clustal-X 1.81, with Gonnet250 matrix and default settings.
Phylogenetic analysis was carried out using Clustal-X 1.81, with PAM350 matrix ((multiple alignment parameters: gap opening set at 10 (default), gap extension set at 2 (0.2 is default)) and the neighbour joining method for calculating the tree (Saitou and Nei, 1987; Hernandez and Ruiz, 1998). The bootstrapped tree was corrected for multiple substitutions as recommended by the program (Kimura, 1983).

Hydro distillation of *C. limon* peel

Samples of lemon flavedo (0.5 g) from green fruits (2x1 cm) were ground in liquid N_2 and used for hydro distillation with ethylacetate as a keeper as previously described (Helsper et al., 2001). After a 1:200 dilution, 2 µl of the ethylacetate phase was injected into a GC-MS using an HP 5890 series II gas chromatograph (Hewlett Packard, Agilent Technologies, Alpharetta, GA, USA) and an HP 5972A Mass Selective Detector essentially as described previously (Bouwmeester et al., 1999b). The GC was equipped with an HP-5MS column (30 m x 0.25 mm internal diameter, film thickness = 0.25 µm) and programmed at an initial temperature of 45 °C for 1 min, with a ramp of 10 °C min^{-1} to 280 °C, and final time of 10 min. Products were identified by comparison of retention times and mass spectra with authentic reference compounds. The α-thujene standard was purchased from Indofine (Indofine Chemical Company Inc., Hillsborough, NJ, USA)

RNA isolation, cDNA library construction, random sequencing and library screening

Plant material from a fruit bearing *C. limon* plant was harvested and frozen directly in liquid N_2. Total RNA for cDNA library construction was isolated from the flavedo layer of 2x1 cm young green fruits, according to a slightly modified RNA isolation protocol for recalcitrant plant tissues (Schultz et al., 1994), by using maximally 2.5 g of tissue per 30 mL RNA extraction buffer. mRNA was extracted from the total RNA using a mRNA purification kit according to manufacturers recommendations (Amersham Biosciences). Of this amount 15 µg was used to construct a custom cDNA UNI-ZAP XR™ library (Stratagene Europe, Amsterdam Zuidoost, The Netherlands).

Mass excision

The *E. coli* strains XL1-MRF' and SOLR were used for mass excision according to the manufacturers recommendations (Stratagene). 150 µL of the primary unamplified library was mixed with 150 µL of XL-1 MRF'cells (OD_{600} = 1), with 20 µL of helper phage (Stratagene). The mix was grown for only 2.5 hours in order to minimise disturbance of the clonal representation. Finally, for 100 single colonies to be picked 1-3 µL of the resulting phagemids was used each time to infect 200 µL of SOLR cells and the next day single colonies were picked from Luria Bertani (LB) plates.

DNA isolation and Sequencing

Plasmid DNA was isolated from overnight grown bacterial cultures using a Qiaprep 96 Turbo kit on a Qiagen Biorobot 9600 according to the manufacturers recommendations (Qiagen GmbH, Hilden, Germany). Between 0.5 and 3 µL of plasmid DNA was used for sequencing isolated clones using Ready Reaction Dye Terminator Cycle mix (Perkin Elmer) and 100 ng of pBluescript SK primer (5'-CGCTCTAGAACTAGTGGATC-3'). Sequencing PCR was performed according to the manufacturers recommendations (Perkin Elmer) in a MJ research PTC Peltier thermal cycler (MJ Research Inc., Watertown, MC, USA). After precipitation and dissolving in TSR buffer (Perkin Elmer), the samples were sequenced on an ABI 310 capillary sequencer (Perkin Elmer). A total of 960 clones were sequenced and analysed for homology to known genes by using the BLASTN and BLASTX programs of the NCBI (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

Full length sequencing and cloning

After sequencing, nine putative terpene synthase genes were identified, representing three different clones. These clones, *B93*, *C62* and *D85* were full length sequenced by designing sequence specific overlapping primers based on the obtained sequence information. On the basis of sequence alignments, sequences that were most distant to each other were selected for further screening of the cDNA library.
Using clones *B93* and *C62* as ^{32}P labelled probes, 75 µL of the custom unamplified cDNA library (Stratagene) from lemon was screened by plaque lifts using Hybond N+ nylon membranes according to the manufacturers recommendations (Amersham Biosciences). Hybridization was performed at 55 °C in buffer containing 10% dextran sulphate (Amersham Biosciences), 1 M NaCl and 1% (w/v) SDS. Filters were washed three times at 55 °C, once in 4 x NaCl/Cit and 0.1% (w/v) SDS and twice in 2 x NaCl/Cit and 0.1% (w/v) SDS. Plaques that were radioactively labelled were picked and using the single clone excision protocol,

separate *E. coli* SolR colonies were obtained from the cDNA library as described in the Unizap-XR manual (Stratagene). After growth and subsequent DNA isolation the clones were sequenced as described above.

cDNA expression in *E. coli*

For putative targeting signal prediction the computer programs TargetP and Predotar were used which gave scores for the most likely localization of the proteins. A description of the interpretation is given on the websites. (http://www.inra.fr/servlets/WebPredotar, http://www.cbs.dtu.dk/services/TargetP/)

The four clones were subcloned in truncated form in order to exclude the putative plastid-targeting signal from being expressed, because this can lead to the formation of inclusion bodies (Williams et al., 1998). The conserved N-terminal amino acid sequence of the RR motif was shown not to be required for functional expression of monoterpene synthases in *E. coli*. Removing this sequence drastically improved the activity of the isolated enzymes (Williams et al., 1998). The clones were truncated and religated in the pBluescript SK vector in frame with the *LacI* promoter for induced expression by isopropyl-1-thio-β-D-galactopyranoside (IPTG) as previously described (Jia et al., 1999). Primers for truncation were designed on the 5'-end of the sequences to include a methionine preceding the RR motif and a restriction site for in frame cloning with the *LacI* promoter. PCR Amplification was carried out using *pfu* polymerase with the T7 primer and a gene specific restriction site containing primer on a MJ research PTC Peltier thermal cycler (94 °C, 30 s; 50 °C, 30 s; 72 °C, 2 min; 30 cycles). The sense primer for *B93* contained a *Pst*I restriction site 5'-GCCAACTGCAGAATGAGGCGATCTGCCGATTACG-3'. The sense primer for *C62* and *M34* was 5'-GCCAGGATCCAATGAGGAGATCAGCAAACTACC- 3', containing a *Bam*HI restriction site. The sense primer for *D85* contained a *Bam*HI restriction site 5'-GCCAGGATCCAATGAGGCGATCTGCTGATTACG -3'. PCR products were digested using the restriction sites introduced by the sense primers and restriction sites in the 3' multiple cloning site of pBluescript, that was included in the PCR fragment by amplification with the T7 primer. The pBluescript expression vectors with the truncated cDNA clones were obtained using standard molecular biological techniques (Sambrook et al., 1989). The clones were fully resequenced after subcloning to check for unwanted changes in the ORF.

For cloning the monoterpene synthases including a His-tag for easy purification, the expression vector pRSET B (Invitrogen corporation) was used for the expression of the four putative full-length monoterpene synthases in *E. coli* (Stratagene: BL21-CodonPlus™-RIL strain), using the original pRSET B vector as negative control for the experiments. For all four clones, primers for amplification of the truncated cDNAs including the RRX_8W motif were designed. PCR amplification was performed for all clones using *pfu* turbo DNA

polymerase (Stratagene) and the same programme on a MJ research PTC Peltier thermal cycler (94 °C, 30 s; 55 °C, 30 s; 72 °C, 2 min; 30 cycles).
For clone *B93* a sense primer including a *Bgl*II restriction site, named B93HISFBGL (5'- AGAGTCAGATCTTAGGCGATCTGCCGATTACG-3') was designed. The clone was amplified using this primer and a T7 primer (5'- GTAATACGACTCACTATAGGGC-3'). In the 3' UTR of the gene another *Bgl*II site was present, providing a PCR fragment after digestion that could be directly ligated to a *Bam*HI digested pRSET B vector after dephosphorylation using calf intestinal alkaline phosphatase.
In the 3' UTR of the *C62* clone a *Sal*I site was introduced to facilitate cloning, by the Quickchange Site Directed Mutagenesis PCR method (Stratagene) according to the manufacturers recommendations and the following program (95 °C, 30 s; 55 °C, 1 min; 68 °C, 10 min; 14 cycles). The complementary primers used were C62FOR (5'- GCAGTTTCAGTCGACGTTGGCCTCCAC-3') and C62REV (5'-GTGGAGGCCAACGTCGACTGAAACTGC-3'). Only the two underlined nucleotides were altered. The resulting 3'UTR modified pBluescript *C62* clone was used as template for cloning into the pRSET B vector. A sense primer including a *Bgl*II restriction site, named C62HISFBGL (5'- CTTGACAGATCTTAGGAGATCAGCAAACTAC-3') was used together with the T7 primer to amplify the cDNA. After purification from the gel, the PCR fragment was digested with *Bgl*II and *Sal*I and ligated to a pRSET B vector fragment digested with compatible *Bam*HI and *Xho*I sites.
For *D85* a sense primer including a *Bgl*II site (5'- AGAGTCAGATCTTAGGCGATCTGCTGATTACG-3') was used together with the T7 primer to amplify the cDNA. After gel purification of the PCR product it was digested with *Bgl*II and *Afl*III restriction enzymes, *Afl*III cuts in the 3' UTR of the cDNA. The digested fragment was ligated to the compatible sites of pRSET B digested with *Bam*HI and *Nco*I.
For subcloning the *M34* clone the sense primer C62HISFBGL and the antisense primer M34HISXHO (5'- TGATCACTCGAGGAATTCGCAACGCATCG-3'), annealing in the 3' UTR of the cDNA introducing an *Xho*I site, were used. After PCR the product isolated from the gel was digested with *Bgl*II and *Xho*I and ligated to PRSET B vector digested with *Bam*HI and *Xho*I.
All the ligations were transformed to *E. coli* strain XL1-blue MRF' supercompetent cells (Stratagene). Isolated DNA from bacterial colonies was fully resequenced in order to check for orientation, mutations and if the gene was integrated in the right frame, resulting in a fusion protein at the N-terminus with a peptide that included an ATG translation initiation codon, a series of six histidine residues (His-tag), and an anti-Xpress (Invitrogen) epitope. Plasmid DNA of the four pRSET B clones and the control (original pRSET B vector) were transformed to BL21-CodonPlus™-RIL competent cells according to the manufacturers recommendations (Stratagene).

Protein expression

The pBluescript expression vectors were induced for protein expression and after centrifugation, the bacterial pellets were dissolved in assay buffer exactly as described previously (Jia et al., 1999).
For induction of protein expression of the His-tag vectors, single colonies were picked from the LB 100 mg L^{-1} ampicillin plates with the BL21 transformations containing the putative terpene synthases and the original pRSET vector. They were transferred to 5 ml LB broth supplemented with 100 mg L^{-1} ampicillin and grown overnight. Aliquots of 0.5 mL were used to inoculate 250 mL conical flasks containing 50 mL LB broth with ampicillin (50 µg mL^{-1}) and chloramphenicol (37 µg mL^{-1}). This was grown at 37 °C with vigorous agitation to OD_{600} = 0.6. For induction of expression IPTG was added to a final concentration of 1 mM and the cultures were grown at 20 °C overnight with agitation at 250 rpm. Proteins were isolated using His-tag purification by passing the lysate over Ni-nitrilotriacetatic spin columns according to the manufacturers recommendations (Qiagen). After washing, the bound protein was eluted using the buffer recommended by the manufacturer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole pH 8, and the eluted protein was supplemented with glycerol to 30% and stored at –70 °C. For protein concentration measurement the proteins were first precipitated in 10% trichloroacetic acid on ice for 15 min, followed by centrifugation for 10 min. The resulting pellet was washed twice with acetone and after drying dissolved in 5 mM Tris, pH 6.8, 0.2% (w/v) SDS and 1% glycerol. Protein concentration was determined using the BCA Protein assay kit using BSA as protein standard reference, according to the manufacturers recommendations (Pierce, Rockford, IL, USA).

Enzymatic characterization of the four recombinant *Citrus* clones

Enzyme assay

Ten µL or less of the eluted His-tag purified protein was used in each assay to check for enzymatic activity. In most cases it was necessary to dilute the enzyme further to guarantee linearity. The assay buffer was a 15 mM MOPSO buffer (pH 7) containing 10% glycerol, 1 mM ascorbic acid and 2 mM DTT. The putative synthases were tested for activity with 2 µM [1-^{3}H]GPP (740 GBq/ mmol) or 20 µM [1-^{3}H]FPP (555 GBq/ mmol). For GPP they were incubated with varying concentrations of either 0.05 to 1.5 mM $MnCl_2$ or 2.5 to 15 mM $MgCl_2$ as cofactors to check their specific bivalent metal ion preference, for FPP only 10 mM $MgCl_2$ was used. The synthases were also tested without addition of metal ions. The reaction was performed in a total volume of 100 µL and before incubation for 30 min at 30 °C with gentle shaking, the assay was overlaid with one ml of hexane. To investigate the linearity of

the assays with time the enzymes were incubated for 0, 10, 20, 30, 45 and 60 minutes at 30 °C. For testing the pH optimum of the enzymes they were incubated in MOPSO buffer with a pH ranging from 6.4 to 7.6, with intervals of 0.3 pH units. Also the affinity for the monovalent ion K^+ was tested at different concentrations of KCl ranging from 0 to 150 mM. All assays were performed in duplicate. After incubation the assays were vigorously mixed and after a short centrifugation step to separate phases, 500 µL of the hexane phase from each sample was added to 4.5 mL Ultima Gold cocktail (Liquid scintillation solution) (Packard Bioscience, Groningen, the Netherlands) for liquid scintillation counting. For K_m determination the enzymes were incubated with GPP concentrations ranging from 1 µM to 180 µM for β-pinene and γ-terpinene synthase, or 0.1 µM to 100 µM for both limonene synthases, at 0.6 mM $MnCl_2$ and pH 7. For some concentrations of [1-^{3}H]GPP buffer controls were used to estimate background levels of hexane soluble radioactivity. After the assays the hexane phase was removed and mixed with about 20 mg of silica to remove any non-specific polar compounds. After centrifugation at 10000 g for 10 minutes, 500 µL of the hexane phase was used for scintillation counting as described above. For the analysis of product formation the same procedure was followed, but in larger volumes. 200 µL of enzyme was used in a total reaction volume of 1 mL, including 10 mM $MgCl_2$, or 0.6 mM $MnCl_2$. For analysis on GC-MS 50 µM GPP, and for analysis using radio-GC 20 µM [1-^{3}H]GPP (740 GBq/ mmol) was used as a substrate. After the addition of a 1 mL redistilled pentane overlay, the tubes were carefully mixed and incubated for 1 h at 30 °C. Following the assay, the tubes were vortexed, the organic layer was removed and passed over a short column of aluminium oxide (Al_2O_3) overlaid with anhydrous Na_2SO_4. The assay mixture was re-extracted with 1 mL of pentane: diethyl ether (80:20), which was also passed over the aluminium oxide column, and the column washed with 1.5 mL of diethyl ether. 100 µL from each sample was added to 4.5 mL Ultima Gold cocktail for scintillation counting.

Samples of the pentane/ ether fraction were analysed using GC-MS as described above and on a radio-GC consisting of a Carlo-Erba 4160 Series gas chromatograph (Carlo-Erba, Milano, Italy) equipped with a RAGA-90 radioactivity detector (Raytest, Straubenhardt, Germany) essentially as described previously (Bouwmeester et al., 1999c).

MDGC-MS

The enantiomeric distribution of the main and the side products produced by the monoterpene synthases, with the cold assays, were analysed using MDGC-MS. The MDGC-MS analyses were performed with a Fisons 8160 GC connected to a Fisons 8130 GC and a Fisons MD 800 quadrupole mass spectrometer and using Fisons MassLab v1.3 (Fisons, Manchester, UK). The system setup was as described previously although the settings were different (Lücker et al., 2001). The fused silica capillary column in GC1 (J&W, Folsom, CA, USA) DB-Wax 20 M

(25 m x 0.25 mm internal diameter (i.d.); film thickness = 0.25 μm) was maintained at 40 °C then programmed to 240 °C at 1 °C min^{-1} (sabinene and pinene preseparation) and at 50 °C then programmed to 240 °C at 3 °C min^{-1} (limonene preseparation) with He gas flow at 3 mL min^{-1}. The fused silica capillary column in GC2 (J&W Cyclodex B (30 m x 0.25 mm i.d.; film thickness = 0.25 μm) was maintained at 45 °C (12 min) then programmed to 200 °C at 5 °C min^{-1} with He gas flow at 3 mL min^{-1}. The compounds of interest were transferred from GC1 to GC2 from 6.6 min to 7.1 min (α-pinene) and 10.2 min to 10.4 min (β-pinene). The fused silica capillary column in GC2 (30 % 2,3-diethyl-6-*tert*-butyl-dimethyl-β-cyclodextrin/PS086 (25 m x 0.25 mm i.d.; film thickness = 0.15 μm)) was maintained at 60 °C (15 min) then programmed to 200 °C at 0.5 °C min^{-1} with He gas flow at 3 mL min^{-1}. The compounds of interest were transferred from GC1 to GC2 from 9.3 min to 9.7 min (limonene) and 11.1 min to 11.5 min (sabinene). The MS operating parameters were ionisation voltage, 70 eV (electron impact ionisation); ion source and interface temperature, 230 °C and 240 °C, respectively.

Results

Monoterpene content of lemon fruits

The monoterpene content of young lemon fruits was analysed using GC-MS. The major monoterpene was identified as limonene (75%), followed by γ-terpinene (11%) and β-pinene (4%). Some *p*-cymene (2%), α-pinene (1%) and myrcene (1%) were also detected. Trace levels below 1% were found of the monoterpenoids α-thujene, sabinene, α-terpinene, (*E*)-β-ocimene, terpinolene, linalool and α-terpineol.

cDNA isolation and sequencing

Random sequencing of a cDNA library made from mRNA isolated from the peel of young lemon fruits resulted in the identification of nine putative monoterpene synthase genes. BLASTX searches using the first 500 bp of the 5' side of the ESTs showed significant sequence homology (all with Expect score below $1.e^{-9}$) with other monoterpene synthases reported in the Genbank ENTREZ database (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). The nine ESTs all proved to be full-length cDNAs and were found to represent three different clones, designated *B93*, *C62* and *D85*. The cDNA library was rescreened with the two most divergent clones as probe under low stringency, and the positive plaques were sequenced. This rescreening yielded one additional putative monoterpene synthase, designated as *M34*, with a high level of identity to one of the already isolated cDNAs. The nucleotide sequences of *B93*, *C62*, *D85* and *M34* have been submitted to Genbank and are available under accession nos AF51486, AF514287, AF514288 and AF514289, respectively.

Sequence analysis

The cDNAs all encoded full-length putative monoterpene synthases from 600 to 606 amino acids long with a calculated molecular mass of around 70 kDa. According to targeting signal prediction programs TARGETP and PREDOTAR they all had a cleavable transit peptide for plastid localization. The scores of the TARGETP program for chloroplast transit peptide were in all cases higher than scores for targeting to other cell compartments. The lengths of the preproteins were predicted to be 22 to 40 aminoacids long. PREDOTAR gave significantly higher scores for plastid localization than for mitochondrial localization.

Table 1 Analysis of sequence identity levels (%) between cDNAs of *Citrus limon* and some other monoterpene synthases

Swiss-Prot accession numbers: *QiMYRS* (*Quercus ilex* myrcene synthase): Q93X23. *Aa(-)βPINS* (*Artemisia annua* (-)β-pinene synthase): Q94G53, *St(+)LIMS* (*Schizonepeta tenuifolia* (+)-limonene synthase): Q9FUW5. In the alignments up to the DDXXD motif, the targeting signal was not taken into account.

		B93	*D85*	*C62*	*M34*
Truncated cDNA[a]	*B93*		84	50	51
	D85			48	49
	C62				97
	St(+)LIMS	42	42	45	46
	QiMYRS	60	60	55	55
	Aa(-)βPINS	49	49	44	45
Targeting signal[a]	*B93*		90	16	16
	D85			16	18
	C62				91
Up to DDXXD motif	*B93*		89	48	50
	D85			49	50
	C62				96
From DDXXD motif	*B93*		78	54	54
	D85			49	50
	C62				98

[a] Truncated cDNA is the cDNA without the supposed targeting signal. Targeting signal is considered as the N-terminal sequence until the RRX_8W motif.

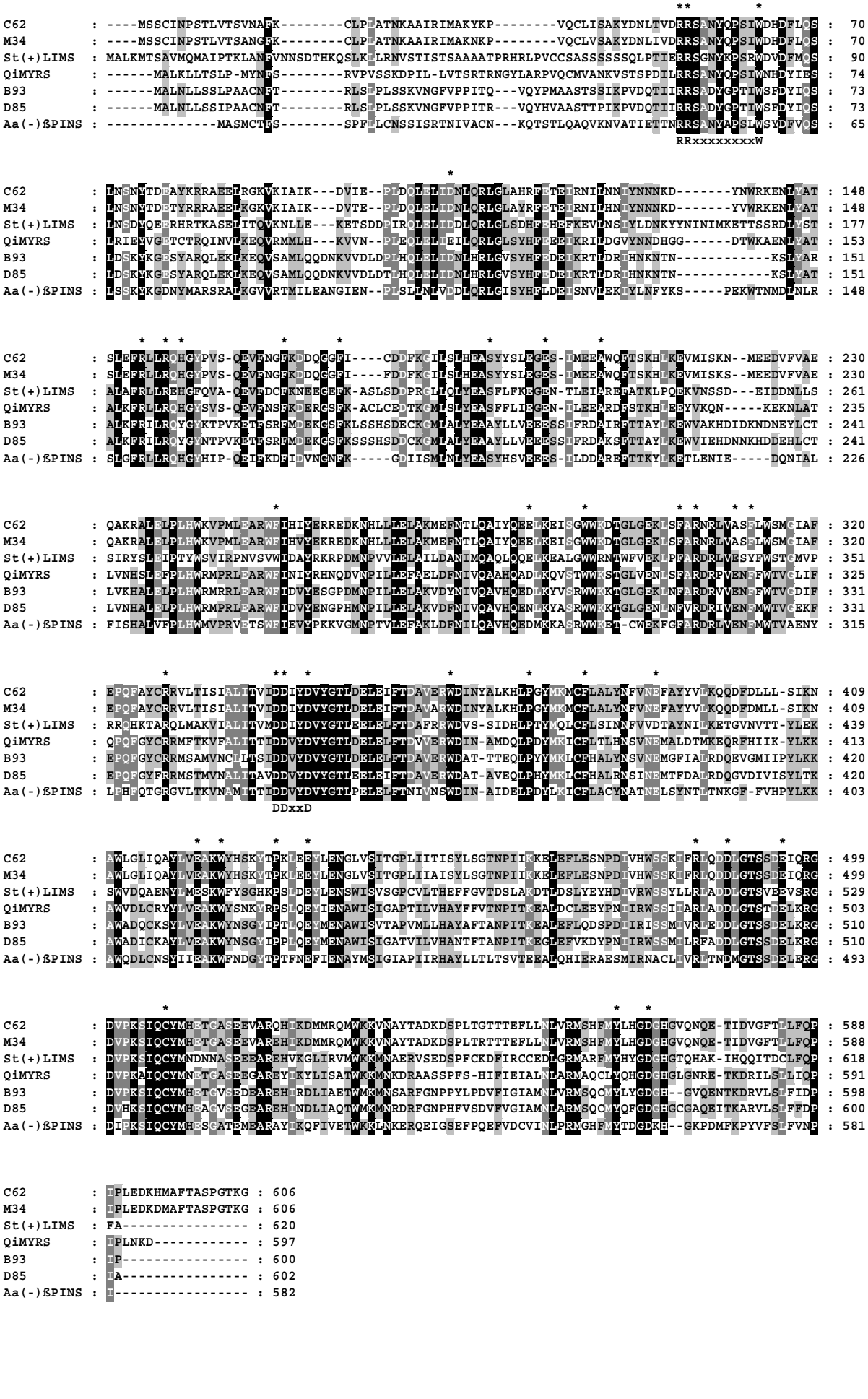
The deduced amino acid sequences of the four lemon cDNAs were aligned with their closest homologues in Genbank: *St(+)LIMS* (*Schizonepeta tenuifolia* (+)-limonene synthase: (Q9FUW5) (Maruyama et al., 2001b)), *QiMYRS* (*Quercus ilex* myrcene synthase: (Q93X23) (Fischbach et al., 2001)) and *Aa(-)βPINS* (*Artemisia annua* (-)-β-pinene synthase: (Q94G53) (Figure 1). The alignment illustrates many conserved regions between these seven monoterpene synthases from different plant species.

The previously reported conserved amino acids for terpene synthases are all found in the four new sequences and they are indicated with an asterix (Bohlmann et al., 1998). The levels of identity to the lemon monoterpene synthases range from 42 to 60%, when the sequences are aligned from the RRX_8W motif onwards, from where significant similarity starts (Table 1). This RRX_8W motif, located at the N terminus, is conserved amongst all the monoterpene

synthases depicted in Figure 1. The sequences of the lemon monoterpene synthases cluster into two separate groups. One group consists of *B93* and *D85*, showing 84% identity. The other group consists of *C62* and *M34* that show 97% identity. Between the groups the identity is not higher than 51%. For the putative targeting signals there is a clear relation between *B93* and *D85*. The identity of the sequences of *B93* and *D85* up to the RRX_8W motif is 90%. They are very different from the targeting signals of *C62* and *M34* (16% identity), which are again very similar to each other (91% identity).

In a phylogenetic analysis the separate clustering within the *tpsb* family of *C62* and *M34* from *B93* and *D85* is clear (Figure 2). The *B93* and *D85* sequences group together with the myrcene synthase from *Q. ilex* and the *A. annua* monoterpene synthases while the limonene synthases from *C. limon* form a distinct branch.

Figure 1. Alignment of deduced amino acid sequences of monoterpene synthases of the *tpsb* family to the lemon monoterpene synthases. *Cl(+)LIMS1* (*C62*, lemon (+)-limonene synthase 1), *Cl(+)LIMS2* (*M34*, lemon (+)-limonene synthase 2), *St(+)LIMS* (*Schizonepeta tenuifolia* (+)-limonene synthase, accession number: Q9FUW5 (Maruyama et al., 2001b)), *QiMYRS* (*Quercus ilex* myrcene synthase, accession number: Q93X23 (Fischbach et al., 2001)), *ClγTS* (*B93*, lemon γ-terpinene synthase), *Cl(-)βPINS* (*D85*, lemon (-)-β-pinene synthase), *Aa(-)βPINS* (*Artemisia annua* (-)-β-pinene synthase, accession number: Q94G53). The alignment was created with the ClustalX program using the Gonnet matrix. Shading indicates conserved identity for the aligned amino acids: black background shading indicates 100% conservation, dark grey shading indicates 80% conservation, and light grey shading indicates 60% conservation. Asterisks indicate residues that are highly or absolutely conserved between all plant terpene synthases (Bohlmann et al., 1998). The highly conserved RRx_8W motif, directly after the supposed plastid targeting signal, and the metal ion-binding motif DDxxD are indicated below the sequence alignments.


C62
M34
St(+)LIMS
QiMYRS
B93
D85
Aa(-)ßPINS
RRxxxxxxxxW
DDxxD

Functional expression of the putative monoterpene synthases in *E. coli*

The putative monoterpene synthases were expressed without the plastid targeting signals in order to prevent inclusion bodies of the expressed protein (Williams et al., 1998). Although the precise cleavage site is not yet known for terpene synthase preproteins, truncation of monoterpene synthases upstream of the conserved tandem arginine motif (RRX_8W) has been demonstrated to result in fully active enzymes (Williams et al., 1998; Bohlmann et al., 1999; Bohlmann et al., 2000). Enzyme activity was verified using radio-GC. Although the pentane fractions of the assays showed the main non-alcoholic products of the synthases, the high activity of aspecific phosphohydrolases in the crude *E. coli* lysates also resulted in production of large amounts of geraniol (data not shown), competing for the radiolabeled substrate.

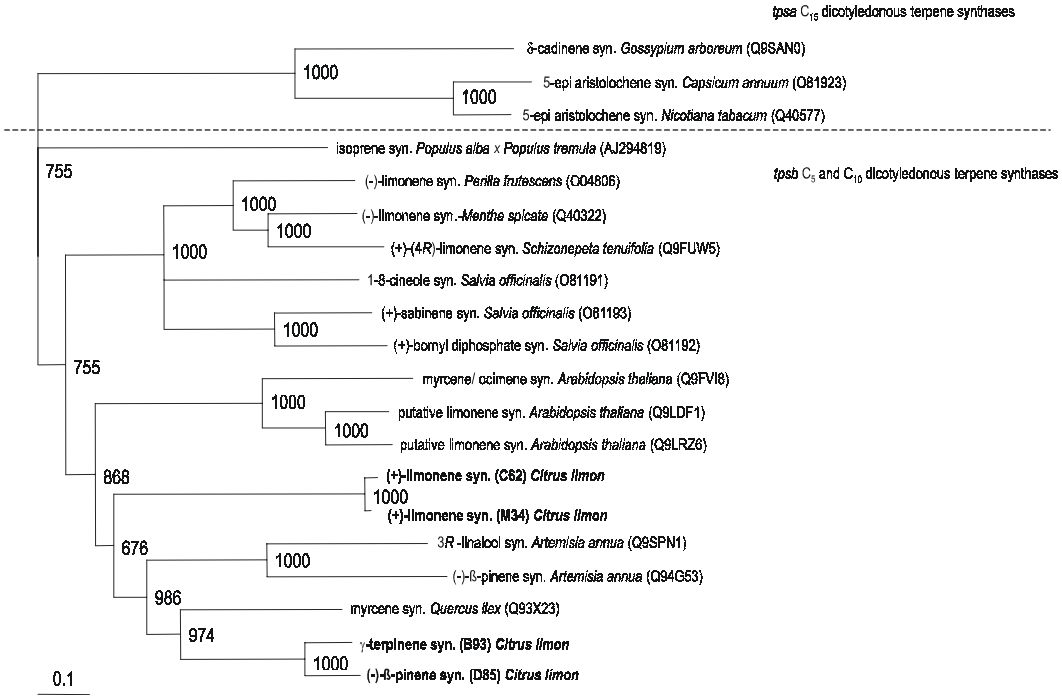


Figure. 2. Phylogram of CLUSTALX alignment of dicotyledonous C_5 to C_{15} terpene synthases using PAM350 matrix and the neighbour joining method. The tree was corrected for multiple substitutions. The sesquiterpene synthases (*tpsa*) were defined as outgroup and the tree was rooted with the outgroup. The lemon synthases are located in the *tpsb* family. Scale bar: 0.1 is equal to 10% sequence divergence. Bootstrap values are given for nodes, and are considered as a value for significance of the branches. Values higher than 850 are likely to be significant.

Therefore the cloning of the synthases truncated at the RRX_8W motif was repeated in the pRSET vector (Invitrogen), which contains a His-tag for purification of the expressed protein. The pRSET vectors were expressed in *E. coli* Bl21-DE3 –RIL cells. This strain contains the RIL-plasmid for expression of tRNA codons that are rare in *E. coli*, to give better expression and accumulation of the protein. In small scale assays, the His-tag purified enzymes were analysed for activity by scintillation counting using [1-^{3}H]GPP and [1-^{3}H]FPP as substrates. The enzymes all proved to be active with GPP and not with FPP (data not shown).

GC-MS analysis

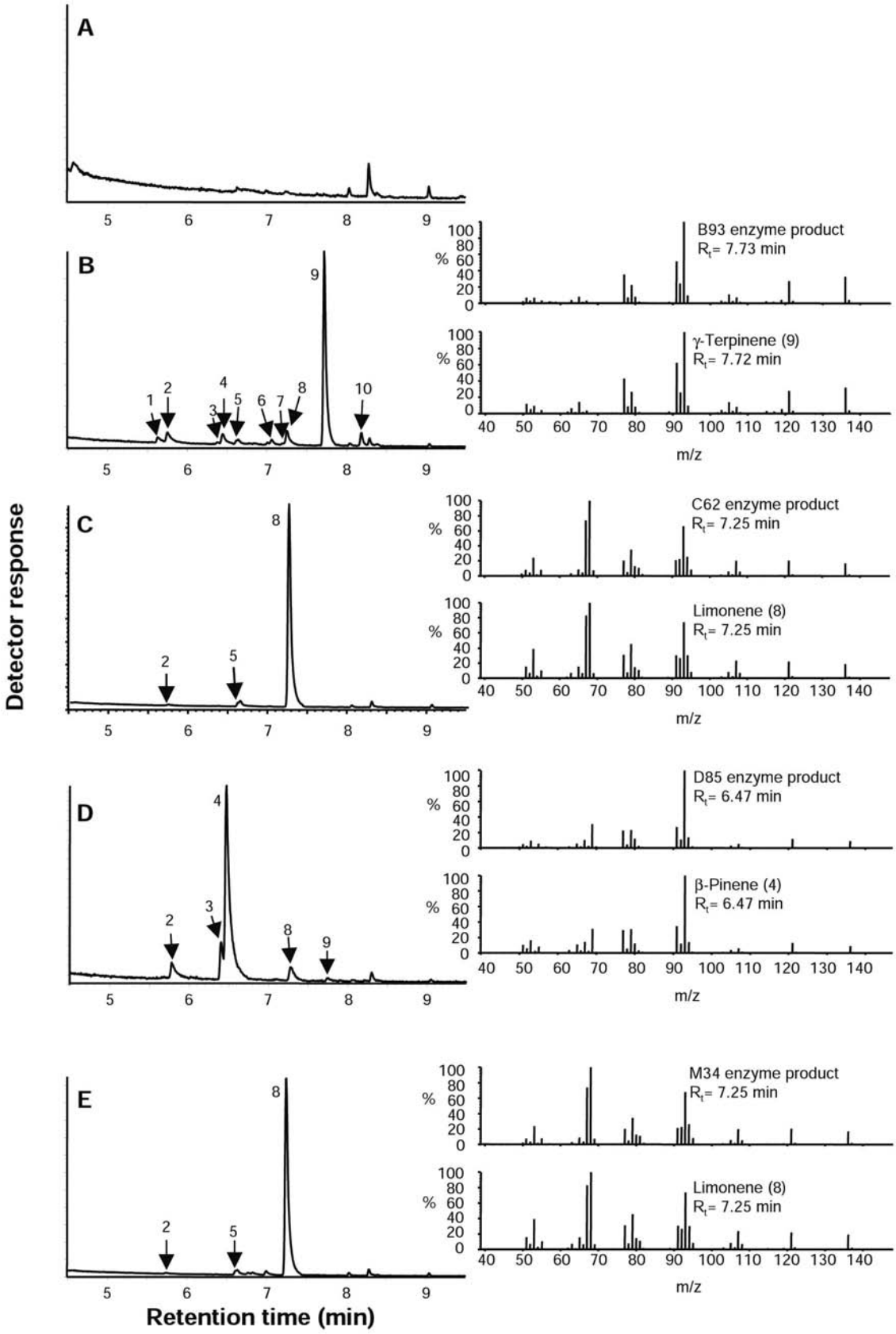
GC-MS analysis demonstrated that the cDNA encoded enzymes produced three different major products (Figure 3). B93 produced γ-terpinene and is therefore designated ClγTS (Figure 3B), C62 and M34 both produced limonene and are designated Cl(+)LIMS1 and Cl(+)LIMS2 respectively, (Figure 3C and 3E) and D85 produced β-pinene and is designated Cl(-)βPINS (Figure 5D). The enantiomeric composition of the products was determined using MDGC-MS, as described in the next section. Also side products and their abundance were determined for each synthase (Figure 3, Table 2). Concentration of the samples showed additional side product traces. No monoterpene products were detected in the pRSET empty vector control (Figure 3A). The major product of ClγTS was γ-terpinene (71.4%), with lower amounts of limonene (9.1%), α-pinene (5.6%), β-pinene (4.7%), α-terpinolene (3.7%), α-thujene (2.5%), α-terpinene (1.7%), myrcene (0.9%), sabinene (0.4%) and a trace of *p*-cymene (Figure 3B, Table 2). Both Cl(+)LIMS1 and Cl(+)LIMS2 produced almost exclusively limonene, (99.15%), with a small amount of β-myrcene (0.85%) and a trace of α-pinene (Figure 3C and Figure 3E, Table 2). The major product of the Cl(-)βPINS enzyme was β-pinene (81.4%), with sabinene (11%), α-pinene (4.1%), limonene (3.5%) and a trace of γ-terpinene as side products (Figure 3D, Table 2).

Table 2: Ratios of products formed by the monoterpene synthases as determined by GC-MS and their corresponding enantiomeric composition as determined by MDGC-MS. The percentages of the products formed by each synthase were determined on the GC-MS without concentrating the samples. tr: trace, ND: not determined

	ClγTS (B93)		Cl(-)βPINS (D85)		Cl(+)LIMS1(C62),Cl(+)LIMS2(M34)	
	(%)	(-):(+)	(%)	(-):(+)	(%)	(-):(+)
α-thujene	2.5	ND				
α-pinene	5.6	62:38	4.1	93:7	tr	13:87
sabinene	0.4	[a]	11.0	87:13		
β-pinene	4.7	2:98	81.4	99.5:0.5		
β-myrcene	0.9				0.85	
α-terpinene	1.7					
p-cymene	tr					
limonene	9.1	80:20	3.5	89:11	99.15	0: 100
γ-terpinene	71.4		tr			
terpinolene	3.7					

[a]: The sabinene in this sample co-eluted with the myrcene on the MDGC-MS preventing accurate determination of the enantiomeric composition.

Figure. 3. GC-MS profiles of products formed by the four heterologously expressed monoterpene synthases. (A) Empty pRSET vector control, (B) B93, (C) C62, (D) D85 and (E) M34. B93 mainly produces γ-terpinene, C62 and M34 produce limonene and D85 mainly produces β-pinene. Peak identities were confirmed using standards, whose mass spectra and retention times exactly matched these products. The mass spectra of the main products and their standards are depicted next to each chromatogram. Monoterpenes are numbered: 1: α-thujene, 2: α-pinene, 3: sabinene, 4: β-pinene, 5: myrcene, 6: α-terpinene, 7: *p*-cymene, 8: limonene, 9: γ-terpinene, 10: terpinolene.


A
B
C
D
E
Detector response
Retention time (min)
B93 enzyme product
Rt= 7.73 min
γ-Terpinene (9)
Rt= 7.72 min
C62 enzyme product
Rt= 7.25 min
Limonene (8)
Rt= 7.25 min
D85 enzyme product
Rt= 6.47 min
β-Pinene (4)
Rt= 6.47 min
M34 enzyme product
Rt= 7.25 min
Limonene (8)
Rt= 7.25 min
%
m/z

Enantiomeric analysis by MDGC-MS

The enantiomeric composition of the monoterpene products was analysed on a multidimensional GC-MS (MDGC-MS) (Table 2). Both Cl(+)LIMS1 and Cl(+)LIMS2 produced exclusively *R*-(+)-limonene, in contrast to ClγTS and Cl(-)βPINS that produced mainly *S*-(-)-limonene as a side product and only a small amount of *R*-(+)-limonene (Figure 4, Table 2). Cl(-)βPINS produced almost exclusively (-)-β-pinene, and 86% e.e. (|%*R*-%*S*|) of (-)-α-pinene. The sabinene side product of Cl(-)βPINS was determined to be 74% e.e. of (-)-sabinene (Table 2). ClγTS produced (-)-α-pinene as a side product with an e.e. of 24%, but (+)-β-pinene was produced with an e.e. of 96%. The enantiomeric composition of the side product sabinene of ClγTS could not be determined with certainty since it co-eluted with the side product myrcene. The α-pinene trace of Cl(+)LIMS2 consisted mainly of the (+)-enantiomer (Table 2).

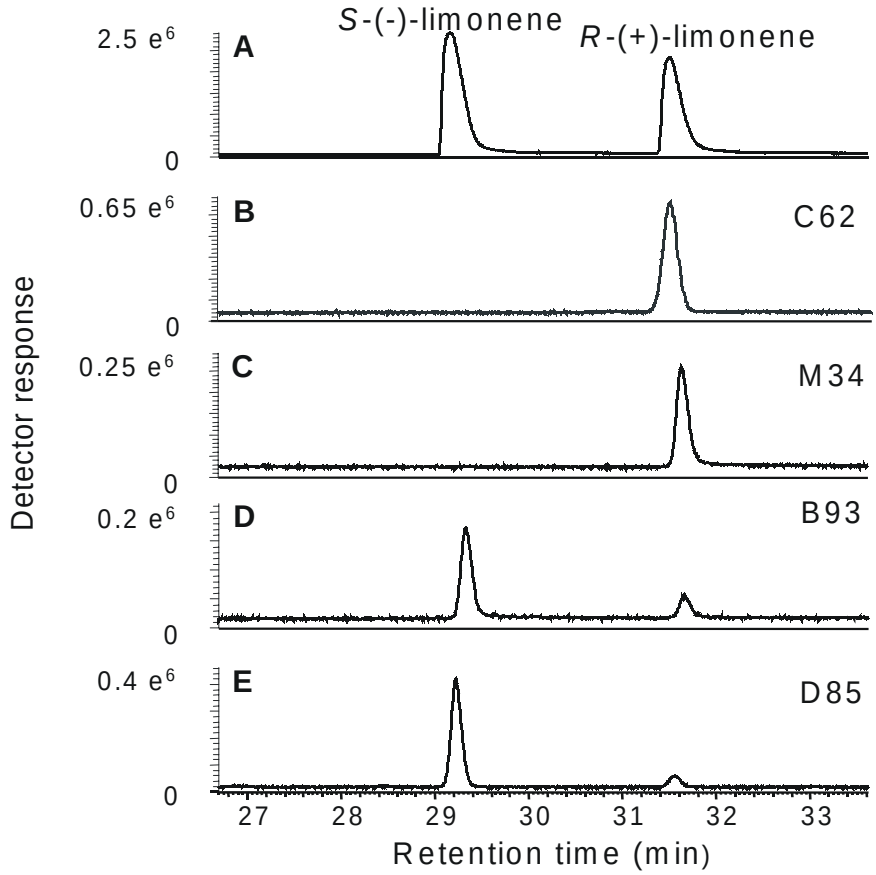


Figure. 4. GC-MS profiles of enantiomers of limonene formed by the different synthases. (A) shows separation of the reference limonene enantiomers. (B) and (C) show that M34 and C62 (Cl(+)LIMS1 and CL(+)LIMS2) produce *R*-(+)-limonene. (D) and (E) show that B93 (ClγTS) and D85 (Cl(-)βPINS) produce predominantly *S*-(-)-limonene as a side product.

Characterization of the heterologously expressed enzymes

The bivalent metal ion cofactor dependency of each synthase was tested with Mn^{2+} and Mg^{2+}. All synthases had around 30 times higher activity with Mn^{2+}. The optimal Mn^{2+} concentration was about 0.6 mM for all four enzymes and higher concentrations inhibited enzyme activity. Mg^{2+} dependency was less pronounced and did not result in inhibition at concentrations up to 15 mM. K^{+} has been reported to strongly enhance the activity of monoterpene synthases from different plant families (Savage et al., 1994), but for the lemon monoterpene synthases, it appeared to be an inhibitor. Maximum inhibition was found for concentrations above 100 mM KCl, when ClγTS was incubated with increasing KCl concentrations (data not shown). The pH dependence was tested for all four enzymes and enzymatic activity was found to be maximal around pH 7 (data not shown). Kinetic properties of the enzymes were determined by incubating with a range of GPP concentrations from 0.1 to 180 μM. The monoterpene synthase enzymes all showed substrate inhibition characteristics because the activity decreased with substrate concentrations above 10 μM.

K_m values for the cyclases were determined ignoring substrate inhibition using an Excel template anemona.xlt (Hernandez and Ruiz, 1998, available from http://genamics.com/software). K_m values were 0.7 μM for both Cl(+)LIMS1 and Cl(+)LIMS2, 2.7 μM for ClγTS and 3.1 μM for Cl(-)βPINS. When the anemona Excel template was used to calculate substrate inhibition kinetics, the K_m for Cl(-)βPINS was 13.5 μM (Figure 5).

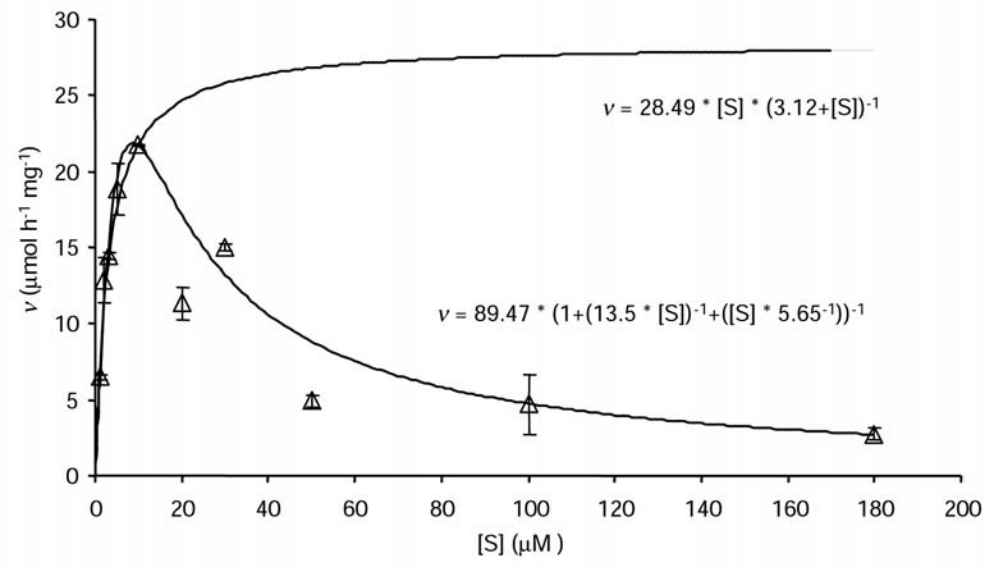


Figure. 5. Cl(-)βPINS enzyme activity curves. Enzyme activities were measured with substrate concentrations up to 180 μM GPP. A Michaelis Menten curve (featuring a K_m of 3.1 μM and an apparent v_{max} of 28.49 μmol h^{-1} mg^{-1}) and a substrate inhibition curve (featuring a K_m of 13.5 μM, an apparent v_{max} of 89.47 μmol h^{-1} mg^{-1} and a K_{si} of 5.65 μM) were fitted to the values obtained.

Discussion

The four monoterpene synthase cDNAs that have been isolated and characterized here account for the formation of more than 90% of the content of lemon essential oil. Most of the monoterpenoids that were found in the young lemon peel are either main or side products of the monoterpene synthases isolated and characterized in the present paper. Only the origin of the trace amounts of linalool, α-terpineol and (*E*)-β-ocimene that are also present in the lemon extract remain unexplained, as they are not a product of any of the synthases presented in this paper. However, the occurrence of the trace of *p*-cymene in the functional analysis of the γ-terpinene synthase could also have resulted from oxidation by air and light of γ-terpinene upon storage of the sample (Chapter 4, Chapter 6).
To isolate these monoterpene synthases from lemon, we used a random sequencing approach on a cDNA library from young lemon flavedo. This method has previously been proven to be successful for the isolation of full length cDNAs, particularly if the source tissue of the library is highly specialized with regard to the process to be studied (Aharoni et al., 2000; Lange et al., 2000; Gang et al., 2001). The levels of identity of the lemon monoterpene synthases indicate that they should be grouped within the *tpsb* clade of the angiosperm monoterpene synthases (Figure 1, and Table 1) (Bohlmann et al., 1998). Although the four lemon cDNAs cluster in the same clade, they clearly form two distinct classes, one containing *B93* and *D85* and the other *C62* and *M34*, because there are large differences both in the putative plastid targeting signals (only 16-18% identity) and the coding sequences (only 48-51% identity), suggesting that they have evolved separately.

This is confirmed by the phylogenetic analysis (Figure 2). The separate clustering of the lemon genes *B93*, *D85*, *Quercus ilex* myrcene synthase and the *Artemisia annua* monoterpene synthases from the limonene synthases *C62* and *M34*, suggests that the two groups of lemon synthases diverged in ancient times, even before *Quercus* and *Artemisia* separated from *Citrus*.

Monoterpene biosynthesis has been shown to be localized in the plastids in plants (Turner et al., 1999; Bouvier et al., 2000), and this is in accordance with the fact that all monoterpene synthases published to date bear an N-terminal transit peptide (Colby et al., 1993; Yuba et al., 1996; Bohlmann et al., 1997; Cseke et al., 1998; Wise et al., 1998; Bohlmann et al., 1999; Jia et al., 1999; Bohlmann et al., 2000; Fischbach et al., 2001; Maruyama et al., 2001b). Monoterpene synthases are nuclear encoded preproteins that are destined to be imported in the plastids, where they are proteolytically processed into their mature forms. Plastid targeting signals are typically rich in serines and threonines and low in acidic and basic amino acids and about 45-70 amino acids long. Usually they show only little homology.

The predictions using PREDOTAR and TARGETP indicate that all the four putative monoterpene synthases contain plastid targeting sequences. The lengths of the predicted targeting signals are rather short but the distance to the RRX_8W motif, common to monoterpene synthases of the *tpsb* clade, from where significant homology starts with other monoterpene synthases is 52 or 55 amino acids long. The RRX_8W motif is supposed to be required to give a functional mature protein and could have a function in the diphosphate migration step accompanying formation of the intermediate linalyl diphosphate before the final cyclisation step catalysed by the monoterpene synthases (Williams et al., 1998). The DDXXD motif, present in all terpene synthases, is supposed to bind the bivalent metal ion cofactor, usually Mn^{2+} or Mg^{2+} and is responsible for the ionization of the diphosphate group of GPP (Tarshis et al., 1994; Lesburg et al., 1997; Bohlmann et al., 1998). The active site domain of sesquiterpene synthases and probably also other terpene synthases is located on the C-terminal part of these proteins starting shortly before the DDXXD motif (Starks et al., 1997). Therefore it was suggested that the C-terminal part of the terpene synthase proteins determines the final specific product outcome (Bohlmann et al., 1999). Less than 10% overall sequence divergence has been shown to result in a significantly different product composition (Bohlmann et al., 1999). Table 1 shows that the identity level before the DDXXD motif between the *B93* and *D85* proteins (*Clγ TS* and *Cl(-)βPINS*) is higher (89%) than after the DDXXD motif (78%), suggesting that these two enzymes, although they are very homologous, are likely to catalyse the formation of two different products.

For the other two homologous protein sequences *C62* and *M34* (*Cl(+)LIMS1* and *Cl(+)LIMS2*), the identity before the DDXXD motif was almost the same as from the DDXXD motif onwards. This makes it likely that these proteins catalyse the formation of identical products.

The characterization of product specificity by functional expression in *E. coli* of the monoterpene synthases of lemon confirmed that both *C62* and *M34* (*Cl(+)LIMS1* and *Cl(+)LIMS2*) encode for enzymes that specifically form a single product, (+)-limonene, with only small traces of myrcene and (+)-α-pinene. Myrcene and α-pinene are trace products that were also described for (-)-limonene synthase from spearmint, but with undetermined stereochemistry (Colby et al., 1993). Although both limonene synthase enzymes produce exclusively (+)-limonene as a main product, the stereoselectivity for the trace coproduct α-pinene is less strong.

The other two monoterpene synthases encoded by *B93* and *D85*, which show less sequence identity, indeed produce different main products, γ-terpinene and (-)-β-pinene respectively. Furthermore these are much less specific in their product formation leading to formation of a number of side products up to 11% of total. It is a common feature of many monoterpene synthases that they are able to form multiple products from GPP as was shown by functional expression of synthases from several species such as spearmint, sage and grand fir (Colby et

al., 1993; Bohlmann et al., 1997; Wise et al., 1998; Bohlmann et al., 1999). The (-)-β-pinene synthase produces almost exclusively the (-)-enantiomer, and its side products show a similar enantiomeric composition, but with less stereoselectivity than the main product.
Considering the high sequence homology of the γ-terpinene synthase, producing an achiral product, to the (-)-β-pinene synthase, it would be expected that all side products would have the same stereochemistry. However, the data show that although the most prevalent side products above 5% have an e.e. for the (-)-enantiomer, there is also a side product with an e.e. of the opposite enantiomer ((+)-β-pinene). Furthermore, the stereoselectivity for most of the side products is even weaker than for the other lemon clones. Remarkably, the (+)-enantiomer of the β-pinene side product is formed in very high e.e. (96%). Other monoterpene synthases have been described that have low stereoselectivity for some of their side products, such as 1,8-cineole synthase and bornyl diphosphate synthase from common sage. The 1,8-cineole synthase produces for most side products an e.e. of the (+)-enaniomers, but for β-pinene an e.e. of the (-)-enantiomer. (Wise et al., 1998). As an explanation, Croteau and coworkers suggested that the *E. coli* host could proteolytically process the enzyme to a form that could compromise substrate and intermediate binding conformations.

In an investigation where monoterpene synthase activity from lemon was partially purified, the preference of Mn^{2+} as a cofactor instead of Mg^{2+} was reported (Chayet et al., 1977). The heterologously expressed enzymes from lemon show the same cofactor preference.
Lemon monoterpene synthases apparently do not prefer Mg^{2+} as the other cloned angiosperm synthases, but Mn^{2+} like the gymnosperm synthases (Bohlmann et al., 1998). These latter enzymes also require a monovalent ion, preferably K^{+} for activity (Savage et al., 1994; Bohlmann et al., 1998), while the lemon enzymes are inhibited by potassium ions. The pH optimum of the lemon synthases is close to pH 7 like other angiosperm synthases, while the gymnosperm synthases show a pH optimum that is generally higher, such as pH 7.8 for the grand fir and lodgepole pine synthases (Lewinsohn et al., 1992; Savage et al., 1994; Bohlmann et al., 1998).

The enzyme activity curves show that the activity decreases dramatically when the substrate concentration increases above 10-50 µM as shown for Cl(-)βPINS (Figure 5). This cannot be caused by product inhibition as the products of the synthases will migrate to the hexane phase used in the assays and are therefore not expected to be interfering with the enzyme. The enzymes show substrate inhibition characteristics, a feature not previously reported for other cloned monoterpene synthases. The observation that the partially purified native monoterpene synthase enzyme fraction from lemon flavedo also showed substrate inhibition at higher substrate concentrations than 5 times the K_m rules out the possibility that this phenomenon is the consequence of changes to the protein due to cloning artefacts (Chayet et al., 1977). An

explanation could be that at higher concentrations, the allylic diphosphates start forming enzymatically inactive 2:1 complexes with metal ions, bound to the enzyme. Recent crystallographic work has shown that both epi-aristolochene synthase and trichodiene synthase contain 3 Mg^{2+} ions in their active site, two of which are chelated by the DDXXD motif of the active site and a third which is liganded by a triad of active site residues (Starks et al., 1997; Rynkiewics et al., 2001).
The K_m values determined for the monoterpene synthases from *C. limon* as determined by Michaelis Menten kinetics are in a similar range as the values for other monoterpene synthases cloned thus far. The limonene synthases have a lower K_m value than the pinene and the γ-terpinene synthases. Although no data are available about relative expression ratios of the four genes, the difference in K_m may explain in part why the level of limonene compared to the other main products in the lemon peel is so much higher.

This report describes the first cloned monoterpene synthase that forms γ-terpinene as a major product. A homodimeric γ-terpinene synthase enzyme, purified from *Thymus vulgaris* produced in addition to the main product also small amounts of α-thujene and lesser quantities of myrcene, α-terpinene, limonene, linalool, terpinen-4-ol, and α-terpineol (Alonso and Croteau, 1991). However the gene encoding this enzyme has so far not been isolated. In addition this is the first report on a (-)-β-pinene synthase cDNA.
Limonene is widely used in beverages and the cosmetics industry, and (+)-limonene has anticarcinogenic properties (Crowell and Gould, 1994). The previously isolated (+)-limonene synthase from *S. tenuifolia* produces, apart from (+)-limonene, also a substantial amount of a non-identified monoterpene side product (Maruyama et al., 2001b). The lemon cDNA encoding (+)-limonene synthase however produces more than 99% pure and exclusively (+)-limonene. Such a pure compound synthesized by a heterologously expressed enzyme could perhaps be a more natural alternative than chemical synthesis and possibly a cheaper alternative than purification from plants.

Acknowledgements

We like to thank B. Weckerle for the MDGC-MS analyses and Dr. Maurice Franssen for critical reading and helpful remarks on the manuscript.

Chapter 4

Modification of monoterpene emission profiles in tobacco by metabolic engineering using three lemon monoterpene synthases

Joost Lücker[1], Wilfried Schwab[2], Bianca van Hautum[1], Jan Blaas[1], Linus H. W. van der Plas[3], Harro J. Bouwmeester[1] and Harrie A. Verhoeven[1]

[1] Business unit Cell Cybernetics, Plant Research International, P.O. Box 16, 6700 AA, Wageningen, The Netherlands,

[2] University of Würzburg, Chair of Food Chemistry, Am Hubland, 97974, Würzburg, Germany

[3] Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD, Wageningen, The Netherlands

Summary

Wild-type tobacco plants emit low levels of terpenoids, particularly from the flowers. By genetic modification of *Nicotiana tabacum* 'Petit Havana' SR1 using three different monoterpene synthases from lemon and the subsequent combination of these three into one plant by crossing, we show that it is possible to increase the amount and alter the composition of the blend of monoterpenoids produced in tobacco. The transgenic tobacco plant line with the three introduced monoterpene synthases is emitting β-pinene, limonene and γ-terpinene from its leaves and flowers. In the flowers this does hardly result in a decrease in the emission of wild type terpenoids. Apparently there is a sufficient amount of substrate geranyldiphosphate accessible to the introduced enzymes to produce a range of new monoterpenes, in addition to the endogenous ones.

Introduction

Plants are producing a wide range of different volatile metabolites by different biosynthetic pathways, each having their own complex regulation (Dudareva and Pichersky, 2000). The regulated emission of volatiles from flowers has been suggested to correlate with the patterns of activity of the organisms that are interacting with the plant such as pollinators, herbivores and predators of the herbivores (Kolosova et al., 2001a; Pichersky and Gershenzon, 2002).
Emission of volatiles has been shown to be regulated at the gene expression level and often displays a nocturnal or diurnal circadian rhythm as was shown by extensive investigations on methylbenzoate emission by snapdragon, tobacco and *Clarkia* (Dudareva et al., 1996; Dudareva et al., 2000; Kolosova et al., 2001a). Terpenoid synthases are to a large extent responsible for the diversity of terpenoid structures involved in floral scent or in herbivore induced volatiles (Bohlmann et al., 1998). Monoterpenes are C_{10} compounds, one of the classes of terpenoids, synthesised from geranyldiphosphate (GPP) via the plastid localised 2*C*-methyl-D-erythritol-4-phosphate (MEP)-pathway (Eisenreich et al., 1997). They are of high economic value as they are products extensively used as flavour and fragrance additives in food and cosmetics (Verlet, 1993). Furthermore they are of interest for their medicinal e.g. anticarcinogenic properties (Crowell, 1999). Their biological significance lies in their involvement in plant-insect, plant-pathogen and plant-plant interactions (Wink, 1999). Monoterpenes can be released in a diurnal rhythm as was shown for rose flowers (Helsper et al., 1998; Helsper et al., 2001). Monoterpene emission is also affected by environmental conditions such as temperature and light (Schuh et al., 1997).
Nicotiana tabacum varieties have been investigated for their emission profiles and were shown to emit mono- and sesquiterpenes. Emission of volatiles from leaves was 30 to 100 - fold lower than from flowers (Andersen et al., 1988; Loughrin et al., 1990).
Genetic modification of terpenoid biosynthesis is a research area that opens up opportunities to study the flexibility of the regulation of this secondary metabolic pathway in plants. When the genome of plants is provided with genes encoding novel enzyme activities, the formation of end products necessarily depends on the metabolome of the transformed plant species. Already several successful approaches of genetic modification of terpenoid pathways have been reported. In rice (Ye et al., 2000), tomato (Romer et al., 2000) and rape (Shewmaker et al., 1999) an increase in the carotenoid level was achieved. By sense and antisense approaches, levels of mono and diterpenoids could be changed (Mahmoud and Croteau, 2001; Wang et al., 2001). Also new monoterpene synthases have been introduced in plants, resulting in a change of the aroma profile, in production of new monoterpenes that are emitted from vegetative and floral tissues or in the accumulation of the corresponding glucoside (Lewinsohn et al., 2001; Lücker et al., 2001; Lavy et al., 2002). However, no reports have been published on the simultaneous introduction of multiple enzymes competing for the same

substrate pool. In this paper we report on the changes of the volatile profile of tobacco by metabolic engineering, using three different monoterpene synthases from lemon and the subsequent combination of these into one plant by crossing. This resulted in an extensive modification of volatile production in leaves and flowers.

Experimental procedures

Plant material

Nicotiana tabacum 'Petite Havana' SR1 (Maliga et al., 1973), a variety widely used in tissue culture was used as starting material for all transformations. Plants were grown under 16 h photoperiod and standard greenhouse conditions.

Cloning of genes in plant expression vectors

From a random sequencing approach on a pBluescript UNIZAP-XR cDNA library (Stratagene), three full length *Citrus limon* monoterpene cyclases, γ-terpinene cyclase, *B93* (Genbank accession number: AF514286), (+)-limonene cyclase 1, *C62* (AF514287) and (-)-β-pinene cyclase, *D85* (AF514288), including a predicted plastid targeting signal, were selected to be transformed to tobacco plants (Lücker et al., 2002). Genbank can be accessed from the NCBI website, http://www.ncbi.nlm.nih.gov/index.html. They were subcloned without the pBluescript multiple cloning sites and the polyA-tail into the kanamycin resistance gene containing pBinPLUS vector (van Engelen et al., 1995). Genes were cloned by Quickchange PCR according to the manufacturers recommendations (Stratagene) followed by resequencing of the mutated vector. The PCR program used was 30 s, 95 °C, 1 min 55 °C, 12 min 68 °C, 14 cycles on a Mastercycler gradient PCR apparatus (Eppendorf Scientific, Inc., Westbury, NY, USA).

For cloning of *B93* first a *Spe*I site was introduced in the 5'UTR using the primers B93F 5'-ggAATTCggC<u>AC*T*Ag*T*</u>ATAgAATCCTTg-3' and its exact complement. For the used primers the altered nucleotides are written in italics and the introduced restriction site is underlined. After selection of the correct clone including the mutation it was cut with *Bgl*II, filled in with Klenow fragment (Gibco-BRL) followed by digestion with *Spe*I. The cDNA insert fragment was ligated, using DNA ligase I (Gibco-BRL) to a pFLAP10 vector fragment (pFLAP10 vector was kindly provided by Dr. A. Bovy, Plant Research International), containing a CaMV d35S promoter and a NOS terminator sequence, digested with *Sal*I, filled in with Klenow fragment followed by digestion with XbaI, to obtain a compatible site for *Spe*I. The pFLAP vector now containing the *B93* ORF was digested with *Pac*I and *Asc*I and ligated to

pBinPLUS vector (van Engelen et al., 1995) digested with the same enzymes resulting in plasmid pJLT2.

For cloning of *C62* first a *Sal*I site was introduced in the 3'UTR using the primers C62F 5'-gCAgTTTCAgTCgACgTTggCCTCCAC-3' and its exact complement. After selection of the correct clone including the mutation it was cut with *Nhe*I and *Sal*I. The cDNA insert was ligated to a pFLAP10 vector fragment that was digested with *Xba*I and *Sal*I. The pFLAP vector was subsequently digested with *Pac*I and *Asc*I and ligated to pBinPLUS digested with the same restriction enzymes, resulting in a plant expression vector pJLT3.

For cloning of *D85* a *Bam*HI site was introduced in the 5'UTR using the primers D85F 5'-ggAATTCggCACgaggaTCCTTgAAATTATATTC-3' and its exact complement. After selection of the correct clone including the mutation it was cut with *Bam*HI and *Afl*III. The cDNA insert was ligated to a pFLAP10 vector fragment that was digested with *Bam*HI and *Nco*I. The resulting vector was digested first with *Asc*I and subsequently partially digested with *Pac*I, since there was also a *Pac*I site at the 3'end of the ORF of the *D85* cDNA. The fragment including the CaMV-35S promoter and the Nos terminator was ligated to pBinPLUS digested with *Pac*I and *Asc*I resulting in the plant expression vector pJLT4. All fragments were isolated from low melting point agarose gel (Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands) using Agar*ACE*® *agarose digesting* enzyme according to the manufacturers recommendations (Promega Benelux b.v., Leiden, The Netherlands). For subcloning steps XLIBlue MRF' supercompetent cells (Stratagene) were used. Plasmid DNA was isolated from *E. coli* cultures using a plasmid DNA isolation robot (Qiagen) as described previously (Lücker et al., 2001).

Restriction enzymes were obtained from Gibco-BRL, apart from *Pac*I and *Asc*I restriction enzymes, which were obtained from New England Biolabs. The sequence mix was obtained from Perkin Elmer.

The pBinPLUS vectors containing the inserts were transformed to *Agrobacterium tumefaciens*, strain LBA4404 competent cells, using a freeze thaw protocol (Hofgen and Willmitzer, 1988). Colonies were checked after transformation by back-transformation to *E. coli XL1* supercompetent cells (Stratagene). Basic DNA manipulations were carried out as previously described (Sambrook et al., 1989).

The three constructs were transformed to *Nicotiana tabacum*. For tobacco transformation a standard leaf disk transformation and regeneration protocol was used essentially as described previously (Horsch et al., 1985). As control, leaf cuttings were also transformed with LBA4404 containing the original pBinPLUS vector. Furthermore some non-transformed leaf cuttings were taken through the regeneration process. Plants rooting on kanamycin (50 mg L^{-1}) containing MS medium, supplemented with 20 g L^{-1} sucrose were transferred to soil in the greenhouse.

RNA analysis

Leaf tissue (young leaf, 2 cm long, not fully developed) was taken from young plants in the greenhouse and directly frozen in liquid N_2.
Total RNA was isolated from 200 mg of frozen tissue ground in liquid N_2 which was vigorously mixed (30 s) with a preheated mixture of 0.6 mL acid phenol (80 °C) and 0.6 mL RNA extraction buffer containing 100 mM NaAc, pH 4.8, 100 mM LiCl, 10 mM EDTA and 10% SDS. Another 0.6 mL of chloroform: isoamylalcohol (24:1) was added and again the mixture was vigorously mixed for 30 s. The mixture was centrifuged at 9400 g for 15 min at 4 °C. 0.6 mL LiCl was added to the aqueous top phase and this mixture was incubated for 1 h at - 80 °C, subsequently thawed and centrifuged at maximum speed and at 4 °C for 15 min. The resulting pellet was washed with 70% ethanol, air dried and dissolved in H_2O.
Northern blotting was performed as described previously (Lücker et al., 2001). Probes used for the Northern blots were the full-length original cDNA clones excised from the pBluescript multiple cloning site. Labeling, hybridization, stripping and subsequent hybridization with a ribosomal cDNA as loading control were carried out as described previously (Lücker et al., 2001).

Crossings and seed analysis

Primary transformants were selfed and crossed. One hundred seeds of control or transgenic plants were plated on solid MS medium supplemented with 10 g L^{-1} sucrose and 100 mg L^{-1} kanamycin to select for transgenics. Plants giving high expression and containing one insertion of the different genes were crossed. First B93-6 was crossed with D85-26 resulting in offspring containing both genes as selected by GC-MS analysis. Such a plant was crossed with C62-21 and the resulting progeny contained a plant with all the three genes inserted in its genome.

Volatile analysis

To measure the release of volatiles by the tobacco plants, the headspace of leaves and flowers was analysed on intact plants in the greenhouse as well as on detached leaves and flowers from the plant. For intact plants, a glass funnel was used to enclose the plant part to be analysed and aluminium foil was used as a seal. A 100 μm polydimethylsiloxane (PDMS)-coated solid phase micro-extraction (SPME) fibre (Supelco, Belfonte, PA, USA) was used in order to capture the volatiles released from the plant tissue. Volatile sampling was carried out during 30 min and repeated several times.

Measurements of the endogenous content of volatiles were performed using tissue ground in liquid N_2, 5 M $CaCl_2$ solution and subsequent SPME headspace analysis as described previously (Lücker et al., 2001). The compounds were identified by comparison of GC retention times and mass spectra with those of authentic reference compounds.

For headspace trapping onto Tenax TA (20/35 mesh, Alltech, Breda, the Netherlands) detached flowers or leaves were used. For flowers four different flower developmental stages were used for volatile sampling (Goldberg, 1988; Koltunow et al., 1990):
Stage 8, corolla elongating; petals green and slightly open,
Stage 10, corolla limb beginning to open; petal tips pink,
Stage 11, corolla limb halfway open; stigma and anthers visible,
Stage 12, flower open; anthers dehisced, corolla limb fully expanded and deep pink.
During the 24 h sampling period all stages developed to the next stage. Five flowers of each stage of the control and of the plant with the three monoterpene synthases were taken for simultaneous analysis of volatile emission. The flowers or leaves were directly placed onto green floral foam blocks (Smithers-Oasis Belgium N.V., Houthalen, Belgium) saturated with water and wrapped in aluminium foil, before they were enclosed in 1 L glass jars closed with a Teflon-lined lid equipped with inlet and outlet. A vacuum pump was used to draw air through the glass jar at approximately 100 mL min^{-1}, with the incoming air being purified through a glass cartridge (140 x 4 mm) containing 150 mg Tenax TA. At the outlet the volatiles emitted by the detached flowers or leaves were trapped on a similar Tenax cartridge. Volatiles were sampled during 24 h. Cartridges were eluted using 3 x 1 mL of redistilled pentane-diethyl ether (4:1). 212 ng (+)-3-carene was added to the eluent, as an internal standard. Of these samples that were either directly used or first concentrated under a stream of nitrogen, 2 µL were analyzed by GC-MS using an HP 5890 series II gas chromatograph equipped with an HP5-MS column (30 m x 0.25 mm i.d., 0.25 µm film thickness) and an HP 5972A Mass Selective Detector as described previously (Bouwmeester et al., 1999b). GC oven temperature was programmed at an initial temperature of 45 °C for 1 min, with a ramp of 10 °C min^{-1} to 280 °C, and final time of 10 min.

Results

Introduction of lemon monoterpene synthases into *Nicotiana tabacum*

Wild type *Nicotiana tabacum* L. 'Petit Havana' SR1 plants were transformed with monoterpene synthase cDNAs isolated from a *Citrus limon* fruit peel cDNA library, previously functionally expressed and characterized in *E. coli* (Lücker et al., 2002). The plants were either transformed with the *B93* (*ClγTS*) cDNA, encoding γ-terpinene synthase,

with the *C62* (*Cl(+)LIMS1*) cDNA, encoding (+)-limonene synthase, or with the *D85* (*Cl(-)βPINS*) cDNA, encoding (-)-β-pinene synthase. Plants that formed roots on kanamycin containing medium were transferred to greenhouse conditions for further examination. The primary transformants had the same phenotype as control plants.

Analysis of the primary transformants

The young developing transgenic plants were analyzed for expression of the cDNAs by Northern blotting. Figure 1 shows that the three monoterpene synthase cDNAs are expressed in leaves of the transgenic plants and that there are large differences in expression levels between the transgenic lines.
For the high expressors, the number of inserts in the transgenic lines was determined using Southern blotting, which showed that some plants contained more than one insert. Cross hybridization with native tobacco genes, possibly terpene synthases, was only visible in the Southern blot hybridised with the *C62* (*Cl(+)LIMS1*) cDNA. One genomic DNA fragment of the same size appeared in the non-transformed control plants as well as in the 12 tested transgenic lines. No cross hybridization with an endogenous gene was detected when *B93* (*ClγTS*) or *D85* (*Cl(-)βPINS*) cDNA probes were used for Southern blotting of the respective plant lines (data not shown).

To simplify phenotypic and genetic analysis of the transgenic lines, lines were selected that showed integration of a cDNA only on a single locus. Seed plating experiments were carried out, after self-pollination of all the primary transformants and the control plants. All germinated seeds of the control plants were sensitive to kanamycin (Table 1). For single loci transformed lines it was expected that the germinated seeds showed Mendelian 1: 3 segregation for kanamycin sensitivity and kanamycin resistance. If the introduced kanamycin resistance gene, included on the T-DNA of the binary vector used for transformation is co-localised with the respective monoterpene synthase cDNAs, lines showing this 1:3 segregation for kanamycin resistance, will also have only one monoterpene synthase insertion. As expected plant lines showing multiple gene insertions by Southern blotting (e.g. B93-8, C62-20, D85-24) did not show a 1:3 segregation for kanamycin resistance (Table 1). The plant lines, B93-6, D85-26 and C62-21, showing one cDNA insertion on the Southern Blot, did show the expected segregation (Table 1). The progeny of the cross between B93-6 and D85-26, each transformed with a different cDNA, but each with a single gene insertion also showed the expected 1:3 segregation for kanamycin resistance (Table 1), indicating that the two different cDNAs were integrated on independent loci. Plant lines B93-6, D85-26 and C62-21 were selected for further analysis of monoterpene production.

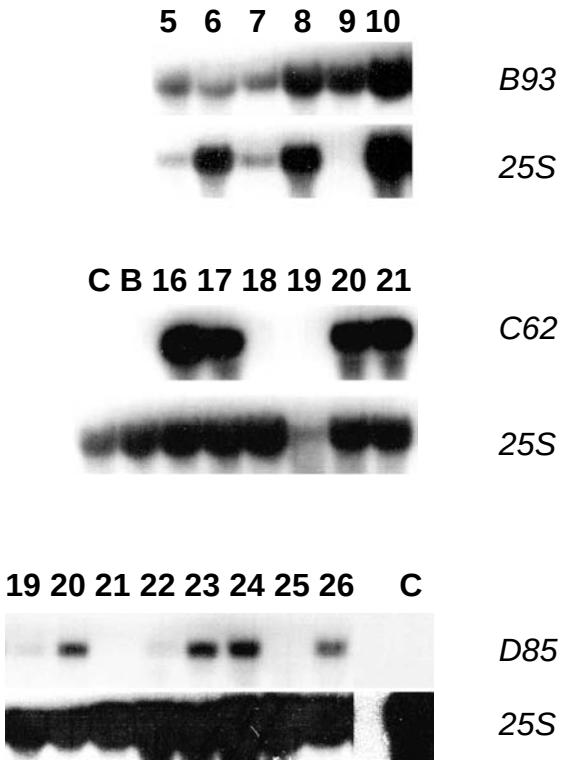


Figure 1:Northern blot analysis of tobacco plant lines transformed with three different monoterpene synthases from lemon. Lines were transformed with single constructs only. Top panels are showing expression in tobacco leaves of the different cDNAs i.e. *B93* (γ-terpinene synthase), *C62* (limonene synthase) and *D85* (β-pinene synthase) in a subset of the different plant lines. The bottom panels are showing a *25S* ribosomal cDNA control to indicate loading differences. C: SR1 non-transgenic control plant, B: empty binary vector transformed line. Transgenic plant line numbers are indicated above the Northerns.

Table 1: **Seed plating experiments on kanamycin containing medium for different transgenic lines of tobacco**

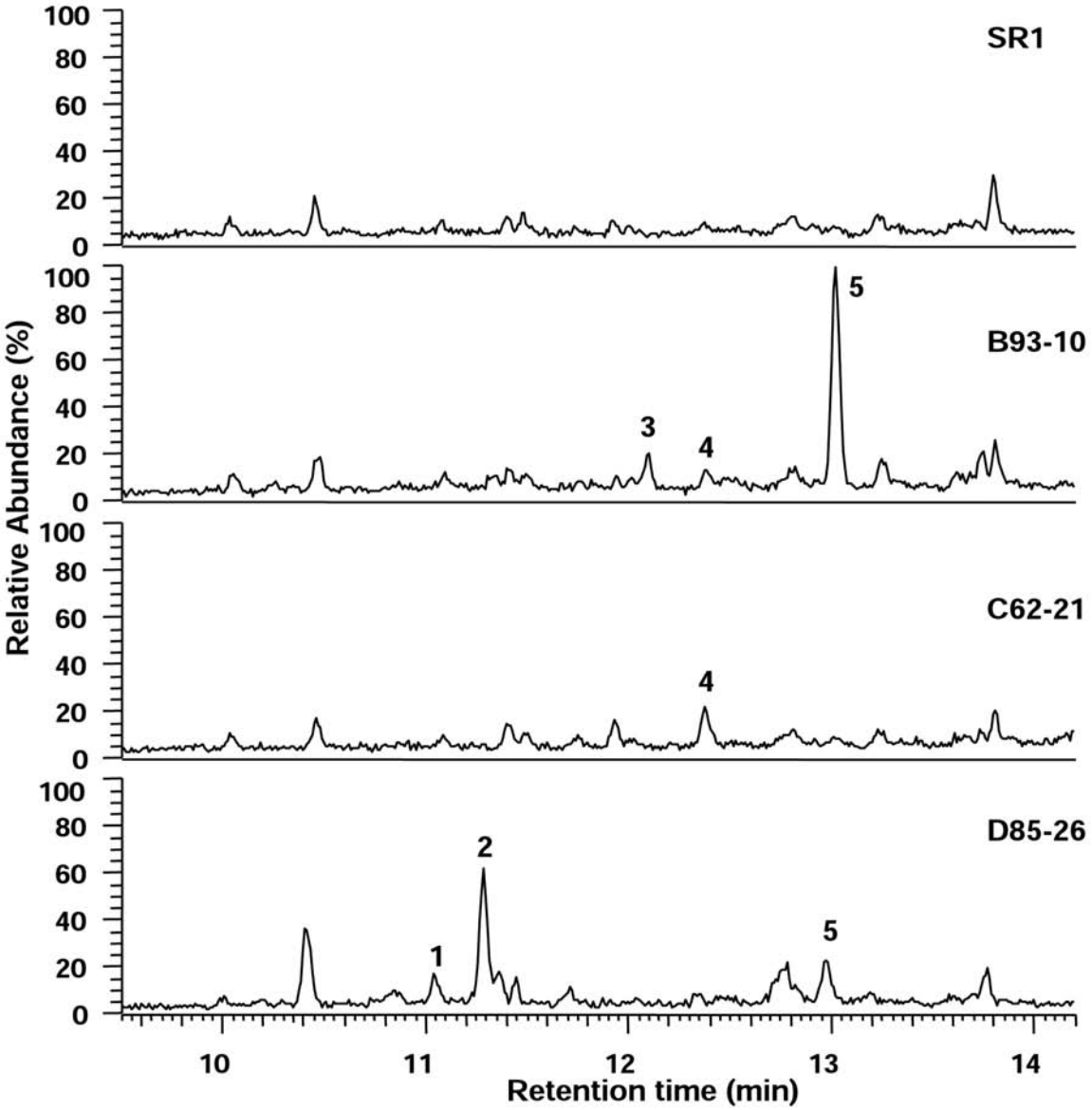
	Plant line	Kan^S	Kan^R	X^2_{KanS}	X^2_{KanR}	X^2_{total}	
	SR-1	100	0				
S1	B93-6	27	89	0.1379	0.0449	0.183	H0
S1	B93-10	24	76	0.04	0.013	0.053	H0
S1	B93-8	6	94	14.44	4.81	19.25	H1
S1	C62-21	23	86	0.6628	0.2209	0.8837	H0
S1	C62-20	2	98	21.16	7.05	28.21	H1
S1	D85-26	30	70	1	0.33	1.33	H0
S1	D85-24	6	94	14.44	4.813	19.25	H1
F1	B93-6 x D85-26	28	72	0.36	0.12	0.48	H0
F1	C62-21 x D85-24	16	84	3.24	1.08	4.32	H1

Seedlings that turn white after germination are kanamycin sensitive. Since all terpene synthase binary constructs also contain a kanamycin resistance gene on the same T-DNA, the number of kanamycin resistance T-DNA insertions is supposed to be equal to the number of monoterpene synthase T-DNA insertions. Both selfings of mother plants (S1) and crosses between mother plants (F1) were made and the progeny of these crosses also showed a Mendelian segregation for the kanamycin gene. For 100 seeds the critical X^2 value for 1 degree of freedom and a P value of 0.05 is 3.841. If H0: Kan S: Kan R=1:3 is true, the transgenic mother plant contains a T-DNA insertion only on a single locus. Plants B93-6, C62-21 and D85-26 were selected for further experiments since these plants were containing only one gene insertion based on these seed plating observations. The progeny of seeds of these plants and also of the cross between B93-6 and D85-26 shows the expected 1:3 ratio for kanamycin sensitivity.

GC-MS analysis

Headspace SPME measurements on $CaCl_2$ extracts of young leaves of the control plants and several transgenic lines were done to verify the formation of new products. Figure 2 shows that in control SR1 leaves no accumulation of monoterpenes could be detected. Transformed plant lines expressing the different constructs however accumulated the expected compounds. Plant lines expressing the *B93* cDNA showed accumulation of mainly γ-terpinene (5), but also trace amounts of α-terpinene (3) and limonene (4). Plant lines expressing the *C62* cDNA accumulated only limonene (4). Plant lines expressing the *D85* cDNA accumulated mostly β-pinene (2), and traces of sabinene (1) and γ-terpinene (5).

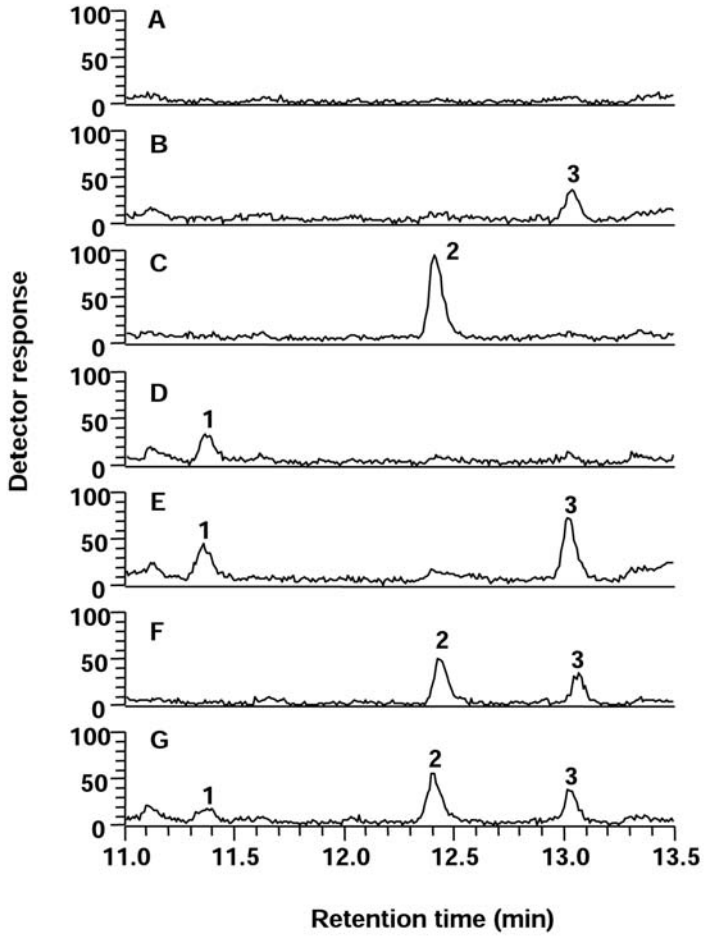
Figure 2: Typical GC-MS chromatograms showing the volatiles of leaves of a wild type tobacco control plant and transformant lines expressing three different monoterpene synthases from lemon. The chromatograms were obtained using headspace solid phase micro extraction (SPME) of the ground leaf material in 2.5 M $CaCl_2$. SR1: non-transformed control plant, B93-10: Transgenic plant line expressing γ-terpinene synthase. C62-21: Transgenic plant line expressing limonene synthase. D85-26: Transgenic plant line expressing β-pinene synthase. Y-axis is showing relative abundance of m/z 67 + 68 + 69 + 79 + 93 + 121 + 136 with 100% level set at 2.01* e^4. All peaks that represent monoterpenes are numbered; they were identified by comparing mass spectra and retention times to authentic standards. 1: sabinene, 2: β-pinene, 3: α-terpinene, 4: limonene, 5: γ-terpinene.



Combination of 3 monoterpene synthases in one plant by crossing

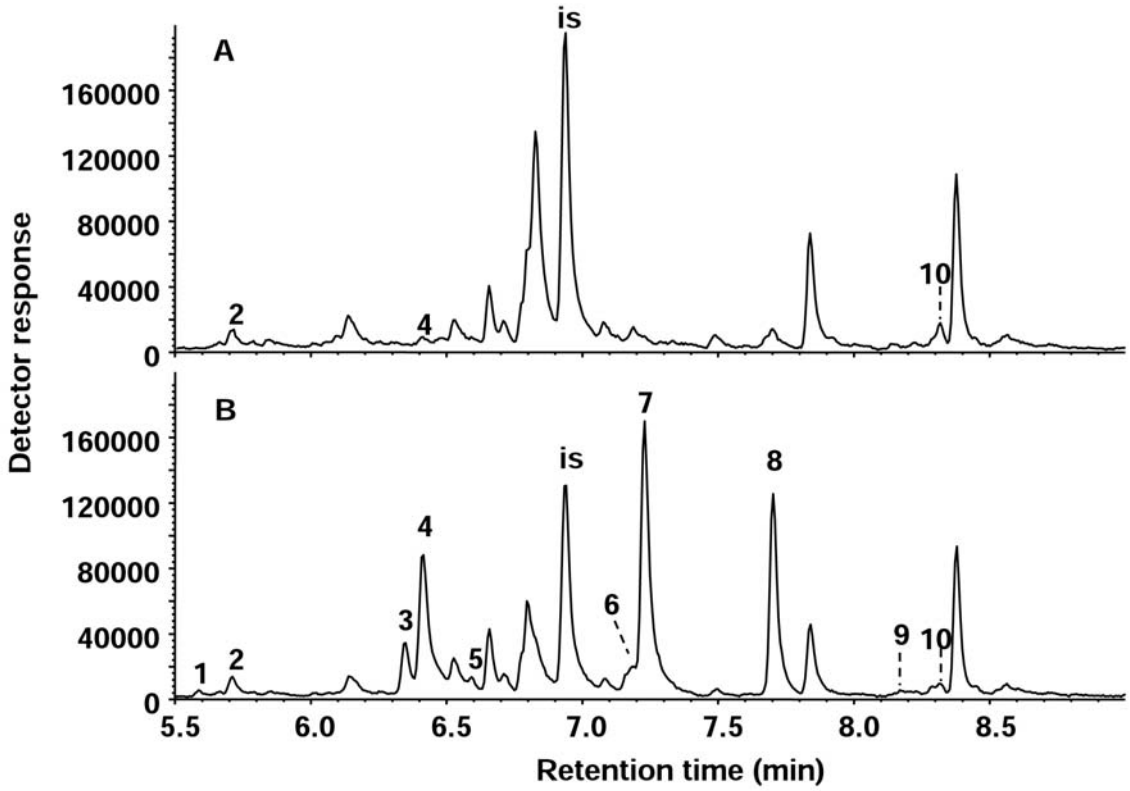
In order to obtain a plant producing several new monoterpenes, crossings were made between primary transformants containing one insert of the three representative monoterpene synthase cDNAs. The first cross was made between plant lines B93-6 and D85-26. Total plant headspace SPME measurements on four week old F1 seedlings resulting from this cross showed emission of either both β-pinene and γ-terpinene, emission of single products or no detectable emission of any of these monoterpenes (data not shown). One plant emitting both β-pinene and γ-terpinene at high levels was used to cross with the limonene producing C62-21 primary transformant. From this cross eight different phenotypes were expected to occur if all the three cDNA insertions are independently inheriting: plants emitting no monoterpenes, plants emitting one of the three main products, plants emitting any combination of two monoterpene synthase main products and plants emitting all three of the main products. Figure 3 shows the results of headspace SPME measurements of the four week old seedlings resulting from this second cross. Seven of the eight predicted phenotypes were indeed detected in a population of 20 plants analyzed, shown in panels A to G (Figure 3). Panel G shows the GC-MS profile of a plant emitting all three main products of the introduced monoterpene synthases.

Figure 3: GC-MS headspace chromatograms measured by SPME of four week old seedlings showing different progeny phenotypes from a cross between an F1 plant of a cross between B93-6 and D85-26 (emitting both β-pinene and γ-terpinene) and C62-21, a primary transgenic line emitting limonene (Panels A to G). The GC-MS trace shows the combination of m/z 67-69,71,79,91,93,105,107,121 and 136. 1: β-pinene, 2: limonene, 3: γ-terpinene. Panel A shows the profile of a plant that lost all three cDNAs in the cross, since there are no monoterpenes emitted anymore. Panels B, C and D show plants that emit only γ-terpinene, limonene or β-pinene respectively. Panels E and F show plants that emit two main products of the introduced monoterpene synthases. The plant in panel E is emitting both β-pinene and γ-terpinene, while the plant in panel F is emitting both limonene and γ-terpinene. Panel G is showing a plant that is emitting all the three main products of the introduced monoterpene synthases.



Headspace analysis using Tenax trapping of young leaves of mature plants of the wild type tobacco and the plant with the three monoterpene synthases (Figure 4) clearly showed emission in the latter plant of the three different main products, β-pinene (4), limonene (7) and γ-terpinene (8) (Figure 4B), all emitted at levels of 150 to 350 ng g^{-1} 24 h^{-1}. The leaves of the control plant emitted traces of the monoterpenoids α-pinene (2), β-pinene (4) and linalool (10) (Figure 4A) at levels of 5 to 15 ng g^{-1} 24 h^{-1}. In the headspace of the transgenic plant also trace amounts were identified of α-thujene (1), myrcene (5), terpinolene (9) and linalool (10) at a level of 5 to 15 ng g^{-1} 24 h^{-1}, while α-pinene (2), sabinene (3) and *p*-cymene (6) were emitted at a slightly higher level of 20 to 50 ng g^{-1} 24 h^{-1} (Figure 4B). In addition, the sesquiterpene β-caryophyllene was emitted by the leaves of both the control and the transgenic plant (66 and 42 ng g^{-1} 24 h^{-1} respectively) (data not shown). Levels of volatiles emitted from young leaves were approximately 5- to 10- fold higher than in older leaves.

Figure 4: Volatile emission profile of detached leaves of tobacco plants sampled for 24h using Tenax trapping of (A) a non transformed control plant and (B) a plant expressing γ-terpinene, (-)-β-pinene and (+)-limonene synthase. Monoterpene peaks identified by mass spectrum and retention time are numbered. 1: α-thujene, 2: α-pinene, 3: sabinene, 4: β-pinene, 5: myrcene, 6: *p*-cymene, 7: limonene, 8: γ-terpinene, 9: terpinolene, 10: linalool. is: internal standard (+)-3-carene (212 ng). Non-monoterpene peaks are not labelled.



Monoterpenes emitted during flower development

Volatiles emitted from different flowering stages were collected for 24 h using Tenax trapping and compared to analyze differences in emitted levels and composition. Control flowers emitted linalool and β-caryophyllene (Figure 5, A-D). In the headspace of transgenic flowers all the main products of the three introduced lemon monoterpene synthases, β-pinene, limonene and γ-terpinene, were detected in all stages (Figure 5, E-H), in addition to the native linalool and β-caryophyllene. Furthermore trace amounts were detected of α-thujene, α-pinene, sabinene, myrcene and *p*-cymene. The levels of linalool emitted from control and transgenic flowers were similar, although emission of linalool in the transgenic flowers seems to peak earlier in development (Figure 5 and Table 2). For the transgenic flowers the levels of limonene and γ-terpinene decreased during flower development, while the level of β-pinene was rather stable and decreased only in the open flower. The β-caryophyllene emission pattern differed from that of linalool. For the control flowers, β-caryophyllene emission steadily increased with flower development, whereas for transgenic flowers it was about 2 to 3- fold lower, during the later developmental stages and somewhat decreased after flower opening (Table 2).

Table 2 Emission patterns of terpenoids from different developmental stages of control and transgenic tobacco flowers

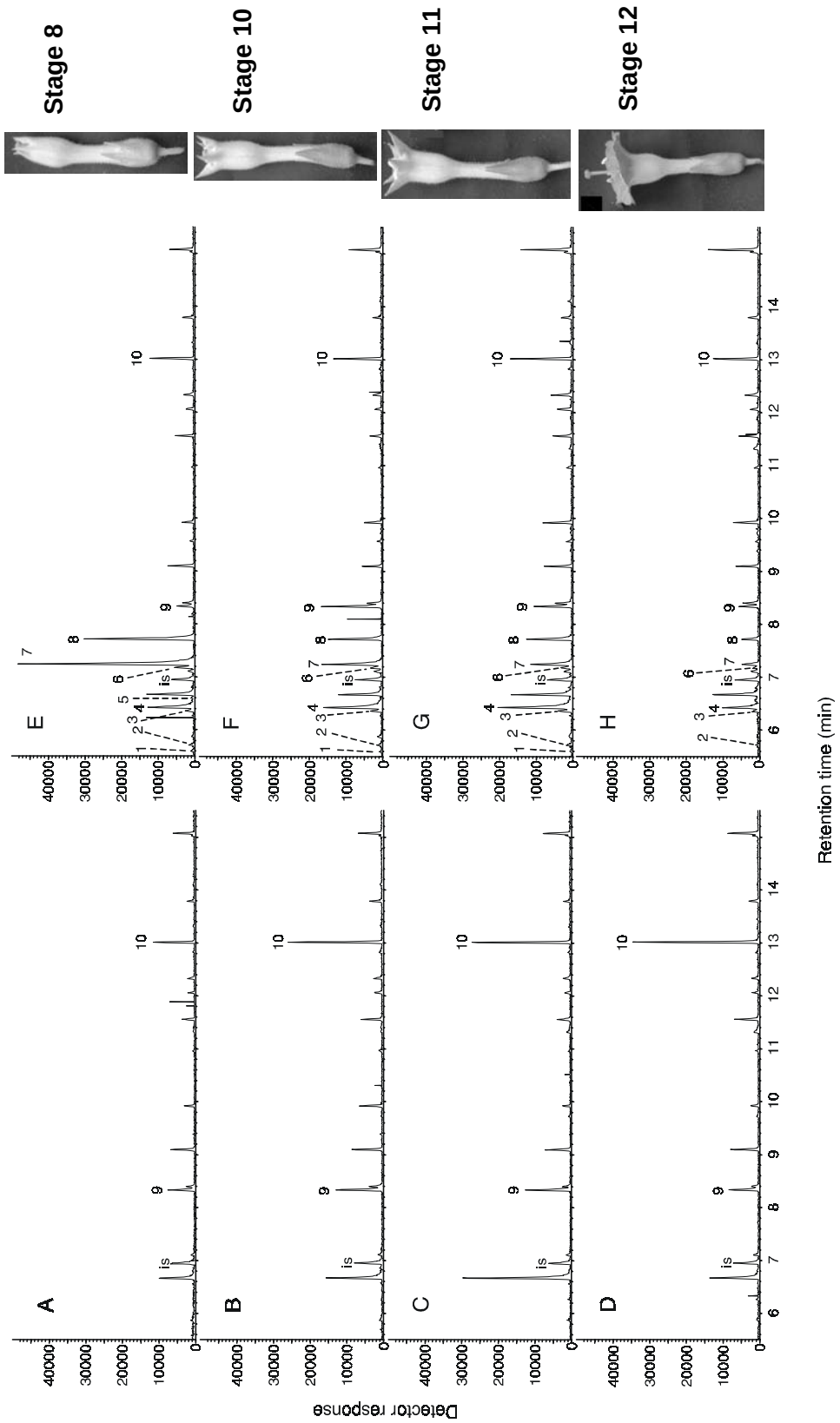
	stage 8 (ng g^{-1} $24h^{-1}$)		stage 10 (ng g^{-1} $24h^{-1}$)		stage 11 (ng g^{-1} $24h^{-1}$)		stage 12 (ng g^{-1} $24h^{-1}$)	
	control	transgenic	control	transgenic	control	transgenic	control	transgenic
α-thujene	0	29	0	13	0	14	0	0
α-pinene	0	30	0	31	0	25	0	9
sabinene	0	49	0	47	0	55	0	28
β-pinene	0	356	0	371	0	378	0	179
p-cymene	0	115	0	57	0	48	0	28
limonene	0	1206	0	321	0	190	0	71
γ-terpinene	0	630	0	237	0	184	0	73
linalool	127	88	188	254	207	139	121	79
β-caryophyllene	182	190	328	171	420	175	468	151

For each stage and plant type 5 flowers were detached and placed on floral foam. Control: non-transgenic SR1 plant, transgenic: plant expressing γ-terpinene synthase, limonene synthase and β-pinene synthase.

Configuration of produced compounds

The enantiomeric composition of the native linalool produced by the tobacco flowers had an enantiomeric excess of 52% *R*-linalool. In the transgenic plants this ratio was still the same. The limonene produced in the limonene synthase plant C62-21 has an e.e. of almost 100% of (+)-limonene, just like the enantiomeric composition of the limonene produced when this enzyme was expressed in *E.coli*. The limonene produced by flowers of several mixed developmental stages of the plant containing all three monoterpene synthases had an enantiomeric excess of 87% (+)-limonene (data not shown).

Figure 5: Volatile emission profiles of different developmental stages of detached tobacco flowers sampled for 24h using Tenax trapping of (A-D) control and (E-H) transgenic plants with the three lemon monoterpene synthases (respectively γ-terpinene, (-)-β-pinene and (+)-limonene synthase). Each time 5 flowers were sampled. The different stages of the flowers, respectively stages 8, 10, 11 and 12 are depicted next to the chromatograms. Monoterpene and sesquiterpene peaks identified by their mass spectrum and retention times are numbered. 1: α-thujene, 2: α-pinene, 3: sabinene, 4: β-pinene, 5: myrcene, 6: *p*-cymene, 7: limonene, 8: γ-terpinene, 9: linalool, 10: β-caryophyllene. is: internal standard (+)-3-carene (212 ng). Non-terpene peaks are not labelled.


Stage 8
Stage 10
Stage 11
Stage 12
A
B
C
D
E
F
G
H
is
Detector response
Retention time (min)

Discussion

This is the first report on the simultaneous expression of multiple introduced terpene synthases in plants. Via this metabolic engineering approach, a large variety of different monoterpene skeletons have been added to the wild type tobacco plants. The total level of the produced monoterpenes is greatly increased and resulted in an extensive alteration of the scent profile emitted from the tobacco plants in the flowers and leaves.

Precursor availability

After genetic modification with three monoterpene synthases from lemon, tobacco plants produced and emitted a complex blend of monoterpenes from both leaves and flowers. As the altered monoterpene emission profile in the tobacco resulted in a drastic overall change in the scent from the plant that can be smelled by the human nose, an approach like this applied to plants that are cultivated for their scent such as roses, could have an interesting commercial value (Vainstein et al., 2001). However, a first attempt with linalool synthase in the cut flower carnation did not lead to the emission of quantities of linalool that could be smelled, probably as a consequence of the low level of the available substrate GPP (Lavy et al., 2002). Another reason for not finding an altered scent profile after genetic modification with a monoterpene synthase was found with petunia plants, transformed with linalool synthase that converted all of the synthesised *S*-linalool into its corresponding non-volatile glucose conjugate (Lücker et al., 2001). Glycosylation of linalool did not occur in the transgenic carnation (Lavy et al., 2002).

Monoterpene formation in a transgenic plant necessarily depends on the availability of the substrate GPP. This in turn is depending on the expression of GPP synthase, and genes earlier in the MEP pathway. However, also exchange of precursor molecules such as IPP between cell compartments can probably occur, which will also influence the level of available substrate (McCaskill and Croteau, 1998). It seems likely that the introduction of monoterpene synthases in a transgenic plant will also lead to a change in the flux to GPP, which may imply that there is less precursor available for GGPP and subsequent diterpene and carotenoid formation.

Nevertheless, considering that large amounts of terpenoids were produced in tobacco leaves and flowers, it seems likely that there are large levels of free substrate available and this seems particularly to be true for young leaves and flower buds. A large pool of available GPP in young tissues would benefit the plant to produce monoterpenes to repel herbivores or attract predators of the herbivores quickly and specifically for young tissues. Whether the geranyl diphosphate precursors are already available in the plants or whether the biosynthesis is upregulated upon expression of the introduced monoterpene synthases remains unknown.

Comparison of sesquiterpene vs monoterpene production levels in transgenic plants

When a fungal trichodiene synthase, a sesquiterpene synthase, was transformed to tobacco plants this resulted only in the accumulation of trichodiene levels of about 5-10 ng g^{-1} of leaf tissue, although there was clearly active trichodiene synthase enzyme present (Hohn and Ohlrogge, 1991). When another sesquiterpene synthase, amorpha-4,11-diene synthase from *Artemisia annua* was transformed to tobacco, this also resulted in only the accumulation of 0.2 to 1.7 ng product g^{-1} fresh weight (FW) (Wallaart et al., 2001). As the accumulated levels are low, there is apparently almost no free farnesyl diphosphate present in these plants.
Our tobacco monoterpene synthase transformations resulted in the emission of much higher levels of new compounds. In the leaves the total level of additional terpenes emitted was maximally 750 ng g^{-1} FW 24 h^{-1} in young leaves and in flowers the levels were ranging from 400 to 2400 ng g^{-1} FW 24 h^{-1}. In other plant transformation experiments using *S*-linalool synthase, transgenic petunia accumulated between 1000-10000 ng g^{-1} FW of the corresponding *S*-linalyl-β-D-glucopyranoside in leaves (Lücker et al., 2001), transgenic tomato accumulated between 165-833 ng g^{-1} FW of *S*-linalool and in addition 199-504 ng g^{-1} FW of 8-hydroxylinalool in the fruit (Lewinsohn et al., 2001), while in transgenic carnation, within mature petals, approximately 400 ng^{-1} g dry weight of the furanoid *trans*-linalool oxide was accumulated, in addition to some non-quantitatied emission of linalool and *cis*- and *trans*-linalool oxide (Lavy et al., 2002). This indicates that the levels of product produced by plants after introduction of a monoterpene synthase by transformation are higher than the levels produced after the introduction of a sesquiterpene synthase. An interesting observation was that in carnation, GPP synthase activity was detected in control plants that do not produce monoterpenes (Lavy et al., 2002), suggesting the presence of a pool of unused GPP. A putative GPP pool available for monoterpene biosynthesis in non-monoterpene producing plants was also suggested in a recent review on terpenes (Chappell, 2002). Alternatively GPP might be 'leaking' from a GGPP synthase during GGPP formation, the precursor for e.g. carotenoids as was suggested to occur in tomato fruit (Lewinsohn et al., 2001).

Competition for substrate in the transgenic plant with three monoterpene synthases

The observation that the levels of the main products of the introduced monoterpene synthases are comparable cannot be explained by the K_m values of the enzymes. The K_m value of the limonene synthase is 4.5 times lower than the K_m of the β-pinene and the γ-terpinene synthase (Lücker et al., 2002). If there would be enough enzyme to convert all the substrate GPP then the level of limonene would be much higher than that of β-pinene and γ-terpinene. In flowers the level of expression of the separate monoterpene synthases is probably not high enough to produce enough enzymes to convert all the available substrate.

In leaves, the levels of compounds produced by the introduced monoterpene synthases are more similar to what would be expected from differences in K_m between the enzymes. As can be seen in Figure 4, the limonene level is higher than those of the other main products indicating that the level of GPP might be a limiting factor for the production in this tissue, and not the activities of the enzymes involved.

Gene expression differences

The genes are independently integrated in the genome, but are all regulated by a CaMV d35S promoter. The introduction of three genes in one plant, all regulated by the same CaMV 35S promoter has been shown to be successful in *Arabidopsis* (Nawrath et al., 1994). In this case the genes were also combined into one plant after successive crossings of the transgenic plants. Position effects due to different genomic integration sites of each T-DNA are likely to account for differences in gene expression and resulting levels of monoterpene emission by the respective introduced enzymes, despite the presence of a common promoter. Use of matrix-associated regions (MARs) such as the chicken lysozyme A element on the borders of the T-DNA construct for transformation might have circumvented these position effects (Mlynarova et al., 1995). The MARs could possibly also decrease spatial and temporal variability of gene expression (van Leeuwen et al., 2001). The emission patterns of native linalool and β-caryophyllene are different from the patterns of the introduced monoterpenes. This is not unexpected, as the native terpenoid synthases are under an endogenous regulation mechanism that will obviously give rise to a gene expression pattern that differs from that of the CaMVd35S induced gene expression.

Possible exchange of precursors between cell compartments

In all analyzed flower developmental stages except the first, the level of β-caryophyllene in the transgenic plants was 2- to 3- fold lower than in the control plant. Possibly there is transport of IPP or GPP precursors formed in the plastids to the cytosol, contributing to the β-caryophyllene accumulation in control plants. Such an exchange of precursors from plastids to the cytosol has been demonstrated in chamomile (Adam and Zapp, 1998). A study on *Mentha x piperita* glandular trichomes, using several ^{14}C radiolabeled substrates even showed that sesquiterpenes are exclusively derived from a plastidic pool of precursors (McCaskill and Croteau, 1995). This supposed transport to the cytosol, might be decreased in our transgenic tobacco plants since most of the IPP/ GPP is used by the introduced monoterpene synthases, which are active at the site of biosynthesis of this precursor and are therefore a better competitor for the GPP than the sesquiterpene synthase residing in the cytosol.

Side products of the monoterpene synthases

In the transgenic tobacco plants the level of *p*-cymene is higher than the levels of α-thujene, α-pinene, α-terpinene and terpinolene (Figure 4, 5). However, the latter products have previously all been reported to be more predominant side products of the γ-terpinene synthase than the *p*-cymene, when this monoterpene synthase was expressed in *E. coli* (Lücker et al., 2002). The higher level of *p*-cymene detected in both leaves and flowers of transgenic plants with the three monoterpene synthases might be caused by further conversion of monoterpenes by native enzymes of tobacco or by non enzymatic oxidation reactions induced by light and air. In an early study on the biosynthesis of aromatic monoterpenes in thyme, it was suggested that *p*-cymene could be formed from γ-terpinene by the action of a putative desaturase enzyme (Poulose and Croteau, 1978). In cold pressed *Citrus junus* oil *p*-cymene concentrations increased upon storage at the expense of e.g. limonene and γ-terpinene (Njoroge et al., 1996). In lemon oil several monoterpenes decreased while *p*-cymene increased in concentration upon UV radiation (Iwanami et al., 1997). Other modifications like hydroxylations described for tomato and carnation transformed with linalool synthase (Lewinsohn et al., 2001; Lavy et al., 2002) were not detected in these transgenic tobacco plants.

Enantiomeric composition of the monoterpenes produced by the tobacco plants

Flowers of the transgenic tobacco plants that were only expressing (+)-limonene synthase produced almost exclusively (+)-limonene, while the plant that in addition to the (+)-limonene synthase also expressed the (-)-β-pinene and the γ-terpinene synthases, produced a lower e.e. of (+)-limonene. This must be the result of the contribution of (-)-limonene produced as side products by the (-)-β-pinene synthase and the γ-terpinene synthase, to the total limonene production in this plant. Around 3% of the total products of (-)-β-pinene synthase and around 7% of the total products of the γ-terpinene synthase, when expressed in *E. coli* was (-)-limonene (Lücker et al., 2002). Since the levels of the main products of the three monoterpene synthases varied in the different transgenic flower development stages, the e.e. of (+)-limonene is expected to vary according to the activity of the three different enzymes as a result of the side product formation. Also the e.e. of (-)-β-pinene is expected to be somewhat lowered in the flowers with the three monoterpene synthases, since (+)-β-pinene was reported to be a side product (almost 5% of total products) of the γ-terpinene synthase, when expressed in *E. coli* (Lücker et al., 2002).

Ecological aspects

The monoterpenes emitted from the transgenic tobacco plants are volatiles that occur in flowers of many plant species (Knudsen et al., 1993) and could therefore attract insect species other than those normally attracted to tobacco. Transgenic plants with altered floral scents have been suggested previously to be ideal models for pollinator behaviour studies, and simultaneously they could be used to study alterations in plant-herbivore interactions (Dudareva and Pichersky, 2000; Pichersky and Gershenzon, 2002). Also changes in tritrophic interactions can be expected such as predator and parasite behaviour (Dicke and van Loon, 2000). These ecological aspects strongly depend on the volatile production.
The opportunity to produce many new monoterpene compounds in one plant makes it feasible to vary levels or even the enantiomeric composition in a plant, creating plants with customised volatile profiles. This gives great opportunities for the improvement of the scent of ornamentals (Bohlmann et al., 1998; Dudareva and Pichersky, 2000; Vainstein et al., 2001).

The question might arise whether tobacco varieties with altered and elevated monoterpene levels would be beneficial for the production of tobacco. Some monoterpenes like (+)-limonene are likely to have an anticancer effect that might decrease the chances of getting cancer by smoking tobacco (Crowell and Gould, 1994). On the other hand the elevation of monoterpene levels might enhance cell permeation (Huang et al., 1999), leading to a facilitated uptake of nicotine resulting in increased addiction to tobacco products.

Acknowledgements

We like to thank Dr. Rajesh Ullanat for providing the modified hot phenol RNA isolation protocol.

Chapter 5

Two step metabolic engineering of monoterpene biosynthesis linking different cell compartments in tobacco leads to subsequent emission of (+)-*trans*-isopiperitenol

Joost Lücker[1], Wilfried Schwab[2], Maurice C.R. Franssen[3], Linus H.W. van der Plas[4], Harro J. Bouwmeester[1], Harrie A. Verhoeven[1]

[1] Business unit Cell Cybernetics, Plant Research International, P.O. Box 16, 6700 AA, Wageningen, The Netherlands,

[2] University of Würzburg, Chair of Food Chemistry, Am Hubland, 97974, Würzburg, Germany,

[3] Laboratory of Organic Chemistry, Wageningen University, Dreijenplein 8 6703 HB, Wageningen, The Netherlands

[4] Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD, Wageningen, The Netherlands

Summary

Terpenoid pathway modification has been shown to be successful in a number of plants. For the first time we now report on metabolic engineering of this pathway involving two subsequent introduced enzymatic steps that are localised in separate cell compartments. A limonene-3-hydroxylase cDNA was isolated from *Mentha spicata*. This cDNA was subsequently used to transform a transgenic tobacco line already possessing three lemon monoterpene synthases producing (+)-limonene, γ-terpinene and (-)-β-pinene. The introduced P450 hydroxylase, targeted to the endoplasmatic reticulum, proved to be functional in the transgenic plants. It hydroxylated the (+)-limonene resulting in the emission of (+)-*trans*-isopiperitenol. The results suggest that intracellular trafficking of monoterpenes from the site of production in the chloroplasts to the endoplasmatic reticulum occurs in the transgenic plant tissue. Some further modifications of the (+)-*trans*-isopiperitenol were also detected, resulting in 1,3,8-*p*-menthatriene, 1,5,8-*p*-menthatriene, *p*-cymene and isopiperitenone emission. A reaction mechanism for these modifications is suggested. The implications for metabolic pathway engineering comprising multiple compartments are discussed.

Introduction

Monoterpenes are a family of secondary metabolites produced in plants that are thought to have many functions in ecology as they are involved in interactions with insects, pathogens and other plants (Harborne, 1991).

They are small fragrant molecules that are commonly used in food, beverages and cosmetics and therefore have a high economic value (Verlet, 1993). There are around 1000 different structures known at present (Haudenschild and Croteau, 1998). Primary monoterpenes are formed via the precursor geranyldiphosphate by the action of monoterpene synthases (Croteau, 1987). These enzymes, including those responsible for the formation of precursors of the monoterpenes that are cloned to date, all carry plastid targeting signals (Haudenschild and Croteau, 1998), and for some it was shown that they were actually localised in plastids (Turner et al., 1999; Bouvier et al., 2000). Secondary modifications of the parent monoterpene olefins, mostly consisting of redox reactions and conjugations, are often initiated by cytochrome P450 catalysed hydroxylation. As a result, one parent olefin leads to the formation of multiple structurally related derivatives bearing an identical oxygenation pattern as initially established by the hydroxylase (Karp and Croteau, 1988). Secondary modifications initiated by these hydroxylases have been reported e.g. in *Mentha* species for the monoterpene (-)-limonene at the C3 or the C6 position (Croteau and Gershenzon, 1994) and in caraway for (+)-limonene at the C6 position (Bouwmeester et al., 1998). Most plant P450 systems are located on the ER (Schuler, 1996; Chapple, 1998), which is present in the microsomal fraction of tissue homogenate. The limonene hydroxylase activity in *Mentha* glandular trichomes was also isolated from the microsomal fraction (Mihaliak et al., 1993).

The different intracellular localisation and the high temporal correlation of activity during development of monoterpene synthases and monoterpene hydroxylases in peppermint (McConkey et al., 2000) and caraway (Bouwmeester et al., 1998), implicate that primary monoterpenes are translocated to the ER. Osmiophilic secretory droplets have been suggested as the means of transport (Bosabalidis, 1996; Bouwmeester et al., 1998).

It is possible that this supposed transport mechanism is not limited to plants that naturally produce secondary modifications of the primary monoterpene olefins. In a previous genetic engineering approach when *S*-linalool synthase was introduced into tomato plants, 8-hydroxy-linalool was detected as a product of further conversion, which must have been a P450 mediated reaction (Lewinsohn et al., 2001). Metabolic engineering of monoterpene biosynthesis in plants has also been achieved in mint, petunia and carnation without any detectable hydroxylation (Krasnyanski et al., 1999; Diemer et al., 2001; Lücker et al., 2001; Mahmoud and Croteau, 2001; Lavy et al., 2002).

In our experiments with tobacco, additional monoterpenes were formed in the transgenic plants in addition to the native linalool. Both leaves and flowers of the engineered tobacco plants were able to synthesise and emit (-)-β-pinene, (+)-limonene and γ-terpinene when all three monoterpene synthases were integrated into a single plant line (Chapter 4). No further conversions for example catalysed by P450 hydroxylases were observed in these plants. Whether this is due to the lack of a transport mechanism to the ER, or due to a lack of suitable (mono)terpene hydroxylases is not clear.
The (-)-limonene-3-hydroxylase and the (-)-limonene-6-hydroxylase isolated from mint species (Lupien et al., 1999), are the only suitable monoterpene hydroxylase cDNAs that have currently been reported for the monoterpenes introduced in the tobacco plants. Although these (-)-limonene hydroxylases were shown to be highly substrate- and regio- specific they were also able to use (+)-limonene as a substrate, albeit with lower efficiency (Karp et al., 1990; Wust et al., 2001). Therefore, these P450s could be used to test whether it is possible to convert the predominantly (+)-limonene produced in the plastids in our transgenic tobacco plants into a hydroxylated product. In this paper we describe the isolation of a limonene-3-hydroxylase cDNA from *Mentha spicata*, its introduction via a subsequent transformation experiment into the transgenic tobacco plants and the resulting additional emitted products.

Experimental procedures

Plant material

As a source for the limonene hydroxylase cDNA, young leaves from the top of non-flowering *Mentha spicata* L. 'Crispa' plants were obtained from the botanical garden of Wageningen University (Wageningen, The Netherlands).

Plant material for transformation was wild type *Nicotiana tabacum* 'Petit Havana' SR1, and a transformed plant line (BCD) containing three different active lemon monoterpene synthases (γ-terpinene synthase (*B93*), limonene synthase (*C62*) and β-pinene synthase (*D85*)) ((Lücker et al., 2002), Chapter 4). New plants were regenerated *in vitro* on MS20 medium supplemented with 1 mg L^{-1} BAP from cuttings of young leaves taken from plants in the greenhouse. The leaf was sterilised in 1.5% sodium hypochlorite solution for 10 min, and washed in sterilised water in three subsequent steps. The regenerated plantlets served as starting material for the transformation and as controls for the experiments.

RNA isolation and cloning of a P450 limonene hydroxylase using RACE PCR

Total RNA was isolated from *M. spicata* L. 'Crispa' leaves, by grinding 100 mg of tissue in liquid nitrogen and using a previously described hot phenol method (Chapter 4). The isolated RNA was dissolved at 60 °C, and was treated for 10 min at this temperature with 1 volume cetyl trimethylammonium bromide (CTAB) buffer (0.2 M Tris, pH 7.5, 50mM EDTA, 2M NaCl, 2% CTAB) in order to remove undesired sugars. Subsequently 1 volume of chloroform-isoamylalcohol (24:1) was added and after mixing the sample was centrifuged for 5 min. To the aqueous top phase 1 ml of pure ethanol was added. After incubation at room temperature for 5 min the sample was centrifuged for 1 min and the pellet was washed with ethanol (70%) before dissolving in double distilled H_2O.

In the initial attempt to amplify the *M. spicata* (-)-limonene-6-hydroxylase, first strand cDNA synthesis was carried out using primer 209 (815SPI*Pst*I) 5'-CGTACTCTGCAGTTAAGGACTTTTATAGAGTGTGG-3', annealing to the 3' end of the open reading frame of the previously described cDNA (GenBank (http://www.ncbi.nlm.nih.gov/) accession number: AF124815, Lupien et al., 1999), with a *Pst*I restriction site overhang and another 6 randomly chosen bases added, and superscript II RT enzyme according to the manufacturers recommendations (Life Technologies, Breda, The Netherlands). By using specific primers for the (-)-limonene-6-hydroxylase and different PCR programs a full length cDNA could not be obtained. However using specific primers F2spic (5'-CATAAGGCAGGAGGAGATCG-3'), R2Spic (5'-TCTCTTACCTCCGCCTGCAC-3') for the middle of the described cDNA and the PCR program; 94 °C, 30 s, 53 °C, 30 s, 72 °C, 5 min, 40 cycles, a cDNA fragment, identical to the published cDNA could be obtained.
Secondly a RACE-PCR approach was followed, that required only one specific primer on the 3' end of the cDNA. RACE-PCR was performed according to the manufacturers recommendations (Clontech laboratories, Inc., Palo Alto, CA, USA) and again 1 μg of total RNA with specific primer 209 was used to prepare the first strand. 5'-RACE PCR was performed at 55 and at 60 °C annealing temperatures, 15 cycles using primer 219; 5'-TTAAGGACTTTTATAGAGTGTGG-3' exactly annealing to the 3'end of the ORF of the (-)-limonene-6-hydroxylase, together with the universal primer mix (UPM) from the RACE-PCR kit. The PCR resulted in several fragments, of which one, *E2*, was 1.6 kb. This fragment was ligated into the pGEM-T easy vector system according to the manufacturers recommendations (Promega Benelux b.v., Leiden, The Netherlands) and subsequent sequencing resulted in the identification of a full length cytochrome P450.
Using targeting prediction programs available on Internet, possible localisation and targeting was analysed using PSORT (http://psort.nibb.ac.jp/form.html) and iPSORT (http://hypothesiscreator.net/iPSORT/#predict).

Alignments were made using the CLUSTALX 1.81 program (Thompson et al., 1997) using standard settings and GENEDOC. Sequence homology levels were analysed using MEGALIGN of the DNASTAR software package (DNASTAR, Inc., Madison, WI, USA).

Construction of vector

For construction of a binary vector, the *E2* open reading frame had to be inserted first in a pFLAP10 vector (kindly provided by Dr. A. Bovy, Plant Research International) containing a CaMV-d35S promoter and a Nos-terminator sequence. For this, PCR primers were used containing restriction site overhangs *Xba*I and *Nco*I. The forward primer used was 5'-TTATATTCTAGATGGAGCTCCAGATTTCGTCG-3' and the reverse primer was 5'-TTATAACCATGGTTAAGGACTTTTATAGAGTGTGG-3'. PCR was performed using *pfu*-turbo (Stratagene Europe, Amsterdam Zuidoost, The Netherlands) and the programme 94 °C 30 s, 55 °C 30 s, 72 °C 2 min 30 s, was repeated for 40 cycles. After purification of the amplified fragment from agarose gel, it was digested using *Xba*I and *Nco*I restriction enzymes and ligated to a pFLAP10 *Xba*I and *Nco*I digested vector fragment. The resulting vector pFLAP*E2* was resequenced to check for correct PCR amplification, digested using restriction enzymes *Pac*I and *Asc*I (NEB, Hitchin, Hertfordshire, England) and ligated to a *Pac*I, *Asc*I digested pCambia1300+ vector (CAMBIA, Canberra ACT, Australia), containing a hygromycin plant resistance gene (*hpt*II) and added *Pac*I and *Asc*I restriction sites (kindly provided by Hans Janssen, Plant Research International), resulting in a vector coded pJLT6. The hygromycin resistance gene was required since the transgenic plant material used for the subsequent genetic modification was already resistant to kanamycin, as a result of the selection marker gene *npt*II that was used for transformation with the monoterpene synthases (Chapter 4). Via a cold shock method the plant expression vector pJLT6 was transformed to *Agrobacterium tumefaciens* strain LBA4404. Colonies were checked after transformation by backtransformation to *E. coli* XL1blue MRF' supercompetent cells (Stratagene). DNA fragments were isolated from agarose gel using a GFX™ PCR DNA and Gel band purification kit according to the manufacturers recommendations (Amersham-Pharmacia Biotech).

Plant transformation

Leaf disks of in vitro grown plant material of tobacco were transformed with LBA4404 containing pJLT6 using a standard plant-transformation protocol (Horsch et al., 1985). Selection for transformants was carried out using 15 mg L^{-1} hygromycin. Leaf disks were inoculated with empty binary plasmid pCambia1300+ as a control. Some leaf disks were not

inoculated with *A. tumefaciens*, but taken through the same regeneration process on non-selecting medium. Rooted plants were transferred to the greenhouse.

RNA isolation for Northern blotting

From the rooting *in vitro* plantlets one small leaf was removed from the top and frozen in liquid N_2. Total RNA was isolated from these leaves by using Tri-Reagent from Sigma according to the manufacturers recommendations (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). RNA gel blot analysis was done using 10 µg total RNA with a sodium phosphate gel electrophoresis and blotting protocol as described previously (Lücker et al., 2001). Labelling of cDNA was done using the RadPrime DNA labelling system (Life Technologies B.V., Breda, the Netherlands). The blots were hybridised with the [α-^{32}P] ATP-labelled 1.5 kb *Xba*I- *Nco*I digested full length ORF of the P450 cDNA. The blots were also hybridised with a 1.8 kb *Nhe*I-*Sal*I C62 limonene synthase cDNA fragment to compare expression of the limonene synthase to the cytochrome P450 limonene hydroxylase. Finally, equal loading of the RNA on the blot was verified by hybridisation with a 25S ribosomal cDNA from potato. Hybridisation, washing and autoradiography were done as described previously (Lücker et al., 2001).

Hydro-distillation of *M. spicata*

0.5 g of *M. spicata* leaf material was ground in liquid N_2 and used for hydro-distillation to ethylacetate as previously described (Helsper et al., 2001). 2 µl was injected into a GC-MS using an HP 5890 series II gas chromatograph and an HP 5972A Mass Selective Detector essentially as described before (Bouwmeester et al., 1999a). The GC was equipped with an HP-5MS column (30m x 0.25 mm i.d., and film thickness of 0.25 µm) and programmed at an initial temperature of 45 °C for 1 min, with a ramp of 5 °C min^{-1} to 120 °C, a ramp of 20 °C min^{-1} to 270 °C, and final time of 5 min.

Synthesis of isopiperitenol from isopiperitenone

10.3 mg of isopiperitenone standard (kindly provided by Haarmann&Reimer, Holzminden, Germany) was added to 1.5 mg $LiAlH_4$ in 0.5 ml redistilled ether and vigorously mixed for 17.5 h. Three small spoons (in total approximately 0.1 g) of $Na_2SO_4 \cdot 10H_2O$ (Merck) were carefully added. The mixture was stirred for another 30 min. One and a half ml of distilled water was added and after mixing vigorously, the mixture was centrifuged at 3500 rpm for 5 minutes. The organic phase was subsequently transferred to a small aluminiumoxide (Al_2O_3) column. The column was eluted with 1 ml ether.

SPME headspace trapping of detached leaves

Fully stretched mature leaves (10-20 cm long) from transgenic and control tobacco plants were collected from the greenhouse. The headspace from the detached leaves was measured by SPME. First the petiole of the leaf was placed in water to prevent desiccation. Then a glass funnel closed with aluminium foil was placed on top of the leaf to create a closed headspace. The PDMS fiber was inserted through the aluminium foil and the headspace was sampled for 30 min.

GC-MS SPME flower headspace measurements *in vivo*

Flowers from several developmental stages were analysed repeatedly. Each time one flower remaining on the plant was covered with a glass funnel and the openings were covered with aluminium foil. The flower headspace was sampled each time for 30 min by capturing volatiles onto a 100 µm PDMS fiber of an SPME needle. Injection was performed by thermal desorption of the SPME fiber in the injector at 250 °C during 1 min using the splitless injection mode with the split valve being opened after 60 s (Verhoeven et al., 1997). GC-MS analysis was performed using a Fisons 8060 gas chromatograph coupled to an MD 800 mass spectrophotometer (Interscience, Breda, The Netherlands). An HP-5 column (50 m x 0.32 mm, film thickness 1.05 µm) was used with He (37 kPa) as carrier gas. GC oven temperature was programmed as follows: 2 min 80 °C, ramp to 250 °C at 8 ° min^{-1} and 5 min at 250 °C. Mass spectra in the electron impact mode were generated at 70 eV.

Tenax trapping

For headspace trapping onto Tenax TA (20/35 mesh, Alltech, Breda, the Netherlands) detached flowers or leaves were used. Simultaneous analysis of emission by different plant lines was performed by headspace trapping for 24 h. The flowers or leaves were directly placed onto green floral foam blocks (Smithers-Oasis Belgium N.V., Houthalen, Belgium) saturated with water and wrapped in aluminum foil, before they were enclosed in 1 L glass jars closed with a Teflon-lined lid equipped with in- and outlet. A vacuum pump was used to draw air through the glass jar at approximately 100 mL min^{-1}, with the incoming air being purified through a glass cartridge (140 x 4 mm) containing 150 mg Tenax TA. At the outlet the volatiles emitted by the detached flowers or leaves were trapped on a similar Tenax cartridge. Volatiles were sampled during 24 h. Cartridges were eluted using 3 x 1 mL of redistilled pentane-diethyl ether (4:1). For quantification, 212 ng (+)-3-carene was added to the elute, as an internal standard. The samples were either directly used or first concentrated

under a flow of nitrogen. 2 µL of the sample was analysed by an automated injector on a GC-MS using an HP 5890 series II gas chromatograph equipped with an HP-5MS column (30 m x 0.25 mm i.d., 0.25 µm film thickness) and an HP 5972A Mass Selective Detector as described previously (Bouwmeester et al., 1999b). GC oven temperature was programmed at an initial temperature of 45 °C for 1 min, with a ramp of 10 °C min^{-1} to 280 °C, and a final time of 10 min.

Synthesis of isopiperitenol from (+)- and (-)-limonene and multidimensional GC-MS (MDGC-MS) measurements

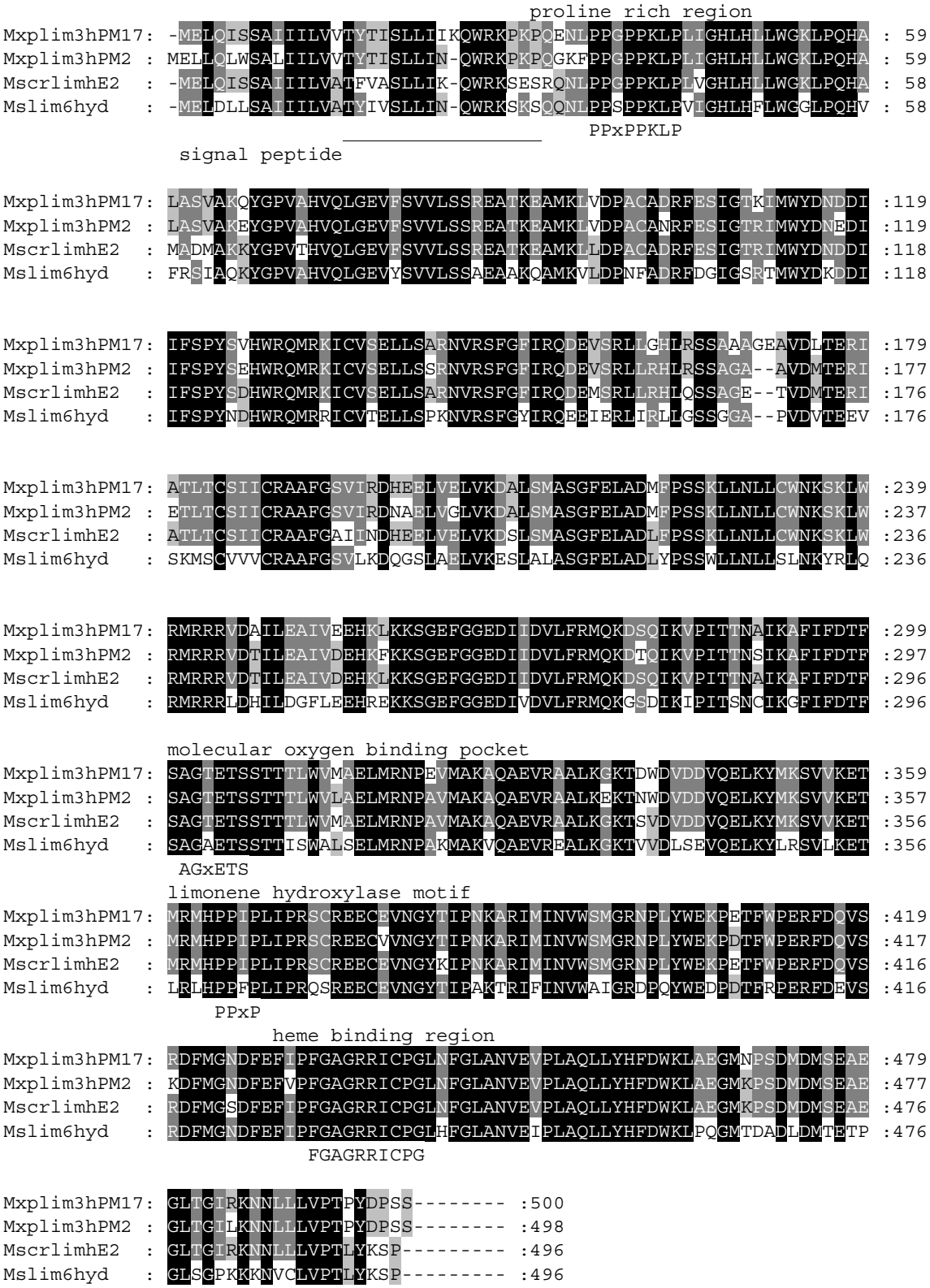
Chemical synthesis of all four enantiomers of isopiperitenol from limonene was performed exactly as described previously and yielded a 40:60 (*cis*: *trans*) mixture of isopiperitenol (Guillon et al., 2000).
MDGC-MS analysis with a DB-wax column in GC1 and an enantioselective column in GC2 was carried out as described previously (Lücker et al., 2001), but with a different temperature program. The DBwax column in GC1 was programmed at an initial temperature of 50 °C which was increased with 4 °C min^{-1} to 240 °C and was kept at this temperature for 10 min. GC2 with the enantioselective column (2,3-di-O-ethyl-6-O-*tert*-butyl dimethylsilyl-β-cyclodextrin/PSO86, 25 m x 0.25 mm i.d., 0.15 µm film thickness) was programmed at an initial temperature of 60 °C for 20 min and was then increased with 1 °C min^{-1} to 200 °C.

Results

cDNA isolation of the *M. spicata* limonene-3-hydroxylase

A (-)-limonene-3-hydroxylase homologue cDNA, *E2*, was isolated from *M. spicata* L. 'Crispa' based on previously obtained sequence information (Lupien et al., 1999). RACE-PCR was used, as initial attempts using specific primers did not result in amplification of a full-length cDNA (data not shown). The resulting full-length P450 that was isolated had a higher percentage of identity based on amino acid sequence to the two published limonene-3-hydroxylases from *Mentha x piperita* (88 and 92% identity) than to the limonene-6-hydroxylase from *Mentha spicata* (71% identity, Figure 1, Lupien et al., 1999). Typical P450 motifs, conserved for most cytochrome P450 enzymes are present in the *E2* cDNA (Chapple, 1998). The iPSORT program indicated that the N-terminal ER- targeting signal peptide was 30 amino acids long; it is underlined in Figure 1. Functional heterologous expression of the isolated P450 cDNA (*E2*) in yeast resulted in the identification of a limonene-3-hydroxylase function (personal communication, Dr. A. Bovy).

Figure 1. Sequence alignment of the *M. x piperita* (-)-limonene-3-hydroxylases Mxplim3hPM17 (AF124816), Mxplim3hPM2 (AF124817), and the *M. spicata* (-)-limonene-6-hydroxylase, Mslim6hyd (AF124815), to the isolated cytochrome P450 from *M. spicata* 'Crispa', MscrlimE2. Functional domains common to all P450 enzymes, the proline rich region, the molecular oxygen binding pocket, the heme binding region (Chapple, 1998) and a limonene hydroxylase specific domain (Schalk and Croteau, 2000) are indicated in the alignment and explained in the discussion. The PSORT and iPSORT predicted non-cleavable N-terminal signal for targeting to the ER is underlined. The alignment was created with the ClustalX program and standard settings. Shading indicates conserved identity for the aligned aminoacids: black background shading indicates 100% conservation, dark grey shading indicates 75% conservation, and light grey shading indicates 50% conservation.


proline rich region
Mxplim3hPM17: -MELQISSAIIILVVTYTISLLIKQWRKPKPQENLPPGPPKLPLIGHLHLLWGKLPQHA : 59
Mxplim3hPM2 : MELLQLWSALIILVVTYTISLLIN-QWRKPKPQGKFPPGPPKLPLIGHLHLLWGKLPQHA : 59
MscrlimhE2 : -MELQISSAIIILVATFVASLLIK-QWRKSESRQNLPPGPPKLPLVGHLHLLWGKLPQHA : 58
Mslim6hyd : -MELDLLSAIIILVATYIVSLLIN-QWRKSKSQQNLPPSPPKLPVIGHLHFLWGGLPQHV : 58
PPxPPKLP
signal peptide
Mxplim3hPM17: LASVAKQYGPVAHVQLGEVFSVVLSSREATKEAMKLVDPACADRFESIGTKIMWYDNDDI :119
Mxplim3hPM2 : LASVAKEYGPVAHVQLGEVFSVVLSSREATKEAMKLVDPACANRFESIGTRIMWYDNEDI :119
MscrlimhE2 : MADMAKKYGPVTHVQLGEVFSVVLSSREATKEAMKLLDPACADRFESIGTRIMWYDNDDI :118
Mslim6hyd : FRSIAQKYGPVAHVQLGEVYSVVLSSAEAAKQAMKVLDPNFADRFDGIGSRTMWYDKDDI :118
Mxplim3hPM17: IFSPYSVHWRQMRKICVSELLSARNVRSFGFIRQDEVSRLLGHLRSSAAAGEAVDLTERI :179
Mxplim3hPM2 : IFSPYSEHWRQMRKICVSELLSSRNVRSFGFIRQDEVSRLLRHLRSSAGA--AVDMTERI :177
MscrlimhE2 : IFSPYSDHWRQMRKICVSELLSARNVRSFGFIRQDEMSRLLRHLQSSAGE--TVDMTERI :176
Mslim6hyd : IFSPYNDHWRQMRRICVTELLSPKNVRSFGYIRQEEIERLIRLLGSSGGA--PVDVTEEV :176
Mxplim3hPM17: ATLTCSIICRAAFGSVIRDHEELVELVKDALSMASGFELADMFPSSKLLNLLCWNKSKLW :239
Mxplim3hPM2 : ETLTCSIICRAAFGSVIRDNAELVGLVKDALSMASGFELADMFPSSKLLNLLCWNKSKLW :237
MscrlimhE2 : ATLTCSIICRAAFGAIINDHEELVELVKDSLSMASGFELADLFPSSKLLNLLCWNKSKLW :236
Mslim6hyd : SKMSCVVVCRAAFGSVLKDQGSLAELVKESLALASGFELADLYPSSWLLNLLSLNKYRLQ :236
Mxplim3hPM17: RMRRRVDAILEAIVEEHKLKKSGEFGGEDIIDVLFRMQKDSQIKVPITTNAIKAFIFDTF :299
Mxplim3hPM2 : RMRRRVDTILEAIVDEHKFKKSGEFGGEDIIDVLFRMQKDTQIKVPITTNSIKAFIFDTF :297
MscrlimhE2 : RMRRRVDTILEAIVDEHKLKKSGEFGGEDIIDVLFRMQKDSQIKVPITTNAIKAFIFDTF :296
Mslim6hyd : RMRRRLDHILDGFLEEHREKKSGEFGGEDIVDVLFRMQKGSDIKIPITSNCIKGFIFDTF :296
molecular oxygen binding pocket
Mxplim3hPM17: SAGTETSSTTTLWVMAELMRNPEVMAKAQAEVRAALKGKTDWDVDDVQELKYMKSVVKET :359
Mxplim3hPM2 : SAGTETSSTTTLWVLAELMRNPAVMAKAQAEVRAALKEKTNWDVDDVQELKYMKSVVKET :357
MscrlimhE2 : SAGTETSSTTTLWVMAELMRNPAVMAKAQAEVRAALKGKTSVDVDDVQELKYMKSVVKET :356
Mslim6hyd : SAGAETSSTTISWALSELMRNPAKMAKVQAEVREALKGKTVVDLSEVQELKYLRSVLKET :356
AGxETS
limonene hydroxylase motif
Mxplim3hPM17: MRMHPPIPLIPRSCREECEVNGYTIPNKARIMINVWSMGRNPLYWEKPETFWPERFDQVS :419
Mxplim3hPM2 : MRMHPPIPLIPRSCREECVVNGYTIPNKARIMINVWSMGRNPLYWEKPDTFWPERFDQVS :417
MscrlimhE2 : MRMHPPIPLIPRSCREECEVNGYKIPNKARIMINVWSMGRNPLYWEKPETFWPERFDQVS :416
Mslim6hyd : LRLHPPFPLIPRQSREECEVNGYTIPAKTRIFINVWAIGRDPQYWEDPDTFRPERFDEVS :416
PPxP
heme binding region
Mxplim3hPM17: RDFMGNDFEFIPFGAGRRICPGLNFGLANVEVPLAQLLYHFDWKLAEGMNPSDMDMSEAE :479
Mxplim3hPM2 : KDFMGNDFEFVPFGAGRRICPGLNFGLANVEVPLAQLLYHFDWKLAEGMKPSDMDMSEAE :477
MscrlimhE2 : RDFMGSDFEFIPFGAGRRICPGLNFGLANVEVPLAQLLYHFDWKLAEGMKPSDMDMSEAE :476
Mslim6hyd : RDFMGNDFEFIPFGAGRRICPGLHFGLANVEIPLAQLLYHFDWKLPQGMTDADLDMTETP :476
FGAGRRICPG
Mxplim3hPM17: GLTGIRKNNLLLVPTPYDPSS-------- :500
Mxplim3hPM2 : GLTGILKNNLLLVPTPYDPSS-------- :498
MscrlimhE2 : GLTGIRKNNLLLVPTLYKSP--------- :496
Mslim6hyd : GLSGPKKKNVCLVPTLYKSP--------- :496

Tobacco transformation and expression analysis of the integrated P450 cDNA

Tobacco plants were transformed with the limonene-3-hydroxylase cDNA under the regulation of a constitutive CaMV d35S promoter. Plants that were rooting on hygromycin containing medium were transferred to the greenhouse. Ten independent transgenic plant lines were obtained by transformation of plant line BCD (possessing already three monoterpene synthases) and coded BCDM. From the control plant line SR1, transformed with the P450 cDNA, eleven independent plant lines were obtained and coded M. As a control, the starting material of the plant lines was also transformed with the empty binary vector.
Introduction of the cytochrome P450 cDNA and its expression was analysed by Northern blotting of leaf total RNA (Figure 2). Expression of the limonene-3-hydroxylase cytochrome P450 cDNA was detected in all the transformed plants although expression was very low in BCDM-5 and 6. The expression of the limonene-3-hydroxylase was highest in the plant line BCDM-8. The Northern blot was subsequently hybridised with the *C62* cDNA as a probe. This verified that all the BCD derived plant lines expressed the limonene synthase cDNA. In the BCD and BCD with empty vector control plants, there was no expression of the P450. Also the SR1 plants transformed with the P450 construct were analysed on the same Northern blot. This showed that their expression level of the introduced P450 was on average higher than the expression level detected in the BCDM plants (data not shown).

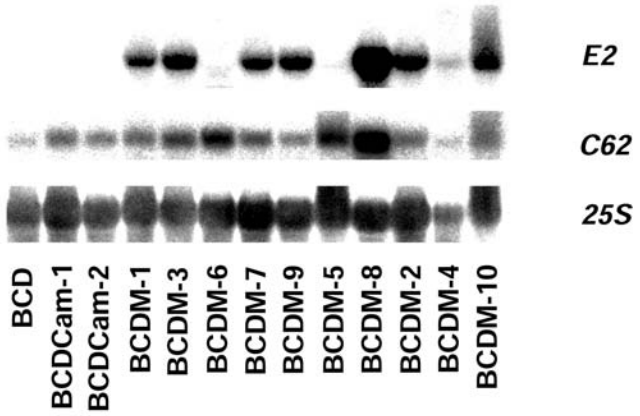


Figure 2. Northern blot analysis of leaves of transgenic tobacco plant lines. The Northern blot was subsequently hybridized with three different cDNA probes: *E2*) *E2* limonene hydroxylase cDNA from *M. spicata* 'Crispa'; *C62*) (+)-limonene synthase cDNA from *Citrus limon*; *25S*) 25S ribosomal cDNA from potato as control for loading differences as described previously (Lücker et al., 2001). Transformed plant lines tested: BCD is expressing three different lemon monoterpene synthases ((+)-limonene synthase, (-)-β-pinene synthase and γ-terpinene synthase). BCDCam is a BCD plant that was subsequently transformed with an empty pCambia1300+ vector. BCDM are BCD plants, subsequently transformed with the isolated limonene-3-hydroxylase cDNA, *E2*.

GCMS analysis using SPME

As the BCDM-8 plant was identified as the plant line with the highest expression, leaves of this plant were analysed by SPME and compared with the controls. The SR1 and the empty binary vector control showed small traces of α-pinene, β-pinene and linalool as described previously (Chapter 4). The M plants showed the same profile as the SR1 controls, as expected (data not shown). Figure 3 shows a typical GC-MS chromatogram obtained from the headspace of leaves of the BCD and BCDM plants. The BCD leaves mainly emitted β-pinene, limonene and γ-terpinene and some traces of other monoterpenes at minor levels (Figure 3A). The chromatogram of Figure 3B shows a very abundant peak in the BCDM-8 plant line at a retention time of 15.95 min (peak 12), not present in the BCD plant (Figure 3A). The mass spectrum of this peak was identical to the mass spectrum of isopiperitenol described in the literature (Lupien et al., 1999). In addition to the isopiperitenol peak, other peaks were detected in the chromatogram of BCDM-8 that were not present in the BCD control. Peak 13 had an identical mass spectrum as peak 12, and is therefore most likely the C3-epimer of isopiperitenol. Using a standard synthesised from isopiperitenone, peak 12 could indeed be identified as *trans*-isopiperitenol and peak 13 as *cis*-isopiperitenol. Flowers of the BCDM-8 plant that were sampled on the intact plant also emitted considerable amounts of *trans*-isopiperitenol (data not shown).

Peak 6 in Figure 3, was tentatively identified as 1,5,8-*p*-menthatriene (KI = 1006) and peak 11 as 1,3,8-*p*-menthatriene (KI = 1115) based on the Kovatz index and *mass* spectrum (Adams, 1995). Peak 14 was identified as isopiperitenone. Also the levels of some monoterpenes present in both the BCD and the BCDM-8 plant were modified in plant line BCDM-8. The levels of β-pinene and γ-terpinene were much lower in the BCDM-8 plant compared to the BCD plant. In contrast, the level of *p*-cymene was elevated in the BCDM-8 plant.

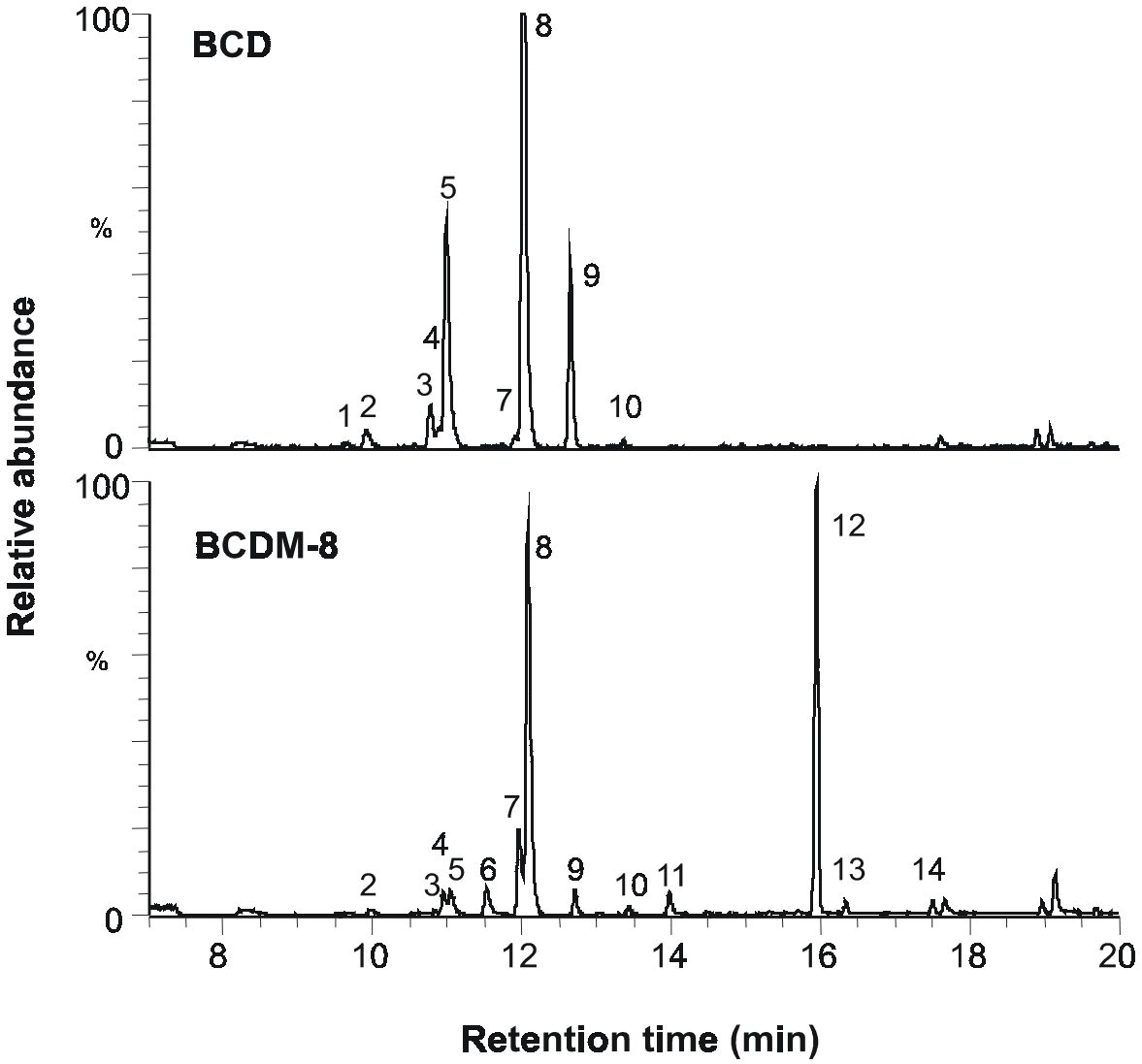
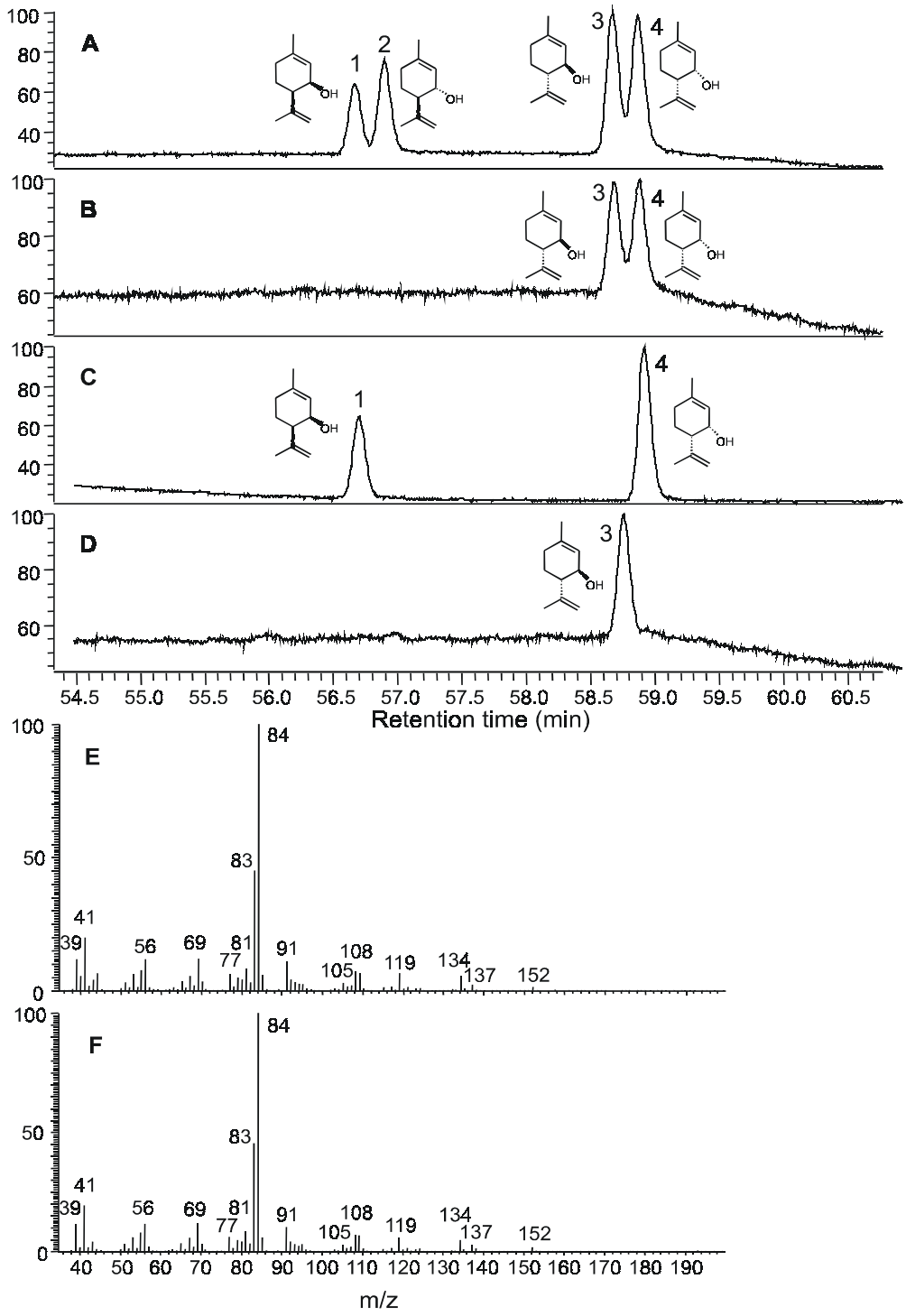


Figure 3. Headspace measurements on detached leaves of tobacco transgenic plant lines BCD and BCDM-8 using SPME. The chromatogram shows the combined *m/z* 68+69+82+84+91+93+119. 1: α-thujene, 2: α-pinene, 3: sabinene, 4: unidentified monoterpene, 5: β-pinene, 6: tentatively identified as 1,5,8-*p*-menthatriene (KI = 1006), 7: *p*-cymene, 8: limonene, 9: γ-terpinene, 10: linalool, 11: tentatively identified as 1,3,8-*p*-menthatriene (KI = 1115), 12: *trans*-isopiperitenol, 13: *cis*-isopiperitenol, 14: isopiperitenone.

Configuration of (+)-*trans*-isopiperitenol

All four synthesized isomers of isopiperitenol, were separated and identified by MD-GC-MS analysis (Figure 4). When the products synthesised from (+)-limonene were run on an enantioselective column (Figure 4B), the (+)-isomers of isopiperitenol could be identified (peaks 3, 4). In order to identify the *cis*- and *trans*- isomers, the synthesised racemic isopiperitenol mixture was first run on a DB-Wax column in the first GC. The first peak representing the *cis* isomers, as previously determined for this column (Werkhoff et al., 1998), was separated on an enantioselective column in the second GC (Figure 4C). Peak 1 was identified as (-)-*cis*-isopiperitenol and peak 4 as (+)-*cis*-isopiperitenol. In addition, synthesis of isopiperitenol from limonene via isopiperitenone was shown to result in a 40: 60 ratio of *cis*-: *trans*- isopiperitenol (Guillon et al., 2000). This ratio is also observed for peaks 1 and 2 that are completely separated (Figure 4A). The isopiperitenol formed in the transgenic plants, was also analysed by MD-GCMS and shown to consist of 99 % enantiomeric excess of (+)-*trans*-isopiperitenol (Figure 4D). The level of emitted (+)-*trans*-isopiperitenol was estimated to be around 400 ng g^{-1} h^{-1}. The isopiperitenol emitted by the leaves also consisted of (+)-*trans*-isopiperitenol (data not shown).

Figure 4. Configuration of the major new monoterpene emitted in the P450 transformed BCDM8 plant as (+)-*trans*-isopiperitenol, by multidimensional GC-MS. A) All four chemically synthesised isomers of isopiperitenol could be separated by MDGC-MS. B) The isopiperitenol chemically synthesized from (+)-limonene separated by the enantioselective column in (+)-*trans*-isopiperitenol (peak 3) and (+)-*cis*-isopiperitenol (peak 4). C) The first peak of the racemic isopiperitenol on the DB-Wax column representing the *cis* isomers (Werkhoff et al., 1998), was transferred to the enantioselective column and separated in (-)-*cis*-isopiperitenol (peak 1) and (+)-*cis*-isopiperitenol (peak 4). D) The isopiperitenol emitted by flowers of the BCDM-8 transgenic plant line could be identified as (+)-*trans*-isopiperitenol (peak 3). E) Mass spectrum of the synthesised standard (+)-*trans*-isopiperitenol F) Mass spectrum of the major new product emitted by flowers of the BCDM-8 plant.


A
B
C
D
E
F
1
2
3
4
OH
Retention time (min)
m/z
84
83
41
39
56
69
81
77
91
105
108
119
134
137
152

Flower headspace analysis of transgenic plant lines using Tenax trapping

Emission of detached open flowers of the same developmental stage was compared for different transgenic lines (Figure 5). The flowers of the BCD plant line did not emit isopiperitenol. Several transgenic lines, transformed with the P450 gene showed emission of the *trans*-isopiperitenol, which eluted at 9.98 min with the GC-MS settings used. It was emitted as one of the major products in the volatile profile (Figure 5B, C and D, peak 9).
Some BCDM plants showed a clear decrease in the emission of the main products of some of the monoterpene synthases. In the case of BCDM3, no emission was detected of limonene and γ-terpinene, and probably as a consequence, no isopiperitenol could be detected (data not shown). Also plant line BCDM7 showed a very low emission of limonene and γ-terpinene, but there was still some isopiperitenol formed (data not shown). In the transgenic plant line BCDM8, the level of γ-terpinene was very low (Figure 5B). The level of γ-terpinene was higher in other transgenic plant lines, although it was always lower than in the BCD plant.
The emission of *p*-cymene from flowers of the BCDM plants emitting isopiperitenol, was higher than that of the BCD flowers, as was already observed for leaves (Figure 3, 5). Especially in the plant lines BCDM2 (Figure 5C) and BCDM4 (Figure 5D) this is obvious. Also in headspace samplings of other flower stages of plants BCDM2 and BCDM4 it was observed that the *p*-cymene level was frequently as high as the limonene level, while in the BCD plant the level of *p*-cymene was never higher than around one third of the emission of either one of the main products of the monoterpene synthases. This increase in *p*-cymene levels was higher for flowers than for leaves of the different transgenic plant lines (data not shown).
Similar to what was observed in leaves of the BCDM8 plant (Figure 3), there were two additional small peaks in the chromatogram of the flowers of the BCDM-4 plant, that were tentatively identified as 1,5,8-*p*-menthatriene (Figure 5D, peak 3) and 1,3,8-*p*-menthatriene (Figure 5D, peak 8). These peaks were also observed after concentration of the flower headspace sample of BCDM8 and BCDM2, but never in any measurement of the BCD plant line (data not shown).
Isopiperitenone, which was observed in the headspace of leaves, was detected in the flower headspace of BCDM4 (Figure 5D) and in BCDM8 and BCDM2 after concentration of the headspace samples. It was never detected in the BCD plant line (data not shown).

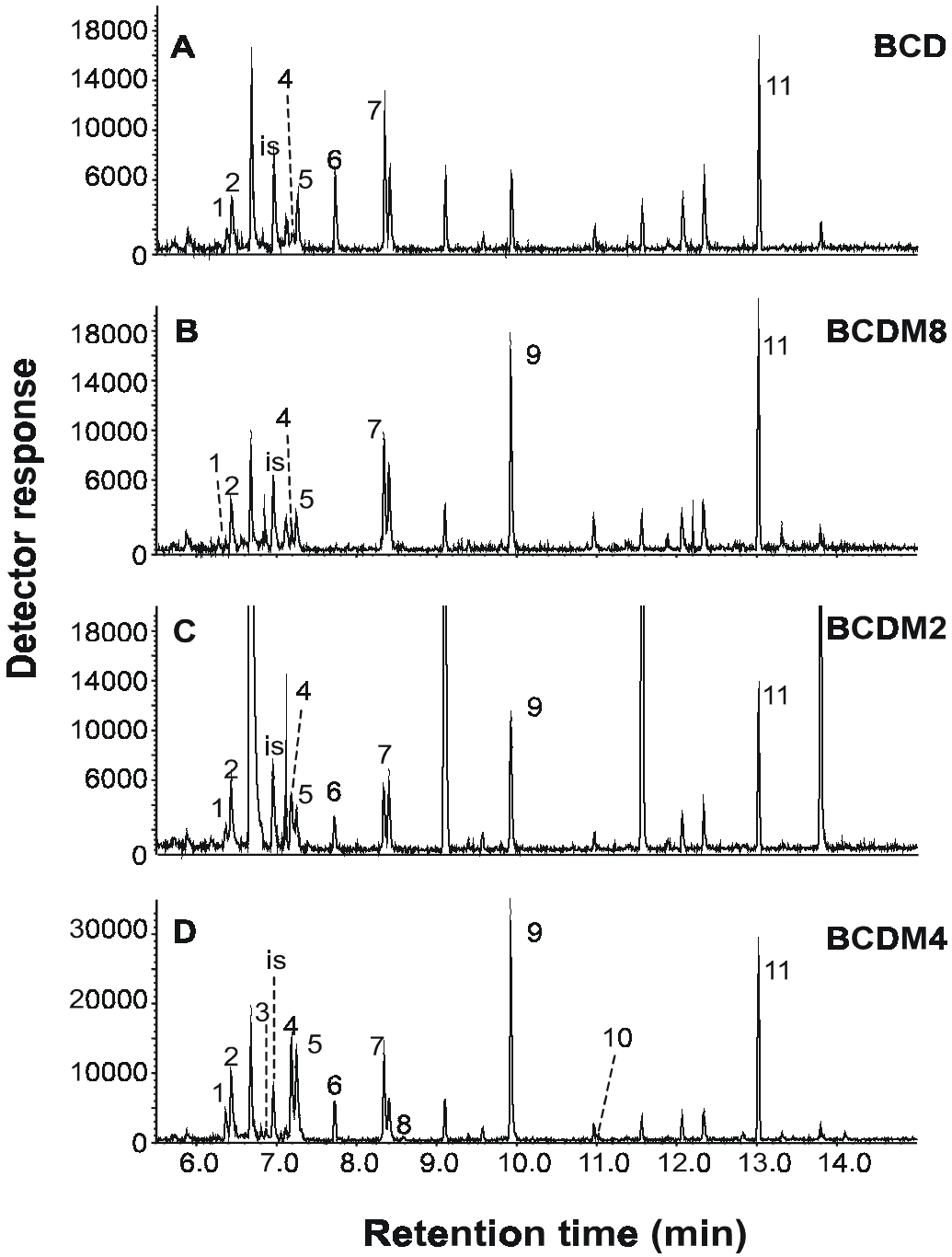


Figure 5. Volatile emission profiles (TIC) of detached flowers of transgenic plant lines sampled for 24 h using Tenax trapping. Each time 4 open flowers were sampled of stage 11 to 12 (Goldberg, 1988; Koltunow et al., 1990). A) Plant line BCD, B) Plant line BCDM8, C) Plant line BCDM2, D) Plant line BCDM4. Monoterpene and sesquiterpene peaks are numbered: 1: sabinene, 2: β-pinene, 3: tentatively identified as 1,5,8-*p*-menthatriene (KI = 1006), 4: *p*-cymene, 5: limonene, 6: γ-terpinene, 7: linalool, 8: tentatively identified as 1,3,8-*p*-menthatriene (KI = 1115), 9: *trans*-isopiperitenol, 10: isopiperitenone, 11: β-caryophyllene. is: internal standard, 212 ng (+)-3-carene. The peak at 9.98 min in panel A is derived from decanal, a natural constituent of tobacco eluting at the same time as isopiperitenol.

Discussion

Metabolic engineering of an additional hydroxylation step to an already introduced monoterpene biosynthesis route in tobacco plants was shown to be functional and resulted in the partial conversion of a large part of (+)-limonene into (+)-*trans*-isopiperitenol. The (+)-trans-isopiperitenol was emitted as a major component of the volatile spectrum of the plants possessing four integrated cDNAs involved in monoterpene biosynthesis. Isopiperitenol is an uncommon compound in the plant kingdom and mostly present as an intermediate, since most of it is converted to other derivatives with the same oxygenation pattern (Guillon et al., 2000). However, in our tobacco plants this occurred only to a small extent.

cDNA isolation and characterisation

The GC-MS profile of the *M. spicata* 'Crispa' contained apart from carvon and carveol also isopiperitenol, indicating that both regioselective (6- and 3-) hydroxylase activities must be present in this plant (data not shown). The gene specific primer, designed on the *Mentha spicata* (-)-limonene-6-hydroxylase sequence (AF124815), could therefore be expected to result in amplification of either a 3-hydroxylase or a 6-hydroxylase, especially as the last 6 bases (5'-GTGTGG-3') on the 3'-end of this primer were identical to the cDNA sequences of the two described *Mentha x piperita* (-)-limonene-3-hydroxylase isoforms (AF124816 and AF124817) which would result in a good annealing with the conditions of the PCR program used. The *E2* cDNA, isolated using RACE-PCR, is probably an isozyme of the previously isolated (-)-limonene-3-hydroxylases (Lupien et al., 1999). Caution must however be taken to the most distant 3'end of the sequence, that is identical to the specific primer used designed on the (-)-limonene-6-hydroxylase of *M. spicata*. This could contain a few altered amino acids, which apparently do not interfere with the activity and functionality of the isolated cDNA.

The regioselectivity motif, identified for the limonene hydroxylases (Schalk and Croteau, 2000), of the isolated *E2* cDNA had the PPIP sequence, indicating that the isolated cDNA was most likely a limonene-3-hydroxylase (Figure 1). The conserved sequence AGxETS is supposed to form the threonine containing binding pocket (A/G)Gx(D/E)T(T/S) for the oxygen molecule required in the catalysis (Von Wachenfeldt and Johnson, 1995). The FGAGRRICPG sequence, conserved for these mint P450s is the supposed heme-binding region (FxxGxxxCxG) (Von Wachenfeldt and Johnson, 1995). The conserved sequence PPxPPKLP is the proline rich region, described as the (P/I)PGPx(G/P)xP motif, which probably acts as a hinge, required for optimal orientation of the enzyme in the ER membrane (Yamazaki et al., 1993). Microsomal P450s, like the limonene hydroxylases (Mihaliak et al., 1993), contain an N-terminal hydrophobic signal sequence for transfer into the ER, which is

not cleaved, but anchored in the membrane and the catalytic domain of the protein is exposed on the cytosolic side of the membrane (Schuler, 1996). Localisation analysis of the isolated P450 cDNA with the PSORT and iPSORT prediction programs indicated with a certainty level of 0.82 that it is localised in the ER, like most plant P450s (Schuler, 1996; Chapple, 1998) and that it had a non-cleavable signal peptide.

Function of the cytochrome P450 enzyme

(-)-limonene is a minor product in peppermint and it predominates (+)-limonene to the extent of about 80%. The (-)-isomer is the preferred substrate for the highly regioselective and substrate specific (-)-limonene-3-hydroxylase of this species, being favoured by a factor 2 over the (+)-enantiomer at saturation (Karp et al., 1990). In the transgenic tobacco plants (+)-limonene is very abundant, and there is only a low level of (-)-limonene produced as a side product of the β-pinene and γ-terpinene synthases (Chapter 4). One major product in the BCDM8 plant line expressing the *E2* cDNA is (+)-*trans*-isopiperitenol with a very high enantiomeric excess of 99%. The lack of (-)-*trans*-isopiperitenol could be explained by the low activity of the monoterpene synthases that produce β-pinene and γ-terpinene as a main product and (-)-limonene as a side product in the BCDM8 plant.

Targeting and substrate availability

Monoterpene synthases show N-terminal plastid targeting peptides. Recently it was shown that a peppermint (-)-limonene synthase was indeed localised in the leucoplasts of secretory cells (Haudenschild and Croteau, 1998; Turner et al., 1999). Monoterpene synthases are probably also localised in chloroplasts of leaf parenchyma cells (Bouvier et al., 2000). Likewise, a plastid targeting signal was observed in the (+)-limonene synthase (*C62*) from lemon (Lücker et al., 2002). The introduced limonene-3-hydroxylase however, is most likely associated with the ER, like other plant P450s (see above). P450 enzymes always function as a complex with an NADPH cytochrome P450 reductase (Schuler, 1996). This reductase appears to be a nonspecific electron donor to cytochrome P450s (Donaldson and Luster, 1991). The fact that the introduced biosynthesis step was functional, implicated that there is sufficient endogenous cytochrome P450 reductase activity present in tobacco, and available for the introduced cytochrome P450. Thus, it can be concluded that the lack of hydroxylated products in the BCD plants was only due to a lack of suitable activity of a P450 hydroxylase. A previously hypothesised transport mechanism of monoterpenes to the ER is therefore likely to be present in tobacco and might be common in plants (Bosabalidis, 1996; Bouwmeester et al., 1998).

Side products and conversions

The use of (+)-limonene as a substrate for the (-)-limonene-6-hydroxylase has recently resulted in the observation that apart from the main product (+)-*cis*-carveol, small levels of (-)-*trans*-carveol, (+)-*trans*-carveol, (+)-*trans*-isopiperitenol and 1,2-*cis*-epoxide were formed (Wust and Croteau, 2002). In the leaves of the BCDM-8 plant a trace of the *cis*-isomer of isopiperitenol was detected. This could be a consequence of the use of the (+)-limonene as the major substrate for the limonene-3-hydroxylase instead of the preferred (-)-limonene, similar to the results obtained for the (-)-limonene-6-hydroxylase. In addition, the introduction of a new monoterpene alcohol in plants might trigger other secondary reactions. It has been shown previously that e.g. hydroxylated monoterpenes can be converted to their respective ketones by cultured suspension cells of *N. tabacum*, governed by an NAD^+- dependent alcohol dehydrogenase (Suga and Hirata, 1990), although the equilibrium in this reversible reaction tends to lie towards the side of the alcohol. The trace of isopiperitenone detected in the headspace of the transgenic plants (Figure 4, 5), indicates that such an endogenous alcohol dehydrogenase activity is indeed present.

Although the small peak of *p*-cymene that was observed in the BCD plants was probably due to oxidation of the terpenes by air (Chapter 4), this cannot explain the substantial increase of *p*-cymene observed after introduction of the cytochrome P450 limonene-3-hydroxylase. It is possible that the *p*-cymene is a product that is derived from (+)-*trans*-isopiperitenol, as depicted in Figure 6. After the formation of (+)-*trans*-isopiperitenol, water could be eliminated, for example catalysed by an endogenous dehydratase, traces of acid or heat (Kobold et al., 1987) resulting in the formation of 1,3,8-*p*-menthatriene, which can isomerise to the more stable *p*-cymene by the action of traces of acid or light by a suprafacial 1,7-hydrogen shift followed by a suprafacial 1,3-H-shift (Smith and March, 2001). Another possibility would be the heat of the GC-injection port (Kobold et al., 1987), but this is probably irrelevant since GC-MS analysis of the samples with injection port temperature set at 150 °C instead of the normal 250 °C did not have an effect. Dehydratase enzymes, which could catalyse such a conversion, have been reported. For example, such an enzyme is present in the shikimate pathway in microorganisms, converting 3-dehydroquinate to 3-dehydroshikimate and water (Herrmann and Weaver, 1999). In plants, this enzyme is present as a bifunctional 3-hydroquinate dehydratase-shikimate dehydrogenase (Herrmann and Weaver, 1999). A partial cDNA of the gene encoding this enzyme was also isolated from tobacco (Bonner and Jensen, 1994). In the BCDM plants two peaks were observed which were tentatively identified as 1,5,8-*p*-menthatriene (KI = 1006) and 1,3,8-*p*-menthatriene (KI = 1115), which could be intermediates in the supposed conversions. The observation that the increase in *p*-cymene is often more clear in flowers than in the leaves of the transgenic

BCDM plants might indicate that the putative dehydratase enzyme is more active in the flowers than in the leaf.

Finally it is possible that novel glycosides could be formed from isopiperitenol in the transgenic tobacco plants, as was shown in petunia plants transformed with a linalool synthase gene (Lücker et al., 2001). However, experiments to check the presence of such glycosides were not carried out.

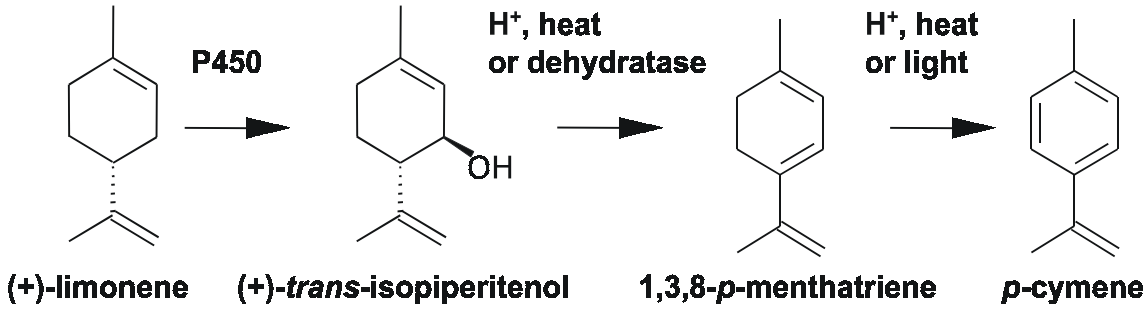


Figure 6. Hypothetical scheme of enzymatic or non-enzymatic conversions occurring in the transgenic tobacco BCDM plants. Conversion of isopiperitenol to *p*-cymene is supposed to be triggered by an endogenous dehydratase and occurs via 1,3,8-*p*-menthatriene and possibly 1,5,8-*p*-menthatriene (not shown). Also heat, acid or light could result in these reactions.

Gene silencing

Figure 4 and 5 show that BCDM-8 emitted lower levels of β-pinene and γ-terpinene than the BCD plant. Also some of the other P450 transformed lines showed a similar decrease in some of the main products of the monoterpene synthases, like BCDM3, which showed only β-pinene emission. This might indicate that gene silencing occurs. All the BCD plants were already expressing three genes regulated by the same CaMV 35S promoter. This promoter is known to cause silencing effects in transgenic plants when multiple copies are present (personal communication, Dr. J.P. Nap). A form of dosage compensation regulates the total accumulative expression level that is reachable by multiple CaMV 35S promoters (Mlynarova et al., 1995). This explains also the higher average level of expression of *E2* observed in the M plant lines, that were only transformed with the pJLT6 (*E2* containing) vector, compared to the average expression of *E2* observed in the BCDM plants (data not shown). The number of inserts of the P450 cDNA in the genome of the BCDM plants after the transformation with the P450 gene was not determined. When multiple copies of the P450 cDNA with the CaMV 35S promoter are integrated, this could therefore affect the expression of the other transgenes. Multiple copies of T-DNA insertions can also result in a lower total expression (Mlynarova et al., 1995).

The present results show that the metabolic engineering of monoterpene biosynthesis involving enzymatic conversions in two separate cellular compartments can be successful. This creates opportunities to build a monoterpenoid biosynthetic pathway from scratch in a desired plant, in order to produce a highly favourable product that has good commercial potential. This approach can for instance be very useful to extensively modify the flower scent profile of commercially interesting cut flowers such as roses.

Acknowledgements

The authors would like to thank the chemical company Haarman & Reimer, for kindly providing the isopiperitenone standard, and prof. dr. Carlo Bicchi for his help in the identification of the (+)-*trans*-isopiperitenol.

Chapter 6

General discussion

During the past five years various reviews described speculations about the possibilities of genetic or metabolic engineering of terpene biosynthesis (McCaskill and Croteau, 1997; Bohlmann et al., 1998; Haudenschild and Croteau, 1998; McCaskill and Croteau, 1998; Little and Croteau, 1999). The opportunities and goals described in these reviews are very diverse, ranging from commercial applications such as improved floral scent of ornamentals and modification of essential oil crops aimed at more valuable oil, to scientific opportunities for ecological studies such as altered herbivore, pollinator and tritrophic interactions. Ideal models would be plant pairs differing in only a single trait (Dicke and van Loon, 2000). Such plants can be obtained efficiently by genetic engineering, since introduction of a desired trait by classical breeding is more time consuming and will usually also introduce unwanted additional traits. There is no doubt that ecological studies on interactions with transgenic plants would eventually lead to commercial applications like better seed set by improved pollination or improved pest and disease resistance. This could open new ways of biological and integrated pest control.

In a number of these reviews it was also discussed that the cellular fate of the monoterpenes biosynthesised in tissues not specifically adapted to secretion and storage of such volatile hydrophobic metabolites is unknown (McCaskill and Croteau, 1997; Haudenschild and Croteau, 1998; McCaskill and Croteau, 1998; Little and Croteau, 1999). Transgenic production of monoterpenes in non-adapted tissues might lead to deposition of oil droplets. As an alternative, the relatively high vapour pressure of the monoterpenes may promote the evaporation from the producing cells to the atmosphere, which is of course highly desirable for scent production. Another possibility is that the volatiles might be retained in the waxy cuticle of the epidermal cell layer. The fate and potential phytotoxicity of an introduced metabolite resulting from genetic modification will need to be determined empirically since is likely to be dependent on the context of where it is introduced (Little and Croteau, 1999).

Various approaches of metabolic engineering of monoterpene metabolism

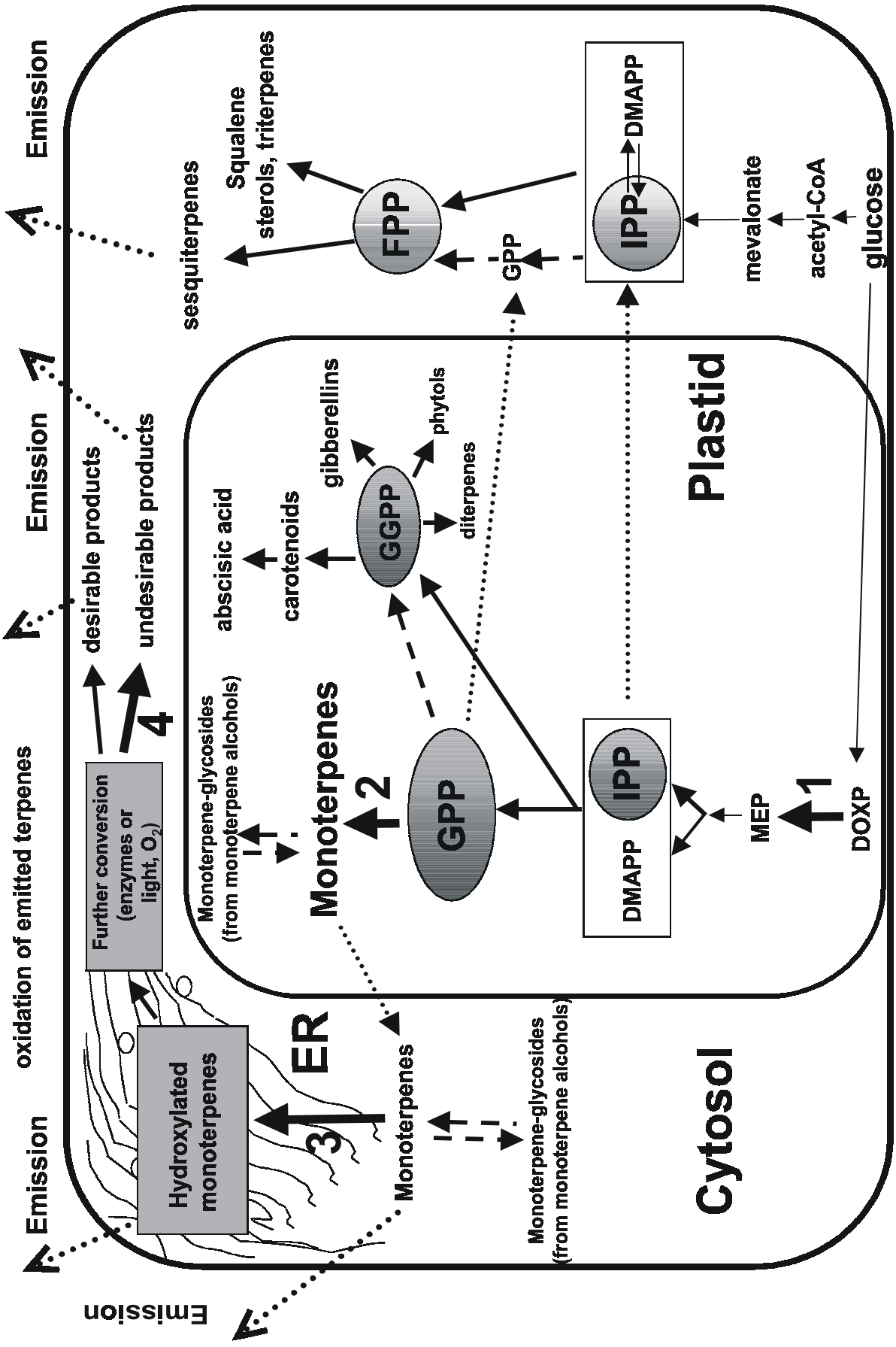
Currently several groups have reported on the genetic modification of terpenoid biosynthesis and this work was recently reviewed (Vainstein et al., 2001; Chappell, 2002; Pichersky and Gershenzon, 2002; Verpoorte and Memelink, 2002). Figure 1 displays a schematic outline of terpene metabolism in plants in order to explain different approaches of metabolic engineering of monoterpene metabolism at target steps in the pathway. The resulting conversions, fluxes to other pathways and the fate of some produced metabolites are also indicated.

For monoterpene biosynthesis, GPP-synthase and monoterpene synthases are considered to be effective points of regulation, but also various steps of the MEP pathway provide multiple

control points to direct precursor flux into the terpene biosynthesis (Haudenschild and Croteau, 1998).

There are a number of approaches that can be used for metabolic engineering. One approach is to increase the total yield of the monoterpenes produced. An increase of 50% of the yield of essential oil was achieved in an experiment with peppermint, where a reductoisomerase of the MEP pathway was overexpressed (Figure 1, #1) (Mahmoud and Croteau, 2001). The same paper reported another approach, which was to improve the quality of the essential oil. To achieve this, the production of an undesired metabolite in peppermint essential oil, menthofuran, was downregulated by the introduction of an antisense construct of the cytochrome P450 enzyme menthofuran synthase (Figure 1, #4) (Bertea et al., 2001; Mahmoud and Croteau, 2001). The level of menthofuran could be reduced by 50% compared to the amount present in the wild type plants under stressed conditions (Mahmoud and Croteau, 2001). Another approach to modify the composition or quality of the essential oil was carried out by transformation of a (-)-limonene synthase (Figure 1, #2) into peppermint. Introduction of this enzyme resulted in small qualitative and quantitative variations in the composition of the essential oil (Krasnyanski et al., 1999). Also in non-essential oil producing plants it might be interesting to introduce or alter the monoterpenes produced, since this might lead to improved defensive properties, against e.g. fungal and insect attack. It could also be used as an approach to bring back scent to cut flowers. This approach was carried out by using the *S*-linalool synthase enzyme from *Clarkia breweri* (Dudareva et al., 1996) producing the corresponding monoterpene alcohol, for introduction in tomato (Lewinsohn et al., 2001) and carnation (Figure 1, #2) (Lavy et al., 2002). In tomato the linalool was only expressed in the fruit using an *E8* promoter and this resulted in high levels of *S*-linalool and 8-hydroxy-linalool, probably as a consequence of the presence of an endogenous P450 enzyme that can hydroxylate linalool. In carnation emission of *S*-linalool was only detected at low levels. The linalool in carnation was probably mostly oxidised to linalool-oxides, which were detected at high levels inside the plant tissue instead of linalool (Lavy et al., 2002).

Figure 1 Schematic outline of terpene metabolism operating in plants. Steps of monoterpene biosynthesis that have been modified by metabolic engineering in this thesis and by other groups are indicated with solid arrows and numbers. Dotted arrows are steps that indicate possible transport of either precursors or volatile terpenes. Dashed arrows indicate steps or locations that are uncertain.


Emission
oxidation of emitted terpenes
Emission
Emission
Emission
sesquiterpenes
Squalene
sterols, triterpenes
FPP
GPP
IPP
DMAPP
mevalonate
acetyl-CoA
glucose
desirable products
undesirable products
Further conversion
(enzymes or
light, O2)
4
Plastid
abscisic acid
carotenoids
gibberellins
GGPP
phytols
diterpenes
Monoterpene-glycosides
(from monoterpene alcohols)
Monoterpenes
2
GPP
IPP
DMAPP
MEP
1
DOXP
Hydroxylated
monoterpenes
3
ER
Monoterpenes
Monoterpene-glycosides
(from monoterpene alcohols)
Cytosol

Chapter 2 shows that also the introduction of the *Clarkia S*-linalool synthase into petunia, a plant without monoterpenes, can lead to the production of high levels of *S*-linalool (Figure 1, #2). The linalool was not detectable in the headspace as it was directly converted by a highly efficient endogenous glycosyltransferase to non-volatile *S*-linalyl-β-D-glucopyranoside (Chapter 2, (Lücker et al., 2001). The same binary vector containing the *S*-linalool synthase cDNA was used to transform microtom tomato and tobacco (data not shown), also resulting in accumulation of a glycosylated form of linalool. However in tobacco, the glycosylation was incomplete since free linalool was detected in the headspace (data not shown). Summarising the results obtained with the transformation of *S*-linalool synthase to different plants by several groups, a pattern emerges in which monoterpene alcohols, produced as a consequence of metabolic engineering, in tissues not adapted to these compounds, are converted into less reactive or less phytotoxic products.

Figure 1 describes the fate of the upregulated levels of the monoterpenes, and shows that they can be either hydroxylated, glycosylated or emitted. Monoterpenes like linalool, at high concentrations, are known to be detrimental to biological tissues (Vaughn and Spencer, 1991; Weidenhamer et al., 1993). While conjugated linalool can be found in abundant levels, indicating that there is a lot of available GPP, free linalool is never detected in high levels in non-adapted transgenic plant tissues. Therefore it seems likely that free linalool, could exert a negative selection pressure against high producing lines.

In Chapter 4 we showed that introduction of three monoterpene synthases, all regulated by CaMV-d35S promoters, in tobacco is possible by successive crossing of primary transgenic lines with single gene inserts that were obtained by three individual transformation experiments (Figure 1, #2). This approach had been used previously for the engineering of a polyhydroxy-butyrate pathway into plants, where three genes performing subsequent steps of the polyhydroxybutyrate pathway, all regulated by a CaMV-35S promoter, were shown to be active in one plant after subsequent crossings (Nawrath et al., 1994). By the introduction of the three monoterpene synthases from lemon to a tobacco plant, three new main products are emitted, which are the cyclic monoterpene olefins γ-terpinene, (+)-limonene and (-)-β-pinene (Figure 2). In addition to the main products a large number of side products are produced: α-thujene (undetermined stereochemistry), (+/-)-α-pinene, (+)-β-pinene, myrcene, (+/-)-sabinene, α-terpinene, (-)-limonene, *p*-cymene and terpinolene (Figure 2). *p*-Cymene was more abundant in the tobacco plants than expected from the heterologous expression of γ-terpinene synthase in *E.coli*, where it was detected as a trace (Chapter 3, Lücker et al., 2002). This is most likely the result of oxidation of some of the introduced monoterpenes, induced by light and air, indicated in Figure 1. This is supported by the fact the *p*-cymene level in essential oils can increase upon storage when they are in contact with air (Njoroge et al., 1996; Iwanami et al., 1997). Oxidation of γ-terpinene could also have caused the formation of a trace of p-cymene in the enzyme assays after heterologouos expressioAnother metabolic

engineering approach that was never carried out previously was to introduce two consecutive steps of the monoterpene biosynthesis pathway in one plant spanning two cell compartments (Figure 1, #2 and #3). This was descibed in Chapter 5 in this thesis. The three monoterpene synthases expressing plant was used for a subsequent transformation experiment in which a cytochrome P450 limonene-hydroxylase from *Mentha spicata* 'Crispa' was introduced in the genome (Figure 1, #3). This resulted in the detection of a new major product in the headspace, which was identified as (+)-*trans*-isopiperitenol. Also some *cis*-isopiperitenol was detected as a side product of the cytochrome P450 enzyme. This latter product was probably formed as a result of the non-optimal binding of the (+)-enantiomer of limonene in the active site of the P450 (Wust and Croteau, 2002).

The relatively unstable (+)-*trans*-isopiperitenol and the other hydroxylated products can give rise to several new products (Figure 2), which may be produced via chemical modification or via enzymatic conversion (Figure 1).

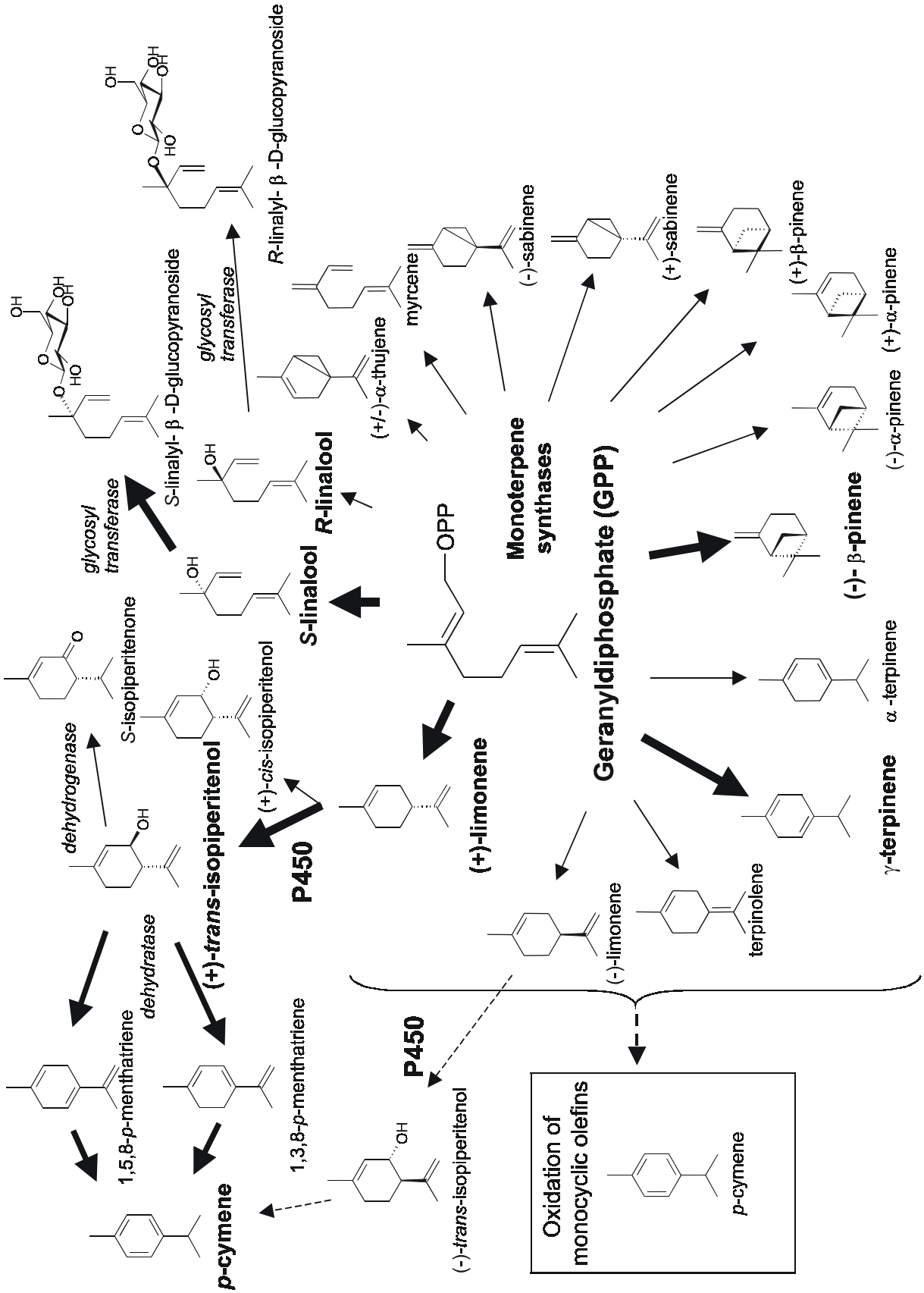
Isopiperitenone was probably formed from isopiperitenol by an endogenous alcohol dehydrogenase of tobacco. It was shown that monoterpene alcohols, such as carveol, can be converted to the corresponding ketone, by cultured suspension cells of tobacco, indicating the presence of endogenous alcohol dehydrogenase activity (Suga and Hirata, 1990). The increase of the *p*-cymene level that was detected can be explained by assuming an endogenous dehydratase activity. Two other products, tentatively identified as 1,3,8-*p*-menthatriene and 1,5,8-*p*-menthatriene were putative intermediates in the dehydratase-initiated conversion of isopiperitenol to *p*-cymene (Figure 2). It is also possible that the accumulating isopiperitenol is partially converted to a glycoside as the endogenous linalool in tobacco was also partially glycosylated (Dr. H.A. Verhoeven, personal communication).

In this way a whole cascade of monoterpenes is created in the transgenic tobacco plants and the volatile profile is extensively altered. All the products and possible conversions are indicated in Figure 2. The cytochrome P450 was most likely localised in the ER in the cytosol where it functions as a complex with an endogenous NADPH-cytochrome P450 reductase (Figure 1). As the monoterpene synthases are supposed to produce monoterpenes in the plastids, this implies the occurrence of a transport mechanism, between the plastids and the cytosol possibly via lipophilic oil bodies. Such a transport mechanism was suggested to be present in plants containing essential oil but is apparently also present in plants not explicitly adapted to the production of monoterpenes (Bosabalidis, 1996; Bouwmeester et al., 1998; Little and Croteau, 1999).

In a recent paper it was postulated that there is probably no IPP isomerase present in the plastidic MEP pathway resulting in a constant ratio of DMAPP to IPP of 1: 5 via this pathway (Rohdich et al., 2002) (Figure 1). In plastids, one molecule of IPP is used for synthesis of GPP together with DMAPP, while three molecules of IPP are used together with one molecule of DMAPP to form GGPP. Therefore there is probably an excess of IPP produced in

the plastids that could be leaking to the cytosol (Figure 1), where IPP isomerase is active. Reports showed that plastidic IPP could be incorporated into FPP in chamomile, but also in sesquiterpenes in peppermint (McCaskill and Croteau, 1995; Adam and Zapp, 1998). Our results suggested the formation of part of the sesquiterpene β-caryophyllene via plastid-derived precursors (Chapter 4). Due to the upregulated formation of monoterpenoids, in the transgenic tobacco plants producing extra monoterpenes, precursors that would normally be transported to the cytosol for the production of β-caryophyllene are not available for the cytosolic IPP-isomerase and FPP-synthase, leading to a decreased formation of β-caryophyllene in these transgenic plants.

Figure 2 Monoterpenes and derivatives formed from GPP after metabolic engineering in tobacco and petunia plants. The various transgenic plants produced during the work described in this thesis, resulted in the production of a large number of new compounds in the plants. Some compounds are very rare in the plant kingdom, like (+)-*trans*-isopiperitenol, one of the major compounds in the volatile spectrum of some of the transgenic tobacco plants (Chapter 5). The main products are indicated by bold arrows.


Geranyldiphosphate (GPP)
Monoterpene synthases
OPP
S-linalool
R-linalool
glycosyl transferase
S-linalyl- β -D-glucopyranoside
R-linalyl- β -D-glucopyranoside
(+/-)-α-thujene
myrcene
(-)-sabinene
(+)-sabinene
(+)-β-pinene
(+)-α-pinene
(-)-α-pinene
(-)- β-pinene
α -terpinene
γ-terpinene
terpinolene
(-)-limonene
(+)-limonene
P450
(+)-trans-isopiperitenol
(+)-cis-isopiperitenol
dehydrogenase
S-isopiperitenone
dehydratase
1,5,8-p-menthatriene
1,3,8-p-menthatriene
p-cymene
(-)-trans-isopiperitenol
Oxidation of monocyclic olefins
p-cymene

Functional implications

Plants in nature are exposed to many organisms that are adapted to specific secondary metabolites and structures of a particular plant. Various forms and levels of resistance to pests have developed during evolution. A first level of resistance is a passive or constitutive one in the form of morphological protection by thorns, spikes, glandular and stinging hairs or an almost impenetrable bark (Wink, 1999). These structures prevent many attacking organisms from accessing the softer nutrient containing tissues. A second layer of defence is present as toxins or protoxins inside the cells, which are lethal for non-host organisms. In addition to preventing access by mechanical means, substances can be emitted continuously to deter pests. A more evolved degree of defence is the active resistance in which the production of certain (toxic) metabolites is upregulated upon beetle infestation or other kinds of wounding, such as in pine trees (Lewinsohn et al., 1991; Lewinsohn et al., 1992). The beetles often infect the plants with fungi they are carrying in their mouthparts that can cause severe damage to a tree. The extra monoterpenes formed might make the resin less soluble and thus less edible; in the meantime they might give the resin a fungicidal activity, since many monoterpenes have a fungicidal effect (Belaiche et al., 1995; Caccioni et al., 1998; Tsao and Zhou, 2000; Aggarwal et al., 2002).
An even more complicated interaction between a number of organisms is present in tritrophic interactions. In this case plants start *de novo* synthesis of volatiles (both terpenoids and non-terpenoids), in order to attract carnivores (predators and parasitoids) of the herbivores that are foraging on the plant (Dicke and van Loon, 2000). Such interactions were shown to occur for instance in cotton, potato, cucumber, *Nicotiana attenuata*, lima bean and *Arabidopsis* (Weissbecker et al., 1997; Bouwmeester et al., 1999b; Baldwin, 2001; van Poecke et al., 2001). Amongst the novel volatiles that are emitted by the plants as a response to herbivory, there are also some monoterpenes such as linalool and ocimene. As it is clear that monoterpenes are involved in ecological interactions, altering the monoterpene composition of a plant might lead to novel interactions, both desired and undesired, that cannot be predicted.

Ecological consequences

The petunia plants in the greenhouse that were accumulating the *S*-linalyl-β-D-glucopyranoside seemed to be less sensitive to infection by mildew than the control. A similar observation was done on the transgenic microtom fruits, which accumulated high levels of linalyl-glycoside. When the fruits were stored in a cold room for a month, the transgenic fruit was still firm and did not show any sign of fungal spoiling, whereas the control fruits got severely infected with fungus. A possible mechanism for this phenomenon is that penetration

of the fungus into the cytoplasm of the transgenic cells might lead to decompartmentalisation of the plant cells, resulting in breakdown of the linalyl-glycoside and subsequent emission of the fungitoxic linalool. Another possibility is that for penetration of the cell wall the fungus needs to release glycosidases, again resulting in breakdown of the linalyl-glycoside.
Apart from effects caused by stored terpene glycosides there were also effects observed on plants emitting new monoterpenes. The transgenic tobacco plants mainly emitting limonene, β-pinene and γ-terpinene were much less visited by herbivorous insects like white flies than other tobacco plants in the same greenhouse compartment. Another observation was that the transgenic tobacco plants seemed highly attractive to fruit flies, which have been described previously to be attracted to limonene (Jacobson, 1982). It would be interesting to test these genetically modified plants in the field to investigate whether different insects visit the transgenic plants than the control plants.

Physiological effects

The introduction of a new flux towards the production of high levels of monoterpenes can alter the natural flux of precursors to other, related pathways. A possible decrease of flux of precursors to the sesquiterpenoid pathway leading to a lower emission of β-caryophyllene was already mentioned above. In this case, the monoterpene synthases compete for the plastidic pool of GPP, and are thus lowering the level of transport of GPP to the cytosol for sesquiterpene biosynthesis. Furthermore, changes in the flux to higher terpenoids such as the carotenoids, gibberellins or abscisic acid could have drastic physiological effects in transgenic plants (Figure 1).
In a carotenoid terpene engineering approach, a phytoene synthase, producing a precursor for carotenoids, was overexpressed in tomato. This resulted in a dwarf phenotype that was explained by a severe inhibition of gibberellin biosynthesis by competition for the common substrate GGPP (Figure 1) (Fray et al., 1995).
Tobacco plants that were homozygous for single monoterpene synthases showed a significant reduction in length of the whole plant compared with the control plants of about 20% (data not shown). The decrease in length was caused by a decrease in internodal length, which was most severe on the internodes just below the inflorescence of the mature tobacco plants. If the endogenous pool of plastidic IPP/ GPP is depleted by the effects of the monoterpene biosynthesis, less IPP/ GPP is available for the synthesis of GGPP and subsequently derived plant growth regulators, such as gibberellins and abscisic acid. A lower level of gibberellins will reduce the length of the internodes, resulting in smaller plants. This hypothesis is further supported by observations on reduction of the internode lengths in transgenic microtom tomatoes. However, the growth reduction could also be caused by a phytotoxic effect of the monoterpenes themselves produced at high levels in a non adapted organ.

The seeds of the transgenic tobacco plants did not show any dormancy after long time storage at room temperature, while the control seed did. A lower level of abscisic acid, or another terpenoid growth hormone, produced in the plastids, could explain this lack of dormancy. During ripening in the seed pods, maternal abscisic acid is transferred to the seeds, resulting in development of dormancy (Bewley, 1997).
The microtom tomato transformation experiment with the *S*-linalool synthase, resulted in a plant line with orange fruit that did not turn red. This indicates a reduced production of carotenoids during fruit ripening. Furthermore the fruit was very soft and looked wrinkled, which could be a phytotoxic softening effect of high levels of linalool, previously described for potato tubers in contact with high levels of linalool (Vaughn and Spencer, 1991). Most of the seeds in these fruits showed precocious germination, indicating a deficiency in abscisic acid uptake during seed maturation, resulting in lack of dormancy (Bewley, 1997). The combined effect in this fruit of the lower carotenoid level (orange colour) and the precocious germination, confirm the presumed lower abscisic acid content, since abscisic acid is thought to be synthesised of carotenoid precursors. Apparently extreme cases of overexpression of monoterpene synthases can lead to phytotoxic effects and a decrease of precursor flux to other terpenoid pathways (Figure 1).

Commercial aspects and goals for the future

As a consequence of the various approaches for the metabolic engineering of monoterpene synthases, several speculative opportunities have become reality, revealing a number of unexpected consequences.
The possibility of the construction of a functional two-step and two-compartment pathway for monoterpene biosynthesis, as shown in Chapter 5, opens up the possibilities of producing any desired flavour or fragrance compound in plants. With combinations of monoterpene synthases and hydroxylating enzymes and different levels of enzyme activity a whole range of changes in scent profiles of a plant can be accomplished, displayed in Figure 2. When this approach is used for ornamental plants, they could be genetically engineered to produce fragrant monoterpenes and monoterpene derivatives according to the wishes of the consumers. However, care has to be taken to carefully examine the resulting plants, since endogenous conversions could result in the production of unexpected metabolites.
The different monoterpenes produced in our experiments gave a different scent to the flowers, depending upon which monoterpene synthase was introduced. The combination of three monoterpene synthases in one plant resulted in the observation of a fruity smell of the tobacco flowers and leaves. But most striking was the menthol-like smell of the plant obtained after transformation with the gene for a cytochrome P450, in which (+)-*trans*-isopiperitenol was emitted in addition to all the other monoterpenoids. Chemically synthesised isopiperitenol

was described to have a pleasant musky-menthol characteristic odour and could become a base material of the perfumery industry (Guillon et al., 2000).

Several effects have been observed on the transgenic plants, which apparently modify the degree of resistance to pests and diseases. Therefore, resistance to certain pests and diseases could be improved by modification with monoterpene synthases. However, before possible effective compounds can be implemented for plant resistance more ecological, molecular and biochemical research is necessary, since the biosynthesis of such a compound might need to be specifically targeted and regulated.

The metabolic engineering of monoterpenes in plants, leading to upregulation and/ or modification of the flavour and fragrance profile of the resulting plants might lead to some additional effects. As monoterpenes have been shown to increase cell membrane permeability in humans (Huang et al., 1999), it could be possible that an increase in flavour, due to monoterpenes, might also increase the uptake of toxic secondary metabolites that are present in many plant species. As already mentioned in Chapter 4, it might be possible on the one hand to produce 'healthier' tobacco, since the (+)-limonene has known anticarcinogenic properties (Crowell and Gould, 1994; Crowell, 1999), while on the other hand, this tobacco could also be more addictive to smokers as it would possibly facilitate the uptake of the addictive toxin nicotine. This once more illustrates that altering monoterpene levels can have various, often opposite and not easily predictable effects, which make extensive research of such effects necessary.

Concluding, this thesis shows that is feasible to obtain an extensive elevation of the monoterpene fragrance profile of a plant without the need to produce the desired volatiles targeted to specialised secretory structures. Although the work that is described in this thesis consists of straightforward approaches of metabolic engineering, it paves the way for more complicated approaches. Now metabolic engineering can be more targeted, for instance to produce a flower specific attractant for a pollinator or to produce wound inducible expression of a specific toxin or a deterrent for defence against insect pests.

Cited literature

Adam KP, Crock J, Croteau R (1996) Partial purification and characterization of a monoterpene cyclase, limonene synthase, from the liverwort *Ricciocarpos natans*. Arch Biochem Biophys **332**: 352-356

Adam KP, Zapp J (1998) Biosynthesis of the isoprene units of chamomile sesquiterpenes. Phytochemistry 48: 953-959

Adams RP (1989) Identification of Essential Oils by Ion Trap Mass Spectroscopy. Academic Press, San Diego, pp 302

Adams RP (1995) Identification of essential oil components by gas chromatography/mass spectroscopy. Allured Publishing Corporation, Carol Stream, pp 469

Aggarwal KK, Khanuja SPS, Ateeque A, Kumar TRS, Gupta VK, Sushil K, Ahmad A, Kumar S (2002) Antimicrobial activity profiles of the two enantiomers of limonene and carvone isolated from the oils of *Mentha spicata* and *Anethum sowa*. Flavour Frag J **17**: 59-63

Aharoni A, Keizer LCP, Bouwmeester HJ, Sun Z, Alvarez HM, Verhoeven HA, Blaas J, van Houwelingen AMML, De Vos RCH, van der Voet H, Jansen RC, Guis M, Mol J, Davis RW, Schena M, van Tunen AJ, O' Connell AP (2000) Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. Plant Cell **12**: 647-661

Alonso WR, Croteau R (1991) Purification and characterization of the monoterpene cyclase gamma-terpinene synthase from *Thymus vulgaris*. Arch Biochem Biophys **286**: 511-517

Alonso WR, Croteau R (1993) Prenyltransferases and cyclases. *In* PJ Lea ed, Enzymes of Secondary Metabolism, Vol 9. Academic Press, London, pp 239-279

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25**: 3389-3402

Andersen RA, Hamilton Kemp TR, Loughrin JH, Hughes CG, Hildebrand DF, Sutton TG (1988) Green leaf headspace volatiles from *Nicotiana tabacum* lines of different trichome morphology. J Agric Food Chem **36**: 295-299

Arctander S (1969) Perfume and flavor chemicals (aroma chemicals). *In*: S Arctander ed, 1 Ed, Vol II, Steffen Arctander's Publications, Las Vegas, pp 904

Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A, Zenk MH (1997) Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. Proc Natl Acad Sci USA **94**: 10600-10605

Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature **406**: 512-515

Auburg S, ALecharny A, Bohlmann J (2002) Genomic analysis of the terpenoid synthase (*At*TPS) gene family of *Arabidopsis thaliana*. Mol Gen Genomics **DOI 10.1007/s00438-002-0709-y**

Baek HH, Cadwallader KR (1999) Contribution of free and glycosidically bound volatile compounds to the aroma of muscadine grape juice. J Food Sci **64**: 441-444

Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant Physiol **127**: 1449-1458

Beckstrom Sternberg SM, Duke JA (1996) CRC handbook of medicinal mints (aromathematics): phytochemicals and biological activities. CRC Press Inc., Boca Raton, pp 458

Belaiche T, Tantaoui EA, Ibrahimy A (1995) Application of a two levels factorial design to the study of the antimicrobial activity of three terpenes. Sci Aliment **15**: 571-578

Bertea CM, Schalk M, Karp F, Maffei M, Croteau R (2001) Demonstration that menthofuran synthase of mint (*Mentha*) is a cytochrome P450 monooxygenase: Cloning, functional expression, and characterization of the responsible gene. Arch Biochem Biophys **390**: 279-286

Bewley JD (1997) Seed germination and dormancy. Plant cell **9**: 1055-1066

Birkett MA, Campbell CA, Chamberlain K, Guerrieri E, Hick AJ, Martin JL, Matthes M, Napier JA, Pettersson J, Pickett JA, Poppy GM, Pow EM, Pye BJ, Smart LE, Wadhams GH, Wadhams LJ, Woodcock CM (2000) New roles for cis-jasmone as an insect semiochemical and in plant defense. Proc Natl Acad Sci USA **97**: 9329-9334

Bohlmann J, Martin D, Oldham Neil J, Gershenzon J (2000) Terpenoid secondary metabolism in Arabidopsis thaliana: cDNA cloning, characterization, and functional expression of a myrcene/(E)-beta-ocimene synthase. Arch Biochem Biophys **375**: 261-269

Bohlmann J, Meyer Gauen G, Croteau R (1998) Plant terpenoid synthases: Molecular biology and phylogenetic analysis. Proc Natl Acad Sci USA **95**: 4126-4133

Bohlmann J, Phillips M, Ramachandiran V, Katoh S, Croteau R (1999) cDNA cloning, characterization, and functional expression of four new monoterpene synthase members of the *Tpsd* gene family from grand fir (*Abies grandis*). Arch Biochem Biophys **368**: 232-243

Bohlmann J, Steele CL, Croteau R (1997) Monoterpene synthases from grand fir (*Abies grandis*). cDNA isolation, characterization, and functional expression of myrcene synthase, (-)-(4*S*)-limonene synthase, and (-)-(1*S*,5*S*)-pinene synthase. J Biol Chem **272**: 21784-21792

Bonner CA, Jensen RA (1994) Cloning of cDNA encoding the bifunctional dehydroquinase.shikimate dehydrogenase of aromatic-amino-acid biosynthesis in *Nicotiana tabacum*. Biochem J **302**: 11-14

Bosabalidis AM (1996) Ontogenesis, ultrastructure and morphometry of the petiole oil ducts of celery (*Apium graveolens* L.). Flavour Frag J **11**: 269-274

Bouvier F, Suire C, d' Harlingue A, Backhaus RA, Camara B (2000) Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plant cells. Plant J **24**: 241-252

Bouwmeester HJ, Gershenzon J, Konings M, Croteau R (1998) Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway. I. Demonstration of enzyme activities and their changes with development. Plant Physiol **117**: 901-912

Bouwmeester HJ, Konings M, Gershenzon J, Karp F, Croteau R (1999a) Cytochrome P-450 dependent (+)-limonene-6-hydroxylation in fruits of caraway (*Carum carvi*). Phytochemistry **50**: 243-248

Bouwmeester HJ, Verstappen FWA, Posthumus MA, Dicke M (1999b) Spider mite-induced (3*S*)-(*E*)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. Plant Physiol **121**: 173-180

Bouwmeester HJ, Wallaart TE, Janssen MH, van Loo B, Jansen BJ, Posthumus MA, Schmidt CO, De Kraker JW, Konig WA, Franssen MC (1999c) Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry **52**: 843-854

Broers STJ (1994) Über die fruhen stufen der biosynthese von isopenoiden in *Escherichia Coli*. PhD thesis. Eidgenössischen Technischen Hochschule, Zürich

Buiatti M, Bogani P (1995) Physiological complexity and plant genetic manipulation. Euphytica 85: 135-147

Burke CC, Wildung MR, Croteau R (1999) Geranyl diphosphate synthase: cloning, expression, and characterization of this prenyltransferase as a heterodimer. Proc Natl Acad Sci USA **96**: 13062-13067

Caccioni DRL, Guizzardi M, Biondi DM, Renda A, Ruberto G (1998) Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. Int J Food Microbiol **43**: 73-79

Cane DE, Du S, Robinson JK, Hsiung Y, Spenser ID (1999) Biosynthesis of Vitamin B6: Enzymatic Conversion of 1-Deoxy-D-xylulose-5-phosphate to Pyridoxol Phosphate. J Am Chem Soc **121**: 7722-7723

Chappell J (2002) The genetics and molecular genetics of terpene and sterol origami. Curr Opin Plant Biol **5**: 151-157

Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu Rev Plant Physiol Plant Mol Biol **49**: 311-343

Chayet L, Rojas C, Cardemil E, Jabalquinto AM, Vicuna R, Cori O (1977) Biosynthesis of monoterpene hydrocarbons from [1-3H]neryl pyrophosphate and [1-3H]geranyl pyrophosphate by soluble enzymes from *Citrus limonum*. Arch Biochem Biophys **180**: 318-327

Chen A, Kroon PA, Poulter CD (1994) Isoprenyl diphosphate synthases: protein sequence comparisons, a phylogenetic tree, and predictions of secondary structure. Protein Sci **3**: 600-607

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4*S*-Limonene synthase from the oil glands of spearmint (*Mentha spicata*): cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. J Biol Chem **268**: 23016-23024

Collu G (1999) Geraniol 10-hydroxylase. A cytochrome P450 enzyme involved in the biosynthesis of terpenoid indole alkaloids in higher plants: an enzymological and molecular study. PhD thesis. University Leiden, Leiden

Croteau R (1987) Biosynthesis and catabolism of monoterpenoids. Chem Rev 87: 929-954

Croteau R, Gershenzon J (1994) Genetic control of monoterpene biosynthesis in mints (*Mentha*: Lamiaceae). Recent Adv Phytochem **28**: 193-229

Crowell PL (1999) Prevention and therapy of cancer by dietary monoterpenes. J Nutr **129**: 775-778

Crowell PL, Gould MN (1994) Chemoprevention and therapy of cancer by d-limonene. Crit Rev Oncogen **5**: 1-22

Cseke L, Dudareva N, Pichersky E (1998) Structure and evolution of linalool synthase. Mol Biol Evol **15**: 1491-1498

de Kraker JW, Franssen MCR, Dalm MCF, de Groot A, Bouwmeester HJ (2001) Biosynthesis of germacrene A carboxylic acid in chicory roots. Demonstration of a cytochrome P450 (+)-germacrene A hydroxylase and NADP(+)-dependent sesquiterpenoid dehydrogenase(s) involved in sesquiterpene lactone biosynthesis. Plant Physiol **125**: 1930-1940

de Kraker JW, Franssen MCR, De Groot A, Koenig WA, Bouwmeester HJ (1998) (+)-Germacrene A biosynthesis: The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. Plant Physiol. **117**: 1381-1392

Dicke M, van Loon JJA (2000) Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomol Exp Appl **97**: 237-249

Diemer F, Caissard Jean C, Moja S, Chalchat Jean C, Jullien F (2001) Altered monoterpene composition in transgenic mint following the introduction of 4*S*-limonene synthase. Plant Physiol Biochem Paris **39**: 603-614

Donaldson RP, Luster DG (1991) Multiple forms of plant cytochromes P-450. Plant Physiol **96**: 669-674

Dudareva N, Cseke L, Blanc VM, Pichersky E (1996) Evolution of floral scent in *Clarkia*: novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. Plant Cell **8**: 1137-1148

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, Bonham C, Wood K (2000) Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant cell **12:** 949-961

Dudareva N, Pichersky E (2000) Biochemical and molecular genetic aspects of floral scents. Plant Physiol **122**: 627-633

Eisenreich W, Rohdich F, Bacher A (2001) Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci **6**: 78-84

Eisenreich W, Sagner S, Zenk MH, Bacher A (1997) Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron Lett **38**: 3889-3892

Espinosa GFJ, Langenheim JH (1991) Effects of sabinene and gamma-terpinene from coastal redwood leaves acting singly or in mixtures on the growth of some of their fungus endophytes. Biochem Syst Ecol **19**:1643-1650

Fahn A (1979) Secretory tissues in plants. Academic Press, London, pp 302

Fischbach RJ, Zimmer W, Schnitzler JP (2001) Isolation and functional analysis of a cDNA encoding a myrcene synthase from holm oak (*Quercus ilex* L.). Eur J Biochem **268**: 5633-5638

Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. Plant J **8**: 693-701

Funk C, Lewinsohn E, Vogel BS, Steele CL, Croteau R (1994) Regulation of oleoresinosis in grand fir (*Abies grandis*). Coordinate induction of monoterpene and diterpene cyclones and two cytochrome P450-dependent diterpenoid hydroxylases by stem wounding. Plant Physiol **106**: 999-1005

Gang DR, Wang J, Dudareva N, Nam KH, Simon JE, Lewinsohn E, Pichersky E (2001) An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. Plant Physiol **125**: 539-555

Gershenzon J, Croteau RB (1993) Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes and diterpenes. *In* TS Moore ed, Lipid metabolism in plants. CRC Press, Boca Raton, pp 340-388

Gershenzon J, Maffei M, Croteau R (1989) Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). Plant Physiol **89**: 1351-1357

Gershenzon J, McConkey ME, Croteau RB (2000) Regulation of monoterpene accumulation in leaves of peppermint. Plant Physiol **122**: 205-213

Goldberg RB (1988) Plants: novel developmental processes. Science **240**: 1460-1467

Guillon J, Rioult JP, Robba M (2000) New synthesis of isopiperitenol, previously isolated from species of *Cymbopogon*. Flavour Frag J **15**: 223-224

Harborne JB (1991) Recent advances in the ecological chemistry of plant terpenoids. *In* JB Harborne, FA Tomas Barberan eds, Ecological chemistry and biochemistry of plant terpenoids. Oxford University Press, Oxford, pp 399-426

Haudenschild C, Croteau R (1998) Molecular engineering of monoterpene production. Genet Eng **20**: 267-280

Hecht S, Eisenreich W, Adam P, Amslinger S, Kis K, Bacher A, Arigoni D, Rohdich F (2001) Studies on the nonmevalonate pathway to terpenes: The role of the GcpE (IspG) protein. Proc Natl Acad Sci USA **98**: 14837-14842

Helsper JPFG, Davies JA, Bouwmeester HJ, Krol AF, van Kampen MH (1998) Circadian rhythmicity in emission of volatile compounds by flowers of *Rosa hybrida* L. cv. Honesty. Planta **207**: 88-95

Helsper JPFG, Davies JA, Verstappen FWA (2001) Analysis of rhythmic emission of volatile compounds of rose flowers. *In* JF Jackson, HF Linskens eds, Analysis of taste and aroma, 1 Ed, Vol 21. Springer, pp 269

Hernandez A, Ruiz MT (1998) An EXCEL template for calculation of enzyme kinetic parameters by non-linear regression. Bioinformatics **14**: 227-228

Herrmann KM, Weaver LM (1999) The Shikimate pathway. Annu Rev Plant Physiol Plant Mol Biol **50**: 473-503

Hill RA (2002) Dictionary of natural products on CD-ROM, version 10:2. Chapman & Hall/CRC

Hill RE, Rowell FJ, Gupta RN, Spenser ID (1972) Biosynthesis of vitamin B6. J Biol Chem **247**: 1869-1882

Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res **16**: 9877

Hohn TM, Ohlrogge JB (1991) Expression of a fungal sesquiterpene cyclase gene in transgenic tobacco. Plant Physiol **97**: 460-462

Hori M (1998) Repellency of rosemary oil against *Myzus persicae* in a laboratory and in a screenhouse. J Chem Ecol **24**: 1425-1432

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227**: 1229-1231

Huang Y, Fang J, Hung C, Wu P, Tsai Y, Huang YB, Fang JY, Hung CH, Wu PC, Tsai YH (1999) Cyclic monoterpene extract from cardamom oil as a skin permeation enhancer for indomethacin: in vitro and in vivo studies. Biol Pharm Bull **22**: 642-646

Iwanami Y, Tateba H, Kodama N, Kishino K (1997) Changes of lemon flavor components in an aqueous solution during UV irradiation. J Agric Food Chem **45**: 463-466

Jacobson M (1982) Plants, insects, and man--their interrelationships. Economic Botany **36**: 346-354

Jia JW, Crock J, Lu S, Croteau R, Chen XY (1999) (3*R*)-linalool synthase from *Artemisia annua* L.: cDNA isolation, characterization, and wound induction. Arch Biochem Biophys **372**: 143-149

Karp F, Croteau R (1988) Role of hydroxylases in monoterpene biosynthesis. *In* P Schreier ed, Bioflavor '87: Analysis, Biochemistry, Biotechnology. Walter de Gruyter, Berlin, pp 173-198

Karp F, Harris JL, Croteau R (1987) Metabolism of monoterpenes: demonstration of the hydroxylation of (+)-sabinene to (+)-*cis*-sabinol by an enzyme preparation from sage (*Salvia officinalis*) leaves. Arch Biochem Biophys **256**: 179-193

Karp F, Mihaliak CA, Harris JL, Croteau R (1990) Monoterpene biosynthesis: Specificity of the hydroxylations of (-)-limonene by enzyme preparations from peppermint (*Mentha x piperita*), spearmint (*Mentha spicata*), and perilla (*Perilla frutescens*) leaves. Arch Biochem Biophys **276**: 219-226

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science **291**: 2141-2144

Kimura M (1983) The neutral theory of molecular evolution. Vol 367. Cambridge Univ. Press, pp 75

Knudsen JT, Tollsten L, Bergstrom LG (1993) Floral scents: A checklist of volatile compounds isolated by head-space techniques. Phytochemistry **33**: 253-280

Kobayashi S, Ding CK, Nakamura Y, Nakajima I (2000) Kiwifruits (*Actinidia deliciosa*) transformed with a *Vitis* stilbene synthase gene produce piceid (resveratrol-glucoside). Plant Cell Rep **19**: 904-910

Kobold U, Vostrowsky O, Bestmann HJ, Kubeczka KH (1987) Strukturaufklärung von C10H14-Dehydroterpenen durch Reaktionsgaschromatographie. Nachweis neuer Terpenkomponenten in etherische Ölen. Liebigs Ann Chem: 557-559

Kolalite MR (1994) Dynamics of ultrastructure in peltate glands *of Nepeta cataria* and *Dracocephalum moldavica* (Lamiaceae) in relation to terpene synthesis. Botanicheskii Zhurnal **79**: 17-27

Kolosova N, Gorenstein N, Kish CM, Dudareva N (2001a) Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. Plant Cell **13**: 2333-2347

Kolosova N, Sherman D, Karlson D, Dudareva N (2001b) Cellular and subcellular localization of *S*-adenosyl-L-methionine: Benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methylbenzoate in snapdragon flowers. Plant Physiol **126**: 956-964

Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB (1990) Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2**: 1201-1224

Krasnyanski S, May RA, Loskutov A, Ball TM, Sink KC (1999) Transformation of the limonene synthase gene into peppermint (*Mentha* x *piperita* L.) and preliminary studies on the essential oil profiles of single transgenic plants. Theor Appl Genet **99**: 676-682

Lange BM, Croteau R (1999) Genetic engineering of essential oil production in mint. Curr Opin Plant Biol **2**: 139-144

Lange MB, Wildung MR, Stauber EJ, Sanchez C, Pouchnik D, Croteau R (2000) Probing essential oil biosynthesis and secretion by functional evaluation of expressed sequence tags from mint glandular trichomes. Proc Natl Acad Sci USA **97**: 2934-2939.

Lavy M, Zuker A, Lewinsohn E, Larkov O, Ravid U, Vainstein A, Weiss D (2002) Linalool and linalool oxide production in transgenic carnation flowers expressing the *Clarkia breweri* linalool synthase gene. Molecular Breeding **9**: 103-111

Lesburg CA, Zhai G, Cane DE, Christianson DW (1997) Crystal structure of pentalenene synthase: Mechanistic insights on terpenoid cyclization reactions in biology. Science **277**: 1820-1824

Lewinsohn E, Gijzen M, Croteau R (1992) Wound-inducible pinene cyclase from grand fir: purification, characterization, and renaturation after SDS-PAGE. Arch Biochem Biophys **293**: 167-173

Lewinsohn E, Gijzen M, Muzika RM, Barton K, Croteau R (1993) Oleoresinosis in grand fir (*Abies grandis*) saplings and mature trees: Modulation of this wound response by light and water stresses. Plant Physiol **101**: 1021-1028

Lewinsohn E, Gijzen M, Savage TJ, Croteau R (1991) Defense mechanisms of conifers: Relationship of monoterpene cyclase activity to anatomical specialization and oleoresin monoterpene content. Plant Physiol **96**: 38-43

Lewinsohn E, Schalechet F, Wilkinson J, Matsui K, Tadmor Y, Nam KH, Amar O, Lastochkin E, Larkov O, Ravid U, Hiatt W, Gepstein S, Pichersky E (2001) Enhanced levels of the aroma and flavor compound *S*-linalool by metabolic engineering of the terpenoid pathway in tomato fruits. Plant Physiol **127**: 1256-1265

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol **50**: 47-65

Little DB, Croteau RB (1999) Biochemistry of essential oil terpenes - A thirty year overview. *In* R Teranishi, EL Wick eds, Flavor chemistry: 30 Years of Progress. Kluwer Academic/ Plenum Publishers, New York, pp 239-253

Lois LM, Campos N, Putra SR, Danielsen K, Rohmer M, Boronat A (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. Proc Natl Acad Sci USA **95**: 2105-2110

Loughrin JH, Hamilton Kemp TR, Andersen RA, Hildebrand DF (1990) Headspace compounds from flowers of *Nicotiana tabacum* and related species. J Agric Food Chem **38**: 455-460

Loughrin JH, Hamilton Kemp TR, Anderson RA, Hildebrand DF (1991) Circadian rhythm of volatile emission from flowers of *Nicotiana sylvestris* and *Nicotiana suaveolens*. Physiol Plant **83**: 492-496

Lücker J, Bouwmeester HJ, Schwab W, Blaas J, van der Plas LHW, Verhoeven HA (2001) Expression of *Clarkia S*-linalool synthase in transgenic petunia plants results in the accumulation of *S*-linalyl-beta-D-glucopyranoside. Plant J **27**: 315-324

Lücker J, El Tamer MK, Schwab W, Verstappen FWA, van der Plas LHW, Bouwmeester HJ, Verhoeven HA (2002) Monoterpene biosynthesis in lemon (*Citrus limon*): cDNA isolation and functional analysis of four monoterpene synthases. Eur J Biochem **269**: 3160-3171

Lupien S, Karp F, Wildung M, Croteau R (1999) Regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha*) species: cDNA isolation, characterization, and functional expression of (-)-4*S*-limonene-3-hydroxylase and (-)-4*S*-limonene-6-hydroxylase. Arch Biochem Biophys **368**: 181-192

Mahmoud SS, Croteau R (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc Natl Acad Sci USA **98**: 8915-8920

Maliga P, Breznovitz A, Marton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nature New Biol **244**: 29-30

Maruyama T, Ito M, Honda G (2001a) Molecular cloning, functional expression and characterization of (E)-beta farnesene synthase from *Citrus junos*. Biol Pharm Bull **24**: 1171-1175

Maruyama T, Ito M, Kiuchi F, Honda G (2001) Molecular cloning, functional expression and characterization of d-limonene synthase from *Schizonepeta tenuifolia*. Biol Pharm Bull **24**: 373-377

McCaskill D, Croteau R (1995) Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha x piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta **197**: 49-56

McCaskill D, Croteau R (1997) Prospects for the bioengineering of isoprenoid biosynthesis. Adv Biochem Eng Biotechnol **55**: 107-146

McCaskill D, Croteau R (1998) Some caveats for bioengineering terpenoid metabolism in plants. Trends Biotechnol **16**: 349-355

McCaskill D, Gershenzon J, Croteau R (1992) Morphology and monoterpene biosynthetic capabilities of secretory cell clusters isolated from glandular trichomes of peppermint (*Mentha piperita* L.). Planta **187**: 445-454

McConkey ME, Gershenzon J, Croteau RB (2000) Developmental regulation of monoterpene biosynthesis in the glandular trichomes of peppermint. Plant Physiol. **122**: 215-223.

Mihaliak CA, Karp F, Croteau R (1993) Cytochrome P-450 Terpene Hydroxylases. *In* PJ Lea ed, Enzymes of Secondary Metabolism, Vol 9. Academic Press, London, pp 261-279

Mills JJ, Chari RS, Boyer IJ, Gould MN, Jirtle RL (1995) Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. Cancer Res **55**: 979-983

Mlynarova L, Jansen RC, Conner AJ, Stiekema WJ, Nap JP (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. Plant Cell **7**: 599-609

Mondello L, Catalfamo M, Cotroneo A, Dugo G, Dugo G, McNair H (1999) Multidimensional capillary GC-GC for the analysis of real complex samples: Part IV. Enantiomeric distribution of monoterpene hydrocarbons and monoterpene alcohols of lemon oils. J High Resoln Chromatogr **22**: 350-356.

Nawrath C, Poirier Y, Somerville C (1994) Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proc Natl Acad Sci USA **91**: 12760-12764

Neirotti E, Moscatelli M, Tiscornia S (1996) Antimicrobial activity of the limonene. Arquivos de Biologia e Tecnologia **39**: 233-237

Njoroge SM, Ukeda H, Sawamura M (1996) Changes in the volatile composition of yuzu (*Citrus junos* Tanaka) cold-pressed oil during storage. J Agr Food Chem **44**: 550-556

Ohnuma S, Koyama T, Ogura K (1993) Alteration of the product specificities of prenyltransferases by metal ions. Biochem Biophys Res Commun **192**: 407-412

Oka N, Ikegami A, Ohki M, Sakata K, Yagi A, Watanabe N (1998) Citronellyl disaccharide glycoside as an aroma precursor from rose flowers. Phytochemistry **47**: 1527-1529

Oka N, Ohishi H, Hatano T, Hornberger M, Sakata K, Watanabe N (1999) Aroma evolution during flower opening in *Rosa damascena* Mill. Z Naturforsch **54**: 889-895

Pare PW, Tumlinson JH (1997) *De novo* biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol **114**: 1161-1167

Park SK, Noble AC (1993) Monoterpenes and monoterpene glycosides in wine aromas. Am Chem Soc **536**: 98-109

Paulsen H, Le-Nguyen B, Sinnwell V, Seehofer F (1985) Synthesis of Glycosides of Mono-, Sesqui- and diterpene alcohols. Liebigs Ann Chem **8**: 1513-1536

Pichersky E, Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr Opin Plant Biol **5**: 237-243

Poulose AJ, Croteau R (1978) Biosynthesis of aromatic monoterpenes. Conversion of gamma -terpinene to *p*-cymene and thymol *in Thymus vulgaris* L. Arch Biochem Biophys **187**: 307-314

Poulter CD, Rilling HC (1981) Prenyl Transferases and Isomerase. *In* JW Porter, SL Spurgeon eds, Biosynthesis of isoprenoid compounds, Vol 1. Wiley, New York, pp. 161

Querol J, Campos N, Imperial S, Boronat A, Rodriguez Concepcion M (2002) Functional analysis of the *Arabidopsis thaliana* GCPE protein involved in plastid isoprenoid biosynthesis. Febs Lett **514**: 343-346

Raguso RA, Pichersky E (1995) Floral volatiles from *Clarkia breweri* and *C. concinna* (Onagraceae): Recent evolution of floral scent and moth pollination. Plant Syst Evol **194**: 55-67

Raguso RA, Pichersky E (1999) A day in the life of a linalool molecule: chemical communication in a plant-pollinator system. Part 1: linalool biosynthesis in flowering plants. Plant Species Biol **14**: 95-120

Rajaonarivony JIM, Gershenzon J, Croteau R (1992) Characterization and mechanism of (4*S*)-limonene synthase, a monoterpene cyclase from the glandular trichomes of peppermint (*Mentha x piperita*). Arch Biochem Biophys **296**: 49-57

Rieder C, Jaun B, Arigoni D (2000) On the early steps of cineol biosynthesis in *Eucalyptus globulus*. Helv Chim Acta **83**: 2504-2513

Rohdich F, Hecht S, Gartner K, Adam P, Krieger C, Amslinger S, Arigoni D, Bacher A, Eisenreich W (2002) Studies on the nonmevalonate terpene biosynthetic pathway: Metabolic role of IspH (LytB) protein. Proc Natl Acad Sci USA **99**: 1158-1163

Rohmer M (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep 16: 565-574

Rohmer M, Knani M H, Simonin P, Sutter B, Sahm H (1993) Isoprenoid biosynthesis in bacteria: A novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J **295**: 517-524

Romagni JG, Allen SN, Dayan FE (2000) Allelopathic effects of volatile cineoles on two weedy plant species. J Chem Ecol **26**: 303-313

Romer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM (2000) Elevation of the provitamin A content of transgenic tomato plants. Nature Biotechnol **18**: 666-669

Rose USR, Manukian A, Heath RR, Tumlinson JH (1996) Volatile semiochemicals released from undamaged cotton leaves. Plant Physiol **111**: 487-495

Rynkiewics MJ, Cane DE, Christianson DW (2001) Structure of trichodiene synthase from *Fusarium sporotrichioides* provides mechanistic inferences on the terpene cyclisation cascade. Proc Natl Acad Sci USA **98**: 13543-13548

Rynkiewicz MJ, Cane DE, Christianson DW (2002) X-ray crystal structures of D100E trichodiene synthase and its pyrophosphate complex reveal the basis for terpene product diversity. Biochemistry-US **41**: 1732-1741

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**: 406-425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, Ed 2nd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Savage TJ, Hatch MW, Croteau R (1994) Monoterpene synthases of *Pinus contorta* and related conifers: A new class of terpenoid cyclase. J Biol Chem **269**: 4012-4020

Schalk M, Croteau R (2000) A single amino acid substitution (F363I) converts the regiochemistry of the spearmint (-)-limonene hydroxylase from a C6- to a C3-hydroxylase. Proc Natl Acad Sci USA **97**: 11948-11953

Schnepf E (1974) Gland cells. *In* AW Roberts ed, Dynamic aspects of plant ultrastructure. McGraw-Hill, NY, pp 331-357

Schuh G, Heiden AC, Hoffmann T, Kahl J, Rockel P, Rudolph J, Wildt J (1997) Emissions of volatile organic compounds from sunflower and beech: Dependence on temperature and light intensity. J Atmos Chem **27**: 291-318

Schuler MA (1996) Plant cytochrome P450 monooxygenases. Crit Rev Plant Sci **15**: 235-284

Schultz DJ, Craig R, Cox-Foster DL, Mumma RO, Medford JI (1994) RNA isolation from recalcitrant plant tissue. Plant Mol Biol Rep **12**: 310-316

Schwarz MK (1994) Terpen-Biosynthese in *Ginkgo biloba*: Eine überraschende Geschichte. PhD thesis. Eidgenössischen Technischen Hochschule, Zürich

Scolnik PA, Bartley GE (1996) A table of some cloned plant genes involved in isoprenoid biosynthesis. Plant Mol Biol Rep **14**: 305-319

Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. Plant J **20**: 401-412

Smith MB, March J (2001) March's Advanced Organic Chemistry: Reactions, Mechanisms and Structure, Ed 5. Wiley Interscience, New York.

Stahl Biskup E, Intert F, Holthuijzen J, Stengele M, Schulz G (1993) Glycosidically bound volatiles - a review 1986-1991. Flav Frag J **8**: 61-80

Starks CM, Back K, Chappell J, Noel JP (1997) Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. Science **277**: 1815-1820

Steele CL, Katoh S, Bohlmann J, Croteau R (1998) Regulation of oleoresinosis in grand fir (*Abies grandis*): Differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in responses to wounding. Plant Physiol **116**: 1497-1504

Suga T, Hirata T (1990) Biotransformation of exogenous substrates by plant cell cultures. Phytochemistry **29**: 2393-2406

Tarshis LC, Yan M, Poulter CD, Sacchettini JC (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-A resolution. Biochemistry-US **33**: 10871-10877

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25**: 4876-4882

Tsao R, Zhou T (2000) Antifungal activity of Monoterpenoids against postharvest pathogens *Botrytis cinerea* and *Monilinia fructicola*. J Essent Oil Res 12: 113-121

Turner G, Gershenzon J, Nielson EE, Froehlich JE, Croteau R (1999) Limonene synthase, the enzyme responsible for monoterpene biosynthesis in peppermint, is localized to leucoplasts of oil gland secretory cells. Plant Physiol **120**: 879-886

Turner GW, Berry AM, Gifford EM (1998) Schizogenous secretory cavities of *Citrus limon* (L.) Burm. f. and a re-evaluation of the lysigenous gland concept. Int J Plant Sci **159**: 75-88

Vainstein A, Lewinsohn E, Pichersky E, Weiss D (2001) Floral fragrance. New inroads into an old commodity. Plant Physiol **127**: 1383-1389

van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A, Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res **4**: 288-290

van Leeuwen W, Hagendoorn MJM, Ruttink T, van Poecke RMP, van der Plas LHW, van der Krol AR (2000) The use of the luciferase reporter system for in planta gene expression studies. Plant Mol Biol Rep **18**: 143a -143t

van Leeuwen W, Mlynarova L, Nap JP, van der Plas LHW, van der Krol AR (2001) The effect of MAR elements on variation in spatial and temporal regulation of transgene expression. Plant Mol Biol **47**: 543-554

van Poecke RMP, Posthumus MA, Dicke M (2001) Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioral, and gene-expression analysis. J Chem Ecol **27**: 1911-1928

van Tunen AJ, Mur LA, Brouns GS, Rienstra JD, Koes RE, Mol JN (1990) Pollen- and anther-specific *chi* promoters from petunia: tandem promoter regulation of the *chiA* gene. Plant Cell **2**: 393-401

Vaughn SF, Spencer GF (1991) Volatile monoterpenes inhibit potato tuber sprouting. Am Potato J **68**: 821-831

Vaughn SF, Spencer GF (1993) Volatile monoterpenes as potential parent structures for new herbicides. Weed Sci **41**: 114-119

Verhoeven H, Beuerle T, Schwab W (1997) Solid-phase microextraction: Artefact formation and its avoidance. Chromatographia **46**: 63-66

Verlet N (1993) Commercial aspects. *In* RKM Hay, PG Waterman eds, Volatile oil crops: their biology, biochemistry, and production. Longman Scientific & Technical, Essex, UK, pp 137-174

Verpoorte R, Memelink J (2002) Engineering secondary metabolite production in plants. Curr Opin Biotech **13**: 181-187

Verwoerd TC, Dekker BM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucl Acids Res **17**: 2362

Von Wachenfeldt C, Johnson EF (1995) Structures of eukaryotic cytochrome P450 enzymes. *In* PR Ortiz de Montellano ed, Cytochrome P450: structure, mechanism, and biochemistry, Vol 2. Plenum Press, New York.

Wagschal K, Savage TJ, Croteau R (1991) Isotopically sensitive branching as a tool for evaluating multiple product formation by monoterpene cyclases. Tetrahedron **47**: 5933-5944

Wallaart TE, Bouwmeester HJ, Hille J, Poppinga L, Maijers NCA (2001) Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. Planta **212**: 460-465

Wang EM, Wang R, De Parasis J, Loughrin JH, Gan SS, Wagner GJ (2001) Suppression of a P450 hydroxylase gene in plant trichome glands enhances natural-product-based aphid resistance. Nature Biotechnol **19**: 371-374

Watanabe N, Nakajima R, Watanabe S, Moon JH, Inagaki J, Sakata K, Yagi A, Ina K (1994) Linalyl and bornyl disaccharide glycosides from *Gardenia jasminoides* flowers. Phytochemistry **37**: 457-459

Weidenhamer JD, Macias FA, Fischer NH, Williamson GB (1993) Just how insoluble are monoterpenes? J Chem Ecol 19: 1799-1807

Weiss EA (1997) Essential oil crops. Cab international, Wallingford, pp 600

Weissbecker B, Schutz S, Klein A, Hummel HE (1997) Analysis of volatiles emitted by potato plants by means of a Colorado beetle electroantennographic detector. Talanta **44**: 2217-2224

Werkhoff P, Guentert M, Krammer G, Sommer H, Kaulen J (1998) Vacuum headspace method in aroma research: Flavor chemistry of yellow passion fruits. J Agric Food Chem **46**: 1076-1093

White RH (1978) Stable isotope studies on the biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*. Biochemistry **17**: 3833-3840

Williams DC, McGarvey DJ, Katahira EJ, Croteau R (1998) Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochemistry **37**: 12213-12220

Wink M (1999) Functions of plant secondary metabolites and their exploitation in biotechnology, Annual Plant Reviews, Vol 3. Sheffield Academic Press, Sheffield, pp 362

Wise ML, Savage TJ, Katahira E, Croteau R (1998) Monoterpene synthases from common sage (*Salvia officinalis*): cDNA isolation, characterization, and functional expression of (+)-sabinene synthase, 1,8-cineole synthase, and (+)-bornyl diphosphate synthase. J Biol Chem **273**: 14891-14899

Wust M, Croteau RB (2002) Hydroxylation of specifically deuterated limonene enantiomers by cytochrome P450 limonene-6-hydroxylase reveals the mechanism of multiple product formation. Biochemistry **41**: 1820-1827

Wust M, Little DB, Schalk M, Croteau R (2001) Hydroxylation of limonene enantiomers and analogs by recombinant (-)-limonene 3- and 6-hydroxylases from mint (*Mentha*) species: Evidence for catalysis within sterically constrained active sites. Arch. Biochem Biophys **387**: 125-136

Yamazaki S, Sato K, Suhara K, Sakaguchi M, Mihara K, Omura T (1993) Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. J Biochem-Tokyo **114**: 652-657

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science **287**: 303-305

Yu SG, Hildebrandt LA, Elson CE (1995) Geraniol, an inhibitor of mevalonate biosynthesis, suppresses the growth of hepatomas and melanomas transplanted to rats and mice. J Nutr **125**: 2763-2767

Summary

Monoterpenes are a large group of compounds that belong to the terpenoid family of natural compounds in plants. They are small, volatile, lipophilic substances of which around one thousand different structures have been identified. Monoterpenes are involved in plant-insect, plant-microorganism and plant-plant interactions. Many monoterpenes, such as menthol, carvone, limonene and linalool, are of commercial interest as they are commonly used in foods, beverages, perfumes and cosmetics and in many cleaning products. In flowers they also contribute to the characteristic scent. Monoterpene synthases and subsequent modifying enzymes such as cytochrome P450 hydroxylases, dehydrogenases, reductases and isomerases are responsible for the production of the variety of different carbon skeletons of monoterpenes that are found in nature. In this thesis the use of genetic engineering to introduce or alter the production of monoterpenes by plants was explored.

Initially, as described in Chapter 2, *S*-linalool synthase from *Clarkia breweri* was introduced in Petunia plants regulated by a constitutive promoter. Expression was obtained in all tissues analysed, but formation of linalool was restricted to leaves, sepals, corollas, stems and ovaries, and could not be detected in nectaries, roots, pollen and style. Although it was expected that the formation of linalool would result in an alteration of the scent of the plants, no linalool was detected in the headspace. Instead, all the *S*-linalool produced was efficiently converted by an endogenous glucosyltransferase present in the petunia tissues to the non-volatile *S*-linalyl-β-D-glucopyranoside. These results showed that genetic engineering of plants for monoterpene biosynthesis is possible, but that it can lead to unexpected conversions of the produced metabolites by endogenous enzyme activities.

In order to obtain new monoterpene synthases for the genetic engineering of plants, a cDNA library was made of the fruit peel of lemon, a plant species producing many different monoterpenes. From this library four different monoterpene synthases were obtained as described in Chapter 3, which together showed to be responsible for more than 90% of the total number of components present in lemon oil. The product specificity of the enzymes could be analysed after heterologous expression in *Escherichia coli*. Two of the four cDNA-encoded enzymes were producing (+)-limonene, the main component present in lemon. One cDNA-encoded enzyme was mainly producing (-)-β-pinene and the fourth cDNA-encoded

enzyme was mainly producing γ-terpinene. The latter two enzymes were both producing traces of multiple side products as well. Also other properties of the heterologously expressed enzymes were determined, which are described in Chapter 3.
Three monoterpene synthases responsible for the production of different main products were chosen for the genetic engineering of *Nicotiana tabacum* 'Petit Havana' SR1, described in Chapter 4. The wild type of this tobacco variety produces one monoterpene, linalool that is only emitted from the flowers. After the transformation with the three monoterpene synthases and subsequent crossings, a plant was obtained that emitted all the three main products of the three introduced monoterpene synthases in addition to the endogenous linalool in the flowers. The levels of limonene, β-pinene and γ-terpinene emitted from the leaves and flowers of the plant were higher than the level of the endogenous monoterpene. Also the side products of the monoterpene synthases were detected. The extensive modification of the volatile profile of the tobacco plants that we obtained indicates that there is a sufficient amount of substrate available to the introduced enzymes.

In Chapter 5 the transgenic tobacco plant emitting the products of three monoterpene synthases, was used in a subsequent transformation experiment in order to modify the already introduced pathway. A second step in the pathway was introduced by transformation of the plant material with a limonene-3-hydroxylase isolated from spearmint, which is supposed to be localised in the endoplasmatic reticulum (ER) in the cytosol of the plant cells, while the primarily introduced monoterpene synthases were most likely localised in the plastids in the transgenic plants. The introduction of the cytochrome P450 monoterpene hydroxylase and the resulting formation of the hydroxylated product of (+)-limonene, (+)-*trans*-isopiperitenol demonstrates that there is intracellular trafficking of limonene from the plastids to the ER in the cytosol. That this trafficking mechanism would be present in plants normally producing these hydroxylated monoterpenes could be expected, but that it is apparently also present in plants not specialised for the production of these compounds is an exciting discovery. Apart from the production and subsequent emission of high levels of (+)-*trans*-isopiperitenol in the headspace of the leaves and flowers, also the further oxidised conversion product isopiperitenone was detected. In addition, an increase in the *p*-cymene level and the formation of the new products 1,3,8-*p*-menthatriene and 1,5,8-*p*-menthatriene were detected. The occurrence of these latter two products and the increase of the *p*-cymene level could be a consequence of the metabolic engineering of the biosynthetic route into a cell compartment not adapted to the production of these compounds. Endogenous enzymes and pH differences were suggested to be the main cause the formation of these products.

Chapter 6 discusses the various strategies followed for the metabolic engineering of monoterpene biosynthesis in this thesis and by other groups. Functional implications are

discussed such as ecological and physiological consequences of the new metabolites for the transgenic plants. The commercial aspects and interesting opportunities for further research are also discussed.

Samenvatting

Monoterpenen zijn geur en smaakstoffen die behoren tot de grote terpenoid familie van natuurlijke componenten die voorkomen in planten. Het zijn kleine, vluchtige en vetoplosbare stoffen waarvan ongeveer 1000 verschillende structuren bekend zijn. Monoterpenen zijn betrokken bij de interactie tussen planten en insecten, planten en micro-organismen en tussen planten onderling. Vele monoterpenen, zoals bijvoorbeeld menthol, limoneen en linalool zijn tevens van commercieel belang omdat ze veel toegepast worden in voedsel en dranken, parfums, cosmetica maar ook in veel schoonmaakmiddelen. In bloemen dragen ze bij aan de karakteristieke geur, die bijvoorbeeld van belang is in snijbloemen, maar waaruit deze nagenoeg verdwenen is. Monoterpeensynthases en monoterpeen modificerende enzymen zoals cytochroom P450 hydroxylases, dehydrogenases, reductases en isomerases zijn verantwoordelijk voor de totstandkoming van de verscheidenheid aan verschillende structuren van monoterpenen die voorkomen in de natuur. In dit proefschrift is gebruik gemaakt van genetische modificatie om ervoor te zorgen dat er een biosyntheseroute voor monoterpenen wordt aangelegd in planten die geen monoterpenen maken of om monoterpenen die al in planten gemaakt worden aan te vullen met nieuwe monoterpenen, die normaal niet in die plant voorkomen. Verder is het ook nog gelukt om, met behulp van eenzelfde werkwijze, één van de geïntroduceerde geurstoffen te veranderen in een geurstof met weer een nieuwe chemische structuur.

In eerste instantie, zoals beschreven is in Hoofdstuk 2, werden Petunia's getransformeerd met een *S*-linalool synthase van *Clarkia breweri* onder de controle van een constitutieve promoter. Expressie van het geïntroduceerde cDNA werd gevonden in alle weefsels die geanalyseerd werden. De vorming van linalool kon echter alleen gedetecteerd worden in bladeren, kelkbladeren, bloembladen, stengels en in ovaria. In de overige geanalyseerde weefsels (nectariën, wortels, stuifmeel en stijl) werd geen linalool gedetecteerd. Alhoewel er verwacht werd dat de ingebrachte vluchtige stof linalool een verandering in het geurstofpatroon van de planten teweeg zou brengen, werd er geen vrij linalool gevonden dat door de plant werd afgegeven. Chemisch onderzoek toonde aan dat al het geproduceerde *S*-linalool met hoge efficiëntie werd omgezet door een endogeen glucosyltransferase in de niet vluchtige stof *S*-linalyl-β-D-glucopyranoside. Deze resultaten laten zien dat het mogelijk is om een biosyntheseroute voor monoterpeen biosynthese aan te brengen in planten maar dat er ook onverwachte verdere omzettingen kunnen optreden van de ingebrachte stoffen door endogene enzymatische activiteiten.

Voor het verkrijgen van nieuwe monoterpeensynthases die gebruikt konden worden voor het genetisch modificeren van planten, werd er een cDNA bank gemaakt van de schil van citroenvruchten. Citroen bevat erg veel monoterpenen. In de cDNA bank werden vier verschillende monoterpeensynthases aangetroffen, die beschreven zijn in Hoofdstuk 3. Deze vier monoterpeensynthases bleken samen verantwoordelijk te zijn voor meer dan 90% van alle componenten die normaliter aangetroffen worden in de essentiële olie van citroen. De productspecificiteit van deze vier enzymen kon worden opgehelderd na heterologe expressie van de cDNAs in *Escherichia coli*. Twee van de vier cDNAs bleken te coderen voor enzymen die als hoofdproduct (+)-limoneen maken, de voornaamste component in citroen. Een ander cDNA codeert voor een enzym dat vooral (-)-β-pineen maakt. Het vierde cDNA codeert voor een enzym dat γ-terpineen maakt als hoofdcomponent. Deze laatste twee enzymen produceerden verder nog kleine hoeveelheden van diverse nevenproducten. Verder werden ook nog andere eigenschappen bepaald van de vier verschillende monoterpeensynthases, die worden beschreven in Hoofdstuk 3.

Drie monoterpeensynthases die alle drie verschillende eindproducten maken werden uitgekozen om de monoterpeenbiosyntheseroute in tabaksplanten te veranderen, zoals beschreven in Hoofdstuk 4. Bloemen van de gebruikte tabaksvariëteit *Nicotiana tabacum* 'Petit Havana' SR1 produceren slechts het monoterpeen linalool. Na transformatie met de drie verschillende monoterpeensynthases en twee opeenvolgende kruisingen werd een planttype verkregen dat alle drie de hoofdproducten van de drie monoterpeensynthases produceerde en afgaf in bladeren en in bloemen. Dit leidde niet of nauwelijks tot een verandering van de emissie van het endogeen geproduceerde linalool door de tabaksbloemen. De hoeveelheden limoneen, β-pineen en γ-terpineen die werden afgegeven vanuit de plantenweefsels waren hoger dan de hoeveelheid afgegeven plant-eigen linalool. Ook emissie van de nevenproducten van de monoterpeensynthases kon gedetecteerd worden. De verkregen uitgebreide verandering van het geurstofprofiel van de tabaksplanten laat zien dat er kennelijk voldoende substraat voor handen is in de planten voor de geïntroduceerde enzymen.

In Hoofdstuk 5 werd de transgene tabaksplant die de eindproducten van drie monoterpeensynthases tot emissie bracht gebruikt als uitgangsmateriaal voor een volgend transformatie-experiment om een nieuwe stap in de biosyntheseroute te introduceren. De monoterpeensynthases zijn enzymen die alle drie naar de plastiden in de plantencel getransporteerd worden waar ze actief zijn. De tweede stap die geïntroduceerd werd in de biosyntheseroute is actief in het endoplasmatisch reticulum (ER) in het cytosol van de cellen. Deze stap in de biosyntheseroute werd ingebracht door de plant die de drie monoterpeensynthases al bevatte, te transformeren met een limoneen-3-hydroxylase enzym dat uit kruizemunt (spearmint) verkregen was. Het introduceren van dit cytochroom P450

monoterpeenhydroxylase en de vorming van het hydroxylatieproduct van (+)-limoneen, namelijk (+)-*trans*-isopiperitenol, liet zien dat er een intracellulair transportmechanisme aanwezig is dat het limoneen geproduceerd in de plastiden, vervoert naar het ER in het cytosol. Het is te verwachten dat een dergelijk transportmechanisme aanwezig is in planten die normaal gesproken ook dit soort stoffen maken, maar dat dit kennelijk ook aanwezig is in een plant waarin dit soort stoffen normaal niet voorkomt is opmerkelijk. Behalve de productie en emissie van grote hoeveelheden (+)-*trans*-isopiperitenol uit bloemen en bladeren werd ook het verder geoxideerde product isopiperitenon aangetroffen. Ook werd er een sterke verhoging van de hoeveelheid *p*-cymeen gevonden en de nieuwe stoffen 1,3,8-*p*-menthatrieen en 1,5,8-*p*-menthatrieen. Het voorkomen van deze laatste twee stoffen en de verhoging van het niveau van *p*-cymeen zou wel eens een gevolg kunnen zijn van het verplaatsen van de metabolische route naar een celcompartiment dat niet aangepast is aan het produceren van deze stoffen. Waarschijnlijk hebben endogene enzymen en/ of de pH de vorming van deze nieuwe, onverwachte producten veroorzaakt.

In Hoofdstuk 6 worden de verschillende werkwijzen besproken voor het veranderen van de metabolische route voor monoterpeenbiosynthese die ondernomen zijn in dit proefschrift, en ook door andere groepen. Verder wordt er gediscussieerd over de functionele implicaties, met name de ecologische en fysiologische effecten van de nieuw ingebrachte stoffen in de transgene planten. Tenslotte worden de commerciële aspecten en interessante mogelijkheden voor vervolgonderzoek besproken.

Nawoord

Na heel wat uurtjes zwoegen is het dan toch eindelijk tijd om een bedankje naar voren te brengen aan een aantal mensen.
Op de afdeling Celcybernetica, die eerder Celbiologie heette heb ik een leuke tijd gehad. Daarom wil ik iedereen bedanken voor de gezelligheid en vriendelijkheid op het lab, de kamer en tijdens de lunch. Maar natuurlijk zorgden ook de koffiepauzes, de borrels en de afdelingsuitjes voor de nodige afleiding. Nuttige tips en motiverende gesprekken zijn altijd een goede drijfveer om er weer een schepje boven op te doen. En uiteraard zijn er meerdere mensen van de afdeling die naast mijn twee copromotoren een steentje bijgedragen hebben aan het tot stand komen van dit werk.
Maar voordat ik van start ga met alle mensen van PRI op te noemen wil ik allereerst mijn promotor Linus van der Plas van de vakgroep Plantenfysiologie hartelijk bedanken. Linus, je motiveerde mij altijd heel erg goed door alles in een positief daglicht te zetten. De laatste maanden van het schrijven van dit proefschrift hebben we erg veel contact gehad en heb je je enorm ingezet om te zorgen dat ik alles af kreeg. Volgens mij moet je nu onderhand een beregoede conditie hebben van al die kilometers die je steeds voor mij op en neer moest fietsen tussen Plantenfysiologie en PRI. Al schoot het soms totaal niet op met schrijven dan nog kon je me toch weer goed op de rails krijgen.
Mijn copromotoren Harrie en Harro wil ik heel erg bedanken voor al hun harde werk om ervoor te zorgen dat ik een beetje in het gareel gehouden werd en me bleef focussen op de belangrijkste punten. Er zijn natuurlijk altijd nog veel meer leuke dingen die je wilt doen maar dat kan gewoon niet allemaal in een AIO periode. Verder hebben jullie beiden heel veel frustraties moeten doorstaan met het steeds maar weer wachten op de steeds weer nieuwe versies van de verschillende hoofdstukken en met het nakijken, verbeteren en omvormen van al mijn soms niet altijd even zinnige teksten. Gelukkig wisten jullie altijd wel weer de juiste dingen eruit te filteren.Verder wil ik jullie nog bedanken voor jullie adviezen wat betreft proefopzet en werkwijze tijdens het dagelijkse werk op het lab. Harro wil ik verder nog bedanken voor het organiseren van het terpenen-clubje waardoor ik erg veel kon leren over de andere kanten van het terpenen-werk en niet alleen beperkt bleef tot het moleculaire vlak. Verder was natuurlijk ook de jaarlijkse barbecue bij jou thuis erg gezellig.
Door het proefschrift bladerend zie ik in hoofdstuk 2 de getransformeerde petunia’s staan. Dan denk ik natuurlijk meteen aan John Franken. John, ik wil je bedanken voor je vrijwillige begeleiding met het transformeren van petunia, de eerste plant die ik ooit heb getransformeerd. Alleen jammer dat we in eerste instantie het verkeerde uitgangszaad gebruikt hadden. Maar dat werd wel duidelijk toen we zagen dat de altijd witte W115 ineens paarse bloemen bleek te hebben.

Arjen van Tunen wil ik bedanken voor de begeleiding in mijn eerste AIO jaar, waarin toch zo'n beetje het AIO project dat je gaat volgen uitgestippeld wordt. Jammer dat je ophield mijn begeleider te zijn want ik vond je begeleiding erg prettig.
Jan Blaas was altijd erg geïnteresseerd en enthousiast en heeft een hoop SPME-metingen gedaan. Verder heeft hij mij ook wel eens geholpen met het verzamelen van een heleboel petuniaweefsel wat ook een hele klus was.
Richard Immink wil ik bedanken voor zijn adviezen wat betreft de opmaak van dit proefschrift en voor de gezellige gesprekken die we af en toe hadden op de gang.
I would like to thank Mazen for being a good friend, his neverending will to help whenever I was very busy again in the lab, and for the amusing conversations we had.
Many thanks to Willi Schwab for all the measurements on the MD-GCMS and the LC-MS-MS. We should go to the Indian restaurant again!
Asaph, thank you so much for your help in the lab, your helpful advices and scientific discussions and it was a very nice pleasure to have you as a room and labmate and as a friend for such a long time. And I hope we will work together again some time.
Also a thanks to Rajesh for his practical help, and the nice Indian food his wife and him cook.
I would like to thank Cinzia for her kindness helping me out with all my questions about the P450s and for helping me with the silly mint plants.

Ik wil Ric de Vos bedanken voor zijn adviezen over hoe je nu het beste die SPME metingen kon doen en wat je nu wel en niet mocht zeggen over die resultaten en voor de discussies over glycosiden.
Dank aan Arnoud voor zijn hulp met de microtom transformatie en talloze adviezen en voor de vectoren pFLAP en pRSET.
Ann, Anja en Iris voor het vervolgwerk dat er mee gedaan is. Gelukkig is het dan toch nog ergens goed voor geweest dat we die planten getransformeerd hebben!
Ingrid, Oscar en Twan wil ik bedanken voor hun praktische adviezen en tips, ik vond het fijn dat jullie altijd tijd voor me hadden als ik weer eens met vragen kwam.
Andries wil ik bedanken voor de koelkast die Asaph en ik mochten bestellen van jou.
Willem Jan de Kogel voor zijn talloze testen met de luisjes, alleen mocht het steeds niet lukken door die lastige kleverige tabaksplanten.
Verder wil ik graag Marloes bedanken voor haar hulp met de synthese van isopiperitenol.
Francel, heel erg bedankt voor je enorme geduld om mij alles uit te leggen over de GCMS-analyse en hoe je enzymassays uitvoert.
Ik wil graag Margo bedanken voor haar geïnteresseerdheid en de hulp met de eiwitgels en de gezelligheid op en ook buiten het lab.
Bert wil ik bedanken voor zijn hulp met de HPLC-zuivering en ook Tjitske voor haar hulp op het lab.

Maurice Franssen ben ik dank verschuldigd voor zijn hulp met de organische kant van de terpenen waar ik soms een beetje te weinig van onthouden bleek te hebben tijdens bio-organische chemie. Een mij wel bekende uitspraak: ‘Je hebt toch wel hier op de universiteit gezeten?’
Ik wil graag de kasmedewerkers bedanken voor het onderhouden van mijn planten, anders hadden ze het nooit overleefd aangezien ik altijd water vergeet te geven aan planten.
Willem Brandenburg voor de botanische vragen die toch af en toe opdoken. En verder voor het opzetten van het MOOG project waar we toch best wel leuke 3-dimensionale eiwitjes konden zien.
Adele van Houwelingen wil ik bedanken voor haar gezelligheid als kamergenoot en tijdens ons gezamelijke congres in Oxford.
Robert Hall, Dirk Bosch en Raoul Bino bedankt voor jullie geïnteresseerdheid in het verloop van mijn AIO project.
Verder heb ik de eer gehad maar liefst vier studenten te mogen begeleiden. Allereerst Bianca die mij heeft geholpen met de Northernblots waarvan er enkele in hoofdstuk vier gebruikt zijn. Vervolgens hebben Helen, Jolanda en Bert mij geholpen met het onderzoek tijdens hun viermaandse stageopdracht. Hiervoor wil ik jullie graag bedanken.
Ook mijn afdelingsgenootjes c.q. Bunkermaatjes ben ik erg dankbaar dat ze toch altijd een bordje eten voor me over hielden als ik weer eens na 22.00 uur thuis kwam.
Ik ben tevens grote dank verschuldigd aan Machiel, Celesta, Johannes, Antine, mijn ouders, oma en Nienke voor hun hulp en steun.
Last but not least wil ik de Windgod bedanken voor het verschaffen van de nodige surfdagen en mijn surfvrienden voor de nodige afleiding en gezelligheid op en om het water!

Het is mooi geweest,

` ,,, /

Joost

Curriculum vitae

Joost Lücker werd geboren op 30 april 1973 te Maasbree. Zijn VWO diploma behaalde hij in 1991 aan het Collegium Marianum te Venlo. In september 1991 begon hij de studie Plantenveredeling en Gewasbescherming op de Landbouw Universiteit Wageningen. Als afstudeerrichting koos hij voor de specialisatie moleculaire biologie. Van september 1995 tot half december 1995 deed hij zijn eerste onderzoekservaring op tijdens zijn praktijktijd in de School of Botany van de University of Melbourne in Australië. Hier werkte hij aan het zelfincompatibiliteitsmechanisme van wilde tabaksplanten. Vervolgens voerde hij in 1996 doctoraalonderzoek uit in Nederland op de Landbouw Universiteit Wageningen bij de vakgroep Plantenveredeling. Hier werkte hij gedurende 8 maanden aan genen die betrokken zijn bij het knolvormingsproces in aardappelplanten. Hij rondde zijn studie af in januari 1997. Aansluitend werd hij aangesteld als Assistent In Opleiding voor de vakgroep Plantenfysiologie van de Landbouw Universiteit Wageningen. Het promotieonderzoek werd uitgevoerd op het CPRO-DLO, nu Plant Research International waar hij gedetacheerd was. Vanaf juli 2002 is hij tijdelijk werkzaam op het RIKILT-DLO in Wageningen, en vanaf november 2002 zal hij werkzaam zijn als postdoctoraal onderzoeker bij de groep van Joerg Bohlmann in het Biotechnology Laboratory van de University of Britisch Columbia, te Vancouver, Canada.

Publications

Lücker J, Bouwmeester HJ, Schwab W, Blaas J, van der Plas LHW & Verhoeven HA (2001) Expression of *Clarkia S*-linalool synthase in transgenic petunia plants results in the accumulation of *S*-linalyl-β-D-glucopyranoside.The Plant Journal **27** (4), 315-324

Lücker J, El Tamer MK, Schwab W, Verstappen FWA, van der Plas LHW, Bouwmeester HJ & Verhoeven HA (2002) Monoterpene biosynthesis in lemon (*Citrus limon*): cDNA isolation and functional analysis of four monoterpene synthases. The European Journal of Biochemistry **269**, 3160-3171

Bachem C, Van Der Hoeven R, Lücker J, *et al.* (2000) Functional genomic analysis of potato tuber lifecycle. Potato Research **43** (4): 297-312