Molecular Regulation of Plant Monoterpene Biosynthesis In Relation To Fragrance

Mazen K. El Tamer

Promotor:

Prof. Dr. A.G.J Voragen, hoogleraar in de Levensmiddelenchemie, Wageningen Universiteit

Co-promotoren:

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

Dr. ir. J.P Roozen, departement Agrotechnologie en Voedingswetenschappen, Wageningen Universiteit

Promotiecommissie:

Dr. M.C.R Franssen, Wageningen Universiteit

Prof. Dr. J.H.A Kroeze, Wageningen Universiteit

Prof. Dr. A.J van Tunen, Swammerdam Institute for Life Sciences, Universiteit van Amsterdam.

Prof. Dr. R.G.F Visser, Wageningen Universiteit

Mazen K. El Tamer

Molecular Regulation Of Plant Monoterpene Biosynthesis In Relation To Fragrance

Proefschrift

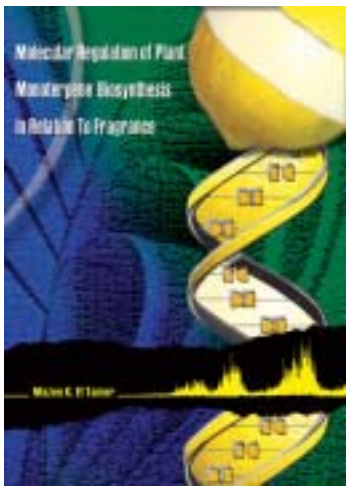
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 27 november 2002
des namiddags te vier uur in de Aula

Mazen K. El Tamer

Molecular Regulation Of Plant Monoterpene Biosynthesis In Relation To Fragrance

Proefschrift Wageningen Universiteit

ISBN 90-5808-752-2



Cover and Invitation

Design: Zeina K. El Tamer

This thesis is dedicated to my Family & Friends

Contents

Abbreviations

ANOVA: analysis of Variance
ATP: adenosine triphosphate
B93: *Citrus limon* γ-terpinene synthase
BAP: 6-benzylaminopurine
BAP-rib: 6-benzylaminopurine riboside
BSA: bovine serum albumin
C62: *Citrus limon* (+)-limonene synthase 1
CIAP: calf intestinal alkaline phosphatase
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
D85: *Citrus limon* (-)-β-pinene synthase
DMAPP: dimethylallyldiphosphate
e.e.: enantiomeric excess ($|\%\ R - \%\ S|$)
EST: expressed sequence tag
FDP: Farnesyl diphosphate
FID: flame ionisation detector
GA_3: gibberellic acid
GC-MS: gas chromatography-mass spectrometry
GDP: geranyl diphosphate
HVS: *Hyoscyamus muticus* vetispiradene synthase
IAA: 3-indoleacetic acid;
IBA: indole-3-butyric acid
i.d: internal diameter
IPP: isopentenyl diphosphate
IPTG: isopropyl-1-thio-ß-D-galactopyranoside
LPP: linalyl diphosphate
M34: *Citrus limon* (+)-limonene synthase 2
MDGC-MS: Multidimensional tandem Gas chromatography-mass spectrometry
NAA: 1-naphtaleneacetic acid
$NADPH_2$: nicotinamide adenine dinucleotide phosphate, reduced form
PG: propylene glycol
TEAS: *Nicotiana tabacum* 5-epi-aristolochene synthase
TIC: total ion count
Tps: plant terpene synthase

Chapter 1

General Introduction

Plant secondary metabolites and their biological functions

Plants have the capacity to synthesize a plethora of low molecular weight compounds that have no obvious role in growth and development and are, therefore, called secondary metabolites. Several tens of thousands of secondary metabolites have already been isolated and their structures determined (Hill, 2002). The number of basic biosynthetic pathways, though, is restricted and distinct. Based on tracer experiments, it is hypothesized that precursors usually derive from basic metabolic pathways, such as glycolysis, Krebs cycle or the shikimate pathway (Bell & Charlwood, 1980; Conn, 1981; Mothes et al., 1985; Luckner, 1990; Dey & Harborne, 1997). There is substantial evidence that most biosynthetic enzymes are quite specific, with regard to substrate and product and have evolved specifically to carry out this task, although they often derive from common progenitors with a function in primary metabolism (Wink, 1999). Some secondary metabolites are produced in many different plant tissues but, in general, their formation is organ-, tissue-, cell- and development- specific. Storage of secondary metabolites - if it occurs- can also be tissue- and cell-specific (Guern et al., 1987).

Cellular biosynthesis

The biosynthesis of secondary metabolites occurs in specific cell compartments. For example, many biosynthetic pathways proceed in the cytoplasm but others, such as the pathway for monoterpenes, occurs in the plastids (Roberts et al., 1981; Wink and Hartman, 1982, Turner et al., 1999; Bouvier et al., 2000). After formation, water soluble compounds are usually stored in the vacuole (Matile, 1978; Boller & Wiemken, 1986) whereas lipophylic substances are sequestered in resin ducts, glandular hairs, trichomes, thylakoid membranes or cuticles (Wiermann, 1981; Wink, 1993; Wink and Roberts, 1998).

Secondary metabolites may also be directly emitted, for example, by flowers (Helsper et al., 1998; Dudareva & Picherky, 2000). Profiles of closely related plants quite often differ substantially, those of unrelated plant groups often show strong similarities. This clearly shows that secondary metabolite patterns are not unambiguous systematic markers but that evolution and selective gene expression are common themes (Wink, 1999). Additional evidence can be derived from the fact that the secondary metabolites do indeed have common functions that are vital for the fitness of a plant producing them. One of the functions is defense against herbivores, pathogens and other plants competing for resources (Vaughn and Spencer, 1993). Secondary metabolites also serve for protection against UV-light or other physical stress (Bieza & Lois, 2001). In addition, they may serve as signal compounds to

attract pollinators and seed dispersing animals or even communicate with other plants and symbiotic micro-organisms (Buee et al., 2000). Plants usually produce a complex mixture of compounds, each of which has its own set of biological activities, which make these mixtures even more powerful for defense, protection and/or communication. In an evolutionary logic, most wild plants were then able to withstand various threats from herbivores, microbes, the physical environment and to communicate signals in an effective manner (van der Fits, 2000).

Plants provide a wide range of secondary metabolites and part of these substances are even biologically active in humans. This is the reason why so many natural products can be used in so many applications, such as the food industry, agriculture, medicine, cosmetics and personal care.

Terpenes

Introduction

The largest class of plant secondary metabolites is that of the terpenoids (or terpenes). Over 36,000 individual structures of this class have been reported (Hill, 2002). The structure of terpenes is extremely variable, exhibiting hundreds of different carbon skeletons. However, this wide structural diversity has a common feature of biosynthesis: All terpenes are derived from the simple process of assembly of 5-carbon atom isoprene units. The categories of terpene compounds are those made up with one (C_5 hemiterpenes) two isoprene units (C_{10} monoterpenes), three isoprene units (C_{15} sesquiterpenes), four isoprene units (C_{20} diterpenes), six isoprene units (C_{30} triterpenes), eight isoprene units (C_{40} tetraterpenes) or even more ($>C_{40}$ polyterpenes) (Chappell, 1995; McGarvey & Croteau, 1995). These categories and their respective biosynthesis within the plant cell are shown in figure 1. Although few terpenoid-derived hormones such as gibberellins (Hedden & Kamiya, 1997) and abscissic acid (Schwartz et al., 1997) have well- established roles in plant development processes, most terpenoids don't have a direct role in such primary plant processes but are thought to serve in ecological roles, providing defense against herbivores or pathogens, attracting animals that disperse seeds or pollen or attracting pathogen predators and even act as germination inhibitors of neighbouring plants (van Beek & de Groot, 1986; Frazier, 1986; Harborne & Tomas-Barberan, 1991; Jansen & de Groot, 1991; Langenheim, 1994; Dicke, 1994; Bouwmeester et al., 1999a; Romagni et al., 2000; Pichersky & Gershenzon, 2002).

However, the function of terpenes is not limited to ecology. Many play important roles in human society, such as the myriad of monoterpene and sesquiterpene flavour and fragrance

agents that are added to foods, beverages, perfumes, soaps, toothpaste, tobacco and other products (Verlet, 1993).

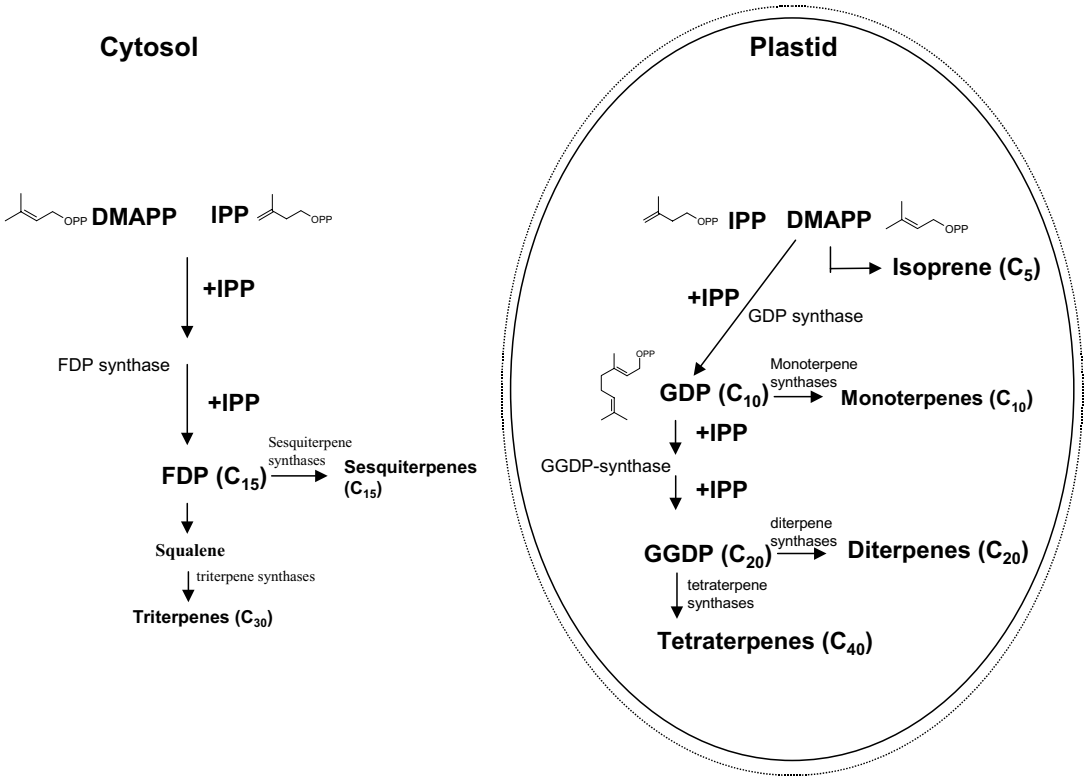


Figure 1. Terpnoid biosynthesis and compartmentation of the different enzymes involved in the different pathways. Monoterpene synthases are located in the plastids. Adapted from Lücker (2002)

Many terpenes find use in industry as raw materials in the manufacture of adhesives, coatings, emulsifiers and specialty chemicals, whilst others, such as limonene are of commercial importance as insecticides because of their low toxicity to mammals and lack of persistence in the environment (Way & van Emden, 2000). Many dietary monoterpenes, including limonene and its active serum-oxygenated metabolite derivatives, perillic acid and dihydroperillic acid (Crowell et al., 1992), have been shown to suppress cancer through their chemopreventive activity during the promotion phase of mammary and liver carcinogenesis (Bardon et al., 1998; Crowell, 1999). This is due to the inhibition of tumor cell proliferation, acceleration of the rate of tumor cell death and/or induction of tumor cell differentiation (Morse & Stoner, 1993; Gould et al., 1994). The treatment of ovarian and breast cancer is also performed by the use of a diterpene from *yew* (*Taxus* spp.) (Hezari & Croteau, 1997). Artemisinin, a sesquiterpene endoperoxide isolated from *Artemisia annua* is proving to be a valuable antimalarial compound (Bouwmeester et al., 1999b).

Biosynthesis of monoterpenes

In the biosynthesis of monoterpenes, the fundamental chain elongation process of terpene biosynthesis is achieved by the condensation of isopentenyl diphosphate (IPP) and a carbon of an allylic pyrophosphate's C5 molecule, dimethylallyldiphosphate (DMAPP) catalysed by a prenyl transferase enzyme called geranyl diphosphate (GDP) synthase (Gershenzon and Croteau, 1993) (Fig.1). This yields the C_{10} prenyl diphosphate, geranyl diphosphate (GDP), as a precursor for the monoterpene biosynthesis in the plant plastid.

A large number of different basic monoterpene skeletons can be formed from GDP (see Figure 2 for examples).

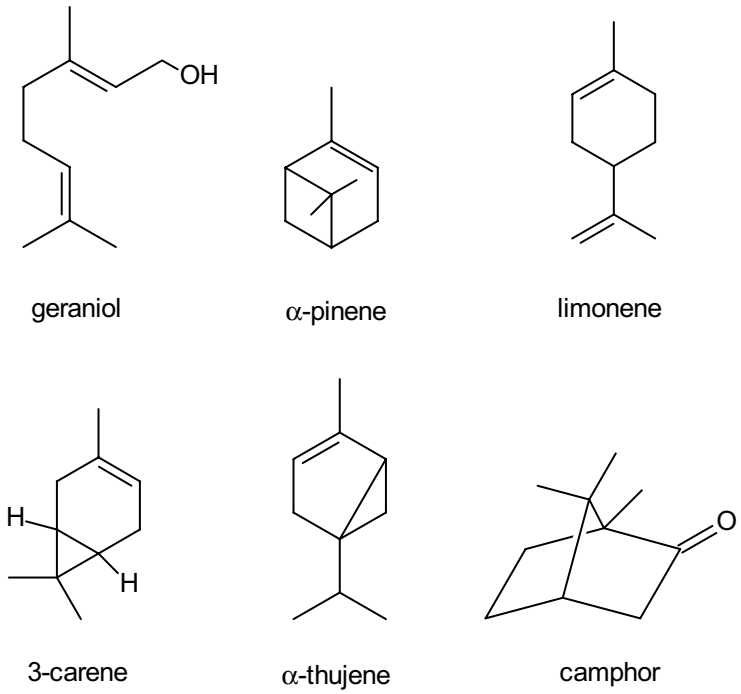


Figure 2. Representative members of various monoterpene subfamily structural types

A widely occurring monoterpene, also reported for *Citrus* spp., is limonene. Figure 3 shows- as an example- the enzymatic cyclisation mechanism responsible for the conversion of GDP to limonene. GDP has a C2-C3 double bond and its ionization, catalysed by a monoterpene synthase, is possible in the presence of a bivalent metal co-factor (such as Mg^{2+} or Mn^{2+}) (Figure 3). The resulting allylic cation-diphosphate anion pair then rearranges to form the enzyme-bound, tertiary allylic isomer, 3R- or 3S-linalyl diphosphate (LPP, depending on the initial folding of the geranyl substrate). After rotation to the cisoid conformer, LPP ionizes and is cyclised in anti, endo-form to the corresponding 4R- or 4S-α-terpinyl cation, the first cyclized intermediate. Finally, abstraction of a proton leads to the formation of limonene.

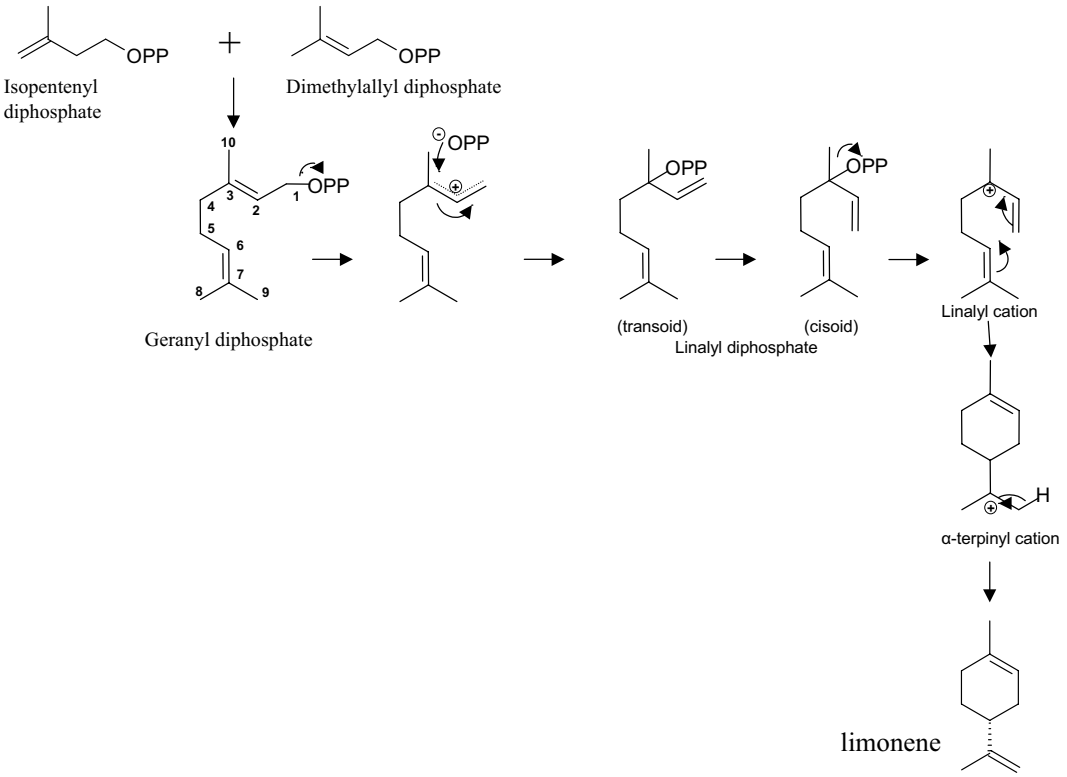


Figure 3. The biosynthesis of monoterpenes. Starting with GPP, followed by a cyclisation to the α-terpinyl cation, the reaction yields the formation of monoterpene olefins for e.g. limonene

The monoterpene synthases produce a wide range of cyclic and acyclic monoterpenes through many different multistep mechanisms involving cationic intermediates, internal additions to the double bonds and hydride shifts (Bohlmann et al., 1998a). The enzymes serve as templates for the conformation and stereochemistry during cyclisation and elegantly protect and stabilise the reactive carbocation intermediates (Starks et al., 1997). The degree of stabilisation of carbocationic intermediates determines what further rearrangements can occur and on how the positive charge is quenched and thus what end products will be formed.

Monoterpene synthase genes

To date, a large number of genes encoding enzymes catalysing the biosynthesis of monoterpenes in plants have been cloned. For example, linalool synthase (Dudareva et al., 1996; Cseke et al., 1998; Jia et al., 1999); (-)-limonene synthase (Colby et al., 1993; Bohlmann et al., 1997); (+)-limonene synthase (Maruyama et al., 2001; Maruyama et al.,

2002); myrcenem and (-)-pinene synthase (Bohlmann et al., 1997); myrcene synthase (Bohlmann et al., 2000; Fishbach et al., 2001) and β-ocimene synthase (Bohlmann et al., 2000); (+)-bornyl diphosphate, 1,8 cineole synthase and (+)-sabinene synthase (Wise et al., 1998); (-)-β-phellandrene synthase, (-)-camphene synthase, terpinolene synthase and (-)-limonene/(-)-α-pinene synthase (Bohlmann, 1999). The enzymes leading to the production of 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 & Croteau, 1998) and have been located in chloroplasts of parenchyma cells (Bouvier et al., 2000) and in leucoplasts of secretory cells (Turner et al., 1999).

Enzyme catalysis mechanism and product specificity

An appreciation of the detailed kinetic mechanism of a wide variety of monoterpene and sesquiterpene synthases and related enzymes, and an understanding of the role of the active site residues in catalysis along with the availability of primary sequence information sets the stage for engineering product formation.

For the engineering of product composition of a specific enzyme, several approaches can be adopted. Site-directed mutagenesis and domain swapping can introduce subtle changes in the amino acid residues at the active site of the enzyme, which can influence substrate binding conformation and the participation of side chain functional groups in catalysis (McCaskill et al., 1997). One could envision that the modification of specific residues to affect the stability of carbocation intermediates could divert the reaction course along alternate routes to novel products. The second approach involves the construction of chimeric enzymes that combine functional domains from different synthases. This approach is suggested by the high level of sequence homology observed for plant isoprenoid synthases. Evolutionary conservation of functional domains in plant isoprenoid synthases (Mau et al., 1994) might accomodate for a third approach known as domain shuffling and suggests that chimeric enzymes with different functional domain sequence can be created to synthesize new products.

Functional domains responsible for a terminal enzymatic step were identified within sesquiterpene synthases. This was rendered possible by swapping exon regions between *Nicotiana tabacum* 5-epi-aristolochene synthase (TEAS) gene and a *Hyoscyamus muticus* vetispiradene synthase (HVS) and by characterizing the resulting chimeric enzymes after expression in *E. coli* (Back & Chappell, 1996). Another group studied the sequence homology

between monoterpene synthases from different plant species, the linalool synthase from *Clarkia breweri* (LIS) and the limonene synthase from *Perilla frutescens* (Cseke et al., 1998). Both studies found that the C-terminal half of the cDNA's, constitutes the terpene synthase portion involved in initial recognition, the binding of the substrate and Mg^{2+} metal ion cofactor in the active site around the DDXXD motif and the initial ionization step (Starks et al., 1997). In addition, the N-terminal half of monoterpene synthases has a primary structure similarity consisting of the RRX_8W motif thought to be involved in the C1, C6 initial ring closure of GDP (Bohlmann et al., 1998b).

The crystal structure of TEAS has been elucidated and provides a basis for understanding the product specificity of terpene synthases, together with more insight in the involvement of aromatic quadrupoles in carbocation stabilisation. TEAS consists entirely of α-helices, short connecting loops and turns and is organized in two structural domains. The crystallographic model of TEAS with the docked farnesyl diphosphate (FDP) substrate suggests that the specificity of the synthases depends on the presence of a particular active site conformation determined by the surrounding layers (Starks et al., 1997). Among the active site residues in TEAS, aromatic amino acids such as Y520, are thought to be playing a key role in the catalysis process leading to the exclusive formation of the sesquiterpene 5-epi-aristolochene from farnesyl diphosphate (Rising et al., 2000). This has been contested since Y520 was conserved within two cDNAs encoding germacrene A synthases recently isolated from chicory. This undermined the conclusion that Y520 is absolutely required for the further cyclization of the enzyme- bound germacrene A to 5-epi-aristolochene (Bouwmeester et al., 2002).

Engineering of terpene metabolism in plants

Metabolic engineering of the monoterpene biosynthesis pathway in plants has been already achieved (Mahmoud and Croteau, 2001, Lücker et al., 2001). Flower scent has almost never been a target trait in commercial flower breeding programs but rather color, longevity and form (Zucker et al., 1998). Some groups have tried to produce fragrance compounds in transgenic plants in an attempt to improve floral scent (Vainstein et al., 2001). For example, in old varieties of carnation, eugenol used to contribute up to 85% of total amount of headspace volatiles but in modern varieties it is below human perception threshold detection levels and therefore these varieties lack the characteristic original fragrance (Clery et al., 1999). However, the introduction of novel genes, encoding enzymes involved in the formation of a specific fragrance-related product, has proved not sufficient by itself. In one

case, the glycosylated form of the final product, which also occurs naturally (Watanabe et al., 1993), did not allow subsequent product volatilization and emission (Lücker et al., 2001). In other cases, the level of precursor had been either limiting to allow product formation (Dudareva & Pichersky, 2000) or the product was emitted at too low levels for human pannelists to detect any olfactory alteration in floral scent (Lavy et al., 2002).

Citrus

Importance

Citrus fruits are among the most widely produced and consumed fruits all over the world (FAO, 1993). These comprise among others of lemons, oranges, mandarins and grapefruits. The production and consumption of Citrus juices in the United States exceed those of all other fruit and vegetable juices combined (Ting & Rouseff, 1986). The (+)-limonene extracted from these fruit peels has cancer chemoprevention properties and Citrus secondary metabolites are also commonly used as additives in the fragrance and flavour industry, such as in candies, liquors and other food products. They are also added as fragrant and hygenic agents in cosmetics (Lota et al., 1999) and are used in aroma therapy (Komori et al., 1995; Lehrner et al., 2000).

Interestingly, monoterpene hydrocarbons constitute 53% of mandarin peel essential oil, 77% of sweet orange peel essential oil (Dugo et al., 1996) and 90% of Citrus lemon L. Burm. peel essential oil (Sawamura et al., 1999). Prior to the start of this thesis, no cloning of any terpene synthase cDNA from *Citrus* sp. was yet reported.

Scope of the thesis

This study aims at widening our understanding about the enzymatic production of monoterpenes in *Citrus*. The aims of the thesis were to isolate monoterpene synthase cDNAs from *Citrus limon L. Burm.* peel and to identify specific domains, and possibly amino acids, involved in product specificity within the active site of these enzymes. In addition, we aimed at investigating factors that might positively contribute to the regeneration of transgenic *Citrus* plants. Finally, we aimed at studying the odor effects of transforming *Nicotiana tabacum* with the isolated *Citrus limon* monoterpene synthases.

For these purposes, we set out to isolate monoterpene synthase cDNAs from lemon, *Citrus limon* L. Burm. that is producing a wide range of monoterpenes. A cDNA library was made of the lemon fruit peel and random sequencing and screening of the library were used to isolate and subsequently characterize monoterpene synthase as described in Chapter 2.

To increase our knowledge about the monoterpene synthase enzymatic function, we wanted to investigate how the product specificity of these enzymes is determined. Therefore, in chapter 3, domain swapping experiments were conducted on three of the *Citrus limon* monoterpene synthases. The results of these experiments and molecular modelling are used to pinpoint amino acids involved in product specificity.

Metabolic engineering of an important crop such as *Citrus* can prove to be of high value to both fruit quality and resistance against biotic diseases. In chapter 4, we have investigated factors that affect the regeneration of *Citrus sinensis* (L.) Osbeck cv. Valencia Late to help in the efficient recovery of transgenic *Citrus sinensis* plants.

An additional objective was to study the concequences of metabolic engineering on olfactory characteristics. Since *Nicotiana tabacum* is a suitable and well characterized model plant that also emits monoterpenes (Loughrin, 1990), we studied the effects of transformation of tobacco with the *Citrus limon* monoterpene synthases. In Chapter 5, we describe the analysis of the fragrance of tobacco Petit Havana SR1 plants transformed with *Citrus limon* monoterpene synthases using human panelists.

Finally, in Chapter 6, the thesis is discussed and suggestions for future work are given.

References

Back K & Chappell J (1996) Identifying functional domains within terpene cyclases using a domain-swapping strategy. *Proc. Natl. Acad. Sci*.(**93**): 6841-6845

Bardon S, Picard K & Martel P (1998) Monoterpenes inhibit cell growth, cell cycle progression, and D1 gene expression in human breast cancer cell lines. *Nutrition and Cancer* (**32**): 1-7

Bell EA & Charlwood BV (1980) *Secondary plant products*. Springer, Heidelberg.

Bieza K & Lois R (2001) An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. *Plant Physiol* (**126**): 1105-1115

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

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

Bohlmann J, Crock J, Jetter R & Croteau R (1998b) Terpenoid-based defenses in conifers: cDNA cloning, characterization, and functional expression of wound-inducible (E)-α-bisabolene synthase from grand fir (*Abies grandis*). *Proc. Natl. Acad. Sci. USA* (**95**): 6756-6761

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

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

Boller T & Wiemken A (1986) Dynamics of vacuolar compartmentation. *Ann. Rev. Plant Physiol.* (**37**): 137-164

Bouwmeester HJ, Verstappen FW, Posthumus MA, Dicke M (1999a) 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 (1999b) Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochemistry* (**52**): 843-54

Bouwmeester HJ, Kodde J, Verstappen FWA, Altug IG, de Kraker JW & Wallaart TE (2002) Isolation and characterization of two germacrene A synthase cDNA clones from Chicory. *Plant Physiol.* (**129**): 134-144

Buee M, Rossignol M, Jauneau A, Ranjeva R, Becard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. *Mol Plant Microbe Interact.* (**13**): 693-8.

Chappell J (1995) Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. *Ann. Rev. Plant Physiol. Plant Mol Biol* (**46**): 521-547

Clery RA, Owen NE, Chambers SF, Thornton-Wood SP (1999) An investigation into the scent of carnations. *J. Essent. Oil Res.* (**11**): 355-359

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ and 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

Conn EE (1981) Secondary Plant Products, in *The Biochemistry of Plants*. Vol. 7, Academic press, New York.

Crowell P, Lin S, Vedejs E & Gould MN (1992) Identification of metabolites of the antitumor agent d-limonene capable of inhibiting protein isoprenylation and cell growth. Cancer & Chemother. *Pharmacol.* (**31**): 205-212

Crowell PL (1999) Prevention and Therapy of cancer by Dietary Monoterpenes. *Symposium on Phytochemicals: Biochemistry and Physiology. American Society for Nutritional Sciences*.

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

Dey PM & Harborne JB (1997) *Plant Biochemistry*. Academic Press. San Diego.

Dicke M (1994) Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant-carnivore mutualism. *J. Plant Physiol.* (**143**): 465-472

Food and Agriculture organization of the United Nations, *Citrus fruit fresh and processed*. Annual statistics, 1993.

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 genetics aspects of floral scents. *Plant Physiol.* (**122**): 627-633

Dugo G, Mondello L & Cotroneo A (1996) Characterization of Italian Citrus Petitgrain oils. *Perfumer & Flavorist* (**21**): 17-28

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

Frazier JL (1986) *The perception of plant allelochemicals that inhibit feeding, in Molecular Aspects of Insect-Plant Associations*. pp 1-42. Plenum Press. New York.

Gershenzon J & Croteau R (1993) *Terpenoid biosynthesis: The basic pathway and formation of monoterpenes, sesquiterpenes and diterpenes*. In TS Moore ed, Lipid Metabolism in Plants. CRC Press. pp 340-388

Gould MN, Moore CJ, Zhang R, Wang B, Kennan WS & Haag JD (1994) Limonene chemoprevention of mammary carcinoma induction following direct *in situ* transfer of v-Ha-ras. *Cancer Res.* (**54**): 3540-3543

Guern J, Renaudin JP and Brown SC (1987) The compartmentation of secondary metabolites in plant cell cultures, in *Cell Culture and Somatic Cell Genetics*. pp 43-76. Academic Press. New York.

Harborne JB & Tomas-Barberan FA (1991) *Ecological Chemistry and Biochemistry of Plant Terpenoid*s. Clarendon Press, Oxford.

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

Hedden P & Kamiya Y (1997) Gibberellin biosynthesis: Enzymes, genes and their regulation. *Ann. Rev. Plant Physiol. Plant Mol Biol* (**48**):431-460

Helsper JPFG, Davies JA, Bouwmeester HJ, Krol AF & van Kampen MH (1998) Circadian

rythmicity in emission of volatile compounds by flowers of *Rosa hybrida* L. cv. Honesty. *Planta* (**207**): 88-95

Henkes S, Sonnewald U, Badur R, Flachmann R & Stitt M (2001) A small decrease of plastid transketolase activity in antisense Tobacco transformants has dramatic effects on photosynthesis and phenylpropanoid metabolism. *The Plant Cell* (**13**): 535-551

Hezari M & Croteau R (1997) Taxol biosynthesis: an update. *Planta Med.*(**63**): 291-295

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

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

Jansen BJM & de Groot A (1991) The occurrence and biological activity of drimane sesquiterpenoids. *Natl. Proc. Rep.*(**8**): 309-318

Komori T, Fujiwara R, Tanida M, Nomura J & Yokohama MM (1995) Effects of Citrus fragrance on immune function and depressive states. *Neuroimmunomodulation* (**3**): 174-180

Langenheim H (1994) Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol.* (**20**):1223-1280

Lavy M, Zuker A, Lewinsohn E, Larkov O, Ravid U & Weiss D (2002) Linalool and linalool oxide production in transgenic carnation flowers expressing the Clarkia breweri linalool synthase gene. *Mol. Breed. In press.*

Lehrner J, Eckersberger C, Walla p, Potsch G & Deecke L (2000) Ambiant odor of orange in a dental office reduces anxiety and improves mood in female patients. *Physiol. Behav.* (**1**): 83-86

Lota ML, de Rocca Serra D, Tomi F, Bessiere JM & Casanova J (1999) Chemical composition of peel and leaf essential oil of *Citrus medica L.* and *C. limonimedica Lush*. *Flavour And Fragrance Journal* (**14**): 161-166

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

Lücker J, Bouwmeester J, 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. *Plant J.* (**27**): 315-324

Luckner M (1990) *Secondary metabolism in Microorganisms, Plants and Animals*. Springer, Heidelberg.

Mahmoud SS & Croteau RB (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

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

Maruyama T, Saeki D, Ito M & Honda G (2002) Molecular cloning, functional expression and characterization of d-limonene synthase from Agastache rugosa. *Biol Pharm Bull*. (**25**): 661-665

Matile P (1978) Biochemistry and function of vacuoles. *Ann. Rev. Plant Physiol.* (**29**): 193-213

McGarvey DJ & Croteau R (1995) Terpenoid metabolism. *Plant Cell* (**7**): 1015-1026

Morse MA & Stoner GD (1993) Cancer chemoprevention: principles and prospects. *Carcinogenesis* (**14**): 1737-1746

Mothes K, Schutte HR and Luckner M (1985) *Biochemistry of Alkaloids*. Verlag Chemie, Weinheim.

Pichersky E & Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology* (**5**): 237-243

Rising KA, Starks CM, Noel JP and Chappell J (2000) Demonstration of germacrene A as an intermediate in 5-epi-aristolochene synthase catalysis. *J. Am. Chem. Soc.* (**122**): 1861-1866

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

Sawamura M, Poiana M, Kawamura A, Itoh T, Song HS, Ukeda H & Mincione B (1999) Volatile components of peel oils of Italian and Japanese Lemon and bergamot. *Ital. J. Food Sci*. (**2**): 121-130

Schwartz SH, Tan BC, Gage DA, Zeevaart JAD & Mccarty DR (1997) Specific oxidative cleavage of carotenoids by vp14 of maize. *Science* (**276**): 1872-1874

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

Ting SV & Rouseff RL (1986) *Citrus and their products. Analysis and technology*. Marcel

Dekker, Inc. New York.

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

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

van Beek TA & de Groot A (1986) Terpenoid antifeedants. Part I. An overview of terpenoid antifeedants of natural origin. *Recueil Des Traveaux Chimiques des Pays-Bas* (**105**): 513-527

van der Fits L, Zhang H, Menke FL, Deneka M & Memelink J (2000) A Catharanthus roseus BPF-1 homologue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene Str and is induced by elicitor via a JA-independent signal transduction pathway. *Plant Mol. Biol.* (**44**): 675-85

Vaughn SF & Spencer GF (1993) *Volatile monoterpenes as potential parent structures for new herbicides*. Weed Science. Champaign, 3rd ed. Weed Science Society of America. Vol. 41:114-119

Verlet N (1993) *Commercial aspects, in Volatile Oil Crops: Their Biology Biochemistry and Production*: pp.137-174. Longman Scientific and Technical, Essex.

Watanabe N, Watanabe S, Nakajima R, Moon JH, Shimokihara K, Inagaki J, Etoh H, Asai T, Skata R & Ina K (1993) Formation of flower fragrance compounds from their precursors by enzymic action during flower opening. *Biosci. Biotech. Biochem.* (**57**): 1101-1106

Way MJ & van Emden HF (2000) Integrated pest management in practice- pathways towards successful application. *Crop Protection* (**19**): 81-103

Wiermann R (1981) Secondary plant products and cell and tissue differentiation, in *The Biochemistry of Plants*, Vol 7: 85-116. Academic Press, New York.

Wink M & Hartmann T (1982) Localization of the enzymes of quinolizidine alkaloid biosynthesis in leaf chloroplast of lupinus polyphyllus. *Plant Physiol.* (**70**): 74-77

Wink M (1993) The plant vacuole: A multifunctional compartment. *J. Exp. Bot.* (**44**): 231-246

Wink M & Roberts MF (1998) Compartmentation of alkaloid synthesis, transport and storage in *Alkaloids: Biochemistry, Ecological Functions and Medical Applications*: 239-262. Plenum, New York

Wink M (1999) *Biochemistry Of Plant Secondary Metabolism*. Annual Plant Reviews, Vol. 2. Sheffield Academic Press. UK.

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

Zuker A, Tzfira T & Vainstein A (1998) Genetic engineering for cut-flower improvement. *Biotech Adv.* (**16**): 33-79

Chapter 2

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

Abstract

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.*

Material and methods

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., 1999a). 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., 1999b).

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.

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.

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.

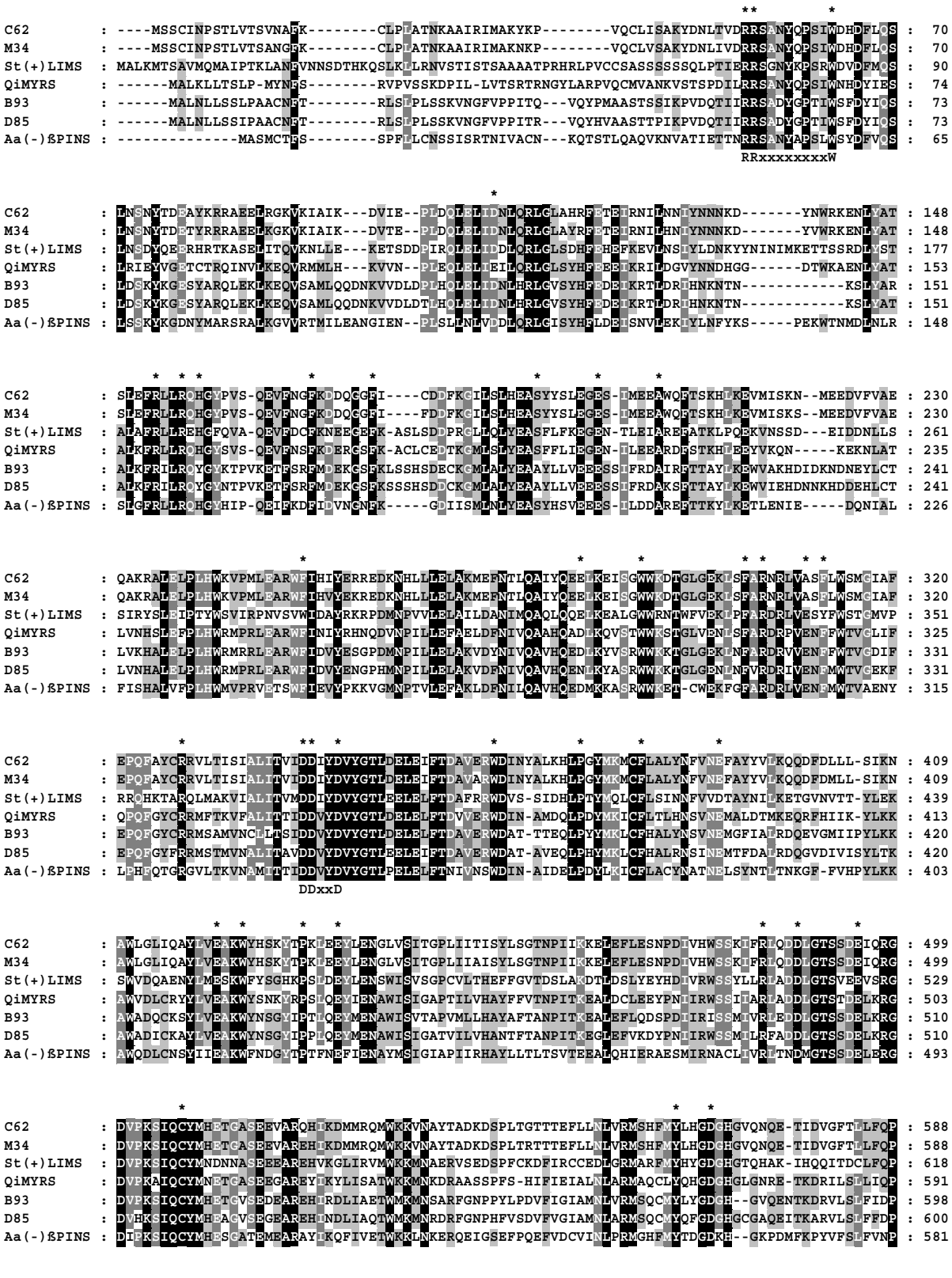

C62
M34
St(+)LIMS
QiMYRS
B93
D85
Aa(-)ßPINS
RRxxxxxxxxW
DDxxD

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)), *Clγ TS* (*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.

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.

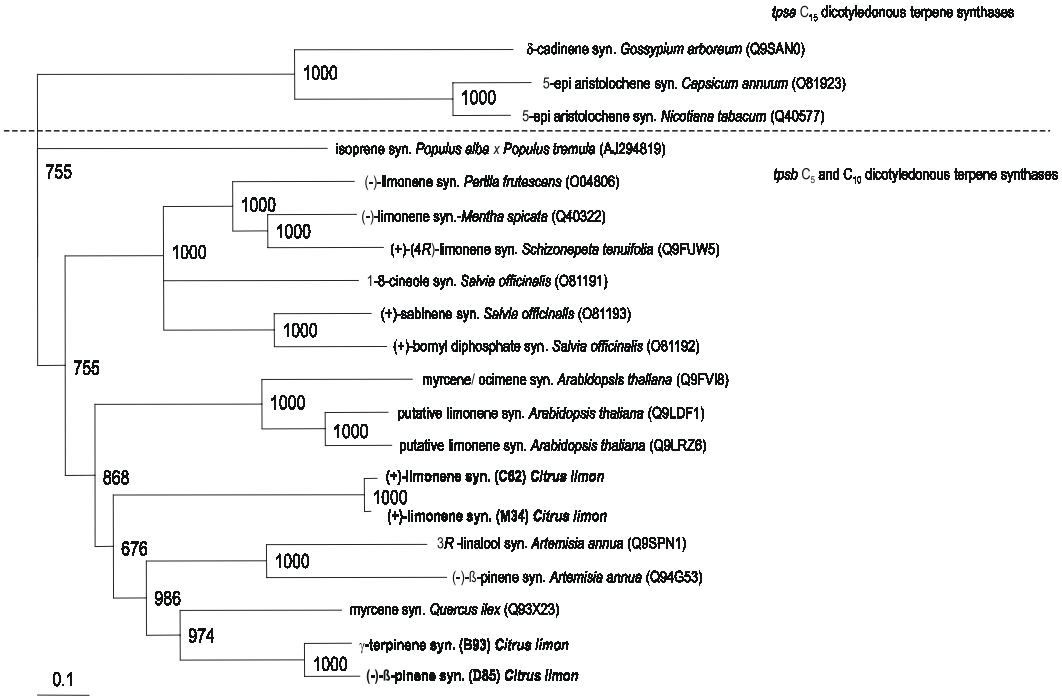


Figure. 2. Phylogram of CLUSTALX alignment of dicotyledonous C_5 to C_{15} terpene synthases using PAM350 matrix and the neighbour joining method. The tree was corrected for multiple substitutions. The sesquiterpene synthases (*tpsa*) were defined as outgroup and the tree was rooted with the outgroup. 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

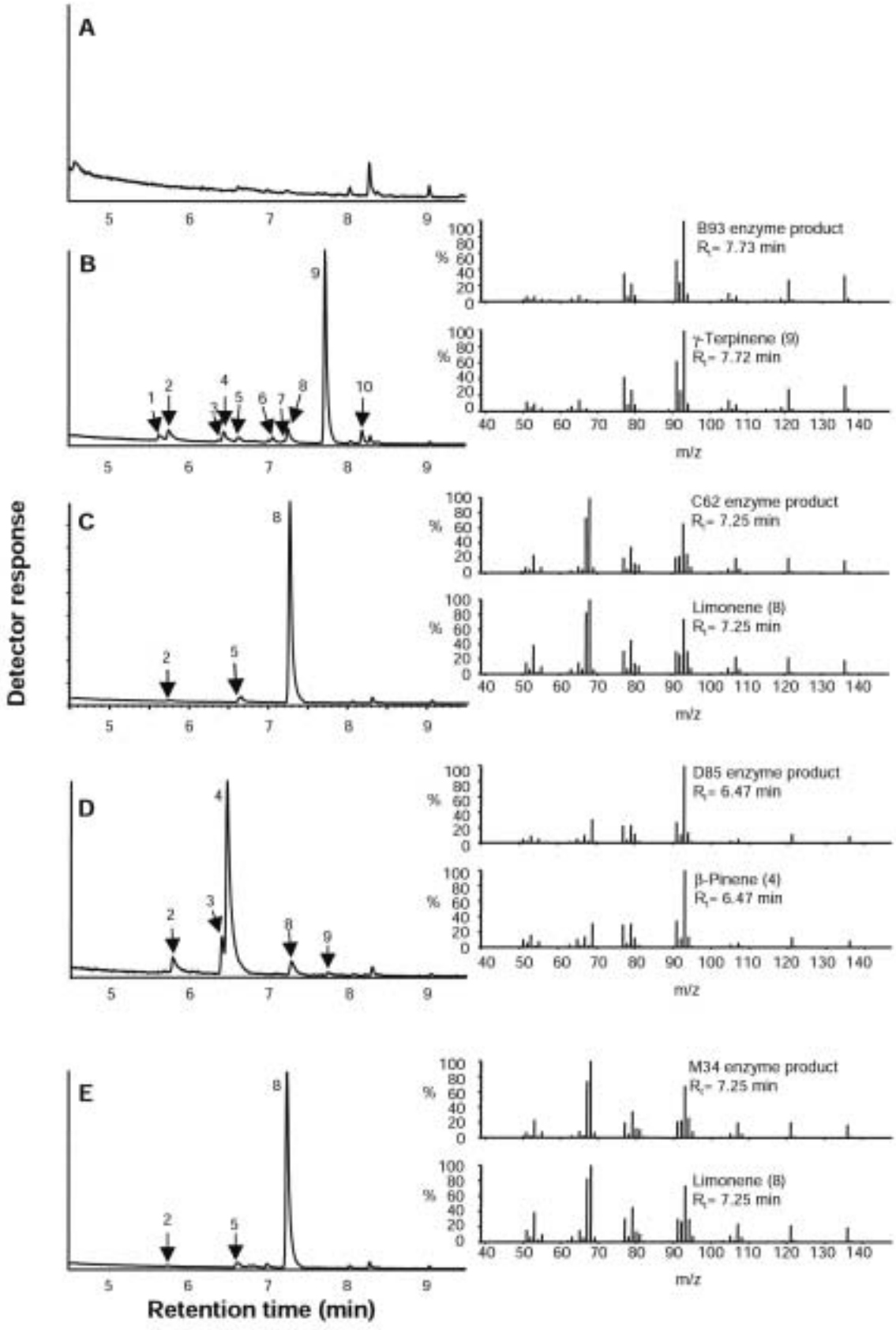
GC-MS analysis demonstrated that the cDNA encoded enzymes produced three different major products (Figure 3). B93 produced γ-terpinene and is therefore designated ClγTS (Figure 3B), C62 and M34 both produced limonene and are designated Cl(+)LIMS1 and Cl(+)LIMS2 respectively, (Figure 3C and 3E) and D85 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-like 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. ND: not determined. -: trace

	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	-	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	-					
limonene	9.1	80:20	3.5	89:11	99.15	0: 100
γ-terpinene	71.4		-			
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 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
R_t= 7.73 min
γ-Terpinene (9)
R_t= 7.72 min
C62 enzyme product
R_t= 7.25 min
Limonene (8)
R_t= 7.25 min
D85 enzyme product
R_t= 6.47 min
β-Pinene (4)
R_t= 6.47 min
M34 enzyme product
R_t= 7.25 min
Limonene (8)
R_t= 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. ($|\%R\text{-}\%S|$) 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).

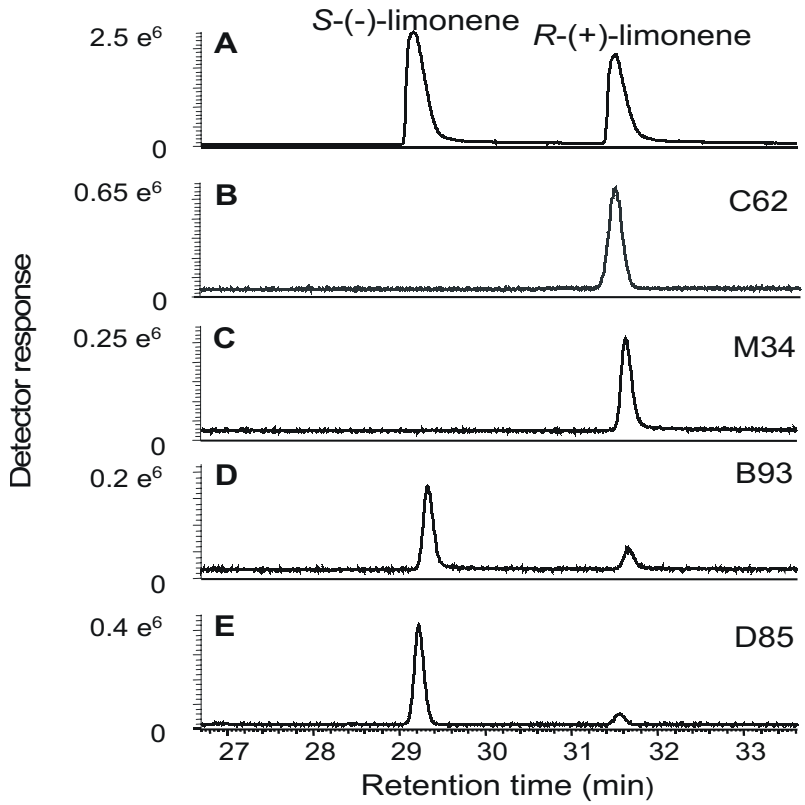


Figure. 4. GC-MS profiles of enantiomers of limonene formed by the different synthases. (A) shows separation of the reference limonene enantiomers. (B) and (C) show that M34 and C62 (Cl(+)LIMS1 and CL(+)LIMS2) produce *R*-(+)-limonene. (D) and (E) show that B93 (ClγTS) and D85 (Cl(-)βPINS) produce predominantly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

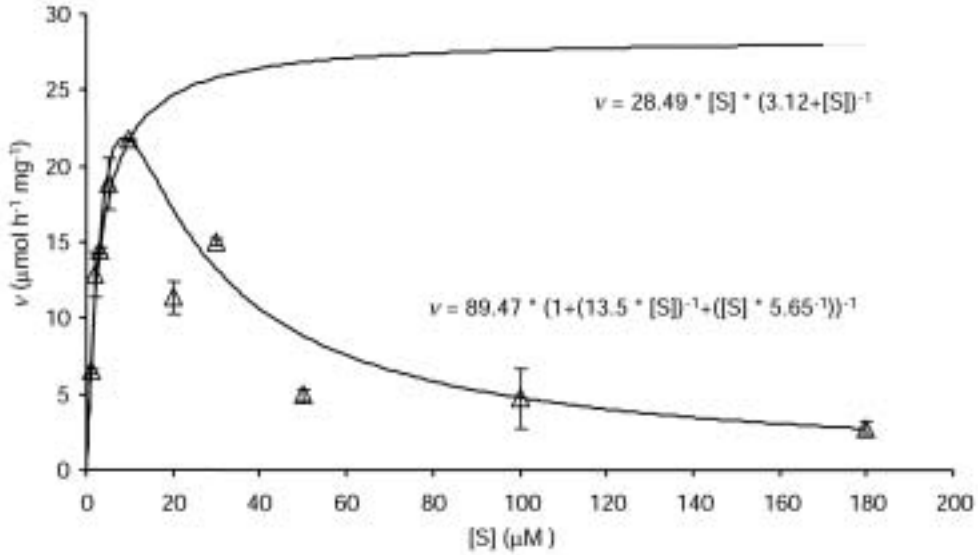


Figure. 5. Cl(-)βPINS enzyme activity curves. Enzyme activities were measured with substrate concentrations up to 180 µM GPP. A Michaelis Menten curve (featuring a K_m of 3.1 µM and an apparent v_{max} of 28.49 µmol h^{-1} mg^{-1}) and a substrate inhibition curve (featuring a K_m of 13.5 µM, an apparent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

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. M.C.R Franssen for critical reading and helpful remarks on the manuscript.

References

Aharoni, A., Keizer, L. C. P., Bouwmeester, H. J., Sun, Z., Alvarez, H. M., Verhoeven, H. A., Blaas, J., van Houwelingen, A. M. M. L., De Vos, R. C. H., van der Voet, H., Jansen, R. C., Guis, M., Mol, J., Davis, R. W., Schena, M., van Tunen, A. J. & O' Connell, A. P. (2000) Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. *Plant Cell* (**12**): 647-661.

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

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W. & Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. (**25**): 3389-3402.

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

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

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

Bouwmeester, H. J., 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, H. J., Verstappen, F. W. A., Posthumus, M. A. & Dicke, M. (1999) Spider

mite-induced (3S)-(E)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. *Plant physiol*. (**121**): 173-180.

Bouwmeester, H. J., Wallaart, T. E., Janssen, M. H., van Loo, B., Jansen, B. J., Posthumus, M. A., Schmidt, C. O., de Kraker, J. W., Konig, W. A. & Franssen, M. C. (1999b) Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochemistry* (**52**): 843-54.

Chayet, L., Rojas, C., Cardemil, E., Jabalquinto, A. M., 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.

Colby, S. M., Alonso, W. R., Katahira, E. J., McGarvey, D. J. & Croteau, R. (1993) 4S-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.

Crowell, P. L. & Gould, M. N. (1994) Chemoprevention and therapy of cancer by d-limonene. *Crit. Rev. Oncogenesis* (**5**): 1-22.

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

de Kraker, J. W., Franssen, M. C. R., de Groot, Æ., König, W. A. & Bouwmeester, H. J. (1998) (+)-Germacrene A biosynthesis: The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. *Plant Physiol*. (**117**): 1381-1392.

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

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

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

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

Gershenzon, J., McConkey M. E. & Croteau R. B. (2000) Regulation of monoterpene

accumulation in leaves of peppermint. *Plant Physiol*. (**122**): 205-213.

Helsper, J. P. F. G., Davies, J. A. & Verstappen, F. W. A. (2001) *Analysis of rhythmic emission of volatile compounds of rose flowers. In Analysis of taste and aroma* (Jackson, J. F. & Linskens, H. F., eds) pp. 269, Springer.

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

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

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

Lange, M. B., Wildung, M. R., Stauber, E. J., 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*. (**97**): 2934-2939.

Lesburg, C. A., Zhai, G., Cane, D. E. & Christianson, D. W. (1997) Crystal structure of pentalenene synthase: Mechanistic insights on terpenoid cyclization reactions in biology. *Science* (**277**): 1820-1824.

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

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, R. M., 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.

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

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

Maruyama, T., Ito, M., Kiuchi, F. & Honda, G. (2001b) Molecular cloning, functional

expression and characterization of d-limonene synthase from Schizonepeta tenuifolia. *Biol. Pharm. Bull*. (**24**): 373-377.

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, M. E., Gershenzon, J. & Croteau, R. B. (2000) Developmental regulation of monoterpene biosynthesis in the glandular trichomes of peppermint. *Plant Physiol*. 122, 215-223.

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.

Rynkiewics, M.J., Cane, D.E. & Christianson, D.W. (2001) Structure of trichodiene synthase from Fusarium sporotrichioides provides mechanistic inferences on the terpene cyclisation cascade. *Proc. Natl Acad. Sci*. (**98**): 13543-13548.

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, E. F. & Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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

Schultz, D. J., Craig, R., Cox-Foster, D. L., Mumma, R. O. & Medford, J. I. (1994) RNA isolation from recalcitrant plant tissue. *Plant Mol. Biol. Rep*. (**12**): 310-316.

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

Steele, C. L., 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.

Tarshis, L. C., Yan, M., Poulter, C. D. & Sacchettini J., C. (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-A resolution. *Biochemistry* (**33**): 10871-10877.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The

ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. (**24**): 4876-4882.

Turner, G. W., Berry, A. M. & Gifford, E. M. (1998) Schizogenous secretory cavities of Citrus limon (L.) Burm. f. and a reevaluation of the lysigenous gland concept, *Int. J. Plant Sci.* (**159**): 75-88.

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

Weiss, E. A. (1997) Essential oil crops, Cab international, Wallingford.

Williams, D. C., McGarvey, D. J., Katahira, E. J. & 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.

Wise, M. L., Savage, T. J., 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.

Yuba, A., Yazaki, K., Tabata, M., Honda, G. & Croteau, R. (1996) cDNA Cloning, characterization, and functional expression of 4S-(-)-limonene synthase from Perilla frutescens. *Arch. of Biochem. Biophys*. (**332**): 280-287.

Chapter 3

Domain swapping of *Citrus limon* monoterpene synthases: Impact on enzymatic activity and product specificity

Mazen K. El Tamer,[1] Joost Lücker,[1] Dirk Bosch,[1] Harrie A. Verhoeven,[1] Francel W.A Verstappen,[1] Wilfried Schwab,[2] Arjen J. van Tunen,[3] Alphons G. J. Voragen,[4] Ruud A. de Maagd[1] & Harro J. Bouwmeester[1]

[1] Plant Research International B.V, P.O Box 16, 6700 AA Wageningen, The Netherlands

[2] Department of Food Chemistry, University of Würzburg, Würzburg, Germany

[3] Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

[4] Department of Agro Technology and Food Sciences, Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands

Accepted in the Archives of Biochemistry and Biophysics

Abstract

Monoterpene cyclases are the key enzymes in the monoterpene biosynthetic pathway, as they catalyze the cyclization of the ubiquitous geranyl diphosphate (GDP) to the specific monoterpene skeletons. From *Citrus limon*, four monoterpene synthase encoding cDNA's for a β-pinene synthase named Cl(-)ßPINS, a γ-terpinene synthase named ClγTS, and two limonene synthases named Cl(+)LIMS1 and Cl(+)LIMS2, were isolated (chapter 2). The aim of our work in this study was to identify domains within these monoterpene synthase enzymes determining the product specificity. Domain swapping experiments between Cl(-)ßPINS and ClγTS, and between Cl(+)LIMS2 and ClγTS were conducted. We found that within the C-terminal domain of these monoterpene synthases, a region comprising 200 amino acids, of which 41 are different between Cl(-)ßPINS and ClγTS, determines the specificity for the formation of β-pinene or γ- terpinene, respectively, while another region localized further downstream is required for a chimeric enzyme to yield products in the same ratio as in the wild type ClγTS. For Cl(+)LIMS2, the two domains together appear to be sufficient for its enzyme specificity, but many chimeras were inactive probably due to the low homology with ClγTS. Molecular modelling was used to further pinpoint the amino acids responsible for the differences in product specificity of ClγTS and Cl(-)ßPINS.

Introduction

Terpenoids form one of the most important classes of plant secondary metabolites consisting of over 36,000 different compounds (Hill, 2002). Throughout the Plant kingdom, terpenoids are known to have a wide range of functions. They can act as defense compounds in plants as they deter herbivores or attract natural enemies of herbivores (Trapp and Croteau, 2001). Plant hormones, such as cytokinin, gibberellin, and abscisic acid, are often derivatives of terpenoids (Rademacher, 2000; Mok and Mok, 2001). In nature, terpenes can function in communication between plants and animals, including humans (Cseke, 1998; Gershenzon and Kreis, 1999). The terpenoids structureconsists of an integral number of five-carbon (isoprene) units. Two such units can form C10 atoms monoterpene structures (i.e. limonene) and three units form the C15 atoms sesquiterpenes (i.e. 5-epi-aristolochene).

Geranyl diphosphate (GDP) formed from the condensation of one molecule of IPP with one molecule of DMAPP is a precursor for monoterpene biosynthesis. In the presence of a metal co-factor (such as Mg^{2+} or Mn^{2+}), monoterpene synthase enzymes use GDP as a substrate for the first step in monoterpene biosynthesis. These synthases act as templates to fix the conformation and stereochemistry during the cyclization process and, upon binding to the hydrophobic substrate, the result is a closed solvent-inaccessible active site pocket that protects and stabilizes reactive carbocation intermediates from attack by water. The degree of stabilization of carbocationic- intermediates determines to what extent rearrangements can occur and thus which monoterpene structures are finally formed (Croteau et al., 1987).

So far only one plant terpene synthase, the tobacco 5-epi-aristolochene synthase (TEAS), has been crystallized and its 3D-structure determined. This structure provides a basis for the understanding of the stereochemical selectivity displayed by this and other terpene synthases, together with more insight in the involvement of acidic and aromatic amino acids in carbocation stabilization (Starks et al., 1997). TEAS was shown to consist entirely of α-helices, short connecting loops and turns, and organized in two structural domains. The crystal structure of TEAS with the docked farnesyl diphosphate substrate suggests that the specificity of the synthases depends on the presence of certain amino acid residues in the active site but also in the surrounding layers (Starks et al., 1997; Rising et al., 2000; Bouwmeester et al., 2002). The study of chimeras derived from homologous proteins having different specificities constitutes a powerful tool to identify functions of structural domains (de Maagd et al., 1996. Back and Chapell (1996) swapped the two structural domains of

TEAS and those of a *Hyoscyamus muticus* vetispiradiene synthase (HVS), both sesquiterpene synthases, and characterized the resulting chimeric enzymes expressed in *E. coli*. The domain swapping between sub-domains within the N-terminal and C-terminal structural domains of the TEAS and HVS resulted in novel enzymes capable of synthesizing the products of either one of the two, or both, parent enzymes. The authors proposed that the evolution of terpenoid synthases is based on recombinations of functional domains encoding parts of genes coding for cyclases. If the active site topology of a protein is in some way altered (e.g. by recombination between genes), the ability to select a single product could be lost, resulting in multiproduct chimeric enzymes (Rynkiewicz et al., 2002). For monoterpene synthases, site-directed mutagenesis or domain swapping experiments, and so far, a crystal structure have not been published.

The isolation of four different monoterpene synthase cDNAs from *Citrus limon* L. Burm. peel, all of which belong to the *tpsb* monoterpene gene family, one encoding a γ-terpinene synthase named ClγTS (GenBank accession number: AF14286), another encoding a β-pinene synthase named Cl(-)ßPINS (AF14288) and two encoding (+)-limonene synthases named Cl(+)LIMS1 and Cl(+)LIMS2 (AF14287 and AF14289, respectively) was recently reported (chapter 2). The main products of the enzymes in assays with GDP as substrate were (-)-ß-pinene, γ-terpinene and (+)-limonene (two cDNAs), respectively, but also a number of minor products were found. Cl(+)LIMS2 and ClγTS are 50% identical at the amino acid level. Cl(-)ßPINS and ClγTS share a higher sequence homology (85% amino acid identity) (Figure 1). The availability of these monoterpene synthases with varying homology and different product specificities presents an excellent opportunity to study product specificity of monoterpene synthases. Three out of these four monoterpene synthases catalyzing the formation of three different major products were used in a study of the effects of domain swapping on product specificity.

Materials and Methods

cDNA clones

Monoterpene synthase cDNAs, encoding Cl(-)ßPINS, ClγTS and Cl(+)LIMS2 truncated and cloned in the multiple cloning site of the pRSETB expression vector (that included an ATG translation initiation codon and a series of six histidine residues (His-tag) (Invitrogen, Groningen) have been described earlier (chapter 2). Further cDNA analysis was conducted using the Megalign software (DNA Star Inc, Madison), and the ClγTS and Cl(-)ßPINS proteins were modeled on the Swissmodel online service (Guex and Peitsch, 1997), using TEAS (Swissprot accession # Q40577) as the template for modelling.

Site directed mutagenesis

In order to create restriction enzyme-recognition sites at the same relative position within the three genes and absent from the pRSETB vector, a site-directed mutagenesis approach was adopted using the Quickchange Site Directed Mutagenesis PCR kit (Stratagene, CA) according to the manufacturers recommendations. The *EcoR* I restriction site of the expression vector pRSETB was substituted with an *EcoR* V restriction site using the sense primer 1 (5'-GAGATCTGCAGCTGGTACCATGGATATCGAAGCTTGATCCGGCTGCTAA-3') and an anti-sense primer 2 (5'-TTAGCAGCCGGATCAAGCTTCGATATCCATGGTACCAGCTGCAGATCTC-3'). The altered nucleotides are underlined. Cl(+)LIMS2 was mutated twice without changing the integrity of the encoded amino acid sequence. Using the sense primer 3 (5'-AGAGGACAAGAACCACCTTTTACTCGAGCTCGCTAAGATGGAGTTTAAC-3') and the anti-sense primer 4 (5'-GTTAAACTCCATCTTAGCGAGCTCGAGTAAAAGGTGGTTCTTGTCCTCT-3'), an *Xho* I restriction site introduced at position 799. Primers 5 (5'-CAATCATTAAGAAGGAACTGGAATTCCTAGAAAGTAATCCAGATATAGTT-3') and 6 (5'-AACTATATCTGGATTACTTTCTAGGAATTCCAGTTCCTTCTTAATGATTG-3') were used to create an *EcoR* I restriction site was at position 1399**.**

Creation of chimeric enzymes by restriction digestion and domain swap approach

The parent Cl(-)ßPINS and ClγTS cDNAs cloned in pRSETB were digested with the enzyme combinations *Nhe* I/*Sac* I, *Sac* I/*Xho* I, *Xho* I/*EcoR* I, *EcoR* I/*EcoR* V and *Xho* I/*EcoR* V. *Nhe* I is a unique restriction site located exclusively in the pRSETB vector and upstream of the cloned monoterpene synthases. The correct size DNA bands were isolated from agarose gel using GFX columns (Amersham Pharmacia Biotech, Freiburg) and ligated to subsequently create the chimeric cDNAs 1 to 8 (Table 1). The parent Cl(+)LIMS2 and ClγTS cDNAs were digested either with *Xho* /*EcoR* I, or *EcoR* I /*EcoR* V or *Xho* I /*EcoR* V. In the same way as described above, the chimeric cDNAs called 9, 10, 11, 12, 13 and 14 were created (Table 1). In addition to restriction digestion analysis, all chimeric genes were sequenced completely.

Gene expression and protein purification

All plasmids containing the chimeric cDNAs, as well as the parental cDNAs including the mutated Cl(+)LIMS2, were used to transform *E. coli* BL21-CodonPlus™-RIL strain (Stratagene, CA), using the original pRSETB vector as a negative control. Clones were inoculated into 5 ml LB supplemented with 100 mg L^{-1} ampicillin and grown overnight. Aliquots of 0.5 ml were taken to inoculate 250 ml Erlenmeyer flasks containing 50 ml LB with ampicillin (50 µg mL^{-1}) and cultures were grown at 37 °C to an OD_{600} of 0.6. For induction of expression, IPTG was added to a final concentration of 1 mM and the cultures were grown at 16°C overnight with shaking at 250 rpm. Cells suspended in buffer were sonicated and proteins were isolated using His-tag purification by passing the lysate over Ni-NTA spin columns (Qiagen, CA). After washing, the bound protein was eluted using 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole pH 8. For protein concentration measurements, 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 (Pierce, Rockford, IL, USA) using BSA as reference, according to the manufacturers recommendations. 150 ng of each of the eluted proteins was loaded on a 12% polyacrylamide gel and the remainder was supplemented with glycerol to 30% and stored at –70 °C until used.

Enzymatic assays and GC-MS analysis

8.3 µg of each of the eluted enzyme proteins and 6 µg of the eluted negative control proteins were used in a total reaction volume of 1 ml of assay buffer containing 15 mM MOPSO

buffer (pH 7), 10% glycerol, 1mM ascorbic acid, 0.6 mM $MnCl_2$, 2 mM DTT and 10 µM GDP [14]. 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 was washed with 1.5 ml of diethyl ether and the organic fractions were pooled. For quantification, 10 ng of Δ-3- carene were added to the eluent as internal standard. 2 µl of each pentane/ diethyl ether fraction were injected into a HP 5890 series II gas chromatograph (Hewlett Packard, Agilent Technologies, Alpharetta, GA, USA) and an HP 5972A Mass Selective Detector essentially as described before (Bouwmeester et al., 1999). The gas chromatograph was equipped with a HP-5MS column (30 m x 0.25 mm i.d., 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.

MDGC-MS

The enantiomeric distribution of the limonene produced by all the parent and the active Cl(+)LIMS2/ClγTS chimeric enzymes, with Geranyl diphosphate assays, were analyzed using MDGC-MS. The MDGC-MS analyses were performed with a Fisons 8160 gas chromatograph (GC1) connected to a Fisons 8130 gas chromatograph (GC2) and a Fisons MD 800 quadrupole mass spectrometer and analyzed with Fisons MassLab software (Version 1.3)(Fisons, Manchester, UK). The system setup was as described previously (Lücker et al., 2001) but with the following settings. The fused silica capillary column in GC1 (J&W, Folsom, CA, USA) DB-Wax 20 M (25 m x 0.25 mm i.d.; film thickness = 0.25 µm) was maintained at 40 °C then programmed to 240 °C at 1 °C min^{-1} with He gas flow at 3 ml min^{-1}. The compounds of interest were transferred from GC1 to GC2 from 2 min to 15 min. The fused silica capillary column in GC2 (30 % 2,3-diethyl-6-*tert*-butyl-dimethyl-ß-cyclodextrin/PS086; 25 m x 0.25 mm i.d.; film thickness = 0.15 µm) was maintained at 40 °C (9 min) then programmed to 200 °C at 2 °C min^{-1} with He gas flow at 3 ml min^{-1}. The MS operating parameters were ionization voltage, 70 eV (electron impact ionization); ion source and interface temperature, 230 °C and 240 °C, respectively.

Results

Construction of the chimeric enzymes

The amino acid sequences of the truncated forms of monoterpene synthases Cl(-)ßPINS, ClγTS and Cl(+)LIMS2 were aligned with TEAS, from which a 3-dimensional structure is available, using the ClustalV algorithm (Figure1). We picked three restriction sites that are either conserved among the genes or could easily be introduced by mutagenesis without changing encoded amino acids, to define four domains for swapping. These domains and the relative homology between the proteins for each domain are shown in Figure 2. Domains I and II together overlap, though not completely, with the N-terminal structural domain of TEAS (Starks et al., 1997). The first 33 amino acids of domain III align with the N-terminal structural domain of TEAS, while the remaining amino acids correspond, along with domain IV, to the second, C-terminal, structural domain of TEAS. DNA fragments corresponding to these domains were swapped by restriction digestion and ligation in the corresponding position of the counterpart cDNAs and transformed to *E. coli*. Further DNA restriction digestion and sequencing revealed that all chimeric cDNAs with the intended swapped domain retained the correct full amino acid sequence and consequently the proteins of expected size (around 65 kDa) were produced upon induction (Figure 3). For all proteins, using GDP as a substrate, all detectable enzymatic products were identified and quantified using GC-MS analysis (Table 1).

Hybrid enzyme-catalyzed product formation

As expected, the β-pinene synthase, Cl(-)ßPINS, produced β-pinene as the major product and the γ-terpinene synthase, ClγTS, γ-terpinene (Figure 4). Both enzymes produced also a number of minor products partially unique for one of the two enzymes (Figure 4, Table 1). When domain I or domain II of Cl(-)ßPINS were separately substituted by their counterparts from ClγTS (chimeras 1 and 2 respectively), the product specificity and enzyme activity remained the same as in Cl(-)ßPINS. The exchange of domain I and domain II together had a similar effect (chimera 3), although with 2.5-fold decrease in the overall major product amount. The reverse substitution of domains I and II together in ClγTS with their counterparts from Cl(-)ßPINS did not change product specificity of ClγTS (chimera 4), γ-terpinene being the major product formed (Table 1).

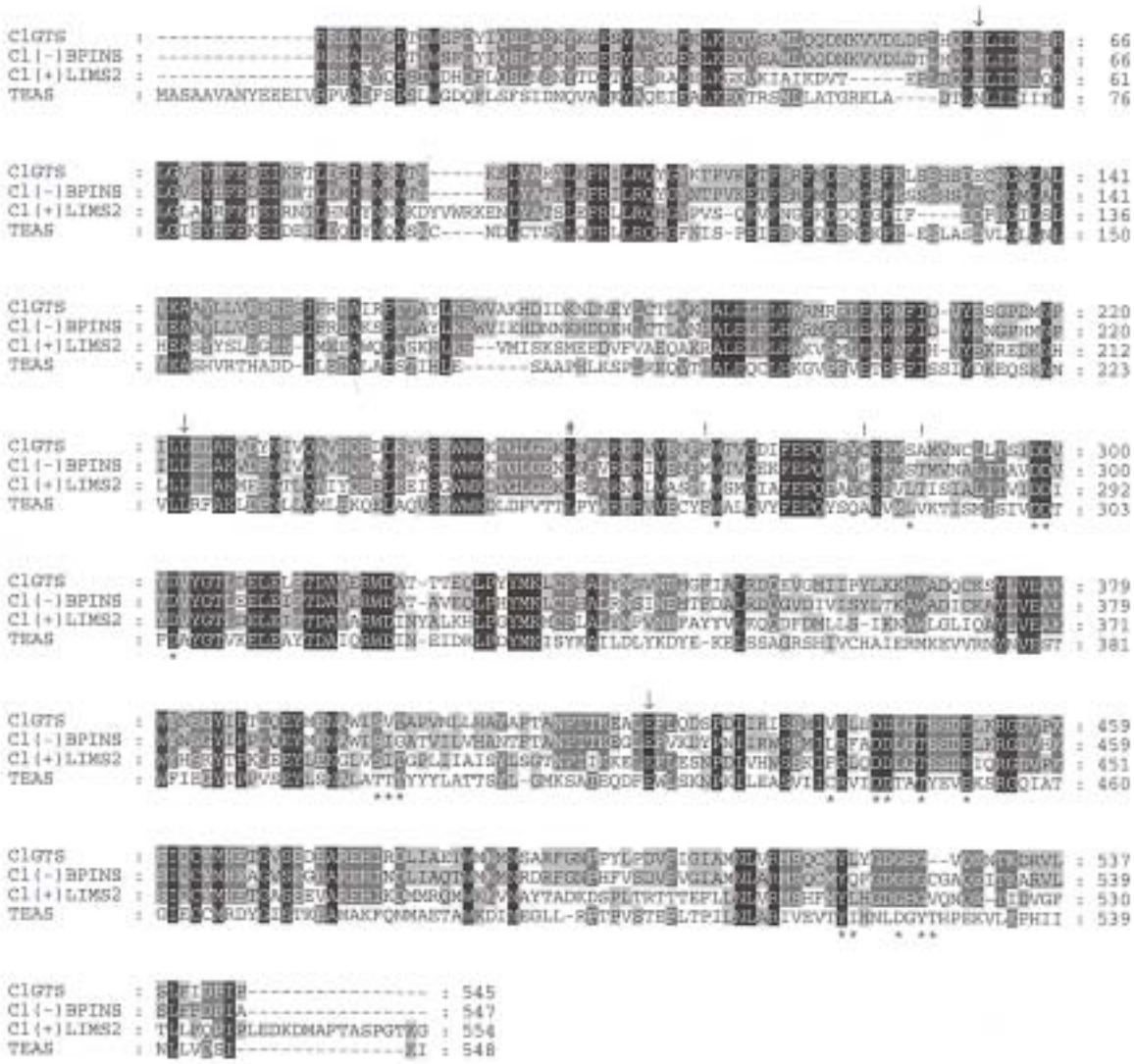


Figure 1: Protein alignment of TEAS, Cl(-)βPINS, ClγTS and Cl(+)LIMS2. Domain borders of the monoterpene synthases used in this study are indicated by ↓. The amino acid residues marked with an asterisk correspond to the ones in TEAS that were hypothesized by Starks *et al.* (1997) to be part of the active site in the catalysis leading to the formation of 5-epi-aristolochene. The borders between the two structural domains of TEAS are indicated by **#**, while amino acids marked by **!** are discussed in the text.

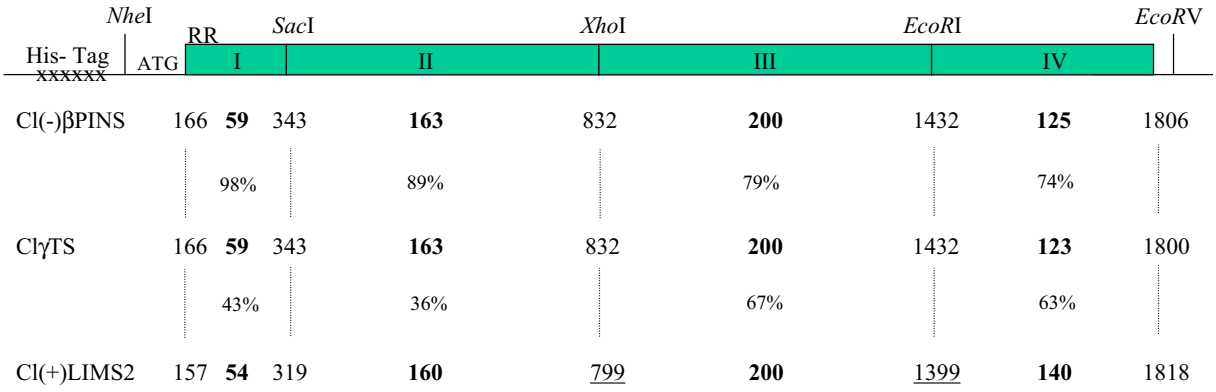


Figure 2: Primary structure of the 3 monoterpene synthases, Cl(-)βPINS, ClγTS and Cl(+)LIMS2, cloned in the pRSETB vector and divided into domains I to IV. The nucleotide positions bordering the domains are indicated. Whenever restriction sites were introduced by site-directed mutagenesis, these positions are underlined. The number of amino acids constituting each domain is given in bold face. The percentage of amino acid identity for each domain between two sets of enzymes (Cl(-)βPINS and ClγTS on the one hand, and ClγTS and Cl(+)LIMS2 on the other hand) is indicated.

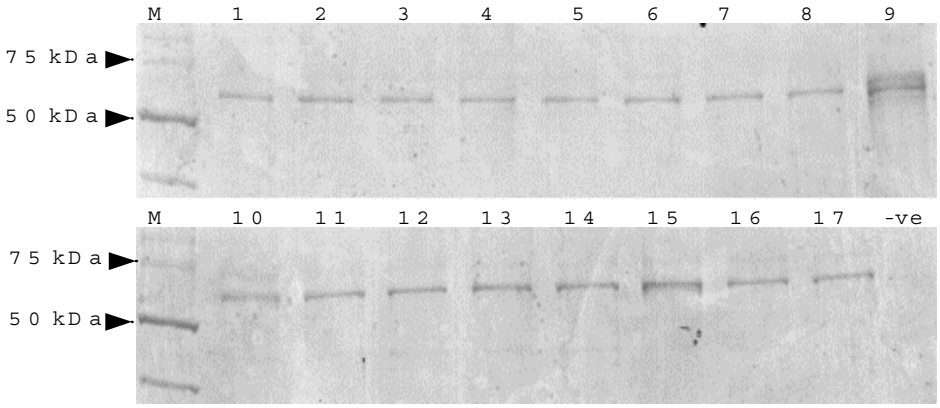


Figure 3. SDS- PAGE analysis of parental and chimeric genes expressed in *E. coli* and purified using Ni-NTA spin columns. M= Marker; lane 1=Cl(+)LIMS2; lane 2=Cl(-)βPINS; lane 3=ClγTS; lanes 4-17=Chimera 1-14. -ve= pRSETB negative control.

Table 1. Product formation of parent and chimeric enzymes generated by swapping of domains I to IV between lemon monoterpene synthases. The hatched columns depict domain composition for wild type β-pinene synthase (Cl(-)βPINS, γ-terpinene synthase (ClγTS), (+)-limonene synthase (Cl(+)LIMS2) and chimeric (1-14) monoterpene synthase cDNAs. Values for products formed are given in ng product.μg^{-1} eluted protein; <0.25=product formation was detectable but below 0.25ng/µg eluted protein; –=product not detectable. Values for limonene are in normal typeface for (-)-limonene and in bold for (+)-limonene.

Name	DI	DII	DIII	DIV	α-Thujene	α-Pinene	Sabinene	β-Pinene	Myrcene	α-Terpinene	ρ-Cymene	Limonene	γ-Terpinene	Terpinolene	α-Terpineol	Total
Cl(-)βPINS					-	2.5	5	30	-	-	-	**0.5**/ 4	-	-	1	43
1					-	2	4.5	28.5	-	-	-	4.25	-	-	-	39.25
2					-	2.5	5.5	29.5	-	-	-	4.25	-	-	-	41.75
3					-	1	1.5	8.5	-	-	-	3.5	-	-	<0.25	14.75
4					0.5	1	0.25	1.25	0.5	<0.25	<0.25	3.25	15	-	<0.25	22.25
5					1.5	1	0.5	1	-	<0.25	-	3.5	2	<0.25	<0.25	10
6					-	1	2.5	6	-	-	-	3.25	-	-	-	12.75
7					-	0.25	0.5	1	-	-	-	2.25	-	-	-	4
8					0.25	0.5	0.25	0.75	-	-	-	3	1.5	-	-	6.25
ClγTS					0.5	1.5	0.25	1.25	0.25	0.25	<0.25	**0.5**/2	16.25	1	0.5	24.25
9					-	-	-	-	-	-	-	-	-	-	-	
10					-	<0.25	-	-	-	-	-	**3.25**/ 0.75	-	-	-	4.25
11					-	<0.25	-	-	-	-	-	**1.5**/ 1	-	-	-	2.75
12					-	-	-	-	-	-	-	-	-	-	-	
13					-	-	-	-	-	-	-	-	-	-	-	
14					-	-	-	-	-	-	-	-	-	-	-	
Cl(+)LIMS2					-	<0.25	-	-	0.5	-	-	**24**	-	-	-	24.75

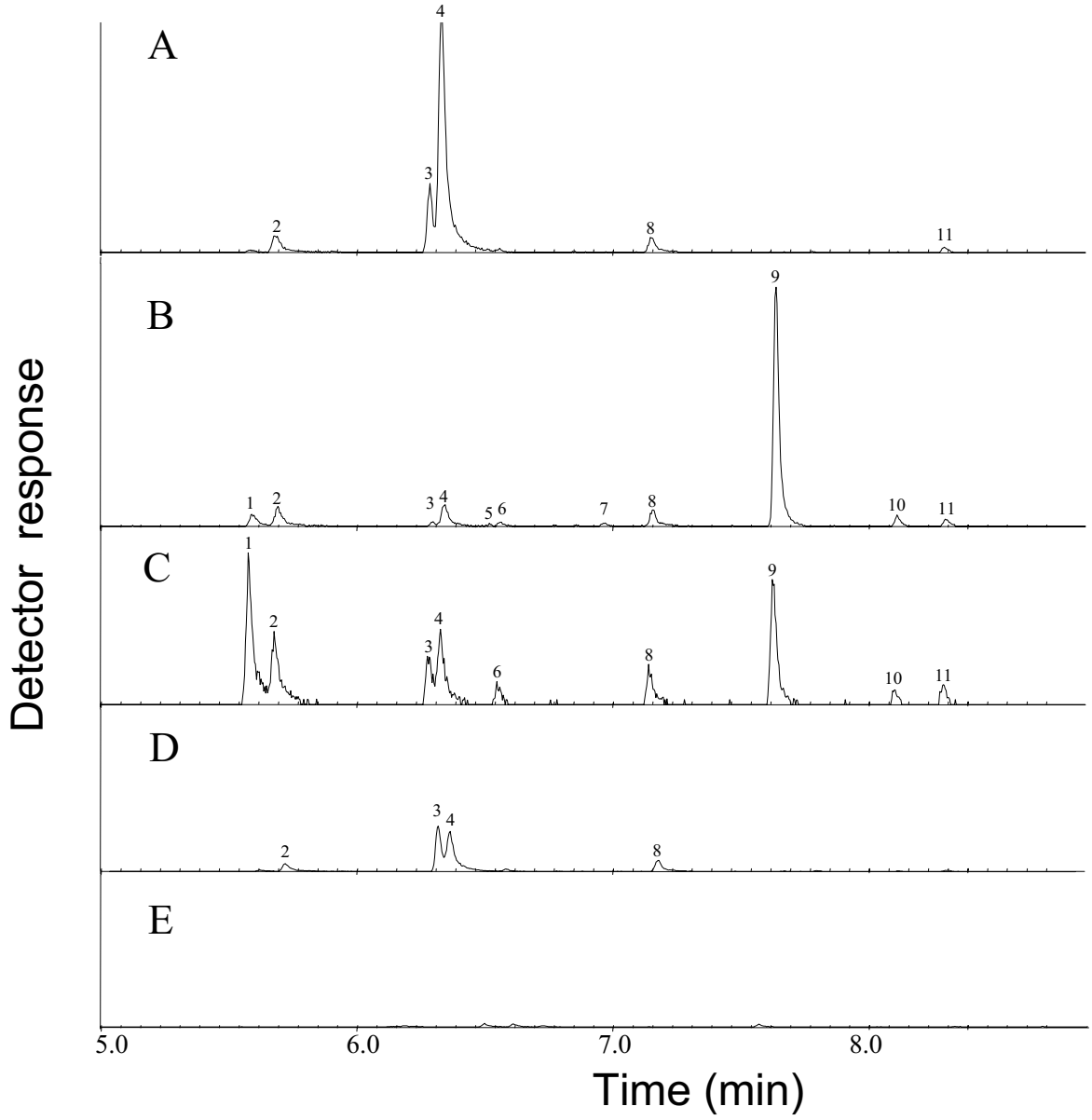


Figure 4: GC-MS chromatograms of *m/z* 93 showing the product profiles of Cl(-)βPINS (A), ClγTS (B), chimera 3 (C) and chimera 4 (D) and empty pRSETB vector negative control (E). Peak identities were confirmed using authentic standards. 1=α-thujene; 2=α-pinene; 3=sabinene; 4=β-pinene; 5=myrcene; 6=α-terpinene; 7=p-cymene; 8=limonene; 9=γ-terpinene; 10=α-terpinolene; 11=α-terpineol.

These results suggest that product specificity is predominantly determined by either the third or fourth domain, or both. The separate replacement of domains III or IV of Cl(-)ßPINS by their respective counterparts from ClγTS (chimeras 5 and 6) resulted in an overall decrease in enzyme activity (9- and 4-fold respectively). But only the replacement of domain III changed the product specificity to γ-terpinene (Figure 4; Table 1). Also, the pattern of minor products of chimera 5 were indicative of the specificity of the parent ClγTS (the presence of, for example, α-thujene, α-terpinene and α-terpinolene). This suggests that while both domains III and IV of ClγTS were required for optimal enzyme function, specificity for the production of γ-terpinene and other related minor products is predominantly determined by domain III. In the reaction catalyzed by chimera 7, where domain III of ClγTS was substituted by its counterpart from Cl(-)ßPINS, no formation of γ-terpinene was observed. In all parent and chimeric enzymes, limonene was always found as a side product.

MDGC-MS analysis confirmed that the limonene cyclase enzyme Cl(+)LIMS2 produced exclusively (+)- limonene as the major product of the enzymatic catalysis, in contrast to Cl(-)βPINS and ClγTS that produced mainly (-)-limonene as a side product and only a small amount of (+)-limonene (chapter 2) (Table 1). In the chimeric enzyme 10, substitution of ClγTS domain III and domain IV by their counterparts from Cl(+)LIMS2 changed product specificity to that of Cl(+)LIMS2, (+)-limonene being the major product formed, although with around 7-fold decrease in product formation. This decrease in enzyme efficiency was accompanied by some decrease in product specificity indicated by the formation of (-)-limonene. Substitution of only domain IV in ClγTS by domain IV from Cl(+)LIMS2 (chimera 11) also led to the formation of some (+)-limonene. Hence, also in these chimeras it seems that domains III and IV are predominantly determining product specificity, here the formation of (+)-limonene by Cl(+)LIMS2. All the remaining chimeric enzymes were inactive and no product was detected using GC-MS analysis.

Discussion

In the present study, we have demonstrated that in monoterpene synthases the C-terminal domains III and IV are essential for proper enzyme activity but that the γ-terpinene specificity of ClγTS is predominantly determined by domain III (see chimera 5 in Table 1). The data suggest that monoterpene synthase product specificity lies within the amino acids of domain III but that the ratio with which the different enzyme-specific monoterpenes, including minor products, are produced by the enzyme is co-determined by amino acids in the fourth domain. This supports the hypothesis that the active site of catalysis previously suggested for sesquiterpene synthases (Starks et al., 1997) is located in a similar region of the monoterpene synthases. Starks *et al*. obtained the 3D-structure of TEAS and have designated the amino acids that are likely to play a key role in the catalysis process leading to the formation of the sesquiterpene 5-*epi*- aristolochene from farnesyl diphosphate. These amino acids are Trp 273, Leu 290, Asp 301, Asp 302, Asp 305, Glu 379, Thr 403 (aligning with amino acids in domain III of the lemon monoterpene synthases) and Cys 440, Asp 444, Thr 448, Glu 452, Thr 519, Tyr 520, Asp 525 and Tyr 527 (aligning with amino acids in domain IV of the lemon monoterpene synthases) (Figure 1).

In contrast to ClγTS and Cl(-)ßPINS, the ClγTS and Cl(+)LIMS2 have a relatively low homology and belong to separate phylogenetic clusters (chapter 2). Domain substitutions between these two enzymes resulted in either a strong reduction in the enzyme efficiency or even complete loss of activity. Also in many of the other chimeras, enzymatic efficiency was reduced. This became more pronounced whenever either of the domains III or IV was substituted separately (Figure 4; Table 1). This suggests that, even though some or all of the amino acids in the first and second domain need not be directly involved in product specificity, proper interactions between amino acids from different domains is essential for enzyme function. A decrease in enzymatic activity was also described in many other studies, such as on site directed mutagenesis (Back and Chappell, 1996; Cane et al., 1996).

The outcome of the GDP cyclization process catalyzed by the monoterpene synthases is determined by the migration of the hydride and quenching of the carbocation in the enzyme-bound intermediate. For the formation of γ- terpinene, a mechanism was proposed by Croteau and co-workers (Loomis and Croteau, 1980) which involves the cyclization of the acyclic GDP, a 1,2- hydride shift (from C-4 to C-8) and the subsequent loss of a proton from C-5 to form the Δ^4 double bond (Figure 5). A similar mechanism, but with formation of a

cyclopropane ring and the loss of the proton from C-6 would yield one of the side products, α-thujene (Loomis and Croteau, 1980). The elimination of a proton from the α-terpinyl cation allows the formation of limonene (Cane et al., 1996). Both wild type Cl(-)ßPINS and ClγTS enzymes, and all their chimeric enzymes produced (-)-limonene as a side product at almost similar rate. If the cyclohexenyl double bond of the α-terpinyl cation undergoes an intramolecular Markovnikov addition, the pinyl cation is formed (Figure 5). Subsequent deprotonation of this pinyl cation produces β-pinene (Little and Croteau, 1999).

In terpene synthases, acidic or aromatic amino acids are known to stabilize positive charges and carbocationic intermediates, whenever positioned around the active site [10]. In the case of acidic amino acids, this is due to the presence of a carboxylate anion. In the case of aromatic amino acids, the π-electrons of the aromatic ring and the phenolic oxygen can readily accept a proton. Thus, they stabilize a cation and participate in the rearrangements of terpene structures (Starks et al., 1997; Dougherty, 1996; Bohlmann et al., 1998; Seemann et al., 2002; Maruyama et al., 2002). The amino acid residues in the enzyme that stabilize the specific position of the positive charge in the carbocationic intermediates are likely to be highly important for product outcome. The high level of amino acid identity between the β-pinene synthase and the γ-terpinene synthase in domain III (80%) forms a good lead to further explore the role of specific amino acids determining product specificity. Of the 41 amino acids of domain III that are different between these two cDNAs, only three (F269M, C283F and A288T) have different physico-chemical properties and are within 3Å of the modeled active site. Of these three, two are aromatic, have carbocationic stabilizing properties and can be involved in deprotonation. Interestingly, they are differentially positioned in the ClγTS and Cl(-)βPINS at positions 283 and 269, respectively (Figure 6). F283 in ClγTS, is substituted by the non-proton abstracting amino acid C283 in Cl(-)βPINS, whereas F269 in Cl(-)βPINS is substituted by M269 in ClγTS. These amino acid substitutions have moved the carbocation stabilizing property from one side of the active site to the other (Figure 6). If the initial spatial positioning of GDP in both enzymes is identical, this change may be responsible for the migration of the double bond in the α-terpinyl cation, resulting in a carbocation at C1 (the pinyl cation), hence allowing the formation of (-)-β-pinene instead of γ-terpinene (Figure 5). Site-directed mutagenesis of these two amino acids should confirm this hypothesis. In addition, in the absence of a crystal structure a domain shuffling approach might not only shed light on the biochemical mechanism of the specificity of monoterpene production by

monoterpene synthases but may also lead to the formation of new monoterpenes that may not arise in 'normal' enzymatic catalysis.

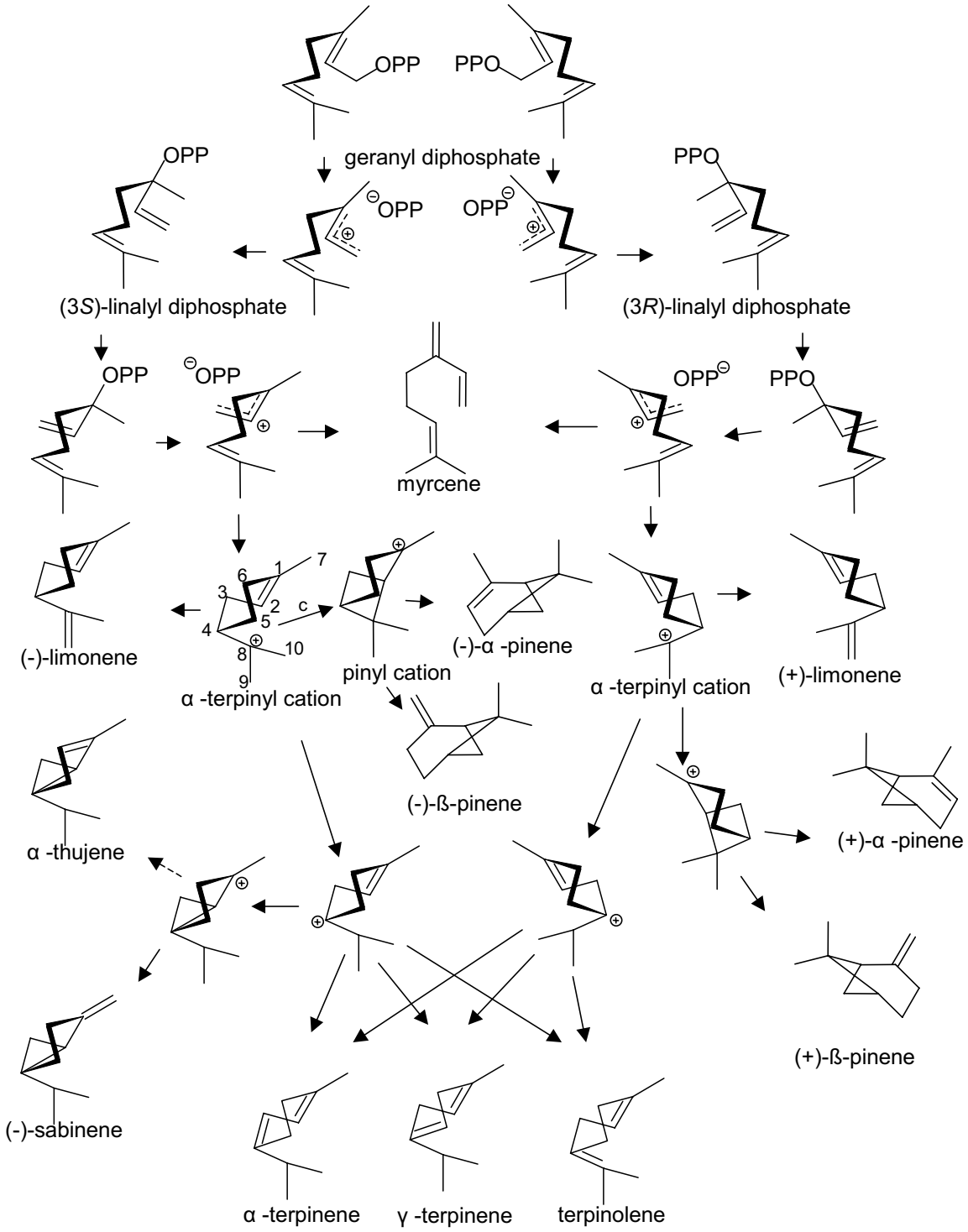


Figure 5. The cyclisation mechanism leading to (+)-limonene, β-pinene or γ-terpinene and other minor products catalyzed by Cl(-)PINS, ClγTS and Cl(+)LIMS2. Redrawn from Croteau et al. (1987).

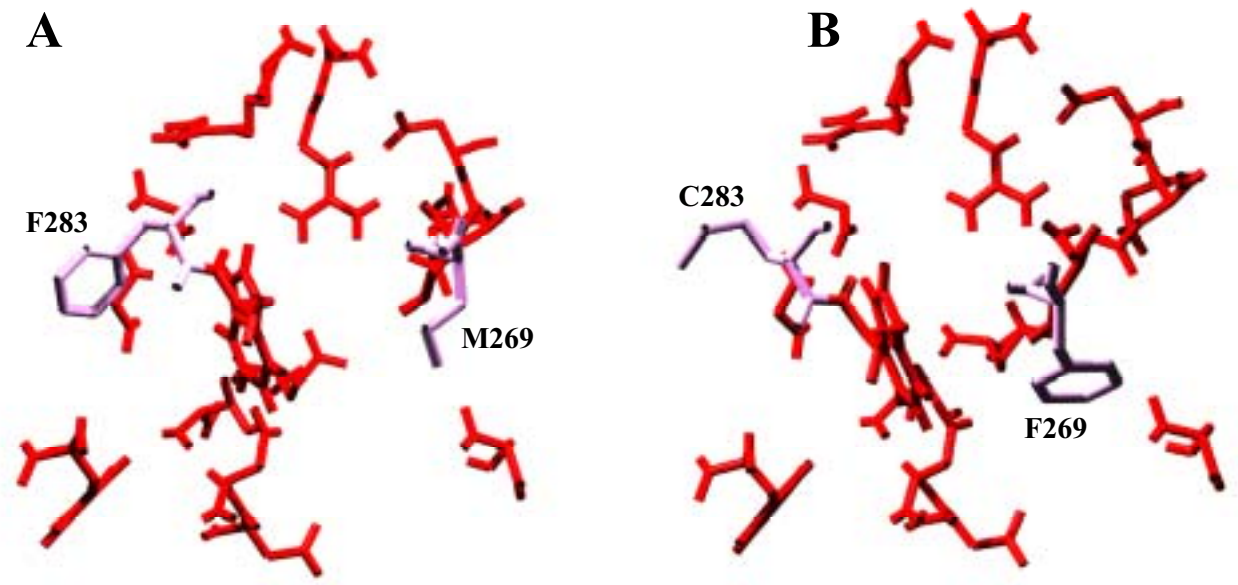


Figure 6. Protein modelling of the ClγTS (A) and the Cl(-)βPINS (B) proteins. The protein sequence of ClγTS and Cl(-)βPINS were modeled on the tobacco 5-epi-aristolochene synthase (TEAS) crystal structure. The amino acid residues shown here in gray were hypothesised by Starks and coworkers (1997) to be involved in the catalysis leading to the formation of 5-epi-aristolochene by TEAS and are mostly conserved in ClγTS and Cl(-)βPINS. Amino acids at positions 269 and 281 in ClγTS (A) and Cl(-)βPINS (B), shown here in white, are discussed in the text.

Acknowledgements

This research was supported by c-DLO Strategic Expertise Research grant. M.K.E.T was partly funded by a Dutch Ministry for Foreign Affairs grant. We like to thank Dr. N.G. Hogenboom for critically reading the manuscript, Dr. M.C.R Franssen for helpful suggestions, J. Blaas, M. Appel and F. Heckel for technical assistance and PPS-Mibiton for their contribution in the Biorobot.

References

Back K, Chappell J, Identifying functional domains within terpene cyclases using a domain-swapping strategy. Proc. Natl. Acad. Sci. 93 (1996) 6841-6845.

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

Bouwmeester HJ, Verstappen FWA, Posthumus MA, Dicke M, Spider mite induced (3*S*)-(*E*)-nerolidol synthase activity in cucumber and lima bean. Plant physiol. 121 (1999) 173-180.

Bouwmeester HJ, Kodde J, Verstappen FWA, Altug IG, de Kraker JW, Wallaart TE, Isolation and characterization of two germacrene A synthase cDNA clones from chicory. Plant Physiol. 129 (2002) 134-144.

Cane DE, Xue Q, Fitzsimons BC, Trichiodene synthase. Probing the role of the highly conserved aspartate-rich region by site directed mutagenenesis. Biochemistry 35 (1996) 12369-12376.

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

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

de Maagd RA, Kwa MSG, van der Klei H, Yamamoto T, Schipper B, Vlak JM, Stiekema WJ, Bosch D, Domain III substitution in *Bacillus thuringensis* delta- endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Applied and Environmental Microbiology 62 (1996) 1537-1543.

Dougherty DA, Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. Science 271 (1996) 163-168.

Guex N, Peitsch MC, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18 (1997) 2714-2723.

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

Gershenzon J, Kreis W, Biochemistry of terpenoids: monoterpenes, sesquiterpenes, diterpenes, sterols, cardiac glycosides and sterol saponins. in: M. Wink (Ed.), Biochemistry of Secondary Metabolism. CRC press, 1999, 222-299.

Little DB, Croteau R, in: Teranishi (Ed.) Flavor Chemistry: 30 years of progress. Kluwer Academic/Plenum Publishers, New York, 1999, pp 239-253.

Loomis WD, Croteau R, in: P.K. Stumpf (Ed.) The biochemistry of plants: A

comprehensive treatise. Vol. 4., Academic press, San Diego, 1980, pp. 363-386.

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

Maruyama T, Saeki D, Ito M, Honda G, Molecular cloning, functional expression and characterization of *d*-Limonene synthase from *Agastache rugosa*. Biol. Pharm. Bull. 25 (2002) 661-665.

Mok DVS, Mok MC, Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 52 (2001) 89-118.

Rademacher W, Growth Retardants: Effects on Gibberellin Biosynthesis and Other Metabolic Pathways. Ann. Rev. Plant Physiol. Plant Mol. Biol. 51 (2000) 501-531.

Rising KA, Starks CM, Noel JP, Chappell J, Demonstration of germacrene A as an intermediate in 5-epi-aristolochene synthase catalysis. J. Am. Chem. Soc. 122 (2000) 1861- 1866

Rynkiewicz MJ, Cane DE, Christianson DW, X-ray crystal structures of D100E trichiodene synthase and its pyrophosphate complex reveal the basis for product diversity. Biochemistry 12 (2002) 1732-1741.

Seemann M, Zhai G, de Kraker JW, Paschall CM, Christianson DW, Cane DE, Pentalenene synthase. Analysis of active site residues by site-directed mutagenesis. J. Am. Chem. Soc. 124 (2002) 7681-7689.

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

Trapp S, Croteau R, Defensive Resin Biosynthesis in Conifers. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 52 (2001) 689-724.

Chapter 4

Regeneration of *Citrus sinensis* (L.) Osbeck cv. Valencia Late: Essential factors involved

Mazen K. El Tamer[1], Ivan J. Tsvetkov[2], Waldo J. de Boer[3], Alphons G. J. Voragen[4], Harro J. Bouwmeester[1] & Frans A. Krens[5]

[1] Wageningen-UR, Plant Research International B.V, Business Unit Celcybernetics, Droevendaalsesteeg 1, Postbus 16, Wageningen, The Netherlands
[2] Institute of Genetic engineering, 2232- Kostinbrod-2, Bulgaria
[3] Wageningen-UR, Plant Research International B.V, BU Biometris, Bornsesteeg 47, 6708 PD, Wageningen, The Netherlands
[4] Wageningen-UR, Department of Agro Technology and Food Sciences, Laboratory of Food Chemistry, Bomenweg 2, Wageningen, The Netherlands
[5] Wageningen-UR, Plant Research International B.V, Business Unit Genetics and Breeding

Abstract

The regeneration potential of *Citrus sinensis* (L.) Osbeck cv. Valencia Late was studied focusing on the influence of the initial germination medium, explant type (source) and of the regeneration medium on shoot regeneration. Results show that the germination on MS30 (Murashige & Skoog based medium) and the regeneration on NNIII (Nitsch & Nitsch based medium) containing a 20:1 cytokinin: auxin ratio supplied with the riboside form of the benzyl amino purine cytokinin are essential for an efficient induction of shoot regeneration. In addition, hypocotyl-derived shoot regenerants incubated on NNRtVIII (Nitsch & Nitsch based medium) showed better rooting than epicotyl-derived shoot regenerants and incubation on other media tested.

Introduction

Citrus sinensis is of high commercial importance worldwide (FAO, 1993). The genus *Citrus* includes a number of species grown for fruit and/or juice products, among which is the orange *C. sinensis* L. Osbeck cv. Valencia Late (Hodgson, 1967).

The largest obstacle for *Citrus sp.* breeding is that, although there are several different *Citrus* genotypes being grown throughout the world, most of them originate from the same genetical background. These arose as chance seedlings or limb or bud mutants that were recognized to be horticulturally superior (Hodgson, 1967), or even interspecific hybrids that underwent somatic mutations and were vegetatively propagated (Scora, 1975; Barrett & Rhodes, 1976; Bowman & Gmitter, 1990). Most *Citrus* scion and rootstock cultivars have not been produced through conventional breeding due to the polyembryonic nature of *Citrus*, because of the presence of nucellar embryos in most *Citrus* species. These adventitious embryos compete with the zygotic embryo for space and nutrients, and frequently the zygotic embryo does not survive (Frost and Soost, 1968; Soost and Cameron, 1975). Thus, the genetic basis of modern *Citrus* cultivars is rather narrow, which makes *Citrus* cultivation very vulnerable to, for example, changes in pathogen virulence or pest outbreaks. In addition, within a species, it is difficult to find enough genetic variation to develop new genotypes adapted to adverse environmental conditions such as saline soils or low-input cultivation systems. On top of this, expanding the genetic variation by interspecific hybridisation may result in the transfer of undesirable traits along with the beneficial ones.

Moreover, many *Citrus* cultivars, which were mainly selected for fruit traits, lack a good root system, which negatively effects the growth rate and disease resistance to soil born pathogens and adaptation to less favorable soil conditions (De Pasquale et al., 1999). Genetic engineering techniques could be useful to produce new *Citrus* varieties and allow the rapid introduction of desirable traits for example, *Citrus sinensis* L. Osbeck cv. Valencia Late and other orange varieties. Many groups have developed a transformation protocol to introduce genes conferring desired properties into these *Citrus* species (Moore et al., 1992; Peña et al., 1995; Yao et al., 1996; Gutierrez et al., 1997; Bond & Roose, 1998; Cervera et al., 1998; Luth & Moore, 1999; Ghorbel et al., 1999; Domingez et al., 2000; Peña et al., 2001), however at low frequencies. A successful regeneration system either through organogenesis or through somatic embryogenesis is an essential preliminary step to develop a more efficient transformation system (Litz et al., 1985; Gmitter et al., 1992).

Several methods of adventitious shoot regeneration have been described (Moore et al. 1992, Peña et al. 1995, Bond & Roose, 1998) and also the induction of somatic embryogenesis in *Citrus* sp. from undeveloped ovules (Kunitake et al. 1991). Furthermore, rooting has been troublesome in the regeneration of *Citrus* and therefore minigrafting was commonly used as a way to rescue shoot regenerants (De Pasquale et al. 1999). In conclusion, further optimization is necessary in order to obtain transgenic *Citrus* plants with a high frequency. In this paper, we describe the effects of the germination medium on which isolated seed embryos were originally grown to collect seedling explants, the explant type and the regeneration medium on the frequency of shoot regeneration. Interestingly, the study shows that the germination medium had a positive effect on later regeneration of both hypocotyl and epicotyl explants. In addition, we also report on an efficient shoot regeneration medium and on an efficient rooting medium of shoot regenerants.

Materials and methods

Plant material

Mature *Citrus sinensis* (L.) Osbeck cv. Valencia Late seeds were removed from fresh fruits and surface sterilized by rinsing in 70% (v/v) ethanol for 1 min followed by 2% (w/v) $NaHClO_3$ in de-mineralized water with a droplet of Tween 20 for 15 min. Seeds were subsequently rinsed three times in sterile distilled water for 10 min and cold pre-treated by incubation at 4^{o}C for 24 hours prior to further handling.

Subsequently, the seed coats were removed aseptically and the intact embryos collected and placed on either MS30 or MSNv germination medium. All the media used in this experiment are summarized in Table 1. Isolated embryos were then incubated in the dark at 27 ^{o} C for 14 days, prior to harvesting hypocotyl and epicotyl stem segments.

Etiolated 0.5 cm hypocotyls and epicotyl stem segments were dissected from 14-day old seedlings and pre-cultured horizontally on either an MS-based (Murashige & Skoog, 1962) medium, MSJB, or a Nitsch & Nitsch-based (Nitsch & Nitsch, 1969) medium, NN III. Petridishes were sealed with parafilm and the cultures maintained at 25 ^{0}C with a 16 hour photoperiod (70 umol.m^{-2}.s^{-1}). After 40 days, the shoot regeneration from the epicotyl and hypcotyl stem segments of *Citrus sinensis* (L.) Ozbeck cv. Valencia Late was assessed on NNIII and MSJB media. The shoot regenerants originating from epicotyls and hypocotyls were transferred onto either NNRt III, NNRt VII, NNRt VIII rooting media or liquid NN-perlite (simulating more closely soil conditions) and cultured for 30 days at 25 ^{0}C with a 16 hour photoperiod (70 umol.m^{-2}.s^{-1}). The rooting frequency of shoot regenerants was then recorded.

Table 1. Different media used in the experiment; MS30 and MSNv were used as germination media. MSJB and NNIII were used as regeneration media. NNRtIII, NNRtVII, NNRtVIII and NN-perlite were used as rooting media. Ac. charcoal= active charcoal

Medium	Auxin ($mg.l^{--1)}$	Cytokinin ($mg.l^{--1}$)	Gibberellin ($mg.l^{-1}$)	Supplement ($mg.l^{-1}$)	Vitamins/ Carbon source ($g.l^{-1)}$	pH/ Gelling agent ($g.l^{-1}$)
MS30					MS Sucrose 30	5.7 Gelrite 4
MSNv	IAA 4	Kinetin 0.4	GA_3 0.7	Ac. charcoal 3000	NN Sucrose 30	5.7 Gelrite 4
MSJB	NAA 0.1	BAP 5			MS Sucrose 30	5.7 Gelrite 4
NNIII	NAA 0.3	BAP-rib 6			NN Sucrose 30	5.7 Gelrite 4
NNRtIII	IBA 0.2		GA_3 0.6		NN Sucrose 20	5.8 Gelrite 3
NNRtVII	IBA 0.2		GA_3 1	Ac. charcoal 2000	NN Sucrose 20	5.8 Gelrite 3
NNRtVIII	IBA 0.2	BAP-rib 1	GA_3 1	Ac. charcoal 2000	NN Sucrose 20	5.8 Gelrite 3
NN-perlite					NN Sucrose 20	5.7 Perlite

Statistical analysis

In each experiment let n be the binomial total (number of plantlets), y, the number of successes (e.g. successful rooting, regeneration) and p, the probability of a successful event.

Probability p is the quantity of interest in the analyses. For p, a binomial distribution is assumed. Binomial data are analyzed using a generalized linear model (McCullagh and Nelder, 1989) which is an extension of the linear model. The generalized linear model applies for response variables that are not normally distributed. An appropriate transformation (called the link function) provides that the linear model holds. By specifying the error distribution, the variance is stabilized. When the response data are proportions, it will usually be appropriate to use the logit function, which conveniently stretches the interval (0, 1) to the whole real line $(-\infty; \infty)$. Effects and interactions are introduced on a logit scale:

$$\text{logit}(p) = \log(p/(1-p)) = \text{grand mean} + \text{main effects} + \text{interactions}$$

An important property of the binomial distribution is that the variance depends on mean p, except for a multiplicative constant φ, called the dispersion factor:

$$\text{var}(y) = \varphi V(p)$$

Usually the variance of y exceeds the nominal variance and dispersion factor $\varphi > 1$. Overdispersion is the norm in practice and nominal dispersion is the exception. In case of overdispersion, the variance is inflated by the dispersion factor φ. Another phenomenon is underdispersion ($\varphi < 1$) which occurs in case of competition. Means or predictions are calculated using the model. All effects were considered to be significant at $P \leq 0.05$ and pairwise differences were obtained using the t-test. All calculations were performed using the statistical program GenStat (2000).

Results and Discussion

The germination frequency of 24h cold pre-treated seed-derived embryos and whole seeds grown on MS30 or MSNv was 90%, in both cases (Figure 1).

Shoot regeneration

After one week of culture, the explants turned dark green and after 14 days, in the case of hypocotyls, and 21 days in the case of epicotyls, shoot formation was clearly visible from the cut ends without callus formation (Figure 2) and the shoot regeneration frequency was recorded on day 40. We investigated the effect of the germination medium (MS30 or MSNv), explant source (hypocotyls or epicotyls) and regeneration medium (MSJB and NNIII on the regeneration potential of explants.

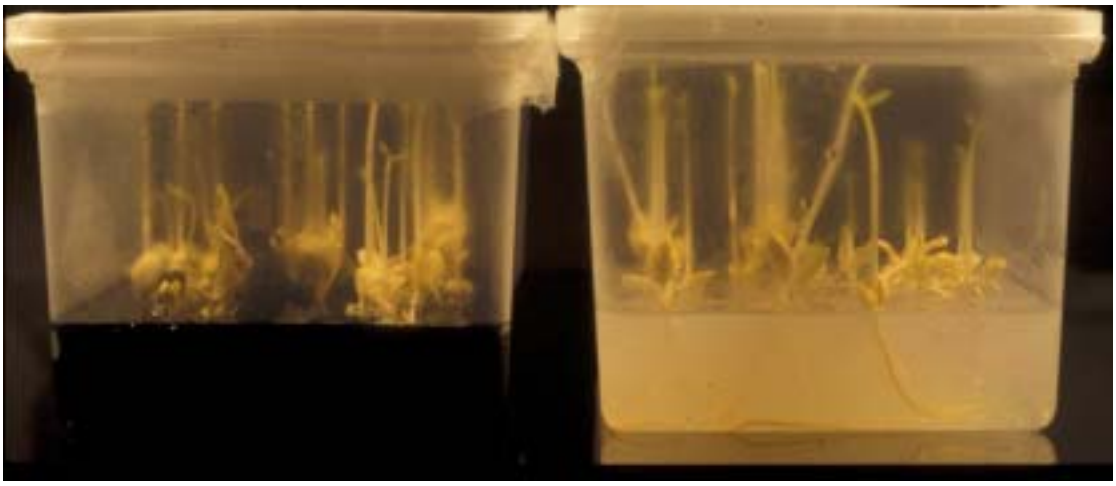


Figure 1. Etiolated seedlings resulting from the germination of de-coated, cold pre-treated *Citrus sinensis* L. Osbeck cv. Valencia Late seeds on MS30 (right) and MSNv (left) following 14 days of incubation at 27 ^{o}C in the dark.

Figure 2. Regeneration of *Citrus* sp. epicotyl stem segments by direct shoot organogenesis after 40 days incubation on NN III

The uptake of auxins, cytokinins and gibberellins present in both germination media is easily feasible because of seed coat removal. Although the role of the vitamins can not be excluded, it is surprising that the presence of hormones in the germination medium has a negative influence on later regeneration. Whenever compared to MSNv, the MS30 germination medium had a positive effect on later regeneration of both hypocotyls and epicotyls in the regeneration media MSJB as well as in NNIII (Table 2). Compared to MS30, there is a decrease of 30-50% of the regeneration frequency of hypocotyls and epicotyls cultured on both MSJB and NNIII media, whenever explants had originated from seedlings that germinated on MSNv. This is an interesting finding and to our knowledge, no such correlation was ever reported.

Table 2. Frequency of shoot regeneration resulting from different treatments.

Germination medium	Explants source	Number explants	Regeneration medium	Regeneration freequency (%)
MS30				
	Hypocotyl			
		10	MSJB	8 (80)
		19	NNIII	17 (89.4)
	Epicotyl			
		40	MSJB	21 (52.5)
		40	NNIII	34 (85)
MSNv				
	Hypocotyl			
		10	MSJB	3 (30)
		10	NNIII	4 (40)
	Epicotyl			
		40	MSJB	9 (22.5)
		40	NNIIII	11 (27.5)

In addition, the calculated prediction means for regeneration frequencies on media MSJB and NNIII (independent of the explant source) based on the model were 42.4 ± 4.4 and 59.5 ± 4.2 respectively, with P= 0.05 and φ =1.4, suggesting that NNIII is promoting shoot regeneration

more than MSJB**.** Hypocotyls have a slightly higher regeneration frequency than epicotyl stem segments, but this is not significant**.**

It was previously shown that upon the induction of somatic embryogenesis leading to plant regeneration, the endogenous level of the cytokinin zeatin-riboside of embryogenic callus cultures was higher (Jimenez et al., 2001).

For the regeneration of transformed *Citrus* stem segments, benzyl amino purine (BAP) is a cytokinin very commonly used (Peña et al., 1995; Peña et al., 1997; Bond & Roose, 1998; Cervera et al., 1998; Luth & Moore, 1999; Domínguez et al., 2000). When testing other media and conditions, none were better in our hands than the ones presented here, however, the results do seem to suggest that the riboside form of BAP might be an important factor (data not shown) in the regeneration efficiency, although other factors such as salts, vitamins and the concentration of NAA cannot be excluded**.**

Rooting

The rooting frequency was evaluated after 30 days of incubation of shoot regenerants on different rooting media. NNRtVIII had the highest effect on the rooting potential of shoot regenerants (Table 3, Fig.3).

Incubating *Citrus* sp. shoot regenerants on an auxin-supplemented medium void of cytokinins or with 5-fold decrease in cytokinin, and with gibberellic acid contribute to the formation of roots (Bouza et al., 1994). GA_3 is known to accelerate the growth and differentiation of plant cells and has been described to promote developmental transitions (Haddon & Northcote, 1976; Kononowicz et al., 1982, Ogas et al., 1997).

No effect of medium was found ($P > 0.23$), where four different ones were tested, whereas the source of the explant (hypocotyl or epicotyl) had a significant effect. The rooting medium NNRtVIII had the highest effect on the rooting ability of shoot regenerants especially for hypocotyls where the rooting frequency was 44%. The dispersion parameter φ was 0.67 suggesting that for root regeneration there was competition and nutrient exhaustion. Maybe using bigger culture pots could have prevented this. Predictions for medium were, NNRtIII 14.3 ±9.2, Perlite 14.3 ± 9.2, NNRtVII 29.0 ± 4.5 and NNRtVIII 35.0 ± 4.7. The probabilities of the largest pairwise differences were about equal to $P= 0.16$.

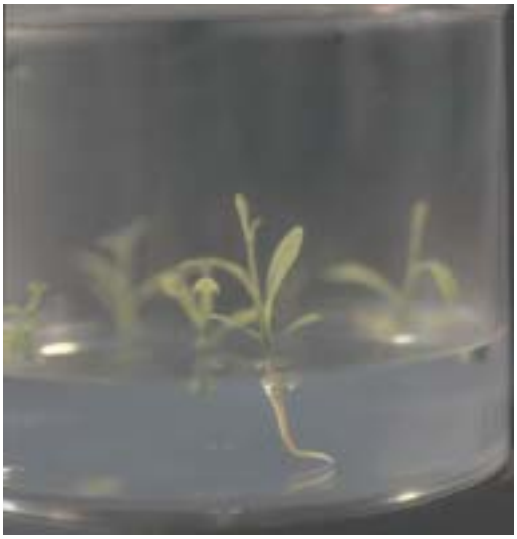


Figure 3. Rooting of shoot regenerants after incubation for 30 days at 25 ^{0}C with a 16-hour photoperiod (70 umol.m^{-2}.s^{-1}) on NNRtIII.

Table 3. Frequency of shoot regenerants originating either from hypocotyl or epicotyl stem segments and rooting on different media.

Explant source	Number of shoot explants	Rooting medium	Rooting freequency (%)
Hypocotyl	7	NNRtIII	1 (14.2)
	50	NNRtVII	19 (38)
	50	NNRtVIII	22 (44)
	7	NN-perlite	2 (28.5)
Epicotyl	7	NNRtIII	1 (14.2)
	50	NNRtVII	10 (20)
	50	NNRtVIII	13 (26)
	7	NN-perlite	-

The results also suggest that the explant type (or source) had a significant effect on the rooting potential of shoot regenerants (P=0.004). The rooting prediction for hypocotyls is significantly higher than for epicotyls, 38.6 ± 4.5 and 21.1 ± 3.8, respectively.

Our results show the influence of growth regulators from the germination medium by the seed- derived embryo on the regeneration potential of hypocotyl and epicotyl explants. An endogenous cellular hormonal imbalance in the seed germination and seedling development stage will result, at a later stage, in lower explant regeneration potential. In contrast to an original hormone- free germination medium (MS 30), there is a decrease in regeneration

potential of up to 57.5% for epicotyls and up to 50% decrease for hypocotyls, whenever auxins, cytokinins and giberellins are added to the germination medium.

In addition, the results confirm the positive effect of a high cytokinin/auxin ratio (20: 1) on the regeneration of shoots from hypocotyls and epicotyls. While high cytokinin concentration in the growth medium seems to induce shoot formation, a moderate amount of auxin is enough to trigger root formation. It is known that cytokinins promote shoot meristem development (Grayburn et al. 1982). The present results show that a decrease of the cytokinin: auxin ratio (5:1) and an increase of endogenous giberellins (GA_3) to the same level as cytokinin promotes the rooting of the explants, especially in hypocotyl- derived explants.

Furthermore, we describe the successful rooting of shoot regenerants (44%) and show that hypocotyl- derived shoot regenerants display a higher rooting reactivity than the epicotyl ones, suggesting a more flexible endogenous balance within hypocotyl- derived shoots. However, only a fraction of the rooted regenerants could be successfully grown to mature plants in the greenhouse. The plants that survived did not differ phenotypically from seed-derived plantlets. Further investigation on suitable plant nutritional balance under greenhouse conditions for a proper survival rate is necessary. In conclusion, the results of our studies on the effect of the germination medium, the explant type, and the medium to enhance the rooting frequencies show that all these factors might contribute positively to the generation of transgenic *Citrus* plants.

Acknowledgements

The authors are grateful to Prof. Arjen van Tunen for his contribution and suggestions, and to Dr. Elma Salentijn for her assistance.

References

Barrett HC & Rhodes AM (1976) A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. *Syst Bot* (**1**): 105-136

Bond J.E & Roose M.L (1998) Agrobacterium- mediated transformation of the commercially important citrus cultivar Washington navel orange. *Plant Cell reports* (**18**): 229-234

Bouza L, Jacques M, Sotta B & Miginiac E (1994) Relations between auxin and cytokinin contents and in vitro rooting of tree Peony (*Paeonia suffruticosa* Andr.). *Plant Growth Regul*. **15** (1): 69-73

Bowman KD & Gmitter FG (1990) Forbidden fruit (*Citrus* sp., Rutaceae) rediscovered in Saint Lucia. *Econ Bot* (**44**): 165-173

Cervera M, Juarez J, Navarro A, Pina JA, Duran-Vila N, Navarro L & Peña L (1998) Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. *Transgenic Res*. (**7**): 51-59

De Pasquale F, Giuffrida S, Carimi F (1999) Minigrafting of shoots, roots, inverted roots, and somatic embryos for rescue of in vitro Citrus regenerants. *J. Amer. Soc. Hort. Sci.* **124**(2): 152- 157

Domíngez A, Guerri J, Cambra M, Navarro L, Moreno P & Peña L (2000) Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep* (**19**): 427-433

Food and Agriculture organization of the United Nations. *Citrus fruit fresh and processed*. Annual statistics, 1993.

Frost, HB & Soost, RK (1968) *Seed reproduction: Development of gametes and embryos*. The Citrus industry, Vol. 2, University of California Press, Berkley: 292-334

Ghorbel R, Juarez J, Navarro L & Pena L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants. *Theor appl genet* **99** (1): 350-358

Gmitter FG, Grosser JA & Moore GA (1992) *Citrus. Biotechnology of perennial fruit crops*. CAB International, Wallingford, Oxon, UK: 335-369

Grayburn WS, Green P.B, Steucek G (1982) Bud induction with cytokinin. A local response to application *Graptopetalum paraquayense*. *Plant Physiol* **69**(3): 682-686

Gutiérrez- E MA, Luth D & Moore GA (1997) Factors affecting Agrobacterium-

mediated transformation in *Citrus* and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep* (**16**): 745-753

Haddon L & Northcote DH (1976) The influence of gibberellic acid and abscisic acid on cell and tissue differentiation of bean callus. J Cell Sci 20(1): 47-55

Hodgson, RW (1967) *Horticultural varieties of Citrus*. The Citrus industry Vol. 1 University of California Press. Berkley: 431-591

Jimenez VM, Guevara E, Herrera J & Bangerth F (2001) Endogenous hormone Levels in habituated nucellar Citrus callus during the initial stages of regeneration. *Plant Cell Reports* **20** (1): 92-100

Kononowicz H, Kononowicz AK, Wasilewska-Dabrowska LD & Kleckowski K (1982) Cytomorphological and biochemical changes in dwarf pea shoots induced by gibberellic acid. *Int. J Biochem* **14**(5): 421-428

Kunitake H, Kagami H, Mii M (1991) Somatic embryogenesis and plant Litz RE, regeneration from protoplasts of 'Satsuma' mandarin (*Citrus unshiu* Marc). *Sci. Hort* (**47**): 27-33

Luth D & Moore G (1999) Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*- mediated transformation. *Plant Cell, Tissue and Organ Culture* (**57**): 219-222

McCullagh P & Nelder JA (1989) *Generalized Linear Models*, 2e edition. Chapman and Hall, London.

Moore GA, Jacono CC, Neidigh JL, Lawrence S.D, Kline K (1992) Agrobacterium- mediated transformation of Citrus stem segments and regeneration of transgenic plants. *Plant Cell reports* (**11**): 238-242

Murashige T & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue culture. *Physiol Plant* (**15**): 473-497

Nitsch J.P, Nitsch C (1969) Haploid plants from pollen grains. *Science* (**163**): 85-87

Ogas J, Cheng JC, Sung ZR & Somerville C (1997) Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana* pickle mutant. *Science* (**277**): 91-4

Peña L, Cervera M, Juárez J, Ortega C, Pina J.A, Durán- Villa N, Navarro L (1995) High efficiency Agrobacterium- mediated transformation and regeneration of Citrus. *Plant Science* (**104**): 183-191

Peña L, Martín- Trillo M, Juárez J, Pina JA, Navarro L & Martínez- Zapater JM (2001) Constitutive expression of Arabidopsis *LEAFY* or *APETALA1* genes in citrus reduces their generation time. *Nature Biotechnology* (**19**): 263-267

Scora, RW (1975) On the history and origins of Citrus. *Bull Torrey Bot Club* (**102**): 369-375

Soost RK & Cameron JW (1975) *Citrus. Advances in fruit breeding*. Purdue University, West Lafayette. Indiana: 507-540

Yao JL, Wu JH, Gleave AP & Morris BAM (1996) Transformation of Citrus embryogenic cells using particle bombardment and production of transgenic embryos. *Plant Science* (**113**): 175-183

Chapter 5

The influence of monoterpene synthase transformation on the odour of tobacco

Mazen K. El Tamer,[1] Monique Smeets,[2] Holthuysen N,[2] Joost Lücker,[1] Ameing Tang,[3] Jacques Roozen,[2,3] Harro J. Bouwmeester[1] & Alphons G.J Voragen[3]

[1]Wageningen-UR, Plant Research International B.V, Droevendaalsesteeg 1, Postbus 16, Wageningen, The Netherlands

[2]Wageningen-UR, ATO B.V, Bornsesteeg 59, Postbus 17, Wageningen, The Netherlands

[3]Wageningen-UR, Department of Agro Technology and Food Sciences, Laboratory of Food Chemistry, Bomenweg 2, Postbus 8129, Wageningen, The Netherlands

Abstract

Monoterpenes are an important class of terpenoids that are commonly present in plant essential oils. These can be extracted from plants and are commonly used in the flavouring and the perfumery industry. Monoterpene synthases are the key enzymes in the monoterpene biosynthetic pathway, as they catalyse the cyclisation of the ubiquitous geranyl diphosphate to the specific monoterpene skeletons. The original aim of our work was to prove the concept that the transformation of monoterpene synthase cDNAs to *Citrus sinensis* leads to changes in the fragrance perception. However, the currently available citrus transformation systems are lengthy and can be troublesome. Tobacco is one of the most studied model plants, easily and efficiently transformed, and is a suitable model to study the release of plant volatiles. Thus, we have isolated monoterpene synthases from lemon, transformed tobacco with these cDNAs and have used human panelists to study the change in fragrance of the transgenic in comparison to the wild type plants. In a triangle test, we found that subjects were capable of smelling significant differences between leaf samples. However, as a result of variability in panel ratings, no significant difference between two sets of transgenic flowers and the wild type SR1 tobacco flowers was found for any of the generated attributes in a descriptive test.

Introduction

The largest class of plant secondary metabolites is that of the terpenoids (or terpenes). Over 36,000 individual structures of this class have been reported (Hill, 2002). Terpenes determine the fragrance of a considerable number of plants and they are commonly present in essential oils (Knudsen et al., 1993; McGarvey & Croteau, 1995). Terpenes are produced by a range of plant organs and play diverse functional roles. They are important for the interaction between the plant and its environment such as for defence against herbivores and pathogens (Mazzanti et al., 1998; Bouwmeester et al., 1999; Senatore et al., 2000), and as attractants for pollinators and seed dispersers (McGarvey and Croteau, 1995; Kosolova et al., 2001; Pichersky & Gershenzon, 2002). In addition, terpenes protect leaves against high-temperature stress damage by enhancing membrane stability under heat stress (Kreuzwieser et al., 1999). Moreover, the terpene essential oil has been shown to have an influence on human behaviour (Ilmberger, 2001). For example, the monoterpenes are of high economic value as they are widely used in products of the flavouring, the perfumery, food and drink, detergent and the cosmetics industry (Verlet, 1993). Monoterpenes have a typical smell and some, for example limonene, display cancer chemoprevention properties (Crowell, 1994; Bardon, 1998).

Terpenes have a unique structure: they consist of an integral number of five-carbon (isoprene) units. Two such units can form a monoterpene (C_{10}), but sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenoids ($>C_{40}$) are also possible (for example rubber) (Gershenzon & Croteau, 1993). Primary monoterpenes are formed from the general precursor geranyl diphosphate (GDP) through the action of monoterpene synthases (Croteau, 1987). Different cyclisation processes catalysed by monoterpene synthases lead to the formation of various monoterpenes (Figure 1). Several monoterpene synthases are able to produce more than one product (McGarvey and Croteau, 1995; Kreuzwieser et al., 1999). Subsequent transformations of the basic skeletons that strongly increase the structural diversity of the monoterpenes include oxidations, reductions and isomerizations (Wust & Croteau, 2002).

For many years, the research into flower fragrance focused on elucidation of structure, coupled with chemical synthesis and production of the large quantities demanded by the industry (Lavy, 2002). Indeed thousands of structures are known (Knudsen et al., 1993; de

Luca & st Pierre, 2000), however, the biochemical pathways leading to their production have been studied in less detail (Dudareva et al., 2000). By introducing genes encoding monoterpene synthases, some research groups have reported on the formation of new monoterpenes or on the increase of already existing terpenes in transgenic plants (Lewinsohn et al., 2001; Lücker et al., 2001; Lavy et al., 2002). To date, none of these studies have reported on the successful alteration of fragrance in plants.

Tobacco is one the world's most studied crop plants, not only because of its economic importance but also because of human health concerning smoking. In addition, the genetics and chemistry of tobacco are well known and hence these plant species are suitable for biochemical and molecular biological studies (Loughrin, 1990). While emission of monoterpenes in rose flowers follows a diurnal circadian rhythm as was shown by Helsper et al. (1998), observations with Nicotiana sylvestris indicate that terpenoid emission does not show circadian rythmicity (Loughrin et al., 1991). In Nicotiana tabacum, the emission of volatiles from leaves was reported to be 30 to 100-fold lower than that from flowers (Andersen et al., 1988). Thus, tobacco offers a suitable model to study the release and perception of plant volatiles through a transgenic approach. Recently, three Citrus limon genes coding for the enzymes γ-terpinene synthase (Genebank accession number: AF514286), (+)-limonene synthase (AF514287) and β- pinene synthase (AF514288) were transformed either separately or together to Nicotiana tabacum 'Petite Havana' SR1 (Lücker et al., submitted). GC-MS analyses showed that the terpenoids expected were produced and emitted by leaves and flowers of the transgenic plants.

In the present study, we investigate the effect of these transgenes on fragrance using human panellists in a triangle and a descriptive test.

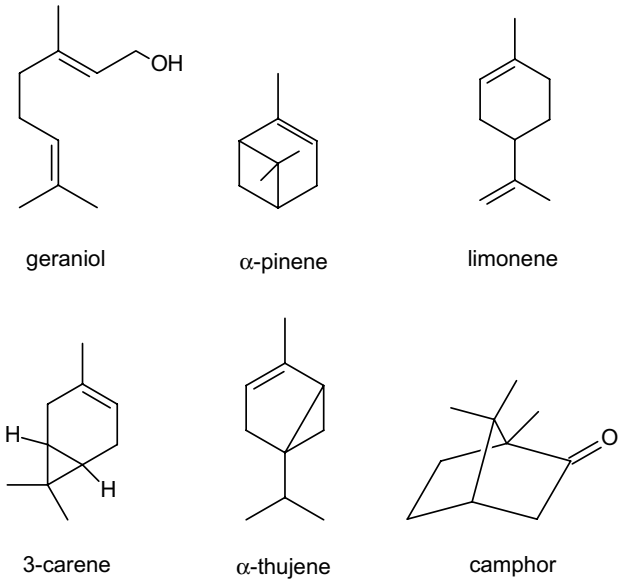


Figure 1: Representative members of various monoterpene structures.

Materials and Methods

Plant material

Wild type *Nicotiana tabacum* 'Petite Havana' SR1 plants (Maliga et al., 1973), plants that were transformed with an empty pCambia vector, plants transformed solely with a β-pinene synthase and plants transformed with a (+)-limonene synthase, β- pinene synthase and γ-terpinene synthase isolated from *Citrus limon* were grown under greenhouse conditions under 16h photoperiod and provided by J. Lücker (Lücker et al., submitted). These sets of plants were referred to as SR1, CAM1, D85 and BCD, respectively.

Headspace trapping of leaf volatiles and GC-MS

Leaf sample of about 4 g (fresh weight) from each SR1, CAM1 and BCD tobacco plants were cut and kept turgescent by placing the leaf petiole in green mulch (Smithers-Oasis Belgium N.V, Houthalen, Belgium). The latter were saturated with water and wrapped with aluminium foil to reduce evaporation. Subsequently, the leaves were placed in a 600 ml glass jar which was then tightly closed with a Teflon-lined lid equipped with an inlet and an outlet (Figure 2A and B). A vacuum pump was used to draw air through the glass jars at 100 $ml.min^{-1}$ for a period of 24h. The incoming air was purified through a glass cartridge (140x 4mm) containing 150 mg Tenax TA (20/35 mesh, Alltech, Breda, The Netherlands). At the outlet, the volatiles emitted by the leaves were trapped on a similar Tenax cartridge.

A **B**

Figure 2. Headspace sampling of whole leaves (A& B)

Alternatively, 4 g of BCD leaves were frozen in liquid nitrogen, stored overnight at -80 ^{o}C, homogenised the next day in 1.5 ml 5M $CaCl_2$, (to inhibit enzymatic reactions; Hinterholzer and Schieberle, 1998), and incubated in a small glass container that had been preheated at 30 ^{o}C, and containing a stir bar. The glass container was then placed in a similar set up as mentioned above, on top of a magnetic stirrer with a speed of 800 rpm and the headspace sampled for 24h. Tenax cartridges were eluted with 3 ml of redistilled pentane/diethyl ether (4:1 v/v) and 2 µl samples were injected on HP5-MS column (30m x 0.25mm i.d., 0.25 µm d_f) and analysed by GC-MS using an HP 5890 series II gas chromatograph combined with HP 5972A Mass Selective detector as previously described (Bouwmeester et al., 1999). GC oven temperature was programmed from 45 ^{o}C (1 min hold) to 280 ^{o}C, at a rate of 10 ^{o}C. min^{-1}, with a final hold of 10 min.

Sensory discrimination test

Twenty employees from Instituut voor Agrotechnologisch Onderzoek (ATO, Wageningen, The Netherlands), who were between the ages of 20-40 years and naïve to the task at hand, were tested in a triangle test. This test is a three-sample test in which all three samples are coded and the subject's task is to determine which one is most different from the other two (Stone & Sidel, 1993). The stimuli used were tobacco leaves placed in 600 ml glass jars with a 7.5 cm screw-top cap. The subject was presented with three jars at a time and proceeded from the left to the right. They were instructed to open each jar and sniff the contents of the jar, then close the jar and continue until all jars had been sampled. They were thereupon asked to indicate which sample smelled most different from the rest. In case of difficulty, they were allowed to smell each of the jars one more time. Each subject completed two trials. In one trial, two of the jars contained leaves from the BCD plant line, and the other one contained leaves from the SR1 control plant line. In the other trial two jars contained leaves from the SR1 control plant line, and the remaining jar contained leaves from the BCD plant. The order of trials was counterbalanced across subjects and the order of samples was randomised so that each sample was used for an equal number of times.

Flower fragrance, screening and selection of panel for descriptive test

Around 1 g of BCD, D85 and SR1 flower samples from four different flower developmental stages (8, 10, 11 and 12) (Figure 3) (Goldberg, 1988; Koltunow et al., 1990) were used as fragrance stimuli. Three-digit codes were generated for each of the samples for use during panel sessions. For each sample, four flowers were placed into a block of green mulch

saturated with water and wrapped with aluminum foil (Fig. 3). The mulch base itself did not have an odor. Mulch and flowers were presented in 600 ml glass jars with screw lids of 7.5 cm diameter. For training purposes, duplicates of flowers of each plant line were used.

Figure 3. Flower presentation method. About 1 g of 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) and stage 12 (flower open; anthers dehisced, corolla limb fully expanded and deep pink) flowers (Koltunow et al., 1990) was presented in a 600 ml glass jar closed with 7.5 cm screw cap to the sensory descriptive panel.

Eleven participants were invited to participate in a separate screening session for a period of 15 min. Participants completed questionnaires about e.g. smoking history, smelling ability, respiratory disease, and occupational exposure to volatile chemicals. They were thereupon tested on their ability to detect terpenes, for which menthone was used as the test stimulus. A binary odorant series consisting of 8 bottles was prepared starting from a 1% (v/v) stock dilution, containing menthone dissolved in propylene glycol (PG: solvent). Participants were offered two plastic squeeze bottles (500 ml with 3cm screw caps perforated by 6 mm holes in the center), each containing 10 ml solution. One bottle contained 10 ml of the odor stimulus, whereas the other contained only the solvent. Participants sniffed from the holes in the center

of the bottle caps while squeezing the bottle. The procedure was started with the bottle which contained the lowest menthone concentration (0.0078% v/v). The participants were instructed to smell each bottle briefly and to select the bottle that contained the odourant (a forced choice procedure). This was repeated 5 times. In case of an error, the experimenter would continue with the next higher concentration (0.016% v/v and so on) until the participant had five hits in a row for the same stimulus. Participants were then tested for their ability to generate odour descriptors for terpenes. Therefore, panellists were instructed to take a careful sniff from two 0.25% solutions of γ-terpinene and β–pinene presented in small glass vials with screw caps and 2.5 cm openings and write down their immediate sensory impressions. Panellists, who were capable of detecting the lowest presented concentration of menthone (0.0078% v/v) without error, and of generating odorant attributes for the above stimuli, were selected for participation in the panel.

Panel training for descriptive test

Panellists participated in three training sessions, which occupied 30 – 45 minutes each.

During *Session 1*, participants sniffed from each of the BCD, D85 and SR1 flower samples and wrote down their sensory impressions. They were instructed not to include associations, memories or feelings, but to restrict themselves to sensory impressions only. The descriptors were collected by the experimenter and discussed among the panel. During *Session 2*, the collected odorant descriptors were presented centrally to the panel. Panellists thereupon smelled each sample and tried to identify the appropriate descriptors for each of the samples. If necessary, new descriptors were generated and definitions for each of the descriptors were agreed upon. During *Session 3*, the participants rated each of the descriptors generated on 100 mm Visual Analogue Scales varying from 'very little' to 'very much' for each of the samples in individual booths using computer software (Fizz, 2002a). Subsequently, they discussed the results and rank-ordered the samples from lowest to highest based on each of the attributes, in an attempt to reach agreement among the panel on the qualitative and quantitative aspects of the descriptors.

Test session and statistical analyses for descriptive test

During the test session, which took place in individual booths, the individual panellists rated each of the tobacco flower samples twice, in random order on the previously selected descriptors. Computerised testing and data collection was performed using Fizz software (Fizz, 2000a). To check whether there were significant differences for each of the generated

attributes, analysis of Variance (ANOVA) (Hair et al., 1987; Fizz, 2000b) was conducted on panelratings.

Results

GC-MS of SR1, CAM1 and BCD whole leaf headspace

According to GC-MS analysis, there were no significant differences between the headspaces of SR1 and CAM1 plants (Fig. 4). SR1 was then considered as a suitable negative control to proceed with further experimentation. However, there were significant differences in the volatile profiles between BCD and SR1 leaves. Major products such as sabinene, β-pinene, (+)- limonene and γ-terpinene, and minor products such as α- thujene, α- pinene and α-terpinolene were detected in the headspace of BCD but not SR1 leaves. In contrast, in the volatile profile of the BCD ground leaves, none of the terpenes mentioned above were detected (Figure 4).

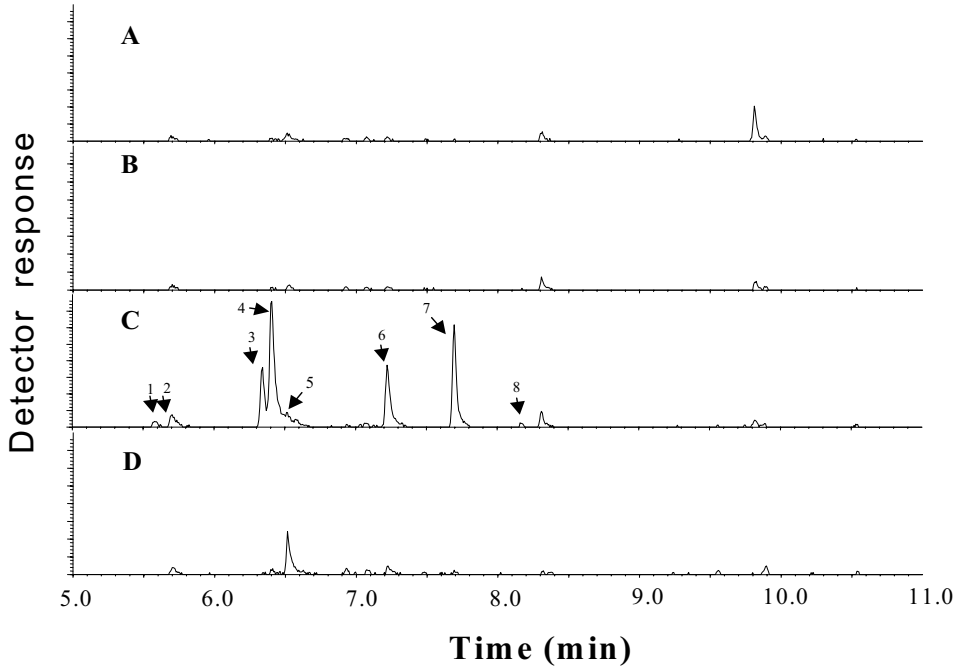


Figure 4: Headspace gas chromatography/ mass spectrometry analysis of whole SR1 (A), CAM1 (B), BCD (C) leaves, and ground BCD (D) leaves.
1=α-thujene,
2=α-pinene,
3=sabinene,
4=β-pinene,
5=β-myrcene
6=limonene,
7=γ-terpinene,
8=α-terpinolene

Sensory discrimination test of BCD and SR1 leaves

Twenty subjects were tested twice, with equal numbers of males and females. Of the 40 scores, 22 were correct (Table 1). In a triangle test the chance probability associated with the test is 1/3. Allowing only 5% risk (α=0.05) to erroneously reject the null hypothesis. The following formula was used: $Z = (k - (1/3)n) / \sqrt{((2/9)n)}$ (Meilgaard, Cicille & Carr, 1991), in

which k is the number of correct answers; n is the total number of presentations, with $k = 22$ and $n = 40$. $Z = 2.91$ exceeds the critical value for Z at $\alpha = 0.05$, which is 1.645. In other words, the null hypothesis that subjects were unable to smell the difference between the leaves from transgenic plants and control plants was rejected with $p < 0.05$. It was observed that with the continuation of the test over time, subjects started to perform better. During the test it was noticed that there were some differences not only across the different samples (transgenic BCD leaves vs. control SR1 leaves) but also between the various samples within one product. For example, differences in intensity were reported between the samples of the BCD leaves. Regardless of these differences, subjects were still capable of smelling the differences across samples with statistical significance.

Table 1. Two sets of triangle test results per subject over time

Subject number	Results for 2 trials (correct=1; Incorrect=0)
1	0-0
2	1-0
3	1-0
4	1-0
5	0-0
6	1-0
7	0-1
8	0-1
9	1-0
10	0-1
11	1-1
12	1-1
13	1-0
14	1-1
15	1-0
16	1-0
17	0-1
18	0-1
19	1-1
20	1-0

Sensory descriptive test of BCD, D85 and SR1 flowers

As shown before, naive subjects were capable of making a significant olfactory discrimination between the leaves of transgenic and control tobacco plants. A descriptive test was conducted to investigate the sensory profile of the transgenic flowers. This experiment had to answer how each of the transgenic BCD and D85 floral profiles differs from that of the control SR1 flowers.

Twenty potential panellists were screened during a 15 min session, after which 7 panellists were selected. Prior to selection, descriptors were given to γ-terpinene and β-pinene. These encompassed 'fresh', 'oil', 'petrol', 'turpentine' and 'herbs', for the first, and 'pine', 'medicine', 'camphor' and 'alcohol' for the latter. The selected panel consisted of four males and three females with ages varying between 26 and 50 years. Six of the panellists did not currently smoke and one smoked cigarettes occasionally. Two panellists reported occasional allergies but not at the time of testing and did not use anti-allergy medication. One of the female panellists reported being pregnant. The selected panellists participated in three training sessions in order to generate sensory attributes and become familiar with the procedures. In a process of elimination, eight descriptors were agreed upon. These descriptors covered the odorant characteristics of all three samples and they were: "Green odor", "sour", "sweet", "stale/moldy", "flowery", "fresh", "prickling" and "overall intensity". In a final test session, the flowers of BCD, D85 and SR1 were evaluated, in duplicate, for each of the attributes. Figure 5 displays the sensory odorant profiles corresponding to each of the plants. Flowers of D85 received highest scores for "overall intensity", "green odor", "prickling", "sour" and "flowery". Although the odorant profile of BCD flowers was almost identical to that of the control SR1 flowers, BCD flowers scored higher on the attributes "sweet", "sour" and "flowery". Control SR1 flowers scored highest on "stale/moldy" and "fresh". Means and standard deviations for each attribute were calculated per flower sample (Table 2). The large standard deviations are associated with the considerable disagreements and differences between panellists in the attribute rating for each of the flower samples. As a result of the disagreements in panellist ratings, no significant difference between the 2 sets of transgenic flowers and the wild type tobacco flowers was found for any of the eight generated attributes (Table 3). The attribute "overall intensity" which had shown the largest difference between D85 flowers and the others in figure 5 also did not reach statistical significance ($p=0.14$).

Table 3 shows that there is a highly significant panellist effect. This supports the large variation between panellists in their attribute rating.

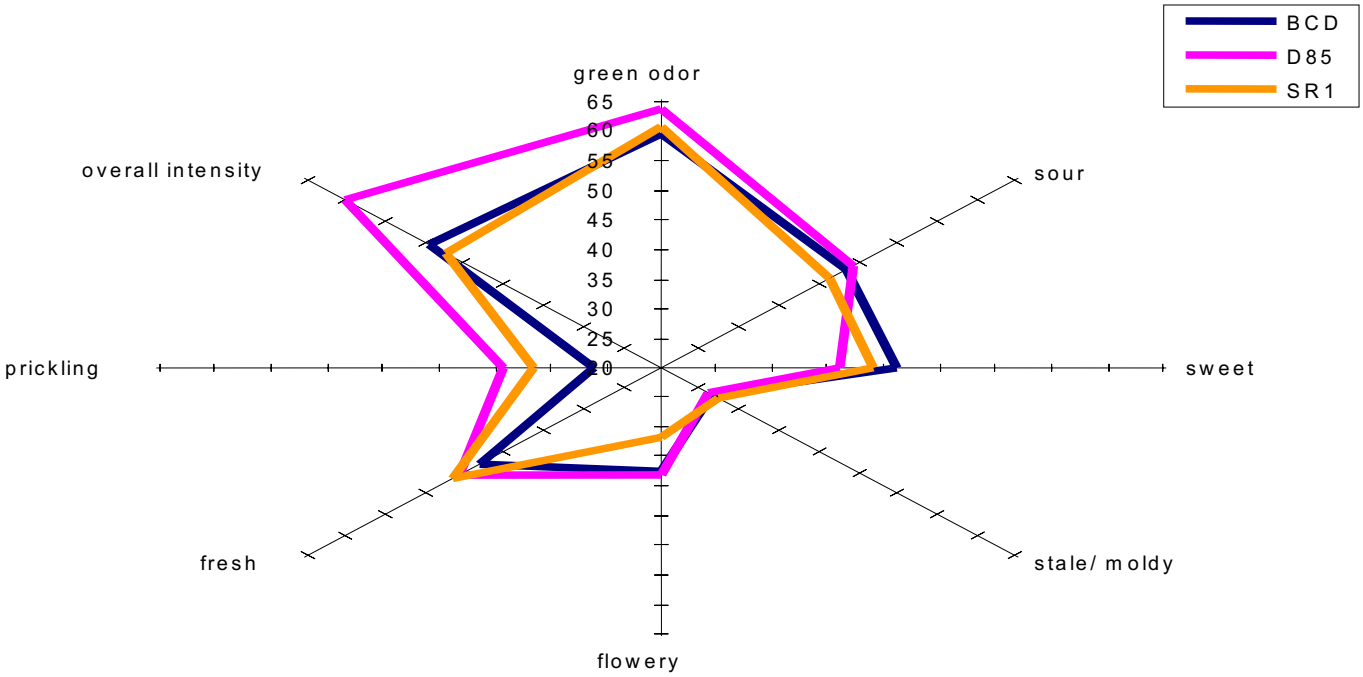


Figure 5: Odorant profiles of tobacco flowers

Table 2: Mean ratings (and SD's) per attribute and product

Product	green odor	sour	sweet	stale/ moldy	flowery	fresh	prickling	intensity
BCD	59.29 (21.26)	43.29 (15.14)	40.93 (19.04)	26.29 (18.59)	37.43 (20.16)	43.21 (22.23)	25.93 (16.68)	49.14 (18.46)
D	63.64 (15.16)	43.93 (16.99)	35.93 (19.99)	25.93 (12.80)	37.93 (22.64)	45.57 (21.67)	34.14 (19.49)	59.79 (13.80)
SR1	60.36 (21.98)	41.21 (17.76)	38.93 (18.18)	27.21 (21.45)	31.57 (18.00)	46.29 (27.00)	31.43 (22.58)	47.36 (24.87)

Table 3: Results from Anova

	Product			Panellist		
Attribute	**Df**	**F-value**	**P-value**	**Df**	**F-value**	**P-value**
Green odor	2	0.27	0.760	6	4.96	0.003**
Sour	2	0.27	0.77	6	11.37	0.001***
Sweet	2	0.98	0.39	6	20.49	0.0001***
Stale/moldy	2	0.04	0.97	6	3.85	0.01 **
Flowery	2	0.96	0.40	6	10.10	0.0001***
Fresh	2	0.19	0.9	6	14.74	0.0001***
Prickling	2	1.37	0.27	6	8.55	0.0001***
Intensity	2	2.20	0.14	6	3.55	0.01*

Note: *significant at 5%; ** significant at 1%; ***significant at 0.1%

Discussion

GC-MS results confirm the success of the tobacco metabolic engineering leading to the formation of a variety of different monoterpene skeletons, thus altering the volatile profile emitted from the transgenic plants (Fig.4). In addition, the sensory discrimination test conducted on BCD transgenic and SR1 control tobacco leaves clearly showed a significant difference between them (Table 1).

In the floral descriptive test, no significant difference between two sets of transgenic flowers and the wild type SR1 tobacco flowers was found for any of the generated attributes (Tables 2 and 3). However, from results in figure 5 we can still predict a trend in the 'sweet' attribute perhaps associated with the presence of (+)-limonene. (+)-Limonene is commonly described as 'mild/light', 'sweet', 'citrus', 'fruity', 'fragrant' (Dravnieks, 1985; Aldrich, 1997).In contrast, panelists had described γ-terpinene as 'fresh', 'oil', 'petrol', 'turpentine' and 'herbs', and β-pinene as 'pine', 'medicine', 'camphor' and 'alcohol'. One panellist described β-pinene as 'sweet'. The other panellist attributes given and literature (pine, woody) (Aldrich, 1997) seem to contradict this. Although the odour of molecules depends on the environment in which they are present, we suggest that the higher 'sweet' attribute value given to BCD is due to the presence of (+)-limonene. In addition, the increase in the 'overall intensity' sensation attributed to D85 flowers might be due to the higher 'prickling' value.

Floral petals, with non-specialised floral epidermal cells recruited for fragrance production and emission (Dudareva & Pichersky, 2000) remain the main source of scent in most plants (Pichersky et al., 1994). However, many modern flower varieties, such as carnation, lack the characteristic original fragrance (Clery et al., 1999). The use of a transgenic approach could lead to the re-introduction of fragrance compounds or could be used to improve the fragrance of ornamental plants and flowers. Flower scent has a composite character which is determined by a complex mixture of low molecular weight volatile molecules. Due to the inaccessibility of this character, to the limitations of human's sense of smell, and to the tightly variable nature of a scent, no simple and efficient methods to screen for genetic variation have been developed yet (Vainstein et al., 2001).

Our results confirm that the evaluation of fragrances is a highly complex matter and the linkage between olfactory sensing and chemical analyses should be established more efficiently (Vainstein, 2001). Many factors have decreased the significance of our results such as the presentation of the samples to the panel. Both leaf and flower samples were

presented in glass jars with screw caps, aiming to balance over time the number of flowers present on the respective plants, volatile release and volatile concentration in the headspace, and the human nose sensitivity. The use of an improved method which guarantees volatile containment or replenishment over time and a change of the volatile extraction method, such as the one suggested by Chida et al. (2002), should be considered. Air streams through the sample containers could be efficient in providing the panel with enough sample to generate more odor descriptors. Also, the use of a list of pre-defined attributes could have been beneficial to the panellists. They had to go into the hardship of generating verbal labels to describe the odours within a very short predetermined time frame. More training sessions could have reduced the disagreements between panellists on their ratings and their differences in experience with the floral odours. Finally, the concept of using human panellists as the 'sensory instrument' directly implies the settling of vaguely defined attributes describing scents such as "flowery", "prickling" etc. In the age of metabolomics and high throughput screening in search of genes involved in the production of floral scent, there is a need for clearer physical attributes to these terms, and for more efficient utilisation of the human nose in the lab to explore new opportunities and to alter floral scent for research purposes. Bylaite et al. (2000) have described the use of GC-FID combined with GC-olfactometry in an attempt to qualitatively and quantitatively determine differences among different parts of lovage (*Levisticum officinale*). We suggest that such an approach, which normally separates mixtures of volatiles into odour active compounds, should be combined with an electronic nose device as previously described by Craven et al. (1996). The experimental challenge is to build extensive databases, with which currently used human scent attributes are correlated with olfactograms and electronic nose patterns.

In order to increase the yield or to alter the composition of plant essential oils, groups have adopted a transgenic plant approach (Mahmoud & Croteau, 2001; Lücker et al., 2001; Lavy et al., 2002, Lücker, *submitted*) and GC-MS analyses have confirmed their successes. Although the goal of this metabolic engineering was -without exception- to change the olfactory characteristics, this was not proven by any of the groups. Our present work was one of the first serious attempts to do so and it is clear that it is a more complex matter than expected. Hence, more research, better trained panels and advanced analytical tools are needed to clearly establish the effects of transformation with genes encoding biosynthetic enzymes on the olfactory properties of the transgenic plants and to help in deciphering the nature of plant fragrance.

References

Aldrich catalogue (1997) *Flavor and Fragrances. Featuring Naturals*. International ed. Sigma-Aldrich, Zwijndrecht.

Bardon S, Picard K & Martel P (1998) Monoterpenes inhibit cell growth, cell cycle progression, and cyclin D1 gene expression in human breast cancer cell lines. *Nutrition and Cancer* (**32**): 1-7

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

Bylaite E, Legger,A, Roozen JP, Venskutonis, P.R. & Posthumus M.A (2000). Dynamic headspace gas chromatography of different botanical parts of lovage (*Levisticum officinale* Koch.). *J. Agric. Food Chem.* (**48**): 6183-6190

Chida M, Sona Y, Nagat H, & Shikata H (2002). *Development of a food volatiles extraction method*. ECRO 2002 Abstract book.

Clery RA, Owen NE & Chambers SF (1999) An investigation into the scent of carnations. *J. Essent Oil Res*. (**11**): 355-359

Craven MA, Gardner JW, Bartlett PN (1996) Electronic noses- Development and future prospects.*Trends Anal. Chem.* (**15**): 486-493

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

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

De Luca V & St Pierre B (2000) The cell and developmental biology of alkaloid biosynthesis. *Trends in Plant Science* (**5**): 168-173

Dravnieks A (1985) *Atlas of Odor Character Profiles*. Institute of Olfactory Sciences, Illinois.

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kosolova 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

Fizz (2002a) Reference manual acquisition version 2.00, Biosystemes, France.

Fizz (2002b) Reference manual calculations version 2.01, Biosystemes, France.

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

Gershenzon J & Croteau R (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

Hair JF, Anderson RE, Tatham RL & Black WC (1987) Multivariate data analysis with readings. 3rd ed. Macmillan Publishing Company, New York.

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

Hill, R.A (2002) *Dictionary of natural products on CD-ROM*, Ed. version 10:2. Chapman & Hall/CRC, New York.

Hinterholzer A & Schieberle P (1998) Identification of the most odour-active volatiles in fresh, hand-extracted juice of Valencia late oranges by odour dilution techniques. *Flavour & fragrance journal* (**13**): 49-55

Ilmberger J (2001) The influence of essential oils on human attention: alertness. *Chemical senses* (**26**): 239-245

Knudsen JT, Tollesten L & Bergstrom GL (1993) Floral scent- a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry* (**33**): 252-280

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

Kreuzwieser J, Schnitzler JP & Steinbrecher R (1999) Biosynthesis of organic compounds emitted by plants. *Plant biol.* (**1**): 149-159

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

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*. *in press*

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

Loughrin JH, Thomas R, 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

LoughrinJH, Hamilton-Kemp TR, Andersen RA & Hildebrand DF (1991) Circadian rhythm of volatile emission from flowers of Nicotiana sylvestris and N. 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, Schwab W, van Hautum B, Blaas J, van der Plaas LHW, Bouwmeester HJ & Verhoeven HA. Modification of monoterpene emission profiles in tobacco by metabolic engineering using three lemon monoterpene synthases. *Submitted*.

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

Mazzanti G, Battinelli L, Salvatore, G (1998) Antimicrobial properties of the linalol-rich essential oil of *Hyssopus officinalis* L. var decumbens (Lamiaceae). *Flavour & fragrance journal* (**13**): 289-194

McGarvey DJ & Croteau R (1995) Terpenoid metabolism. *The Plant cell* (**7**): 1015-1026

Meilgaard M, Cicille GV, & Carr BT (1991). *Sensory Evaluation Techniques*, 2nd Ed. CRC Press, Boca Raton.

Pichersky E, Raguso RA, Lewinsohn E & Croteau R (1994) Floral scent production in Clarkia (Onagraceae). I. Localization and developmental modulation of monoterpene emission and linalool synthase activity. *Plant Physiol*. (**106**): 1533-1540

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

Senatore F, Napolitano F, Ozcan M (2000) Composition and antibacterial activity of the essential oil from *Crithmum maritimum* L. (Apiaceae) growing wild in Turkey. *Flavour and fragrance journal* (**15**): 186-189.

Stone, H. & Sidel, J.L. (1993). *Sensory Evaluation Practices*, 2nd Ed. Academic Press, Inc. San Diego.

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

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

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

Chapter 6

General discussion

Citrus terpenes, terpene synthases and molecular evolution

Citrus fruits are among the most widely produced and consumed fruits all over the world (FAO, 1993). Terpenes provide defense against herbivores or pathogens and attract animals that disperse seeds or pollen (Bouwmeester et al., 1999; Pichersky & Gershenzon, 2002). Citrus terpenes are also commonly used as additives in the fragrance and flavour industry, such as in candies, liquors and other food products. They are also added as fragrant and hygienic agents in cosmetics (Lota et al., 1999).

In this thesis, we aimed at studying factors that might positively contribute to the regeneration of transgenic *Citrus sinensis* (L.) Osbeck cv. Valencia Late plants. Since tobacco is one the world's most studied crop plants, we also aimed at investigating the effect of *Nicotiana tabacum* transformation with *Citrus limon* monoterpene synthases on fragrance using human panellists. Nevertheless, the major objective of this thesis was to isolate cDNAs coding for monoterpene synthases from *Citrus limon* and to identify amino acids involved in product specificity within the active site of these enzymes. We managed to isolate and characterize from *Citrus limon*, four cDNAs encoding monoterpene synthases belonging to the plant terpene synthase b (*tpsb*) family. These cDNAs encoded a β-pinene synthase (Cl(-)ßPINS), a γ-terpinene synthase (ClγTS), and two limonene synthases (Cl(+)LIMS1 and Cl(+)LIMS2) (chapter 2). From a phylogenetic analysis, the separate clustering within the *tpsb* family of Cl(-)ßPINS and ClγTS from Cl(+)LIMS1 and Cl(+)LIMS2 becomes clear (Figure 1). The ClγTS and Cl(-)ßPINS sequences group together with the myrcene synthase from *Quercus ilex* and the *Artemisia annua* monoterpene synthases while the limonene synthases from *Citrus limon* form a distinct branch. Different domain swapping experiments between Cl(-)ßPINS and ClγTS, and between Cl(+)LIMS2 and ClγTS were conducted (chapter 3). It was found that within the same putative C-terminal structural domain of monoterpenes synthases, a region comprising 200 amino acids, of which 41 are different between Cl(-)ßPINS and ClγTS, determines the specificity for the formation of β-pinene or γ-terpinene, respectively. Two aromatic amino acid residues, which are within 3Å of the modelled active site and are differentially positioned in the ClγTS and Cl(-)βPINS at positions 283 and 269, respectively, were suggested to be involved in the final deprotonation leading to the formation of either β-pinene or γ- terpinene.

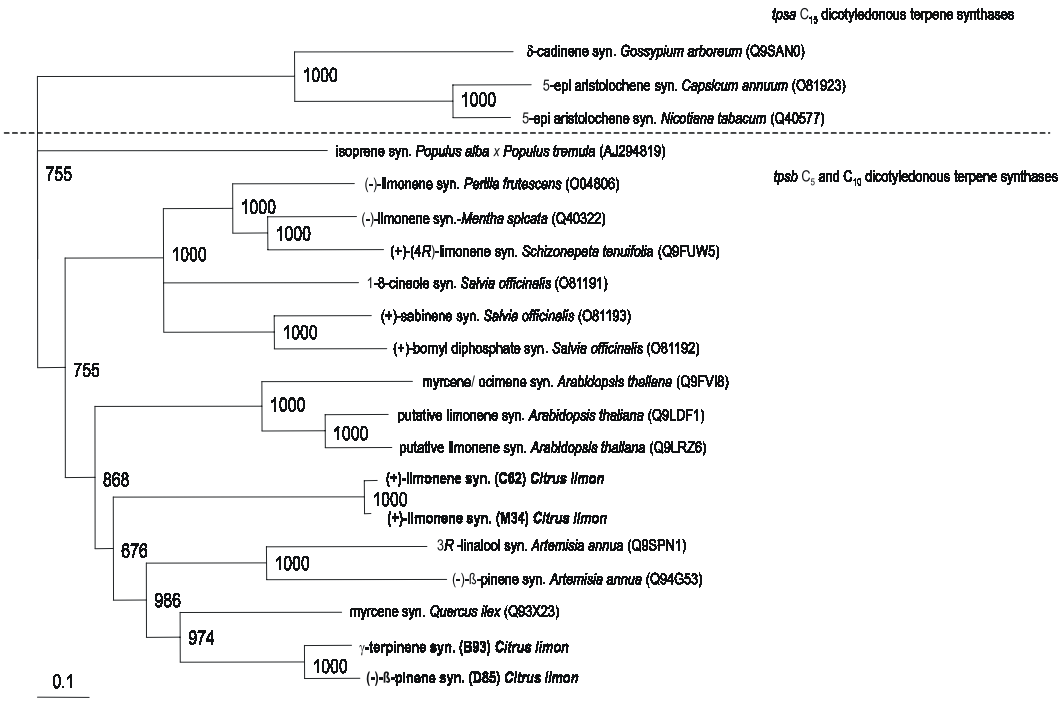


Figure 1. Phylogenetic tree of plant terpene synthases, including Cl(+)LIMS1 (C62), CL(+)LIMS2 (M34), Cl(-)βPINS (D85) and ClγTS (B93)

The magic begins when one starts to gaze at the amazing number of structural terpene derivatives, how the original diversity might arise by the action of a single biosynthetic step catalysed by a terpene synthase (Greenhagen & Chappell, 2001) and when one considers the evolution processes behind the "different" synthases. It was postulated that the terpene synthases share a common evolutionary origin, since they all share specific sequence motifs (McGarvey & Croteau, 1995; Bohlmann et al., 1998, Trapp & Croteau, 2001). Furthermore, the conservation of genomic organisation throughout the large multigene superfamily encoding plant monoterpene, sesquiterpene and diterpene synthases, especially with respect to the distinct pattern of intron loss, provides a compelling argument for the common evolution of terpene synthases (Trapp & Croteau, 2001). Amino acid sequence relatedness of plant terpenoid synthases, the *Tps* gene family, allows its subdivision into six subfamilies, designated *Tpsa* through *Tpsf*, each distinguished by sharing a minimum of 40% identity among members (Bohlmann et al., 1998). Many terpenoid synthases are encoded by multiple-gene copies risen by duplication (Facchini et al., 1992; Back & Chappell, 1995; Chen et al., 1995) that provided the basis for diversification. Since genes of secondary metabolism are not

essential for growth and development (Hartmann, 1996), they may tolerate more functional mutations than genes involved in primary metabolism, which seems responsible for the huge product diversity. Moreover, increased diversity in terpenoid chemistry may prove beneficial in ecological interactions with competing plants, as well as pathogens, herbivores, and pollinators (Bohlmann et al., 1998). Walsh (1995) gave statistical evidence that if a duplicated gene acquires an even slightly advantageous function, then it is unlikely to become nonfunctional in subsequent evolution.

Crystal structure and enzymatic evolution

Crystal structure analyses reveal significant three-dimensional similarities between farnesyl diphosphate (FDP) synthase (Tarshis et al., 1994) and sesquiterpene cyclases (Starks et al., 1997; Lesburg et al., 1997), in spite of very limited sequence similarities (Figure 2). These similarities in tertiary structure may have evolved convergently as a consequence of common reaction mechanisms.

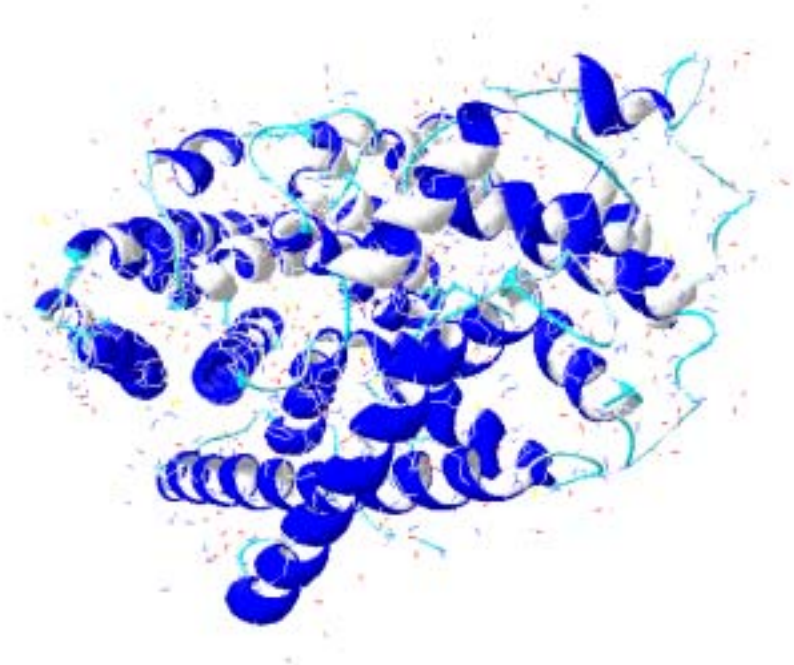


Figure 2. 3D-model of ClγTS, based on the TEAS model, consisting of α-helices and loops.

The crystallised tobacco 5-epi-aristolochene synthase, a sesquiterpene synthase, has shown that it is organised into two structural domains. The backbone NH_2-terminal domain (residues 36-230) aligns structurally with the catalytic cores of glycoamylase (3GLY) from *Aspergillus awamori* and an endoglucanase (CelD) from *Clostridium thermocellum*. Todate, the function

of this domain remains unknown. The COOH-terminal domain alligns with avian FDP synthase (Starks et al., 1997). These observations suggest that terpene synthases originated from a single ancestral gene perhaps composed from these ancient, non plant enzymes and have undergone successive cycles of duplication and divergence (Cseke et al., 1998).

In addition to duplication and divergence, additional terpene synthases may have been created by a process of recombination between terpene synthase genes, or domain swapping (Gilbert, 1980; Doolitle, 1995). Similarities between the N-terminal domains of limonene synthase (LIMS) from *Abies grandis* and copalyl diphosphate synthase (CPS) from *Arabidopsis thaliana* were assumed to have been due to preferential sequence conservation of a region that may have been present in the ancestral terpene synthases but whose function is conserved only in these enzymes and not in other terpene synthases. In addition, a linalool synthase isolated from *Clarkia breweri* was postulated to have been a composite gene resulting from a discrete recombination between the first half of a CPS-like gene and the second half of an LIMS-type gene (Cseke et al., 1998). These two processes- duplication followed by divergence and duplications followed by domain swapping- could explain the extraordinarily large number of diverse terpene synthases found in present-day plants.

Illegitimate recombinations or exon shuffling (Gilbert, 1980; Gilbert, 1987; van Rijk et al., 2000; van Rijk et al., 1999), unfixed spontaneous events of recombinations of genes sharing little or no homology, were observed in tomato (Chen et al., 1997). Such non-sense recombinations occur when an exon from one gene is connected to an exon from another gene. Analysis of protein sequences and 3D-structures has revealed that many proteins are composed of a number of discrete domains and some of these domains are evolutionary mobile, spreading during evolution and now occurring in otherwise unrelated proteins (Bork, 1992; Doolittle, 1995). These mobile domains are characterised by their ability to fold independently, which is essential because it prevents misfolding when they are inserted into a new protein environment (Kolkman & Stemmer, 2001). These self-contained and functional domains are typically compact and cysteine-rich modules.

Enzymes have selectable structural phenotypes with defined activity. Similarly like in the case of RNA (Gutell et al., 1994; Schuster et al., 1997), we suggest these features may be generally mapped by folding algorithms or by positional covariance (i.e patterns of correlated sequence substitution) in comparative sequence analysis. Eyre-Walker (1996) proposed that, for proteins translated at the same rates, selection to reduce translational misincorporations should be higher in longer genes because the energy cost of producing dysfunctional peptides will be proportional to their length, therefore enhancing the necessity of reliable protein

synthesis. Some amino acids, such as glycine, in addition to being less bulky than others (eg. Alanine), allow more conformational flexibility and are therefore known to be disfavoured in specific protein structures like α-helices which could influence the overall 3-dimensional structure of the enzyme. (Matthews et al., 1987).

***Citrus limon* monoterpene synthases, molecular diversity and thermodynamic evolution**

From an evolutionary point of view, we suggest that in *Citrus limon*, the monoterpene synthases have also evolved from a common ancestral predecessors possibly as hypothesised for TEAS in *Nicotiana tabacum* (Starks et al., 1997). We suggest that Cl(+)LIMS2 was created due to a gene duplication event of Cl(+)LIMS1, or vice versa, and has undergone a limited mutation divergence. Although we cannot rule out the possibility that this duplication occurred because a high concentration of limonene is conferring an evolutionary advantage, perhaps later another recombination or further mutation may bring about a new product. We suggest that ClγTS has originated in the same way as LIMS followed by gene duplication and that one of the copies has recently diverged to the Cl(-)βPINS (Fig.1, Fig.3). The domain swapping experiments and the modelling carried out in chapter 3 suggest that just a few amino acid changes were sufficient to bring about this new catalytic activity.

Enzymatic safety mechanism?

In chapter 3, all chimeric enzymes created between ClγTS and Cl(+)LIMS2 were either dysfunctional or had considerable reduced enzymatic activity. A question posed here is whether a system selected for the preservation of the cellular metabolome might be genetically coded for and laying within the enzyme's structural characteristic. This should, then, be considered whenever attempting to alter an enzyme's product specificity. Both transcription and translation processes require a substantial input of energy in terms of adenosine triphosphate (ATP). Furthermore, the biosynthesis itself is costly, demanding ATP or reduction equivalents, i.e nicotinamide adenine dinucleotide phosphate (reduced form) ($NADPH_2$) (Wink, 1999). Such an evolution-selected safeguard mechanism also might be of value in times of substrate scarcity. The question is important since the generation of molecular diversity has become a fundamental element in stratagies for pharmaceutical development and also for deciphering structure-function relationships (Bailey, 1999).

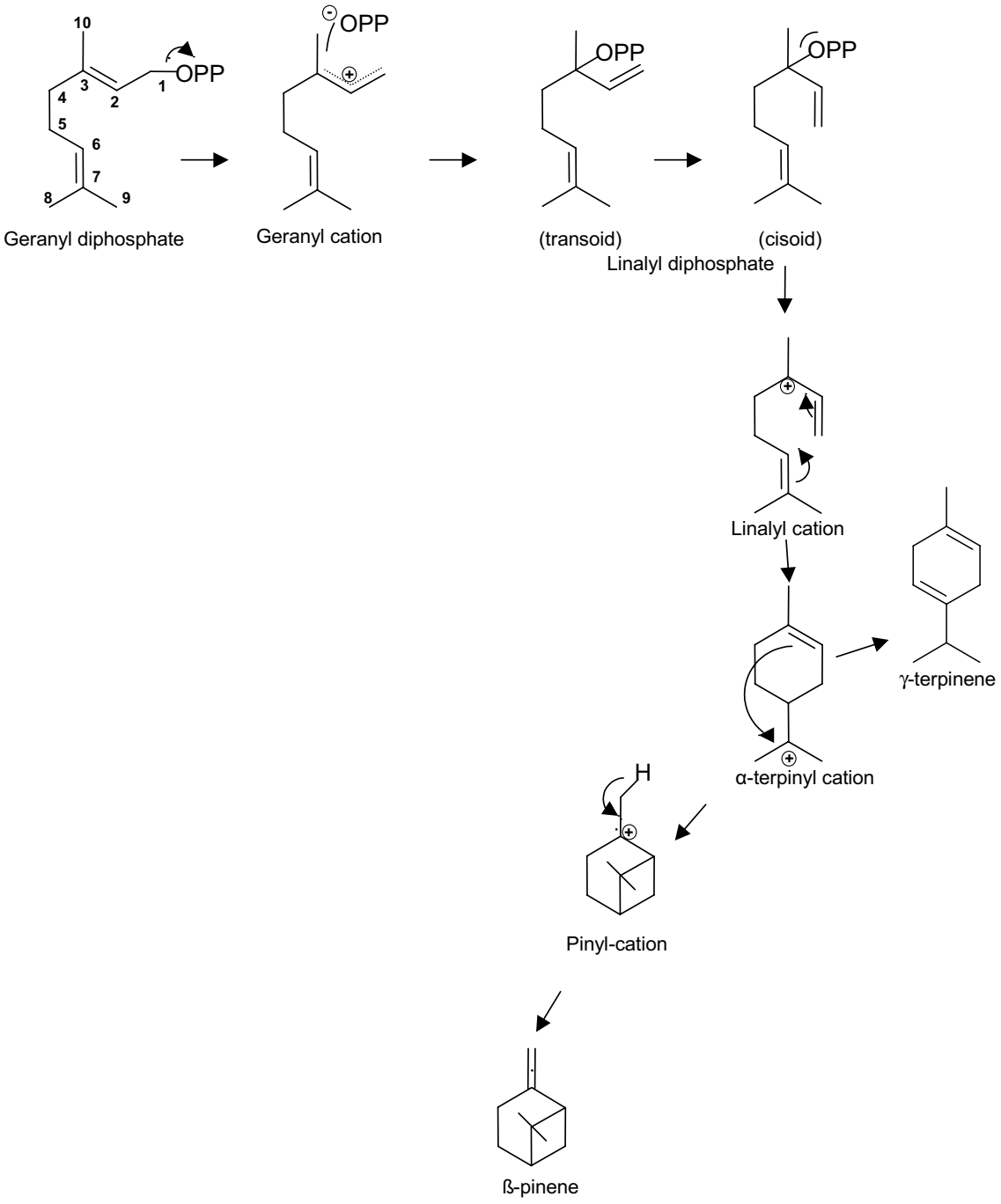


Figure 3. Conversion by monoterpene synthases of GPP, via linalyl diphosphate, to either γ-terpinene or β-pinene. OPP indicates the diphosphate group.(Modified after Little and Croteau, 1999)

Thermodynamics and enzyme evolution

Though studies have focused on the diversification and, sometimes, origin of terpene synthases, the evolutionary constraints imposed by structural properties intrinsic to these enzymes remain poorly characterised. Entropic stabilisation provides an explanation why glycine, for example, occurs less frequently within α-helices than any other amino acid (Chou & Fasman, 1978), since each such glycine represents a source of instability. It was predicted that functional enzymes have evolved to minimise frustrated intramolecular interactions with respect to random heteropolymer sequences. This principle of minimal frustration (PMF) (Bryngelson et al., 1995) is a generic response of enzymes to any selection pressure requiring well-ordered structure. A better understanding of these interactions is fundamentally important to understand the origin of enzyme structures and functions. The intelligent and rational design of novel enzymes could start with investigating statistical patterns in the primary structure (Schultes et al., 1999), which are correlated with minimal frustration, to create new functions.

The transgenic plant fragrance approach

Trying to tap into the metabolic mechanism of terpenoid biosynthesis inside the plant, whether to increase the yield or to alter the composition of the essential oil, many groups have adopted a transgenic plant approach in order to reach that aim (Dudareva et al., 1996; Lewinsohn et al., 2001; Mahmoud & Croteau, 2001; Lücker et al., 2001; Lavy et al., 2002, Lücker, 2002). For citrus, a number of groups have developed a transformation protocol (Peña et al., 1995; Bond & Roose, 1998; Luth & Moore, 1999; Domingez et al., 2000). However, all these protocols suffer from low transformation efficiencies. In order to find factors that might improve the transformation efficiency of *Citrus sinensis* (L.) Osbeck cv. Valencia Late, the interactive effects within the regeneration of *Citrus sinensis* have been studied in chapter 4. We focused on the influence of the initial germination medium, explant type and of the regeneration medium on shoot regeneration. We have shown that the germination on MS30 medium and the regeneration on NNIII medium containing a 20:1 cytokinin: auxin ratio supplied with the riboside form of the benzyl amino purine cytokinin are essential for an efficient induction of shoot regeneration. In addition, hypocotyl-derived shoot regenerants incubated on NNRtVIII showed better rooting than epicotyl-derived shoot regenerants and incubation on other media tested.

In chapter 5, the aim of our work was to study the floral fragrance of tobacco Petit Havana SR1 plants transformed with *Citrus limon* monoterpene synthases using trained

human panelists. In a triangle test, we found that naive subjects were capable of smelling differences across leaf samples with statistical significance. However, as a result of disagreements in panel ratings, no significant difference between two sets of transgenic flowers and the wild type SR1 tobacco flowers was found for any of the generated attributes in a descriptive test with trained panellists. GC-MS analysis provides quantifiable means to study the alteration of plant volatile emission, however, scent has a composite character which is determined by a complex mixture of low molecular weight volatile molecules. Due to the inaccessibility of this character, to the limitations of human's sense of smell, and to the tightly variable nature of a scent, no simple and efficient methods to screen for genetic variation have been developed yet (Vainstein et al., 2001). Nevertheless, up untill now, our tobacco trials were the first to report on a difference in smell between wild type and transgenic plants.

Since we are starting to unravel the intricate connections of different terpene biosynthetic pathways, it has been noted that sometimes in the plant metabolic engineering of monoterpene biosynthesis, undesirable traits such as further metabolic conversion or phenotypes such as dwarfing are also introduced into the plant (Fray et al., 1995; Lücker, 2002). This undoubtedly may come at a price from an ecological point of view. To restrict the influence of the metabolically undesirable "transgenic" effect on the delicate balance between the plant and the environment, scientists should take into consideration the different interacting metabolic fluxes and try to prevent that any of these other pathways is negatively affected.

Ecological sustainability, metabolic engineering and future perspective

For the moment and in line with a sustainability principle, it will be possible to revert to a transgenic approach in order to save plant species threatened with extinction either due to a newly introduced and highly adapted pest or due to a decrease in the respective pollinating insect population. This is necessary to either induce an artificial defence system or to widen the range of pollination vectors and hence improve seed dispersal. Alternatively, as a last resort for plant conservation, we might also transgenically divert the metabolic flux away from enzymes causing the formation of metabolites attracting pests and herbivores or inducing the germination of parasitic weeds simply in depleting the pool of substrate needed by these enzymes. This can be achieved by introducing genes coding for enzymes that compete for the same substrate pool.

Despite advances, the evolution of molecules in nature still remains vastly unexplored because of the need of an appropriate morphospace with which to compare evolved molecules by statistical approaches (Caetano-Annollés, 2002). Although major recent progress has focused on the early molecular processes creating new genes, further explorations of evolutionary forces that govern the fixation of new enzyme protein structures and subsequent sequence evolution should unravel their genesis mechanism. In the future, adopting a more exhaustive and integrated approach in phytochemical research will undoubtedly have a deep impact on our fundamental perception about the metabolism, evolution and ecology.

References

Back K & Chappell J (1995) Cloning and bacterial expression of a sesquiterpene cyclase from *Hyoscyamus muticus* and its molecular comparison to related terpene cyclases. *J. Biol. Chem.* (**270**): 7375-7381

Bailey JE (1999) Biochemical engineering. Molecular, cellular, and process frontiers. *Curr. Opin. Biotechnol.* (**10**): 115-116

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

Bond J.E & Roose M.L (1998) Agrobacterium- mediated transformation of the commercially important citrus cultivar Washington navel orange. *Plant Cell reports* (**18**): 229-234

Bork P (1992) Mobile modules and motifs. *Curr. Opin. Struct. Biol.* (**2**): 413-421

Bouwmeester HJ, Verstappen FW, Posthumus MA, Dicke M (1999) 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

Bryngelson JD, Onuchic JN, Socci ND & Wolynes PG (1995) Funnels, pathways, and the energy landscape of protein folding: A synthesis. *Proteins* (**21**): 167-195

Caetano-Annolés G (2002) Evolved RNA secondary structure and the rooting of the universal tree of life. *J. Mol. Evol.* (**54**): 333-345

Chen XY, Chen Y, Heinstein P & Davisson VJ (1995) Cloning, expression, and characterization of (+)-delta-cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. *Arch. Biochem. Biophys.* (**324**): 255-266

Chen JJ, Janssen BJ, Williams A & Sinha N (1997) A gene fusion at a homeobox locus: alterations in leaf shape and implications for morphological evolution. *Plant Cell* (**9**): 1289-1304

Chou PY & Fasman GD (1978) Empirical predictions of protein conformation. *Annu. Rev. Biochem.* (**47**): 251-276

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

Domíngez A, Guerri J, Cambra M, Navarro L, Moreno P & Peña L (2000) Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep* (**19**): 427-433

Doolittle RF (1995) The multiplicity of domains in proteins. *Annu. Rev. Biochem.* (**64**): 287-

314

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

Eyre-Walker A (1996) Synonymous codon bias is related to gene length in *Escherichia coli:* selection for translational accuracy? *Mol. Biol. Evol.* (**13**): 864-872

Facchini PJ & Chappell J (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. *Proc. Natl. Acad. Sci. USA* (**89**): 11088-11092

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

Gilbert W (1980) Why genes in pieces? *Nature* (**271**): 501

Gilbert W (1987) The exon theory of genes. *Cold Spring Harbor Symp. Quant. Biol*. (**52**): 901-905

Greenhagen B & Chappell J (2001) Molecular scaffolds for chemical wizardry: Learning nature's rules for terpene cyclases. *Proc. Natl. Acad. Sci. USA* (**98**): 13479-13481

Gutell RR, Larsen N & Woese CR (1994) Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* (**58**): 10-26

Hartmann T (1996) Diversity and variability of plant secondary metabolism: a mechanistic view. *Entomol. Ex. Appl.* (**80**): 177-188

Hill, R.A (2002) *Dictionary of natural products on CD-ROM*, Ed. version 10:2. Chapman & Hall/CRC, New York.

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*. *In press*

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, 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

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 (2002) Metabolic engineering of monoterpene biosynthesis in plants (ph.D thesis). Wageningen University, Wageningen.

Luth D & Moore G (1999) Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*- mediated transformation. *Plant Cell, Tissue and Organ Culture* (**57**): 219-222

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

Matthews BW, Nicholson H & Becktel WJ (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Natl. Acad. Sci. USA* (**84**): 6663-6667

McGarvey DJ & Croteau R (1995) Terpenoid metabolism. *Plant Cell* (**7**): 1015-1026

Murashige T & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue culture. *Physiol Plant* (**15**): 473-497

Nitsch J.P, Nitsch C (1969) Haploid plants from pollen grains. *Science* (**163**): 85-87

Peña L, Cervera M, Juárez J, Ortega C, Pina J.A, Durán- Villa N, Navarro L (1995) High efficiency Agrobacterium- mediated transformation and regeneration of Citrus. *Plant Science* (**104**): 183-191

Pichersky E & Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology* (**5**): 237-243

Raillard SA, Krebber A, Yonghong C, Ness JE, Bermudez E, Trinidad R, Fullem R, Davis C, Welch M, Seffernick J, Wackett LP, Stemmer WPC & Minshull J (2001) Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. *Chemistry & Biology* (**8**): 891-898

Schultes E, Hraber PT & LaBean TH (1999) A parametrization of RNA sequence space. *Complexity* (**4**): 61-71

Shuster P, Stadler PF & Renner A (1997) RNA structures and folding: from conventional to new issues in structure predictions. *Curr. Opin. Struct. Biol.* (**7**): 229-235

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

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

Trapp SC & Croteau RB (2001) Genomic organization of plant terpene synthases and molecular evolutionary implications. *Genetics* (**158**): 811-832

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

Van Rijk AF, van den Hurk MJ, Renkema W, Boelens WC, de Jong WW & Bloemendal H (2000) Characteristics of super alphaA-crystallin, a product of in vitro exon shuffling. *FEBS Lett.* (**480**): 79-83

Van Rijk AA, de Jong WW & Bloemendal H (1999) Exon shuffling mimicked in cell culture. *Proc. Natl. Acad. Sci. USA* (**96**): 8074-8079

Walsh JB (1995) How often do duplicated genes evolve new functions? *Genetics* (**139**): 421-428

Wink, M. (1999) *Biochemistry of plant secondary metabolism*. Annual plant reviews, Vol. 2, CRC press.

Summary

Citrus belongs to an important economical group of crops. Fruits have a distinguished and pleasant taste partly due to the presence of terpenes. Furthermore, these terpenes potentially play a role in the resistance against pathogens such as insects and fungi. The aim of this thesis was therefore to study the regulation of terpene biosynthesis in *Citrus limon* L. Burm. Therefore, monoterpene synthase cDNAs from *Citrus limon L. Burm.* peel were isolated by a cDNA library-screening approach and to identify specific domains, and possibly amino acids, involved in product specificity within the active site of these enzymes. In addition, we aimed at investigating factors that might positively contribute to the regeneration of transgenic *Citrus sinensis* (L.) Osbeck cv. Valencia Late plants. Because transforming Citrus was not practical, we aimed at studying the odor effects of transforming a model plant, *Nicotiana tabacum*, with the isolated *Citrus limon* monoterpene synthases.

In chapter 2, the isolation, expression and characterization of four new cDNAs belonging to the terpene synthase b sub-family (*tpsb*) family (Bohlmann et al., 1998) and coding for monoterpene synthases in *Citrus limon L. Burm*. has been described in detail. A random sequencing approach was applied to a cDNA library from mRNA isolated from the peel of a young developing fruit. These monoterpene synthase cDNAs were isolated and all four cDNAs could be functionally expressed in *E. coli* after removal of their plastid targeting signals. The main products of the enzymes in assays with geranyl diphosphate (GDP) as substrate were (+)-limonene (two cDNAs), (-)-ß-pinene and γ-terpinene and these enzymes were named Cl(+)LIMS1, Cl(+)LIMS2, Cl(-)βPINS and ClγTS, respectively.

In chapter 3, the functional differences which were present within the domains of the monoterpene synthases and leading to product specificity in the enzymatic catalysis, have been studied. Domain swapping experiments were conducted as well between Cl(-)ßPINS and ClγTS as between Cl(+)LIMS2 and ClγTS. We showed that within the same putative C-terminal structural domain of monoterpene synthases, a region comprising 200 amino acids, of which 41 are different between Cl(-)ßPINS and ClγTS, determines the specificity for the formation of β-pinene or γ- terpinene, respectively, while another region localized further downstream is required for a chimeric enzyme to yield products with the same ratio as in the wild type ClγTS. For Cl(+)LIMS2, both domains together appear to be sufficient for its enzyme specificity, but probably due to the low homology with ClγTS, many chimeric enzymes were inactive.

In order to find factors that might improve the transformation efficiency of *Citrus sinensis* (L.) Osbeck cv. Valencia Late, the interactive effects within the regeneration of

Citrus sinensis have been studied (Chapter 4). The latter was focused on the influence of the initial germination medium, of the explant type (source) and of the regeneration medium on shoot regeneration. Results show that the germination on MS30 (Murashige & Skoog, 1962) and the regeneration on NNIII (Nitsch & Nitsch, 1969) containing a 20:1 cytokinin: auxin ratio supplied with the riboside form of the benzyl amino purine cytokinin, are essential for an efficient induction of shoot regeneration. In addition, hypocotyl-derived shoot regenerants incubated on NNRtVIII (Nitsch & Nitsch based medium) showed better rooting than epicotyl-derived shoot regenerants and incubation on other media tested.

Finally, with the help of a human panel, the floral fragrance effect of transforming *Nicotiana tabacum* either together with the Cl(+)LIMS1, Cl(-)βPINS and ClγTS genes, or singly with the Cl(-)βPINS gene has been investigated (Chapter 5). In a triangle experimental design, the panel was able to discriminate significantly between the smell of a transgenic plant and a wild type plant. However, a descriptive panel had difficulties to generate accurate sensory descriptors for smells, and to reach consensus on the precise meaning of chosen attributes in a predetermined small number of panel sessions.

Nevertheless, this thesis illustrates an example where the combination of molecular biology, cell biology and biochemistry, and sensory analysis offers an integrated approach as a first step in trying to unravel the world of plant fragrance emission and perception.

Samenvatting

Citrus soorten zijn een economisch erg belangrijke groep van gewassen. De vruchten hebben een opvallende, aangename smaak, die deels door de aanwezigheid van terpenoiden wordt veroorzaakt. Daarnaast spelen die terpenoiden mogelijk een rol in de resistentie tegen belagers, zoals insecten en schimmels. Het doel van dit proefschrift was daarom het bestuderen van de regulatie van de terpeenbiosynthese in de Citrus soort citroen (*Citrus limon* L. Burm). Daartoe werden terpeen synthase cDNAs uit citroen geïsoleerd en hun productspecificiteit bestudeerd. Daarnaast zijn de factoren onderzocht die van belang zijn voor de regeneratie van *Citrus sinensis* (L.) Osbeck cv. Valencia Late. Aangezien transgene Citrus binnen dit project niet praktisch was is tenslotte met tabak als model, getransformeerd met de citroen terpeen synthases, bestudeerd wat de gevolgen van deze transformatie voor de geureigenschappen zijn.

In Hoofdstuk 2 wordt de isolatie, heterologe expressie en karakterisering van vier nieuwe monoterpeen synthases beschreven. De cDNAs zijn geïsoleerd door middel van random sequencing van een cDNA bank die was gemaakt van de schil van jonge citroenvruchten. De geïsoleerde monoterpeen synthases behoren alle tot de tps-b subfamilie en konden in *Escherichia coli* functioneel tot expressie worden gebracht na verwijdering van het targeting signaal. De hoofdproducten van deze enzymen met geranyl difosfaat (GDP) als substraat waren (+)-limoneen (twee cDNAs; Cl(+)LIMS1 en Cl(+)LIMS2), (-)-ß-pineen (Cl(-)βPINS) en γ-terpineen (ClγTS).

In Hoofdstuk 3 is bestudeerd waarop de verschillen in productspecificiteit van de geïsoleerde monoterpeensynthases berusten. Zogenaamde domain swapping experimenten, waarbij stukken van twee cDNAs werden uitgewisseld, werden uitgevoerd met Cl(-)ßPINS en ClγTS en met Cl(+)LIMS2 en ClγTS. Er werden diverse chimere enzymen gemaakt die nog steeds het substraat GDP konden omzetten. Door het bestuderen van de productvorming van deze chimere enzymen hebben we laten zien dat in het C-terminale gedeelte van de enzymen een domein van 200 aminozuren (waarvan er 41 verschillen tussen Cl(-)ßPINS en ClγTS) de productspecificiteit bepaalt. Voor Cl(+)LIMS2 wordt de productspecificiteit ook door het C-terminus bepaald, maar veel van de chimeren van Cl(+)LIMS2 en ClγTS waren inactief, waarschijnlijk doordat de twee cDNAs te weinig homoloog waren.

Voor het onderzoek naar factoren die bepalend zijn voor een efficiënte transformatie van *Citrus sinensis* (L.) Osbeck cv. Valencia Late, is de regeneratie onderzocht (Hoofdstuk 4). Dit onderzoek richtte zich op de invloed van het kiem-medium, het weefseltype en het scheut-regeneratie medium. Kieming op MS30 (Murashige & Skoog, 1962) en regeneratie op NNIII (Nitsch & Nitsch, 1969) met 20:1 cytokinine : auxine gaven de beste scheutregeneratie.

Hypocotyl-afgeleide scheutjes bewortelden beter op NNRtVIII (medium gebaseerd op Nitsch & Nitsch) dan epicotyl-afgeleide scheutjes of andere bewortelingsmedia.

Tenslotte is met behulp van een menselijk panel bepaald wat het effect is van de transformatie van tabak met de Cl(+)LIMS1, Cl(-)βPINS en ClγTS cDNAs op de geur van die planten (Hoofdstuk 5). In een directe vergelijking werd een significant verschil geconstateerd in de geur van transgene tabak vergeleken met het wildtype. Toen vervolgens werd geprobeerd met behulp van descriptors dit significante verschil ook te beschrijven bleek er zoveel variatie te bestaan tussen de panelleden in hun waardering dat er geen significante verschillen konden worden aangetoond.

Desalniettemin, laat dit proefschrift zien hoe de combinatie van moleculaire biologie, celbiologie, biochemie en sensorisch onderzoek geintegreerd kunnen worden om een begin van inzicht te krijgen in de regulatie van plantengeur en de waarneming daarvan door de mens.

Nawoord

"Ok Mazen, lab time is up, there is life still after a PhD". These were the words that suddenly reminded me that nearly 4 years of research should be wrapped up. What followed was a frantic race against time and resource depletion to deliver this thesis. Coming to the end of my PhD trip, I find myself thankful to many that were involved in the widening of my knowledge and personal development, and with whom I shared special moments during the last 4 years.

Two gentlemen were at the heart of my research project. Fons Voragen and Arjen van Tunen. Dear Promotor, I will always remember the words "Mazen, people trust you" from day one. Being already initiated for years earlier on the value of that trait among few others, it was never put to me so straight forward as you did. These few words meant a lot to me: Be very responsible in satisfying expectations. I am grateful to the fact that you have Guided me through a challenging period of re-organization and mergers, through periods where normally scientific focus can be compromised...but we have finally overcome, together. You were always there for me when I needed you and afterwards I was speechless. Thank You.

Arjen, I had already decided to join Cell Biology (the seed of Cell Cybernetics) for a PhD the moment we had our initial talk. I had the feeling that this research was tailor-made based on my interest...after all, Citrus is my favorite fruit! Your outgoing vision and solution-oriented professionalism reminded me for four years that there is enough room for innovation, that there is no space for unnecessary hesitation and that extracting the best out of any challenging situation was the only lively option. For this, gentlemen, I've always had a warm feeling inside of me in CPRO-DLO (currently Plant Research International).

Jolanda, thank you for fitting me in the schedule of our boss whenever I needed, even if it meant an afsprakje tussen twee afspraken, and for the positive karma you add to that office!

The atmosphere in Cell Cybernetics was unforgettable, mainly due to all the friends I've encountered and had the pleasure to work and socialize with. In the early years, I've enjoyed so much the work discussions and going out with all of you! This contributed a lot for me to value the close link between a team. Now just like addiction, I actively seek such a close team link anywhere I go. Margo and Froukje, ik dank jullie zo veel, for you have been so caring and along with Marianne, Edgar, Helen & Ivo, Roy & Sabine, Marieke, Rob, Bart, Diane, Roel, Jeroen, Helene, Basten, Rita, Fieke, Jolanda and Carin I attended the course "Working and living in Wageningen 101". Tjitske thanks for the help! I feel very connected to you all!

To my flat mates Monique, Gea, Ilse, Tiny, Renée and Richard: Thank You for your hospitality!

Talking about living, I would like to thank the bus driver maatjes of ligns 84 and 86 for making my travel to work and back home an enjoyable experience during the last 4 years!

If there is anything I've learned about group work in PRI, it's that your group gets bigger and fluid with time. In the initial period of the PhD, I enjoyed the work with Frans and Elma. Frans, I've met your lovely family over dinner and I would like to thank you and Elma for the close cooperation. I appreciate the meticulous nature of the initial guidance you provided me with. Harrie, thank you for helping with the formalities of this work, literature and the 3-D enzyme modelling. Jan and Gerard thank you for your help and explanation!

Following the formation of Cell Cybernetics, I had the pleasure to join Harro's group. Harro, I thank you so much for the way you guided this group, including me. You made me feel again as part of a team and, somehow, I was very happy once the thesis writing started because of all the beautiful discussions we both had. I can still trace the development of these drafts and...I realise. We had nice outings together with the group. The delicious barbecues that Marianne and yourself held at your house are specially to remember. Cinzia! Thanks for the nice talks we had together during the weekends and I hope RNA isolation is at its best! Iris and Iris, Jan-Willem, Francel, Per, Marloes, Hester, Rada, Olca thank you for sharing a nice common feeling specially brought to us by the Guru himself.

Jacques, I appreciate your desire to always reach out towards new scientific horizons with an open mind against many odds. I found you to be a craftsman in suggesting solutions, and I also thank you for the many useful discussions we had together even over lunch.

Monique and Nancy, thank you for your Help within the constraints of a very short time!

Working during late night hours and starting 18.00, a special mood settles over Cell Cybernetics. Asaph! Joost! Thank you for becoming more than casual neighbors. To me, there was a bridge between our rooms. Of course, the length of that bridge varied and was sometimes diverted by the presence of lekker dropjes on the way (the last source of nutrition during the night) but still a daring Light was protruding out of our rooms sweeping over the darkness of the corridors...ever reminding us.

Arnaud and Luis, I spent amazing time going out together and meeting your lovely families. I still remember our laughs, our comments and reaction to many things that occured during dinners and trips to Arnhem, Utrecht and Wageningen! The words "Masen" and "geltamer" still resonate in my ear. Luis, I still keep your door tag. Arnaud... thanks for refining my computer skills with your kind advice concerning the thesis layout!

Ivan and Raj, thank you for the unforgettable time we spent together inside and outside the lab. I feel so much I like to repeat that. Tetty, Wiwim, Herti, Marina, Salva and Eddy. Yes, my Nederland was mostly the lab space ("Ooo welcome welcome to your Lab!") and the road

leading back to the house. Later on, it extended to the desk and computer...Thank you, for I enjoyed a lot your company, specially during the weekends! "You miss me don't you!?"
Petra, thanks for re- allocating me to that lab and I wish you a lot of success in Groningen!
Dion, I enjoyed sharing very gezellig talks with you and sharing the office with you is quite a story! I learned from you through your willingness to professionally and amicably discuss matters and through your DOW "experience". Sorry if I was socially slipping away during the thesis writing... but it was a one-time life experience. Mieke, besides the fact that we were roommates in Cell Cybernetics, we share a common quality of making people sick once they see us eating lekker fruit dropjes! Catarina, you happy mother, thanks for tolerating the heavy dropjes consumption within what seemed to be a limiting time frame. Nicolai and Rumyana, thanks for showing the way lab persons can still succeed in the ordinary life and I wish you lots of happiness together with Sophia and many other beautiful babies!
Ruud and Dirk, thank you for being part of a functional team along with Harro. I've learned a lot from you, also about you, and it feels clear: We have provided.
Robert, thank you for your understanding and ethical fairness. Within the intricate lines of the CPRO-PRI re-organization I always had a feeling "Robert knows how to get us through this!".
Raoul, I thank you for the dinners held at your house each year. It was a pleasure to get to meet your lovely family, Madelon, Ruben (the table tennis champion!) and Ilana, and also mingle with collegues in the BU at needed moments. Not only I appreciated the degree of openness and transparency displayed at each business unit meeting to reach our set goals, but also the sensibility to professional and personal urgencies of the BU members. I wish you success with yet a new and demanding challenge: Biosciences.
Beste mensen van Sodexho, ik dank jullie wel voor het lekker Food with Attitude! You made all the difference in my "outside"-eating experience! I thank Hans, Willem and Egberd for the level of tolerance shown after the building's opening hours and giving me lifts back home.
Alenka, Rommert, Emmy & Jan, thank you for the nice dinners we shared together and, Manuela, Raffaella, D for the invitations for different events. Arend, thank you for the beautiful scientific discussions we both had in the office for they made me reach out and "visualise the picture". Ans, Margriet and Renée, thank you for the help, advice and kindness during these 4 years. Eugene, Ton, Thres, Geert, Paulien, Lydia, Leny, Peter (nice jokes!), thank you for your friendliness and care! Ankie, Nora, Marianne, Erik, Luud and Ariette! You make the life of visiting scientists an enjoyable experience in Plant Research International. I know mine was and I Thank You! Lia, Jennie, Margot and Dinie, a smile on my face was guaranteed after each time we spoke, Thank you ☺. Hana and Ilse, thanks for the sweets!!!

I am grateful to the Dutch Ministry for Foreign Affairs for partly funding me. The PRI Post and Library staff members, the Dean's office for foreign students (Ankie! We did manage to go out for a cup of coffee 4 years later!), the studenten zaken, the PhD office and secretariat in WU and The Netherlands embassy in Lebanon: Thank You for your dedication and help!
Nic, that live high tree pushing the limits of the possible...while still connected, thank you for your Model Vision and restless efforts in making it happen in Plant Research International (dat zijn wij allemaal). We all understood...and from there on, it was just a matter of choice.
Peter, thank you for the help you provided me with computers and software. Mariame, thank you for your time and care to talk each time we met on the stairs. Jack, Thank you for a great computer/ communication job even under time pressure!
Louis, thank you for your valuable help with the computer. It made me RUN! I wish you all enlightenment in your new quest. Yvonne, thank you for your kindness and good luck too!
Cornelles, Thank You for your brotherly help, facilitating my living here in Wageningen.
Ameing, Hala it was a pleasure to work with you. I hope you have enjoyed the stay in PRI and that the supervision was positively contributing to that stay. Success in a surely bright Future!
I Thank grandfather for his support and special attention to my thesis. With you, I'm Happy!
Zeina, my beloved sister, friend and Plant Research International collegue. I thank you so much for you have shown me the meaning of true commitment in persuing Visions and Aims. Professionally, I saw you jump straight from one challenge to another, restlessly. We have been through a lot together in our life and you took care of me very well here in Wageningen. It's you who always says "the greatest obstacle in Life is not ignorance but rather the illusion of Knowledge"! We both like to let our mind fly, you still keep an eye on Life's reality. It is a rare occasion where I feel reminding you about the beauty of your mind and the importance of a sound and realistic judgement in stearing your amazing energy to where it is now needed.
Father and Mother, we never left each other did we? Surely not. You Inspire me and I thank and love you so much for the continous unconditional support I had from You. From the soft handhold, to the studies and talks of Life till early morning hours, to the warm and caring voice on the phone (each one of you with his accustomed style of course) I derived more than you imagine. I know that not being close to you, the last years were hard for you, especially that you were used to provide to us abundantly but...I hope I'm on my way to become a better man. Father, do you think they can feel what we both came to stand for?

Mazen

Curriculum Vitae

Mazen K. El Tamer was born on November 4th 1970 in Beirut, Lebanon. He graduated with a B.E degree in Agriculture Engineering and a B.Sc. degree in Agriculture and Food Sciences from the American University of Beirut in 1995. After his university studies, from April 1995 till August 1996, he worked with the Food and Agricultural Organization (FAO) and several agricultural companies as an expert agriculture engineer. He also worked as a food scientist in the Food and Beverage department of a consulting company. From August 1996, he studied for his M.Sc. degree in Biotechnology at Wageningen University and successfully completed his M.Sc. thesis in January 1998 in the department of Molecular Biology. From May 1998 till July 2002 he worked as a PhD researcher on a joint research project between the department of Food Chemistry in Wageningen University and the Business Unit Cell Cybernetics in Plant Research International B.V. The thesis research was on the cDNA cloning, characterization and regulation of monoterpene synthases isolated from *Citrus limon* in relation to plant fragrance.