From Plant to Plastic

Esmer Jongedijk

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Metabolic Engineering of Plant Monoterpenes for Biobased Commodity Chemicals

Esmer Jongedijk

Propositions

1. In principle, nowadays every natural compound of choice can be produced using metabolic engineering.
   (this thesis)

2. Identifying candidate biosynthetic genes solely based on sequence homology is misleading; adding a co-expression analysis, even with a limited number of samples, is much more efficient.
   (this thesis)

3. The often-used term 'plant terpenes' needs to be used carefully, as volatile compounds commonly attributed to plants (Salvador et al. 2017, Food Chem. 229), may actually be derived from associated microbes (Peñuelas et al. 2014, Sci. Rep. 4:6727).

4. Suggested manipulations of trichome formation in plants for metabolic engineering purposes (e.g. Singh et al. 2016, Plant Biotechnol. J.) are ignoring the reported tight links of trichome formation with plant physiological responses (Huchelmann et al. 2017, Plant Phys. 175; Ishida et al. 2008, Annu. Rev. Plant Biol. 59) that will impose challenges.

5. Teamwork is essential to win the game, independent of whether this game consists of sequential enzymatic conversions or is a sports match.

6. 'Survival of the fittest' also holds for music, the age-long survival of classical music confirms its higher 'fitness' compared to the volatile hit-list music.

Propositions belonging to the thesis entitled:
"From Plant to Plastic: Metabolic engineering of plant monoterpenes for biobased commodity chemicals"

Esmer Jongedijk,
Wageningen, 13 April 2018

From Plant to Plastic

Metabolic engineering of plant monoterpenes for biobased commodity chemicals

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From Plant to Plastic

Metabolic engineering of plant monoterpenes for biobased commodity chemicals

Esmer Jongedijk

Thesis

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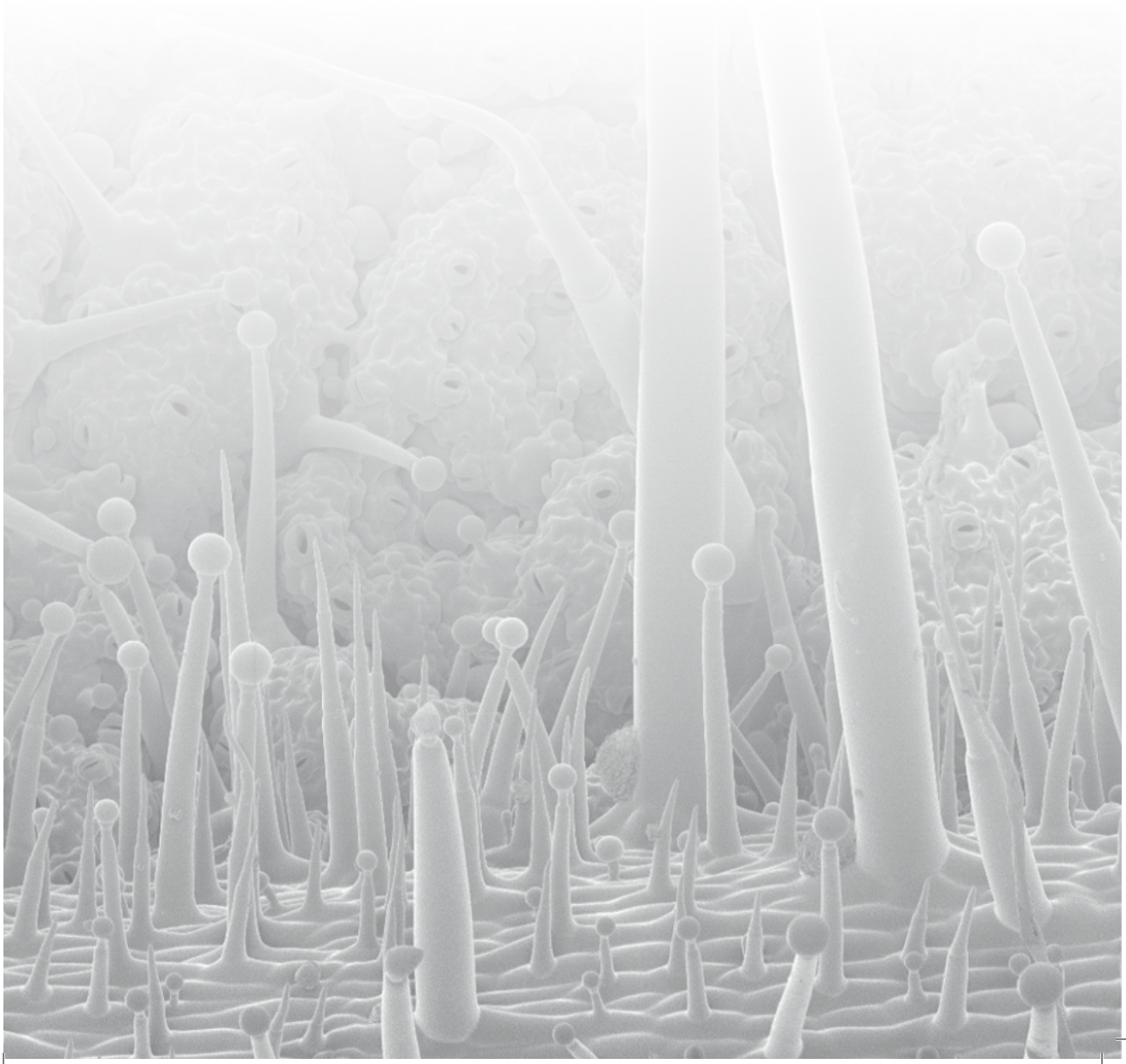
Table of Contents

Chapter 1
General introduction



Renewable commodity chemicals

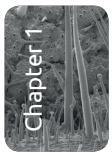
The use of fossil resources increasingly raises concerns on a worldwide scale. Oil supplies are not infinite. There is a continuous difference between forecasted demand for oil and maximum production, and this difference will increase even more in the near future (Sasaki et al., 2005; Owen et al., 2010). Besides, the use of fossil feedstocks is accompanied with environmental changes caused by greenhouse gas emissions (Owen et al., 2010). These drawbacks create and require the need for sustainable alternatives. In order to transfer from fossil to renewable resources in a biobased economy, new knowledge concepts need to be developed. For example, to replace petrochemistry-based platform chemicals that require extensive chemical modification, e.g. oxidation or amination, biobased compounds containing desired functional groups could be deployed. An example of an extensively modified building block is terephthalic acid (TA), which is currently mainly produced from petrochemical sources by oxidation of *para*-xylene (Rezaei and Sajadi, 2015). Global TA demand is expected to reach 65 million ton in 2018, predominantly for the production of polyethylene terephthalate (PET) (Collias et al., 2014). In the Netherlands, billions of PET bottles are sold on an annual basis (Van Zundert, 2006). PET is the fourth largest synthetic polymer in global annual production (Lithner et al., 2011). Therefore, the production of TA from natural sources (bio TA) is a potentially attractive approach. There is need for renewable biobased feedstocks that can be replenished, naturally or via agricultural techniques, within the timeframe of their consumption (Collias et al., 2014). Plants are such a renewable feedstock, and could serve as an alternative source for the production of bio-based fuels, chemicals and polymers.

Plant monoterpenes

The plant kingdom contains a rich variety of compounds. To sustain their growth and maintenance, plants synthesize compounds, such as sugars, lipids and amino acids, so called primary metabolites. Besides this group of molecules, plants produce a wide range of secondary metabolites, mainly low molecular weight compounds with diverse chemical structures that have specific important functions in the plant, but are not crucial for plant (short term) survival. The largest and most diverse group of plant secondary metabolites are the terpenes (Borghi et al., 2017).

In this thesis, applications of terpenes for human society are explored. Terpenes include several molecules that traditionally are used in food and cosmetics for their flavour and fragrance properties. One well-known example is (-)-menthol, a mint flavouring agent isolated from *Mentha* oil (Lange, 2015a), which is used as an ingredient in candy and cosmetics. Another example is (+)-limonene, a citrus fragrance isolated from waste of the citrus juice industry, which is added to soft drinks, candies, household cleaning

products and cosmetics. For example, >90% of available hair shampoos and >90% of available tooth pastes at the biggest retailer of the Netherlands contain limonene (November 2017, personal observation). Recently, the industry has commenced to use plant terpenes, bulk produced, as substitution for oil-based chemicals, for example as fuel additives and building block of commodity chemicals and polymers. An example is the terpene farnesene, that is used as a fuel additive (George et al., 2015). In 2016, the first 12 hour flight took place, from Toulouse in France to Hong Kong, on fuel supplied with 10% renewable farnesene produced by microorganisms (hydrogenated to farnesane) (DeNardo, 2016). Another example of a terpene that is being used as commodity is limonene, which is used as a solvent and as flavour and fragrance ingredient and can be used for building blocks for biopolymers (Colonna et al., 2011; Firdaus et al., 2011; Jongedijk et al., 2016).



The class of terpenes can be divided in sub-classes, based on molecular structure. Monoterpenes are composed from two isoprene C5 units, which provides their backbone C10 structure. Other classes of terpenes are hemiterpenes (C5), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) and polyterpenes (>C40) such as rubber (Lange, 2015b). The low molecular weight classes of terpenes, which are often volatile, have specialized functions in plant signalling, defence and reproduction (Lücker et al., 2002; Seo, 2003; Kappers et al., 2005; Borghi et al., 2017). By emitting terpenes, plants are capable of attracting enemies of plant herbivores that aid the plants' defense mechanisms, so called 'cry for help'. For example when Arabidopsis was engineered to emit the sesquiterpene (3*S*)-(*E*)-nerolidol and the homoterpene 4,8-dimethyl-1,3(*E*),7-nonatriene (*(E)*-DMNT) derived from it, it was capable of attracting carnivorous predatory mites (*Phytoseiulus persimilis*), a natural enemy of herbivorous mite species (Kappers et al., 2005).

Monoterpenes are volatile, colourless, lipophilic substances. For example the Henry's law constant, which describes partition of solutes between a watery phase and the headspace, of limonene is only 0.048 M/atm (Leng et al., 2013), indicating that 95% of limonene would partition into the headspace and evaporate. Monoterpene backbone biosynthesis usually occurs via the methylerythritol 4-phosphate (MEP) pathway in the plastids of the plant cell, via the common monoterpene precursor geranyl diphosphate (GPP) (Fig. 1) (Dudareva and Pichersky, 2006). GPP synthase (GPPS) combines the two C5 units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) in a head-to-tail fashion (Fig. 1). GPP is subsequently converted by monoterpene synthases to acyclic (e.g. geraniol) and cyclic (e.g. limonene) monoterpenes (Degenhardt et al., 2009).

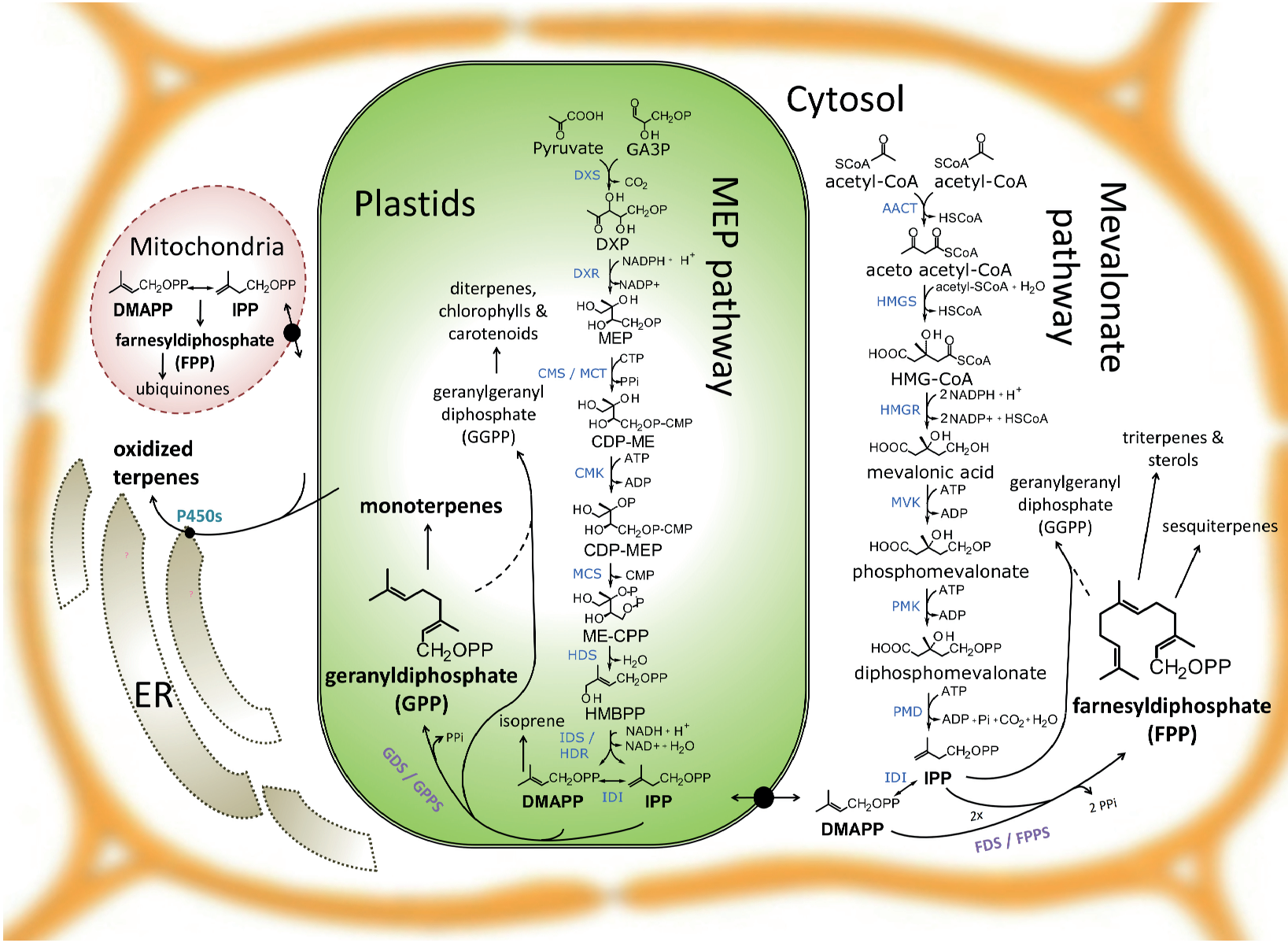

Cytosol
Plastids
Mitochondria
DMAPP
IPP
farnesyldiphosphate
(FPP)
ubiquinones
Pyruvate
GA3P
DXS
DXP
DXR
MEP
MEP pathway
diterpenes,
chlorophylls &
carotenoids
geranylgeranyl
diphosphate
(GGPP)
CMS / MCT
CDP-ME
CMK
CDP-MEP
MCS
ME-CPP
HDS
HMBPP
IDS /
HDR
isoprene
monoterpenes
geranyldiphosphate
(GPP)
GDS / GPPS
oxidized
terpenes
P450s
ER
acetyl-CoA
acetyl-CoA
AACT
aceto acetyl-CoA
HMGS
HMG-CoA
HMGR
mevalonic acid
MVK
phosphomevalonate
PMK
diphosphomevalonate
PMD
IDI
IPP
DMAPP
2x
2 PPi
FDS / FPPS
Mevalonate
pathway
geranylgeranyl
diphosphate
(GGPP)
triterpenes &
sterols
sesquiterpenes
farnesyldiphosphate
(FPP)

Figure 1 Terpene biosynthesis in plants. Abbreviations: MEP methylerythritol 4-phosphate, ER endoplasmatic reticulum, GA3P D-glyceraldehyde 3-phosphate ,CoA coenzyme A, DXP 1-deoxy-D-xylulose-5-phosphate, DXS 1-deoxy-D-xylulose-5-phosphate synthase, DXR 1-deoxy-D-xylulose-5-phosphate reductoisomerase, CDP-ME 4-diphosphocytidyl-2-C-methylerythritol, CMS / MCT 4-diphosphocytidyl-2-C-methylerythritol synthase / 2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase, CDP-MEP 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, CMK 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol kinase, ME-CPP 2-C-ethyl-D-erythritol-2,4-cyclodiphosphate, MCS 2-C-ethyl-D-erythritol-2,4-cyclodiphosphate synthase, HMBPP (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate, HDS 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase, IDS isopentenyl diphosphate / dimethylallyl diphosphate synthase, HDR 4-hydroxy-3-methylbut-2-enyl-diphosphate reductase, AACT aceto acetyl-CoA thiolase, HMG 3-hydroxy-3-methylglutaryl-CoA, HMGS 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR 3-hydroxy-3-methylglutaryl-CoA reductase, MVK mevalonate kinase, PMK phosphomevalonate kinase, PMD diphosphomevolonate decarboxylase, IDI isopentenyl diphosphate isomerase, DMAPP dimethylallyl diphosphate, IPP isopentenyl diphosphate (Rodriguez-Concepcion and Boronat, 2002; Miziorko, 2011; Banerjee and Sharkey, 2014)

An alternative pathway that also produces DMAPP and IPP, the mevalonate pathway, is situated in the cytosol, and is used as backbone biosynthesis pathway for other terpenes and sterols, via the intermediate farnesyl diphosphate (FPP) (Fig. 1) (Degenhardt et al., 2009). Some exceptions exist in nature to the usual monoterpene biosynthesis route via GPP. For example the *Z*-form of GPP, neryl diphosphate (NPP), can be used as substrate (Sun et al., 2016), two C5 units can be combined in non-head-to-tail fashion to form irregular monoterpenes (Demissie et al., 2013), or parts of the route can take place in the cytosol instead of in the plastids (Sun et al., 2016).

Natural functionalization

After backbone biosynthesis, monoterpenes are usually modified by the plant to form thousands of different, specialized structures (Lücker et al., 2002). Examples of enzymes from classes that are known to perform these modifications are cytochrome P450s, dehydrogenases and glycosyl transferases. These modifications change the behaviour of the compound in the plant cell, as the compounds' volatility, polarity and lipophilicity are changed. The monoterpene is functionalised for its specific action in plant ecology, which may involve transport, storage or evaporation from the cell. A well-studied example of a monoterpene that plays an important role in the ecology of plants is the monoterpene linalool, a floral scent compound that attracts pollinators (Parachnowitsch et al., 2013). In *Actinidia chinensis* flowers, linalool is glycosylated according to a daily pattern, with peak glycosylation between midnight and noon, and a peak release of the free volatile in the afternoon (Green et al., 2012). Presumably, in this way the plant restricts linalool release to moments when pollinators are present. To control monoterpene biosynthesis and storage, plants have intricate regulation mechanisms, in which often hormones are involved. For example, in Norway spruce monoterpenes are stored in defence-related resin ducts in the stem. When the tree is attacked by stem boring insects or fungal pathogens, or is treated with the hormone methyl jasmonate,

formation of resin ducts takes place, accompanied with transient induction of monoterpene synthase transcript accumulation (Martin et al., 2002; Faldt et al., 2003).

Besides specific functional groups, many monoterpenes have a chiral centre. Limonene synthases usually are capable of synthesising specifically the (-)- or (+)-form of the terpene. The specific functional groups and chirality are important for the application of terpenes. For example, (+)-limonene has a fresh citrus smell, while (-)-limonene has a turpentine-like smell. When limonene is hydroxylated at the 7-position, the product perillyl alcohol is formed, which has a sweet woody smell and is less volatile and hydrophobic than limonene. The natural diversity in chirality and functional groups of terpenes delivers opportunities for the smart design of commodity chemicals. Hereto, terpenes with structural similarity to a commodity chemical of interest can be used. Additionally, the structure of commodity chemicals is now limited by the possibilities of organic chemistry. Terpenes usually have natural enantiopurity (exclusively the (-)- or (+)- form). Enzymatic biosynthesis opens up routes to new materials with new, maybe chiral, properties. In this way, biobased, existing or new, commodity chemicals could be designed with the least number of synthetic steps, and mild chemistry, to design a sustainable production process.

Specialized tissues

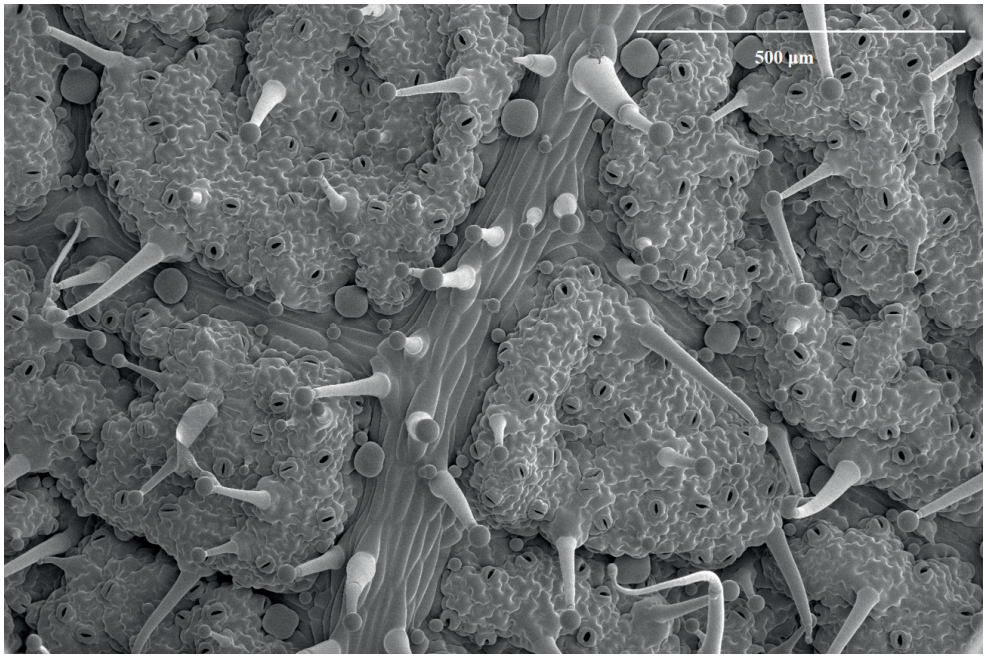


Figure 2 Trichomes on the surface of a *Salvia dorisiana* leaf. Glandular trichomes synthesize and store monoterpenes.

These useful chemical properties are naturally delivered by plant monoterpene biosynthesis. However, as monoterpenes serve in the plant as signalling molecules, plants usually do not naturally produce large amounts of them, in general less than 1% of the plant dry weight (Namdeo, 2007). Moreover, monoterpene biosynthesis usually takes place only in specialised plant tissues related to their ecological function. For example, in *Salvia dorisiana,* which we study in this thesis, (-)-limonene and it's oxidised derivatives are synthesised and stored in the glandular trichomes on the leaf surface (Fig. 2). Upon insect attack, the cuticle of the glandular trichomes may break, which causes directed release of the there-stored defence compounds (Glas et al., 2012). In citrus species, (+)-limonene is stored in secretory cavities in the peel of the fruit.

Heterologous production platforms

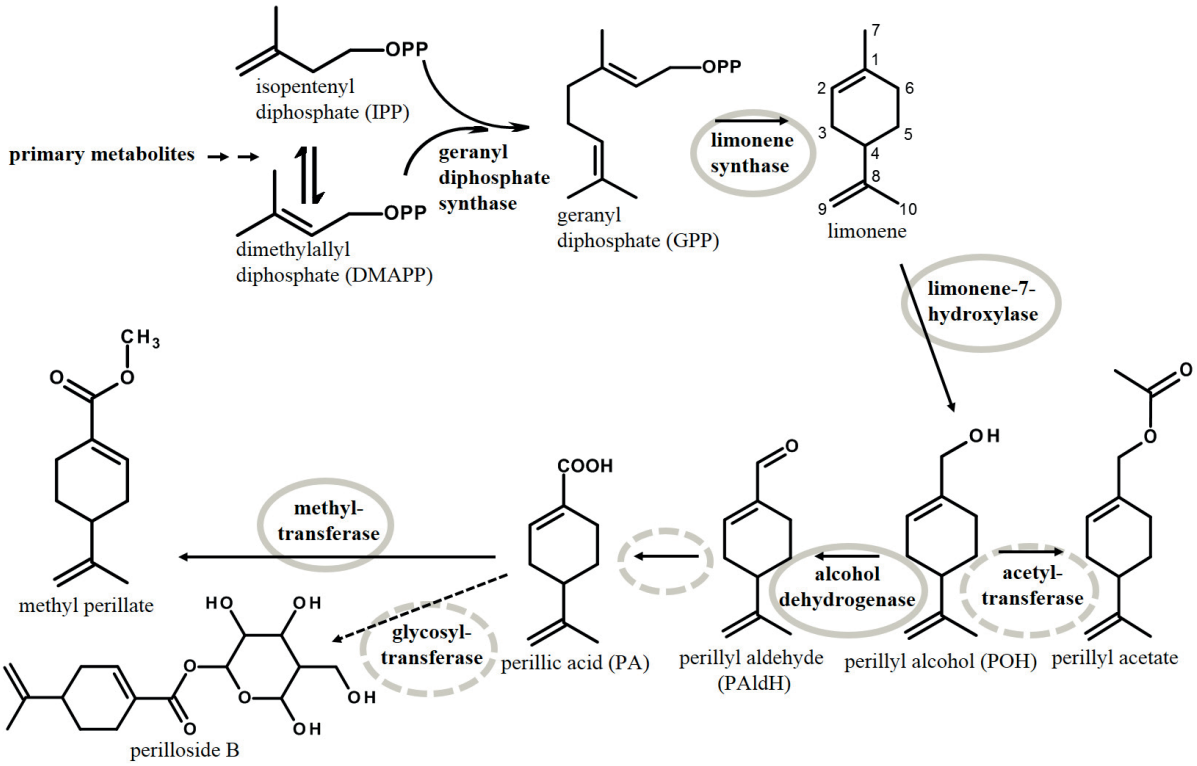
Production and harvesting of terpenes from specialised plant tissues that produce only low amounts can be laborious and usually is not very economic. Besides, the natural production might be confined to plant species that are not common agricultural crops. However, it may be possible to produce large and pure amounts of terpenes, and hence might be worthwhile doing so, by transferring the biosynthesis to a heterologous production system like micro-organisms. Well-known examples of the production of terpenes in micro-organisms are the production in yeast of the sesquiterpenes artemisinic acid (Paddon et al., 2013), a precursor for the antimalarial drug artemisinin, and farnesene (Meadows et al., 2016), a fuel additive and versatile industrial commodity chemical, with titers of 25 and 130 g / L respectively. The plant species *N. benthamiana* has also been demonstrated to be a suitable heterologous production host. For example biosynthesis of the monoterpene geraniol (Dong et al., 2016) and the sesquiterpene costunolide (Liu et al., 2011) has been reconstructed in this plant.

The heterologous production of terpenes as mentioned above requires profound knowledge of their natural biosynthesis in the plant. The genes encoding the biosynthetic enzymes are to be isolated from the plant and transferred to a suitable production host. The shape of the terpene backbone and functional groups can be designed as required, by means of the addition of corresponding biosynthetic enzymes. The new host could be subjected to further engineering whenever required. A well-known engineering strategy is to enhance the production of precursor compounds. Expression of a truncated form of HMGR (Fig. 1), for example, can increase the flux to the precursor farnesyl diphosphate (FPP) both in plants and microbes, resulting in increased sesquiterpene production (Martin et al., 2003; Peralta-Yahya et al., 2011; Cankar et al., 2015). In *Escherichia coli*, addition of a plant GPP-synthase to the microbial system enhanced production of limonene (Willrodt et al., 2014). Other engineering strategies are closing down the synthesis of undesired side-products (Cankar et al., 2015), and removal of the (sometimes toxic) end-product. Capturing of volatile products from a liquid microbial

culture can reduce toxicity (Jongedijk et al., 2015). In plants, volatile products are often derivatised to non-volatile glycosides, as a potential detoxification mechanism (Chapter 5) (Liu et al., 2011; Dong et al., 2016).

The perillic acid pathway

The perillic acid (PA) pathway produces useful monoterpenes for humans (Scheme 1). Products from this pathway are reported to be present in the species *Perilla frutescens* and *Salvia dorisiana*, both from the Lamiaceae family (Chapter 5) (Halim and Collins, 1975; Tabata, 2000). The first postulated step in this pathway is the biosynthesis of limonene (Scheme 1). Limonene itself has versatile applications (Jongedijk et al., 2016). Limonene is then further functionalised by hydroxylation on the C7-position, and oxidation to perillic acid. In plants, perillic acid is reported to be further derivatised by glycosylation (Fujita and Nakayama, 1993; Fujita et al., 1995). A side product of the intermediate perillyl alcohol is produced by acetylation. Also, a methylated form of PA has been reported in Salvia, where functionalisation with the methyl-ester group on the 7-position leads to methylperillate (MPA) (Chapter 5) (Halim and Collins, 1975).



Scheme 1 Perillic acid biosynthesis pathway

Methylperillate shares similarity in structure to the PET plastic building block terephthalic acid. The perillic acid pathway has not been fully elucidated (Ohara et al., 2003; Mau et al., 2010; Sato-Masumoto and Ito, 2014) (Scheme 1). A large scale production of limonene and methylperillate would be an attractive alternative to the use of present restricted resources and would enable their use for bulk applications as solvents and polymer building blocks.

Thesis outline

This thesis focusses on a new concept for the production of biobased commodity chemicals. The objective is to replace petrochemistry-based platform chemicals that require extensive chemical modification, by biobased compounds already containing desired functional groups. To study this we use the *Salvia* monoterpene methylperillate as a model biobased source for the commodity chemical terephthalic acid. Terephthalic acid production from oil requires extensive chemical modification, and methylperillate is a biobased molecule with desired functionalities, but is not present in large amounts in nature. New knowledge was required, for which a multi-disciplinary approach was used (Fig. 3).

In **Chapter 1** (this introduction) I introduce plant terpenes and the perillic acid pathway, and the goal to be able to use monoterpenes from the perillic acid pathway as a substitution for oil-based chemicals (Fig. 3).

Chapter 2 of this thesis reviews production and use of limonene, the first dedicated precursor from the perillic acid pathway. Limonene has a wide range of applications for humans, and has already been produced by heterologous expression of plant synthases in different micro-organisms. Limonene production hosts and the engineering techniques are described that were applied to harvest maximum amounts of limonene from these microbes. Future perspectives of monoterpene production in micro-organisms are discussed as well in this mini-review.

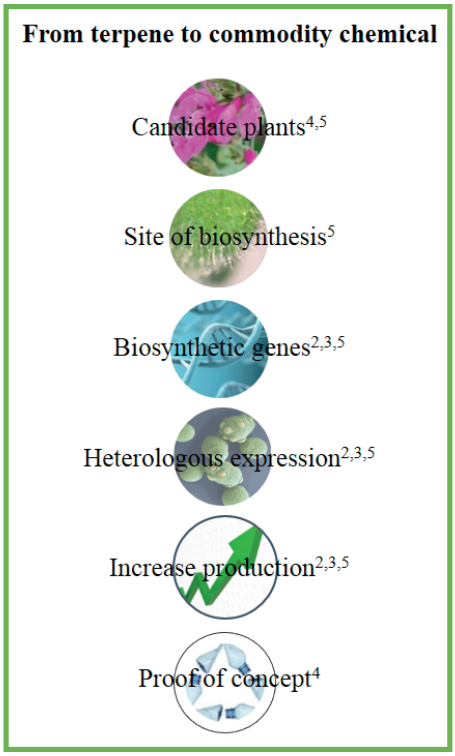


Figure 3 Approach from terpene to commodity chemical, as followed for this research. Superscript numbers indicate chapter numbers of this thesis.

The volatile and anti-microbial properties of limonene make it a challenge to produce it in a microbe. This challenge is faced in **Chapter 3**, where production of limonene in the yeast *Saccharomyces cerevisieae* is demonstrated. A harvesting system is reported to capture the volatile product and to avoid toxicity.

In order to apply limonene as a direct precursor for sustainable biobased polymers, additional functional groups are preferred. The limonene-derived monoterpene methylperillate carries a methyl-ester group on the 7-position. It has naturally relevant functional groups for the synthesis of the PET plastic building block terephthalic acid. In **Chapter 4** methylperillate is used as a starting material for the synthesis of terephthalic acid. The described route was designed to improve sustainability.

Production of the monoterpene methylperillate in a micro-organism or other heterologous platform requires elucidation of the methylperillate biosynthetic pathway and characterization of the enzymes involved. Methylperillate is naturally produced in *Salvia dorisiana*, not a common agricultural crop. Its biosynthesis in specialized tissues, the Type IV-like glandular trichomes, is described in **Chapter 5**. A genomics approach was taken to identify the enzymes involved in methylperillate biosynthesis. The transcriptome of trichomes and other tissues was sequenced, and candidates selected by comparative expression analysis. Necessary enzymes for methylperillate biosynthesis are identified and characterized. Methylperillate synthesis is established in the model heterologous plant host *Nicotiana benthamiana*.

Finally, I discuss the general relevance of the strategy described in my thesis in **Chapter 6**. Encountered obstacles are analysed and future perspectives given. Therewith this thesis describes a new way to produce sustainable high-value commodity chemicals.

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Chapter 1

Chapter 2
Biotechnological production of limonene in microorganisms

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JENS SCHRADER, HARRO BOUWMEESTER
AND JULES BEEKWILDER

Applied Microbiology and Biotechnology (2016) 100:2927-2938

Abstract

This mini review describes novel, biotechnology based, ways of producing the monoterpene limonene. Limonene is applied in relatively highly priced products, such as fragrances, and also has applications with lower value but large production volume, such as biomaterials. Limonene is currently produced as a side-product from the citrus juice industry, but the availability and quality are fluctuating, and may be insufficient for novel bulk applications. Therefore complementary microbial production of limonene would be interesting. Since limonene can be derivatized to high value compounds, microbial platforms also have a great potential beyond just producing limonene. In this review, we discuss the ins and outs of microbial limonene production in comparison with plant-based and chemical production. Achievements and specific challenges for microbial production of limonene are discussed, especially in the light of bulk applications such as biomaterials.

Keywords: Limonene, Biomaterial, Monoterpene, Microbial production, Metabolic engineering, Toxicity

Introduction

Limonene is a well-known cyclic monoterpene. It is an olefin hydrocarbon ($C_{10}H_{16}$), which can occur in two optical forms. (+)-Limonene is one of the most important and widespread terpenes in the flavor and fragrance industry. Limonene (in both optical forms) has been found in more than 300 plant essential oils (DNP 2015) from very diverse species including orange, lemon, mint and fir. Its biosynthesis has been well-described in the plant kingdom. Limonene has been detected naturally in trace amounts in the headspace of microbes (Effmert et al. 2012; Heddergott et al. 2014; Hung et al. 2013), however to our knowledge no corresponding biosynthetic mechanism has been identified. By transformation with plant limonene synthases, microorganisms such as yeast and bacteria have been engineered to produce limonene. In this work, biotechnological production of limonene for application as commodity chemical is reviewed. Others have reviewed general aspects of production of terpenes in microbes and plants (Aharoni et al. 2006; Duetz et al. 2003; Kirby and Keasling 2009; Vickers et al. 2014; Wang et al. 2015). Recently Lange (2015) reviewed the biosynthesis and biotechnology of limonene for flavor and fragrance applications.

New applications of limonene for fuel and biomaterials ask for large and stable production volumes. Metabolic engineering strategies, like overexpressing precursor pathway enzymes, have been applied for the purpose of increasing limonene titers, which are at the moment still far from the maximal theoretical yield. Crucial in such strategies is the overproduction of geranyl diphosphate (GPP), the direct precursor of

limonene. New opportunities to increase yield will be discussed, including novel strategies for capturing the product from the microbial cultures and possibilities for relieving limonene toxicity. When successful, these optimization strategies could result in a role for limonene-based products in the bio-based economy.

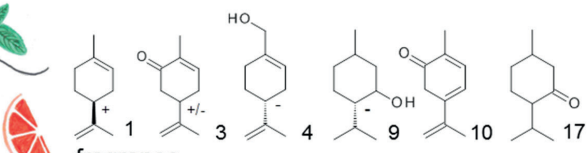
Applications and products from limonene

Limonene has a wide variety of applications, which differ in volume, quality requirements and price (Ciriminna et al. 2014) (Fig. 1). Traditionally (+)-limonene is used as a flavoring compound in citrus flavored products such as soft drinks and candy, and as a fragrance ingredient in household cleaning products and perfumes (Duetz et al. 2003). As a flavor and fragrance ingredient, limonene has a relatively high price because of the quality requirements in this field. For this application, chirality is important. (+)-Limonene (also called R- or d-limonene) has a pleasant, orange-like odor whereas the (-)-form (also called S- or l-limonene) has a more harsh turpentine-like odor with lemon note (Friedman and Miller 1971). Limonene has minor applications in other products. For example, it is used as insecticide (Ciriminna et al. 2014) and is being investigated for medical applications due to its anti-microbial and anti-cancer properties (Inouye et al. 2001; Miller et al. 2010).

Potentially, limonene can also be used for larger scale applications, for example as an alternative to so-called BTEX (benzene, toluene, ethylbenzene and/or xylene) solvents that are used in substantial volumes for oil and gas production (Fischer 2013). In addition, jet fuel replacements can be supplemented with limonene (Renninger et al. 2008). For solvent and fuel applications large volumes of limonene at a low price would be required, however exact numbers have to our knowledge not been reported.

The structure of limonene is very suitable for chemical modifications, due to the two available double bonds and possibility for hydroxylation (Wilbon et al. 2013). Modifications are important for many applications. Natural derivatives of limonene, mainly oxidized forms (Fig. 1), are used in particular for flavoring. For instance, the mint flavoring agent (-)-menthol is isolated from *Mentha* oil (Lange 2015). But limonene is also suitable for (additional) chemical modifications (Fig.1). For example, after complete hydrogenation, limonene can be added to diesel, to lower the cloud point and decrease its viscosity (Tracy et al. 2009). Modifications usually increase the price of the product, for example limonene is sold at 9-10 $/kg, while (-)-menthol makes 15-40 $/kg (Lange 2015; Stuart Clark IV 1998).

Compared to the traditional use of limonene as flavor and fragrance ingredient, its application in the chemical industry has not received much attention in the scientific literature. However, biomaterials will be increasingly important to replace traditional, petrochemical-based materials (Langeveld et al. 2010). Several types of biomaterials



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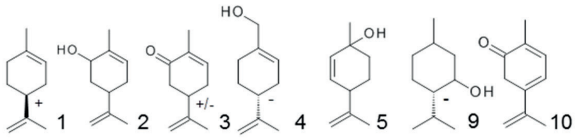
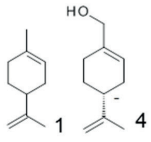
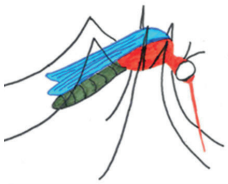
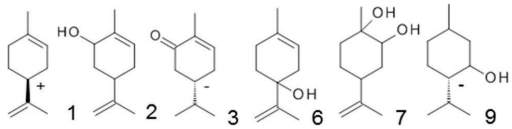


flavour

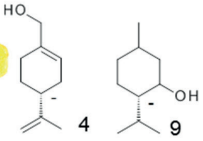


anti-microbial

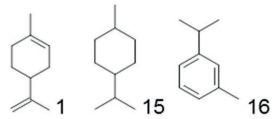




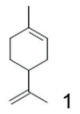
insect attractant / repellent



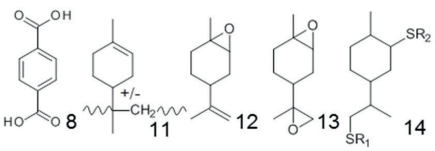
medicine



fuel



solvent



biomaterial

Figure 1 Applications of limonene and limonene derived molecules made by plants (1, 2, 3, 4, 8, 9, 10), microbes (1, 2, 3, 4, 5, 6, 7, 8, 12) and/or chemical synthesis (1, 3, 11, 12, 13, 14, 15, 16, 17) (Duetz et al 2003, Lerin et al 2010, Duetz et al 2001, Bowen 1975, Weldon et al. 2011, Tripathi 2009, Lange 2015, Colonna 2011, Ciminno 1998, Ciriminna 2014, Firdaus 2011, Tracy 2009). 1 Limonene; 2 Carveol; 3 Carvone; 4 Perillyl alcohol; 5 p-mentha-2,8-diene-1-ol; 6 p-mentha-1,8-dien-4-ol; 7 p-menth-8-ene-1,2-diol; 8 Terephthalic acid; 9 Menthol; 10 Dehydrocarvone; 11 Polylimonene; 12 Limonene mono-epoxide; 13 Limonene di-epoxide; 14 Product of thiol di-addition (R1 and R2 are thiol-side groups e.g. 2-mercaptoethanol, methyl thioglycolate or thioglycerol); 15 1-isopropyl-4-methylcyclohexane; 16 m-cymene; 17 menthone; + and – indicate where a single enantiomer is used; +/- means either enantiomer can be used, but not as a mixture.

can be made from limonene. A widely applied polymer of limonene, polylimonene (Piccolyte C115), is made from citrus oil (Cimmino et al. 1999). It is used as a resin in adhesives, thermoplastics for the food packaging industry and electro-conductive parts, and as a masticatory agent in chewing gum. Terpene resins have suitable compositions for medical purposes such as drug delivery (Barros et al. 2007). Epoxidation of limonene yields limonene mono- or di-epoxide (Fig. 1), which can subsequently be polymerized (Ciriminna et al. 2014). Limonene epoxide polymers are used in metal coatings, varnishes and printing inks (Firdaus et al. 2011). Attaching two thiols to limonene (Fig. 1) facilitates polymerization to for example limonene/fatty-acid based polyesters (Firdaus et al. 2011). These are used as sealants and adhesives (Ciriminna et al. 2014). For many limonene-based biopolymers chirality is important. Enantio-pure limonene production from plants or microbes provides opportunities for chiral polymers with applications as chiral purification, nonlinear optics or conducting materials (Firdaus et al. 2011). Limonene can also be converted to terephthalic acid (Fig. 1), which is used as building block for polyethylene terephthalate (PET) plastic (Colonna et al. 2011). PET is a widely used packaging material. The worldwide production of PET in 2009 was approximately 13 million tons (Colonna et al. 2011). Clearly, for commercial use of limonene in such biomaterials, affordable and reliable production at large volumes would be required.

Current production of limonene

Limonene is available from various sources. Most limonene currently on the market is (+)-limonene, produced as a side-product from the citrus juice industry. Citrus oil can contain 70-98% of (+)-limonene (Sokovic et al. 2010; Tranchida et al. 2013). It is produced in more than 60,000 ton/year (Lange 2015). Availability of citrus oil has been under pressure lately. Important citrus production areas in Brazil and the USA have been infested by the bacterial disease Huanglongbing (HLB), which has led to a drop in yield and a reduced area for citrus production (Hodges and Spreen 2012). Prices for citrus fruit, citrus oil and limonene are therefore fluctuating and increasing. Currently there are no successful disease management strategies for control of HLB, and therefore

availability of citrus-derived limonene is expected to continue to decrease. Besides that, part of the citrus-derived limonene is not food-grade as it may contain significant amounts of pesticides (Nichkova et al. 2009), which limits the application in food and household products. Another source of limonene is turpentine, from which racemic limonene (referred to as dipentene) is produced at 450 ton/year in the US (Thorp 2010). Fully synthetic limonene can be made by Diels-Alder addition of two isoprene units. A process based on this addition has been described, which converts scrap tire rubber to limonene (Hanson et al. 1999). The scale at which fully synthetic limonene is produced is limited (Lange 2015). For limonene that needs to be food-grade or enantio-pure, not all current sources are suitable.

Biotechnological production of limonene may complement current production systems. Microbial production would reduce dependence on the citrus industry, and can convert raw materials like glucose or glycerol, which are available from a large variety of agricultural sources, to limonene. To avoid competing claims with food production, microbial biotechnology is developing ways to deploy biomass from non-food sources as a feedstock for microbial cultures (Rumbold et al. 2009). Microbially produced limonene and its derivatives may in many cases be labeled as 'natural', which has consequences for market prices (Serra et al. 2005). Microbial limonene synthesis is enantio-specific, which is necessary for applications in flavor and fragrance products and for chiral polymers. Moreover, oxidation by biocatalysts can be integrated in microbial production systems (Alonso-Gutierrez et al. 2013). The large volumes of limonene necessary for biobased solvents, fuel additives and materials would make microbial production systems valuable on the longer term.

Costs of production of limonene in microbes have not been reported yet. For the related sesquiterpene farnesene, a jet fuel substitute, the costs for manufacturing in yeast have been reported to be as low as US$ 1.75 per liter (McCoy 2015). Clearly, if prices of microbially-produced limonene drop to the same level, they reach the same order of magnitude as the current price for citrus-derived limonene. Farnesene yields of more than 100 g / L culture have been reported (Pray 2010). To our knowledge the highest reported limonene titer so far was 1.35 g limonene per L culture (Willrodt et al. 2014), and would need to increase still two orders of magnitude to reach the current price of plant-derived limonene.

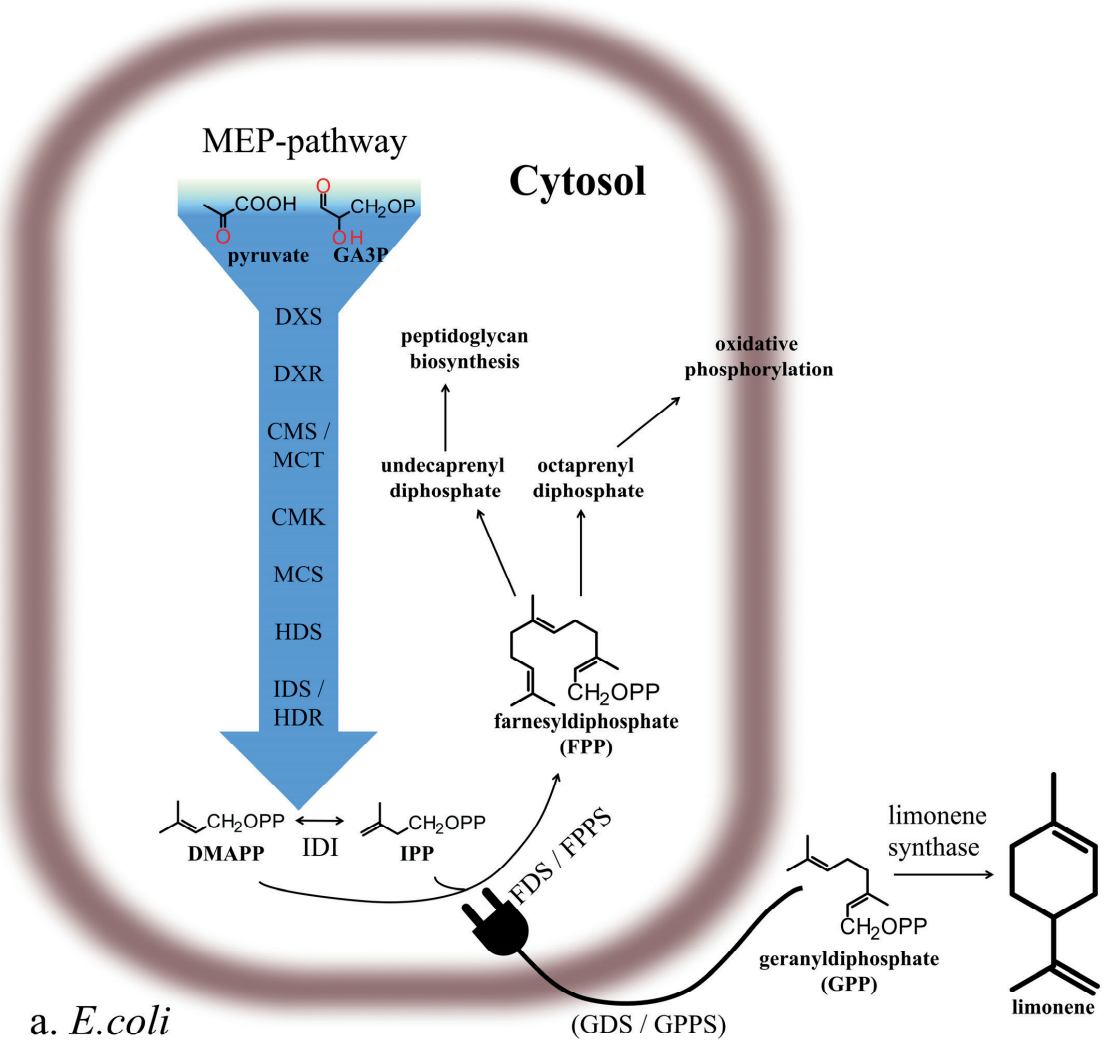
Biosynthesis of limonene

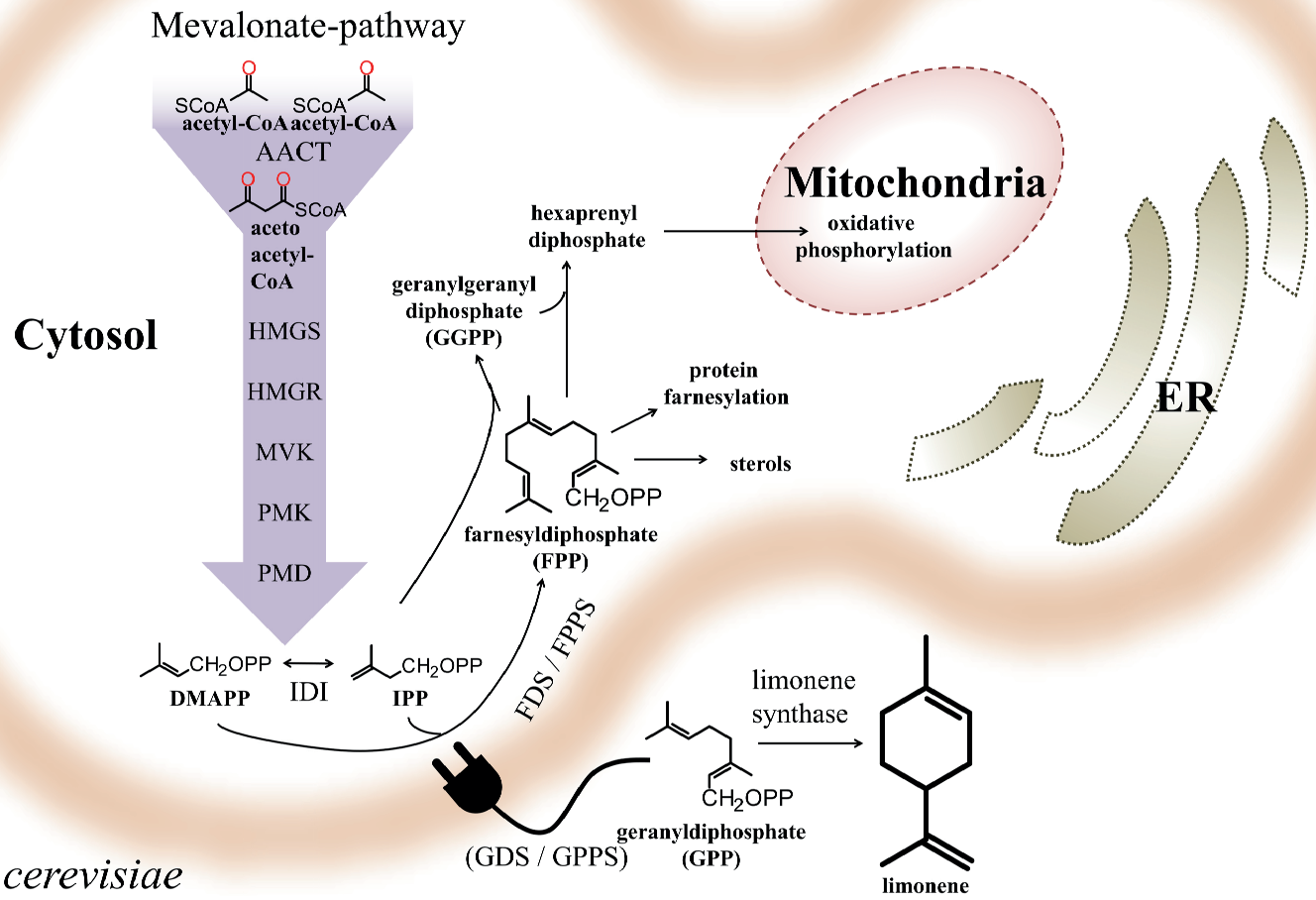
Optimization of microbial limonene production can be inspired by plants. Plants produce and store limonene in specialized structures. In citrus species, (+)-limonene is stored in secretory cavities in the peel of the fruit (Voo et al. 2012). These cavities are located in the outer, colored region of the peel, the flavedo. Biosynthetic genes for the

production of limonene have been found to be highly expressed in epithelial cells that are lining the secretory cavities. The required enzymes were shown to be localized in organelles present in these cells, called leucoplasts (Turner and Lange 2015). Leucoplasts are plastids, and differ from chloroplasts in that they lack photosynthetic machinery. In plant species of the *Lamiaceae* family, (-)-limonene and its derivatives accumulate in glandular trichomes, small structures on the surface of the leaves (Voirin and Bayet 1996). The enzymes involved in limonene biosynthesis and downstream oxidation are active in the secretory cells of these glandular trichomes (Turner et al. 1999; Turner and Croteau 2004). Limonene is stored in the subcuticular cavity of the trichome. The high concentrations of limonene found in the subcuticular cavities prove that trichomes should be considered as true cell factories (Lange and Turner 2013; Tissier 2012). Both in trichomes and in secretory cavities, limonene is stored outside the plant cells. If limonene concentrations in unspecialized plant cells become very high, the plant responds by emission of limonene into the air (Aharoni et al. 2005) or the glycosylation of limonene oxidation products (Fujita and Nakayama 1993; Lucker et al. 2001).

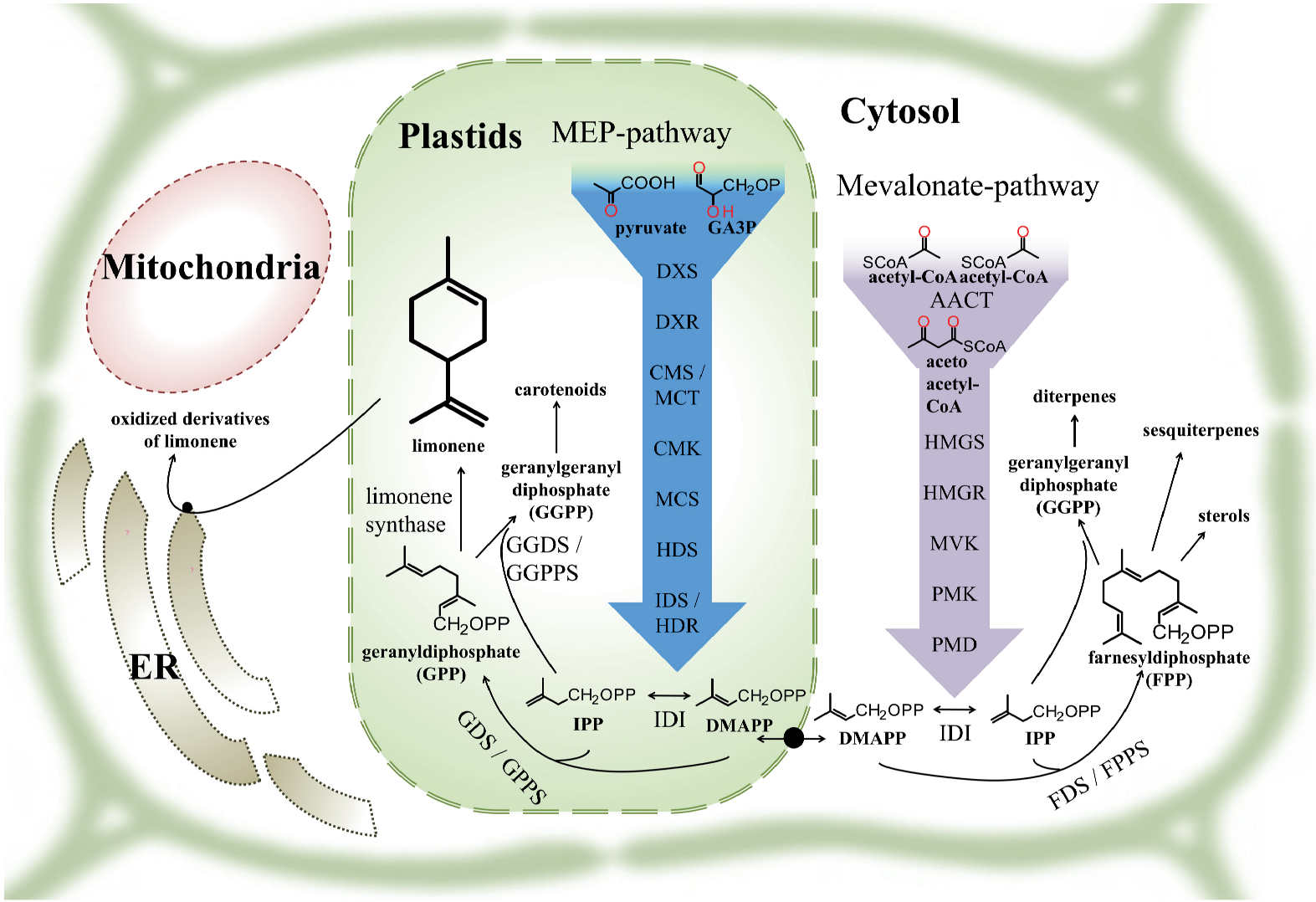
In plants, the biosynthesis of terpenes can proceed through two distinct isoprenoid-synthesizing pathways, which have been reviewed extensively (Banerjee and Sharkey 2014; Miziorko 2011; Rodriguez-Concepcion and Boronat 2002). Limonene is produced by limonene synthases from the substrate GPP (Fig. 2) (Lücker et al. 2002). GPP is a C_{10} compound that originates from the methylerythritol 4-phosphate (MEP) pathway. The MEP pathway produces the C_5 units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), that are condensed to form GPP, facilitated by a prenyltransferase enzyme, GPP synthase. The two C_5 units are also the building blocks for higher isoprenoids, for example geranylgeranyldiphosphate (GGPP), the C_{20} building block for the carotenoid pathway and diterpenes. The MEP pathway operates in the plastids. A parallel pathway, the mevalonate pathway, operates in the cytosol (Fig. 2), and also delivers the same two C_5 building blocks. This pathway is mostly used to supply the C_{15} substrate farnesyl diphosphate (FPP), for biosynthesis of sesquiterpenes and sterols.

Figure 2 Terpene backbone biosynthesis in microorganisms and plants. Plug indicates plug-in of limonene biosynthesis in microbial hosts. Abbreviations: MEP methylerythritol 4-phosphate, ER endoplasmatic reticulum, GA3P D-glyceraldehyde 3-phosphate ,CoA coenzyme A, DXS 1-deoxy-D-xylulose-5-phosphate synthase, DXR 1-deoxy-D-xylulose-5-phosphate reductoisomerase, CMS / MCT 4-diphosphocytidyl-2-C-methylerythritol synthase / 2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase, CMK 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol kinase, MCS 2-C-ethyl-D-erythritol-2,4-cyclodiphosphate synthase, HDS 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase, IDS isopentenyl diphosphate / dimethylallyl diphosphate synthase, HDR 4-hydroxy-3-methylbut-2-enyl-diphosphate reductase, AACT aceto acetyl-CoA thiolase, HMGS 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR 3-hydroxy-3-methylglutaryl-CoA reductase, MVK mevalonate kinase, PMK phosphomevalonate kinase, PMD diphosphomevolonate decarboxylase, IDI isopentenyl diphosphate isomerase, DMAPP dimethylallyl diphosphate, IPP isopentenyl diphosphate (Banerjee and Sharkey 2014; Miziorko 2011; Rodriguez-Concepcion and Boronat 2002)





b. *S. cerevisiae*


Plastids
MEP-pathway
Cytosol
Mevalonate-pathway
Mitochondria
ER
pyruvate
GA3P
DXS
DXR
CMS / MCT
CMK
MCS
HDS
IDS / HDR
limonene
limonene synthase
carotenoids
geranylgeranyl diphosphate (GGPP)
GGDS / GGPPS
geranyldiphosphate (GPP)
oxidized derivatives of limonene
IPP
IDI
DMAPP
GDS / GPPS
acetyl-CoA
AACT
aceto acetyl-CoA
HMGS
HMGR
MVK
PMK
PMD
diterpenes
sesquiterpenes
sterols
geranylgeranyl diphosphate (GGPP)
farnesyldiphosphate (FPP)
FDS / FPPS

c. *C. limon*

Limonene synthases

It is relevant for microbial production efficiency to use an appropriate limonene synthase. Synthases can differ in their product- and enantio-specificity and performance in micro-organisms. Synthases that convert GPP to limonene have been identified in 27 plant species from 9 plant families (Table S1). Most limonene synthases produce almost exclusively limonene, but in some cases, limonene is one of several products (Lucker et al. 2002). Synthases often produce one predominant enantiomer of limonene, either (-) or (+). Remarkably, enantio-specificity can differ between limonene synthases from the same plant family. For example within the *Lamiaceae* family, limonene synthase from *Perilla frutescens* makes predominantly (-)-limonene (Jongedijk et al. 2015), while limonene synthase from *Lavandula angustifolia* produces predominantly (+)-limonene (Landmann et al. 2007). Interestingly, a limonene synthase from the glandular trichomes of the wild tomato *Solanum habrochaites* has been described that deploys neryl diphosphate (NPP) as a substrate to make limonene, instead of GPP (Schilmiller et al. 2009). NPP is also a C_{10} diphosphate, but with a different stereochemistry than GPP, and is made by an NPP synthase (Kang et al. 2014). NPP derived monoterpenes have also been found in soybean, indicating that NPP-dependent monoterpene biosynthesis may occur in more species (Zhang et al. 2013).

All known limonene synthases carry a plastid transit peptide, which mediates localization to the plastid, but is not present in the final, active form of the enzyme. Proper microbial expression of the limonene synthase requires removal of the transit peptide, which is not functional in microbes and may interfere with correct folding of the protein. Indeed, removal of the transit peptide improved limonene yields 4- to 8-fold in yeast (Jongedijk et al. 2015). In all the microbial production systems described below the predicted transit peptide was removed.

Producing limonene in microorganisms

Several choices have to be made when engineering a microbe for the production of limonene. This starts already with the choice of the microorganism, which may have consequences for the possibilities of feedstocks, possibilities for engineering, and system properties like solvent tolerance. The production of limonene in microorganisms can in principle be achieved by simply expressing a plant limonene synthase (Carter et al. 2003), but this has resulted in disappointing yields, likely due to the low availability of GPP in microorganisms. For an economically successful production of limonene in microbes, a metabolic engineering approach is required, directed at increasing the availability of GPP. Metabolic engineering choices that have been described, and remaining challenges, will be discussed in more detail.

Microbial hosts used for limonene production

Naturally, microorganisms carry only one of the isoprenoid precursor pathways, either the MEP or the mevalonate pathway. In *Escherichia coli* and *Saccharomyces cerevisiae*, the two hosts commonly used for metabolic engineering of monoterpene production, only minor amounts of GPP precursor are available. Mostly, this GPP is a by-product from endogenous prenyltransferases, as a short-lived intermediate to longer-chain isoprenoids (Burke and Croteau 2002) (Fig. 2). *E. coli* has a cytosolic MEP pathway, which normally functions to produce C_{15} FPP and higher polyprenyl diphosphates, which are used for biosynthesis of peptidoglycan, a cell wall component, and for production of ubiquinone for oxidative phosphorylation (Erhardt et al. 2014; Swiezewska and Danikiewicz 2005). (Table 1).

Table 1 Microbial strains engineered to produce limonene

Host	**Engineering design**	**Limonene synthase origin, (+/-)-limonene, accession number**	**Maximal limonene yield per L culture & recovery method**	**Reference**
E. coli BLR (DE3) codon +	• *Abies grandis* tGPPS	*Mentha spicata*, (-), L13459	5 mg/L (-)-limonene; steam distillation	(Carter et al. 2003)
E. coli DH1 ΔacrAB	• HMGS and tHMGR from *Staphylococcus aureus* • codon optimised MVK, PMK and PMD from *S. cerevisiae* • AACT and IDI from *E. coli* • tGPPS from *Abies grandis* • one plasmid containing the mevalonate pathway genes and one plasmid with tGPPS and tLS • *A. borkumensis* efflux pump (YP_692684)	*M. spicata*, (-), L13459, codon optimised	57 mg/L (-)-limonene; dodecane organic phase	(Dunlop et al. 2011)
E. coli DH1	• HMGS and tHMGR from *Staphylococcus aureus* • codon optimised MVK, PMK and PMD from *S. cerevisiae* • AACT and IDI from *E. coli*	*M. spicata*, (-), accession number not clear, codon optimised	430 mg/L (-)-limonene; dodecane organic phase	(Alonso-Gutierrez et al. 2013)

	• tGPPS from *Abies grandis* • one plasmid			
E. coli BL21(DE 3)	• HMGS and tHMGR, MVK, PMK and PMD from *S. cerevisiae* • AACT and IDI from *E. coli* • tGPPS from *Abies grandis* / GPPS from *Streptomyces sp.* strain KO-3988	*M. spicata*, (-), L13459, codon optimised	1.35 g/L (-)-limonene; diisonoyl-phtalate organic phase	(Willrodt et al. 2014)
Synechocystis sp. PCC 6803	• DXS, IDI and CrtE from *Synechocystis*	*Schizonepeta tenuifolia,* enantioselectivity not clear, AF282875	56 µg/L culture/day ; gas stripping	(Kiyota et al. 2014)
Synechococcus sp. PCC 7002	• Wild-type and Δ*glgC* background were compared	*M. spicata,* (-), Q40322, codon optimised	4 mg/L in wild-type background ; dodecane organic phase	(Davies et al. 2014)
S. cerevisiae AE9	• Yeast FPPS (ERG20 K197G) mutated to partly produce GPP	*Perilla frutescens,* (-), KM015220 and *Citrus limon*, (+), AF514287	0.49 mg/L (-)-limonene, 0.12 mg/L (+)-limonene; headspace trapping	(Jongedijk et al. 2015)
S. cerevisiae EPY210C	• tHMGR from *S. cerevisae* • upc2-1 transcription factor	*M. spicata,* (-), L13459 and *C. limon,* (+), AF514287, codon optimised	1.48 mg/L (-)-limonene dodecane organic phase	(Behrendorff et al. 2013)

GPPS geranyl diphosphate synthase, t truncated, LS limonene synthase, HMGS 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR 3-hydroxy-3-methylglutaryl-CoA reductase, MVK mevalonate kinase, PMK phosphomevalonate kinase, PMD diphosphomevolonate decarboxylase, AACT aceto acetyl-CoA thiolase, IDI isopentenyl diphosphate isomerase, KO knock-out, DXS 1-deoxy-D-xylulose-5-phosphate synthase

While *E. coli* engineering has so far resulted in the highest yields of limonene, other microorganisms may offer advantages. *Saccharomyces* encodes a cytosolic mevalonate pathway (Fig. 2), that supplies FPP for biosynthesis of sterols (Takami et al. 2012) and for protein farnesylation (Dolence and Poulter 1995), and supplies GGPP for ubiquinone biosynthesis (Meganathan 2001). In yeast, fewer reports on limonene production are available and the titers reached are still lower than in *E.coli* (Table 1). However, yeast is more suitable for the co-expression of terpene synthases with subsequent oxidizing enzymes, which are often membrane-bound plant P450 enzymes with their corresponding NADPH-cytochrome P450 reductases (Gruchattka et al. 2013). Yeast has other advantages compared to *E. coli* in terms of tolerance to pH, osmotic pressure and culture infections (Gruchattka et al. 2013).

Among the rare microbial species that do produce monoterpenes are *Streptomyces* species and cyanobacteria, many of which produce the monoterpene methylisoborneol (Giglio et al. 2011; Komatsu et al. 2008). Cyanobacteria are photosynthetic microorganisms, which are able to use CO_2 and light as sources for limonene production (Davies et al. 2014). Using CO_2 as a carbon source could in principle contribute to a highly sustainable production platform.

Creating a GPP pool in microorganisms

A well-known strategy in the microbial engineering of terpene production is to overexpress mevalonate or MEP pathway enzymes with the terpene synthase. For example in the cases of the microbial production of the sesquiterpenes trans-β-farnesene (a jet fuel substitute) and amorphadiene (a precursors of the antimalarial artemisinin), this was achieved by overexpressing the mevalonate pathway together with the sesquiterpene synthases in an industrial yeast strain (George et al. 2015) (Martin et al. 2003).

For limonene production, this strategy has been successfully applied to *E. coli* by adding a mevalonate pathway (Alonso-Gutierrez et al. 2013; Dunlop et al. 2011; Willrodt et al. 2014). In *E. coli,* the level of limonene formation correlated with acetate formation, which was described as a side-effect of the heterologous mevalonate pathway (Willrodt et al. 2014). In the cyanobacterium *Synechocystis* sp. PCC 6803, enzymes from the MEP pathway were added, resulting in modest improvements of limonene titers (Kiyota et al. 2014). This might have to do with the (unknown) product specificity of the prenyldiphosphate synthase crtE. An additional engineering strategy used to increase limonene production, includes overexpression of a truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR). HMGR is the key regulatory enzyme of the mevalonate pathway and truncation by deletion of its N-terminus will overcome feed-

back inhibition of this pathway (Alonso-Gutierrez et al. 2013; Behrendorff et al. 2013; Dunlop et al. 2011; Willrodt et al. 2014).

One strategy to promote GPP availability is to express truncated versions of microbial GGPP or FPP synthases. These truncations can lead to enzymes that predominantly produce GPP (Narita et al. 1999). However, several disadvantages of this concept have been reported, for example a negative influence on reaction kinetics (Reiling et al. 2004) or formation of by-products caused by solvolysis of GPP in the enzymatic pocket (Fischer et al. 2011; Jongedijk et al. 2015)

As an alternative to mutant enzymes, a true GPP synthase can be introduced, which has so far only been described for plants and for *Streptomyces* (Willrodt et al. 2014). Many plant GPP synthases have been reported, however not all of them are equally suited for microbial metabolic engineering. Most convenient for this purpose are homodimeric synthases, such as those from *Arabidopsis* (Bouvier et al. 2000) and *Abies* (Burke and Croteau 2002). Also heterodimeric GPP synthases have been described, for example from peppermint *Mentha x piperita* (Chang et al. 2010), but balanced biosynthesis of the two subunit genes is still challenging. Importantly, most GPP synthases appear to mediate also FPP and/or GGPP biosynthesis in vitro (Burke and Croteau 2002). Such enzymes may play a dual role in the biosynthesis of mono- and diterpenes, as for example has been reported for tomato GPP synthase (van Schie et al. 2007). For reasons of efficiency, a synthase that produces exclusively GPP would be advantageous for a monoterpene biosynthetic microorganism. Therefore, many reports on limonene biosynthesis in microbes have used the *Abies grandis* GPP synthase (Alonso-Gutierrez et al. 2013; Carter et al. 2003; Willrodt et al. 2014), which produces predominantly GPP (Burke and Croteau 2002). Tuning of the expression and solubility of GPP synthases in the microorganism appeared to have strong effects on productivity (Alonso-Gutierrez et al. 2013), demonstrating the importance of this enzyme for a successful limonene production system.

Capturing produced limonene from fermentation systems

In microbial production systems, downstream processing usually constitutes a considerable part of the costs. The recovery of limonene from fermentation systems needs attention due to its high volatility and anti-microbial nature (Jongedijk et al. 2015; Leng et al. 2013). Several capturing methods have been reported on lab scale, including culture extraction, solvent overlay, solid-phase micro extraction (SPME), an adsorbent polydimethylsiloxane bar (Twister®), continuous headspace trapping and gas stripping to a coldtrap (Behrendorff et al. 2013; Ignea et al. 2014; Jongedijk et al. 2015; Kiyota et al. 2014; Vararu et al. 2015). Not all of these methods are suitable for industrial scale recovery of limonene. Most suitable for also larger production-scale are the ones in

which limonene is continuously removed during culturing, for example by a two-phase system or by headspace removal. These strategies prevent product inhibition and toxicity effects and avoid evaporative loss of the produced limonene. For example, Brennan et al. (2012) showed that using an overlay of dibutyl phthalate could increase the minimal inhibitory concentration of limonene to yeast by up to 702-fold, and thus alleviate its toxicity. In cyanobacteria, an overlay of dodecane enhanced limonene recovery (Davies et al. 2014). In the absence of a solvent layer, it is important to continuously trap limonene from the culture headspace, in order to minimize losses but also possible toxic effects. Jongedijk et al. (2015) showed that continuous capturing of limonene from a yeast culture headspace results in an 8-fold higher limonene yield, compared to extraction by a solvent overlay.

While volatility and toxicity limit the choice of methods for limonene recovery from microbial production systems, this choice may also depend on the subsequent application of limonene. If limonene needs to be obtained as a pure essential oil, for example for fragrance application, it might be preferable to use a solvent-free system. If limonene is to be used as an additive to a fuel or solvent, it might be preferable to use this matrix already as overlay during culturing (Brennan et al. 2012).

Toxicity and solvent tolerance

High titers of limonene are in principle incompatible with the fact that limonene exerts strong toxic effects on cells (Andrews et al. 1980; Uribe and Pena 1990). Limonene is highly lipophilic (Griffin et al. 1999), which causes accumulation of limonene in biological membranes. Disruption of the cell membrane integrity as well as inhibition of essential membrane functions are the mechanism underlying the general toxicity of solvents such as monoterpenes (Sikkema et al. 1994). The mechanisms of microbial solvent tolerance, especially in *Pseudomonas* species have been studied in detail (reviewed e.g. by (Segura et al. 2012); (Ramos et al. 2015)), although mostly with regard to organic solvents other than monoterpenes. Changes in the cellular energy homeostasis, alterations of cell membrane structure, increased formation of chaperones, induction of proteins dealing with reactive oxygen species and activation of efflux pump systems are the main cellular responses observable after exposure of microbes to organic solvents. Especially *Pseudomonas putida* shows extraordinarily high tolerance towards many organic molecules (Ramos et al. 2015) and has been demonstrated to serve as a suitable microorganism for processes with high amounts of externally added limonene (Mirata et al. 2009).

In microbial limonene production a high limonene export rate is required to avoid intracellular accumulation. A screen for efflux pumps that increase tolerance of *E. coli* towards added limonene identified the AcrAB pump of *E. coli* as well as an efflux pump

of *Alcanivorax borkumensis* (Dunlop et al. 2011). Expression of the latter in an *E. coli* strain producing non-growth inhibiting amounts of limonene resulted in a 30% higher product concentration, probably due to reduced feedback inhibition of the limonene synthesis pathway. Tolerance of *S. cerevisisae* towards limonene and other monoterpenes could be enhanced by expression of a fungal efflux pump that was demonstrated to be involved in monoterpene resistance of the bark beetle-associated pine tree pathogen *Grosmannia clavigera* (Wang et al. 2013). Recently, evolutionary engineering of *S. cerevisiae* demonstrated a truncation of a tricalbin protein with a probable function in cell wall integrity regulation to confer a drastic improvement in tolerance towards limonene and other monoterpenes (Brennan et al. 2015).

A recent report (Chubukov et al. 2015) may reveal why externally added limonene at a concentration of about 0.025% (v/v) completely inhibits growth of *E. coli*, whereas α-pinene is tolerated at much higher concentrations (Dunlop et al. 2011) and more than 0.04% limonene (v/v) can be produced de novo from glucose by *E. coli* (Alonso-Gutierrez et al. 2013). Chubukov and colleagues identified limonene hydroperoxides as the main toxic compounds present in externally added limonene and could furthermore demonstrate that a single amino acid change in the alkyl hydroperoxidase AhpC alleviates this toxicity. An *E. coli* strain expressing this variant, AhpCL177Q, which is probably able to reduce the limonene hydroperoxides to less toxic monoterpene alcohols, still displayed more than 50% of its maximal specific growth rate in a culture containing 10% limonene (v/v) (Chubukov et al. 2015). The authors also demonstrated that addition of 2% non-oxidized (anaerobically stored) limonene (v/v) did not lead to a clear reduction of the *E. coli* growth rate. When considering that limonene hydroperoxides are rapidly formed from limonene if stored under an oxygen-containing atmosphere (Karlberg et al. 1994), interpretation of most of the toxicity data from the publications described herein is difficult due to the lack of knowledge about the hydroperoxide content of the limonene used. Moreover, some of the efflux pumps might show transport activity towards limonene hydroperoxide, complicating direct comparisons between different proteins. It was nevertheless demonstrated that the AcrAB efflux pump is essential for the high resistance of the AhpCL177Q-expressing *E. coli* strain towards the limonene/limonene-hydroperoxide mixture (Chubukov et al. 2015). Although the limonene hydroperoxide issue might have strong impact on limonene biotransformation processes using growing cells, the authors furthermore stated that in limonene production processes the toxicity of limonene hydroperoxides will only be relevant in long lasting fermentations. Clearly, the control of cellular export and catabolism of limonene is important for reaching high limonene productivity in microbial systems.

Conclusions

Limonene, which is now mostly used as a fragrance, has a wide variety of potential applications as a bulk material. A stable and scalable source of limonene is needed for production of biomaterials, solvents and fuels. Microbial production could meet that demand, but still needs significant engineering efforts. As yet, titers produced by various microbial systems would need to be improved at least two orders of magnitude for a price competitive with plant-derived limonene. The difference in productivity between plants and microbes may be explained by the adaptations of plant cells for limonene production. These include the natural presence of the precursor geranyl diphosphate (GPP) in specialized compartments, and the ways plants protect their cells from toxic effects by modification and/or extracellular storage of limonene. However, several research groups successfully engineered a GPP pool in microbes, using engineered microbial enzymes or plant GPP synthases. Microbes can suffer from the presence of limonene due to its anti-microbial properties. However, promising results have been reported to alleviate toxicity of limonene for microorganisms by capturing the product from the culture and increasing solvent tolerance. Further studies to increase the GPP pool, and alleviating toxicity effects of limonene on the used host, would make it possible to maximally exploit microorganisms to produce limonene for new bulk applications, such as solvents and biomaterials.

Supplemental files to this chapter can be downloaded from:
http://www.wageningenseedlab.nl/thesis/ejongedijk/SI/

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Chapter 3
Capturing of the monoterpene olefin limonene produced in *Saccharomyces cerevisiae*

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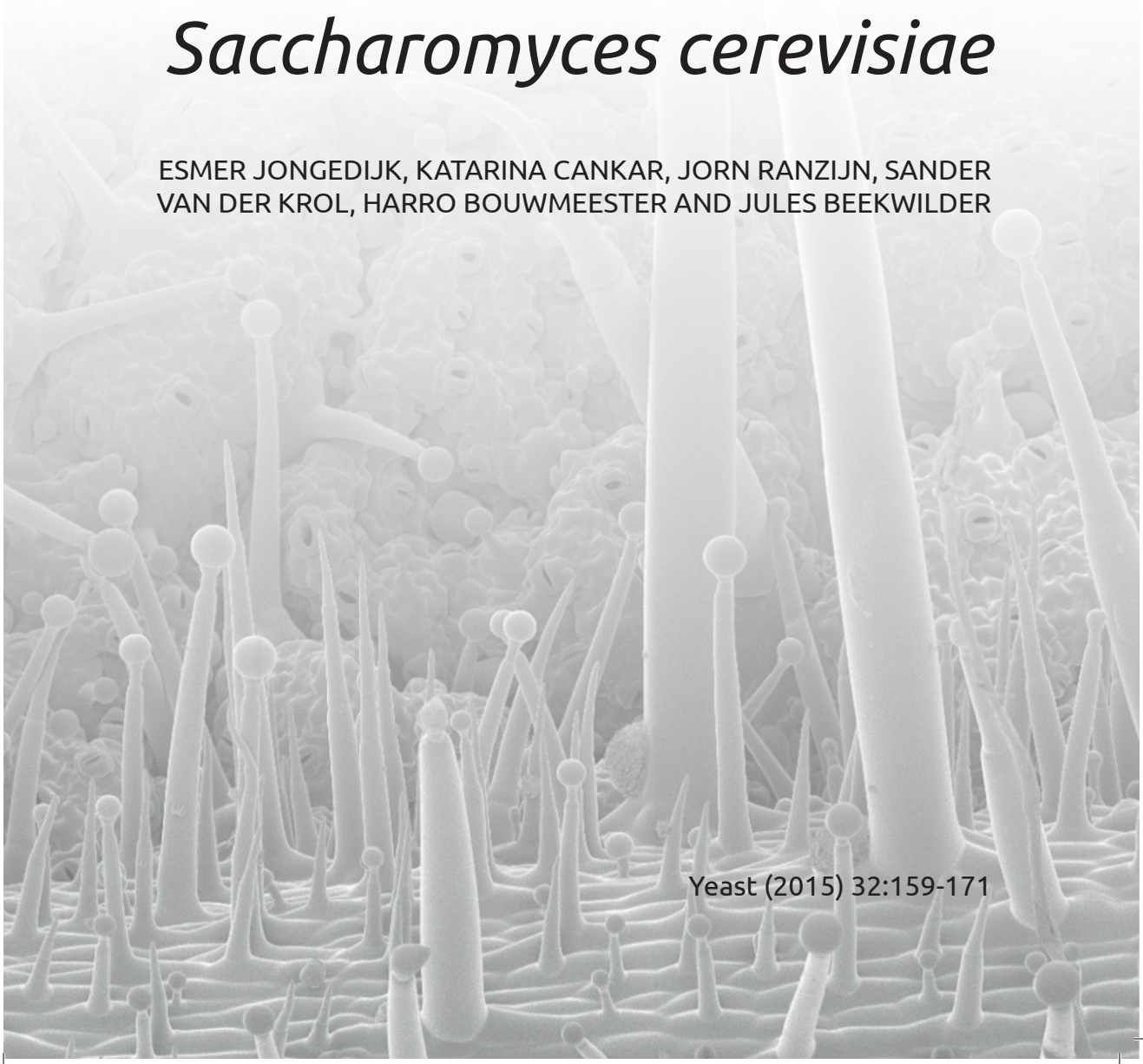

Yeast (2015) 32:159-171

Abstract

Monoterpene olefins such as limonene are plant compounds with applications as flavouring and fragrance agents, as solvents and potentially also in polymer and fuel chemistry. We engineered baker's yeast *Saccharomyces cerevisiae* to express a (-)-limonene synthase from *Perilla frutescens* and a (+)-limonene synthase from *Citrus limon*. Both proteins were expressed either with their native plastid targeting signal, or in a truncated form where the plastidial sorting signal was removed. The yeast host strain for expression was AE9 K197G, which expresses a mutant Erg20 enzyme. This enzyme catalyses the formation of geranyl diphosphate, which is the precursor for monoterpenes. Several methods were tested to capture limonene produced by the yeast. Extraction from the culture medium by pentane, or by addition of $CaCl_2$ followed by solid-phase microextraction, did not lead to detectable limonene, indicating that limonene is rapidly lost from the culture medium. Volatile terpenes like limonene may also be trapped in a dodecane phase added to the medium during fermentation.. This method resulted in recovery of 0.028 mg/L (+)-limonene and 0.060 mg/L (-)-limonene in strains using the truncated Citrus and Perilla synthases respectively. Trapping the headspace during culturing of the limonene synthase expressing strains resulted in higher titers, at 0.12 mg/L (+)-limonene and 0.49 mg/L (-)-limonene. These results show that the volatile properties of produced olefins require specific methods for efficient recovery of these molecules from biotechnological production systems.

Gene bank numbers: KM015220 (Perilla limonene synthase, this study); AF317695 (Perilla limonene synthase, [Yuba et al. 1996]), AF514287.1 (Citrus limonene synthase, [Lucker et al. 2002])

Abbreviations: SPME: solid phase microextraction; GPP: geranyl diphosphate; rpm: rotations per minute; FPP: farnesyl diphosphate; GC-MS: gas chromatography mass spectrometry; DMAPP: dimethylallyl diphosphate; IPP: isopentenyl diphosphate; IDI: isopentenyl diphosphate isomerase; MEP: methylerythritol phosphate

Introduction

Monoterpenes are C10 compounds that consist of two isoprene units. About 900 different types of monoterpenes have been identified in nature [DNP 2012]. They may occur as hydrocarbon olefins, or may be modified by oxidation to monoterpene alcohols or acids. The majority of natural monoterpenes are found in plants where they form the main constituent(s) of essential oils. In plants, monoterpenes are produced in plastids in which the methylerythritol phosphate (MEP) pathway supplies the two isoprene units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Fig. 1). In the plastids, these two isoprene units are combined to form geranyl diphosphate (GPP) by

GPP synthase. GPP is the universal precursor for the biosynthesis of all monoterpenes, and its conversion to the monoterpene skeleton is mediated by monoterpene synthases [Degenhardt *et al.*, 2009].

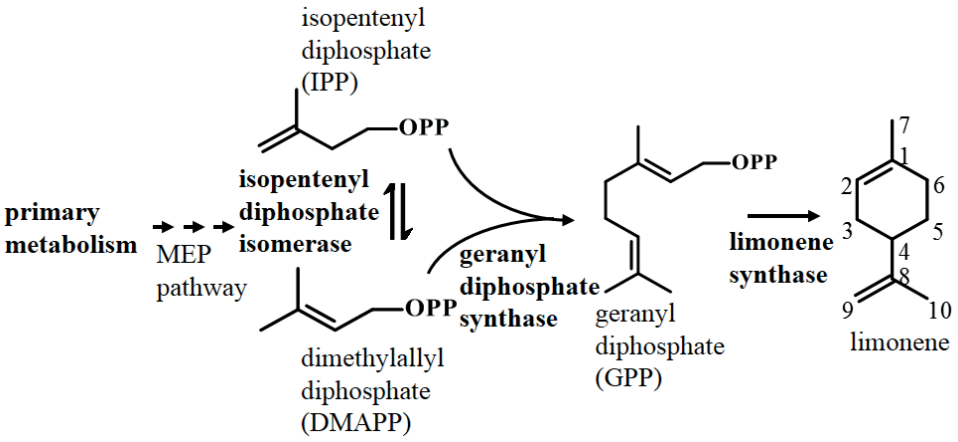

Figure 1 Biochemical pathway of limonene biosynthesis in plants.

Limonene is a monoterpene olefin that is well known for its citrus-like olfactory properties. It has a well-documented antimicrobial activity [Brennan *et al.*, 2013; Zhang *et al.*, 2014] , and is therefore often included in detergents and soaps. Limonene may be oxidized to several products of interest, including menthol, carvone and perilla alcohol, which have wide applications as flavouring agents and preservatives [Duetz *et al.*, 2003; Mars *et al.*, 2001]. Due to its unsaturated cyclic structure limonene has potential as a precursor for biopolymers [Wilbon *et al.*, 2013]. Limonene has been proposed as an additive for diesel [Tracy *et al.*, 2009] and as a component for jet fuel replacements [Brennan *et al.*, 2012] .

Natural sources of limonene include orange oil, derived from Citrus species, in which it is present as the (+)-enantiomer, and oils from Lamiaceae such as *Mentha* spp. and *Perilla frutescens*, in which the (-)-enantiomer is present [Duetz *et al.*, 2003]. Limonene is mainly produced as by-product of citrus juice processing with a production volume of ~50.000 tonnes per year [Braddock, 2013]. Limonene value may increase when new potential applications in polymer and fuel industry lead to a higher demand which cannot solely be met by the citrus industry. Moreover, essential oils produced from citrus materials may contain significant amounts of pesticides [Nichkova *et al.*, 2009], which limits the application in food and household products. Such problems could be circumvented by an industrial scale production of limonene in yeast.

In recent years, novel concepts for production of terpenes for applications as flavouring, pharmaceuticals and fuel have been explored. In particular sesquiterpenes (C15) have

been produced in fermentation systems to high yields [Scalcinati *et al.*, 2012; Westfall *et al.*, 2012]. Sesquiterpenes differ in several aspects from monoterpenes. Sesquiterpenes usually have a relatively low toxicity towards microorganisms and do not rapidly evaporate from fermentation systems [Heeres *et al.*, 2014]. Moreover, they can easily be separated from an organic fermentation phase such as dodecane by distillation. Monoterpenes, on the other hand, are highly volatile, which complicates their recovery from fermentation systems. Partition of solutes over the watery phase and culture headspace is defined by their Henry's law constant (K_H). The K_H of monoterpene olefins is low. For example, at 25°C, the K_H of limonene is only 0.048 mol/L/atm [Leng *et al.*, 2013], indicating that over 95% would evaporate to the headspace, and thus be lost in an aerated shaker flask. Moreover limonene is poorly tolerated by microorganisms such as yeast [Brennan *et al.*, 2013]. Limonene has a low minimum inhibitory concentration (MIC) of 0.44 mM for *S. cervisiae* in a solvent-free system [Brennan *et al.*, 2012]. Therefore, limonene may need to be continuously removed from a culturing system in order to sustain growth.

Production and capture of monoterpenes by expressing plant terpene synthases in yeast has been hardly explored because yeast does not naturally produce monoterpenes or its precursor GPP. Yeast mediates its isoprenoid biosynthesis by the mevalonate (MVA) pathway, which, like the MEP pathway in plants, also produces IPP and DMAPP. In yeast these building blocks are then converted to the C15 molecule farnesyl pyrophosphate (FPP) by activity of FPPsynthase Erg20. Recently, a modified Erg20 enzyme (K197G) has been shown to produce partly GPP instead of FPP, and a strain where wild-type Erg20 has been replaced by this mutant Erg20 enzyme has been employed for the production of geraniol and linalool by a geraniol synthase from basil [Fischer *et al.*, 2011].

In this work we explore capturing of limonene from a monoterpene producing yeast strain. Using the *ERG20* mutant strain AE9 K197G with plant genes encoding a (+)-limonene synthase, from *Citrus limon*, and a (-)-limonene synthase, from *P. frutescens*, yeast is engineered to produce both enantiomers of limonene. We compare different methods for capturing limonene from these strains, and demonstrate that optimization of the capturing method for a volatile product considerably contributes to its effective yield during production in a fermentation system.

Materials and methods

Chemicals

(-)-Limonene was purchased from Merck-Schuchardt (Hohenbrunn, Germany). Pentane and ethyl acetate were obtained from Biosolve (Valkenswaard, The Netherlands). (±)-linalool and cis-nerolidol were purchased from Fluka Chemika (SanktGallen,

Switzerland). Geranyl acetone, n-dodecane and geraniol were purchased from Sigma Aldrich (StLouis, USA).

Construction of plasmids and strains

The *C. limon* (+)-limonene synthase was amplified and subcloned in the pGEMTeasy vector (Promega) from the pBlueC62 plasmid previously described [Lucker *et al.*, 2002] with primers from start and stop codon, ClLS-F and ClLS-R (Table 1) and transformed to *E. coli* DH5α.

P. frutescens (-)-limonene synthase was amplified from cDNA from young leaves of a green *P. frutescens* variety, purchased from Chiltern Seeds (Wallingford, UK). The *P. frutescens* plants had a perillyl aldehyde chemotype [Tabata, 2000]. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from the RNA using the SMART^{TM}RACE cDNA Amplification Kit (Clontech) according to manufacturer instructions. The limonene synthase candidate cDNA was amplified with primers overlapping the start and stop codon PfLS-F and PfLS-R (Table 1), ligated in the pGEMTeasy vector, and transformed to *E. coli* DH5α. Positive clones for ClLS and PfLS were analyzed by sequencing (Macrogen Europe, The Netherlands). The cloned gene encoded a PfLS protein with 96% amino acid identity to the *Perilla frutescens* limonene synthase gene published by Yuba et al. with GenBank accession number AF317695 (supplemental Fig. S1) [Yuba *et al.*, 1996]. The PfLS protein contained the RRX_8W and DDXXD motifs, typical for monoterpene synthases [Bohlmann *et al.*, 1998; Williams *et al.*, 1998].

For the *in vitro* assay, the plastid targeting signal of the proteins was removed, by using a new forward primer for the ClLS and PfLS, starting from the RRX_8W motif (Supplemental Fig. S1) [Lucker *et al.*, 2002]. These truncated versions of the monoterpene synthases (CltLS and PftLS) were amplified with restriction site primers CltLS-SalF, PftLS-SalF, CltLS-NotR and PftLS-NotR (Table 1) and cloned in the pCDF-duet (Novagen, Madison, USA) expression vector, in-frame with the HIS-tag under the T7 promoter, using restriction enzymes *Sal*I and *Not*I. The presence of the insert was confirmed by sequencing. For expression, plasmids pCDF-duet, pCDF-duet-PftLS and pCDF-duet-CltLS were transformed to the *E.coli* BL21RIL strain (Stratagene, Santa Clara, USA).

For expression in yeast, constructs expressing full length terpene synthase proteins or expressing truncated proteins (without plastid targeting) were prepared in the pESC-HIS-CPR plasmid. This plasmid contains the NADPH-cytochrome P450 reductase gene *AtCPR1* from *Arabidopsis thaliana*, which has been amplified from plasmid pUDE172 [Koopman *et al.*, 2012], using primers with *Sal*I/*Xho*I restriction sites CPR1-SalF and CPR1-XhoR (Table 1). The gene was cloned in the commercially available pESC-HIS

(Agilent, Santa Clara, USA) plasmid under control of the Gal1 promoter. The *P. frutescens* and *C. limon* limonene synthase genes were amplified using primers with *Not*I/*Pac*I restriction sites PfLS-NotF, PfLS-PacR, ClLS-PacR and ClLS-NotF for full length, and PftLS-NotF and CltLS-NotF for truncated versions (Table 1) from plasmids pGEMT-PfLS and pBlueC62, and introduced into the *Not*I and *Pac*I restriction sites present following the GAL10 promoter of pESC-HIS-CPR, to yield pESC-HIS-CPR1-PfLS, pESC-HIS-CPR1-PftLS, pESC-HIS-CPR1-ClLS, pESC-HIS-CPR1-CltLS respectively.

The yeast strain *Saccharomyces cerevisiae* AE9 K197G [Fischer *et al.*, 2011] was kindly provided by dr. Fischer from Strasbourg University and was maintained on SD medium (1.7 g/L yeast nitrogen base (w/o aminoacids; Difco), 5 g/L $(NH_4)_2SO_4$, 20 g/L D-glucose, 20 g/L purified Bacto agar) supplemented with uracil, histidine and leucine. The strain was transformed with plasmids pESC-HIS-CPR1, pESC-HIS, pESC-HIS-CPR1-PfLS, pESC-HIS-CPR1-ClLS, pESC-HIS-CPR1-PftLS and pESC-HIS-CPR1-CltLS using lithium acetate [Gietz and Schiestl, 2007], resulting in strains *Sc*-CPR1, *Sc*-pESC, *Sc*-PfLS, *Sc*-ClLS, *Sc*-PftLS and *Sc*-CltLS, respectively (Table 1). After transformation cells were selected on synthetic medium for 3–4 d at 30°C on SD minimal medium supplemented with uracil and all amino acids except histidine.

In vitro assay terpene synthases

5 mL starter cultures of recombinant BL21 with pCDFduet, pCDFduet-PftLS and pCDFduet-CltLS were grown overnight at 37°C, 250 rpm in LB medium with 1% glucose supplemented with spectinomycin (50 µg/mL) and chloramphenicol (50 µg/mL). 250 µL starter culture was diluted in 25 mL 2xYT medium with antibiotics and incubated at 37°C and 250 rpm until the optical density at 600 nm was 0.6-0.8. After addition of 25 µL 1M IPTG, cultures were incubated overnight at 18°C and 250 rpm. Cultures were centrifuged for 15 min at 3400 rpm and supernatant discarded. Pellets were resuspended in 1 mL ice-cold buffer A (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1.4 mM β-mercaptoethanol). About 0.2 g zirconium/silica beads 0.1 mm (Biospec products, Bartlesville, USA) were added to each pellet and the cells lysed by shaking for 10 s in a FastPrep machine (FP120 Bio101 Savant) at speed 6.5. After cooling of the tubes on ice for 2 min, they were shaken again for 10s. Subsequently, lysates were centrifuged for 10 min at 13,000xg and 4ºC, and supernatants were immediately used for enzyme assays. A mix of 32.5 µL 100 mM Tris pH 7.4, 800 µL assay buffer (15 mM MOPSO, 12.5% (v/v) glycerol, 1 mM ascorbic acid, 0.1% Tween 20, 1 mM $MgCl_2$, 2mM DTT, pH 7.0) and 20 µL sodium orthovanadate 250 mM was prepared in a glass tube, and 100 µL of the cell supernatant and 5 µL geranyl diphosphate 10 mM were added. The mix was immediately covered with an overlay of 1 mL pentane, and incubated at 30°C for 1h, under gentle agitation. The tubes were vortexed well and

centrifuged for 5 min at 3400 rpm. The pentane phase was collected, dried over anhydrous Na_2SO_4, and injected into a 7890A gas chromatograph (Agilent technologies) equipped with a mass selective detector (model 5975C, Agilent technologies), scanning from 45-450 m/z. Splitless injection of 1 µL sample was performed at 250°C on a Zebron ZB-5MS column (30 m x 0.25 mm, 0.25µm thickness, Phenomenex) at a helium flow rate of 1 mL/min. The temperature program was 2.25 min at 45°C, then 40°C min^{-1} until 300°C, then 3 min at 300°C. When using dodecane the detector was switched off at RT 10 min to prevent saturation. Compounds were identified by comparing the retention time and mass spectrum with a mix of authentic standards including terpinolene, γ-terpinene and (-)-limonene..

Yeast culturing for production

Single colonies were inoculated in 5 mL SD-galactose medium (1.7 g/L YNB, 5 g/L $(NH_4)_2SO_4$, 20 g/L galactose) to generate precultures and grown in a shaking incubator at 300 rpm 30°C for 48-72 h. Cultures were started by inoculating 50 mL SD-galactose with the preculture to a final optical density at 600 nm of 0.05 in 250 mL Erlenmeyer flasks, and were grown at 30°C and 300 rpm for the indicated time. For all culturing experiments, the optical density at 600 nm was between 6 (headspace set up) and 10 (dodecane set up) at the final time of sampling.

Collecting volatiles

In one set of experiments (Fig. 2a), 50 mL cultures were transferred from the 250 mL Erlenmeyer flasks to a separation funnel after three days of growth, and extracted using 5 mL pentane by vigorous shaking during 2 min and separation of pentane in the funnel, followed by centrifugation of the pentane phase (5 min, 3500 x g) to further clarify the pentane phase. Pentane was further prepared for GC-MS analysis as described above.

In a second set of cultures (Fig. 2b), the 50 mL SD-galactose medium was overlayed with 5 mL n-dodecane during fermentation in the 250 mL Erlenmeyer flasks, as described in [Cankar *et al.*, 2011].

A third set of 50 mL cultures were grown (Fig. 2c), and at regular intervals, 0.5 mL samples were taken from the 250 mL Erlenmeyer flasks and immediately transferred to GC-MS vials. To each vial, 0.5 mL of a 5 M $CaCl_2$ solution was added, and the vial was immediately capped. Each sample was incubated at 50°C for 10 min, and was subsequently automatically extracted and injected into the GC-MS using a Combi PAL autosampler (CTC Analytics AG). Headspace volatiles were extracted by exposing a 65 µm polydimethylsiloxane-divinylbenzene SPME fiber (Supelco, Bellefonte, USA) to the vial headspace for 20 min without agitation and heating at 50°C. The fiber was inserted into a GC 8000 (Fisons Instruments) injection port (with a 1 mL liner) and

volatiles were desorbed for 1 min at 250°C (splitless). Chromatography was performed on an HP-5 (50 m×0.32 mm×1.05 µm) column with helium as carrier gas (37 kPa). The GC interface and MS source temperatures were 260°C and 250°C, respectively. The GC temperature program began at 80°C (2 min), was then raised to 150°C at a rate of 5 °C/min, raised to 250°C with 18°C/min and finally held at 250°C for 2 min. Mass spectra in the 45 to 400 m/z range were recorded on an MD800 electron impact MS (Fisons Instruments) at a scanning speed of 2.8 scans/s, a solvent delay of 2 min and an ionization energy of 70 eV. The chromatography and spectral data were evaluated using Xcalibur software (http://www.thermo.com).

A fourth set of cultures (Fig. 2d) was used for trapping volatiles using the method for trapping plant volatiles described in [van Herpen *et al.*, 2010]. Precultures were diluted in 50 mL of SD-galactose medium to an OD600 of 0.05 and were cultured in Rundrand-Glas 100 cuvettes of 500 mL (Weck) equipped with in and outlets for gas in a 30°C chamber. The cultures were stirred at 700 rpm using a Teflon stir bar. During cultivation, air was constantly refreshed through Steel sorbent cartridges (89 mm ×6.4 mm O.D.; Markes) containing 200 mg Tenax TA 20/35 for collection of volatiles. Tenax cartridges were conditioned for 60 min at 285°C under a nitrogen flow of 30 psi using a TC-20 multi-tube conditioner and were kept airtight with brass caps until use. Air was sucked through the containers with a flow rate of 100 mL/min for the indicated time through the Tenax cartridges to trap culture-produced volatiles. Incoming air was purified with a second Tenax cartridge. Before GC-MS analysis, the cartridges were dried for 15 min at room temperature with a nitrogen flow of 15 psi.

Headspace of cultures trapped on Tenax cartridges was analyzed with a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham). Before thermodesorption, traps were flushed with helium at 30 mL/min for 3 min to remove moisture and oxygen. After flushing the collected volatiles were desorbed from the Tenax cartridges at 220°C (Ultra; Markes, Llantrisant) for 10 min with a helium flow of 30 mL/min. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes, Llantrisant) at a temperature of 35°C. Volatiles were injected on the analytical column (ZB-5MSI, 30 m ×0.25 mm ID, 1.0 µm – film thickness, Zebron, Phenomenex) injecting at split ratio 62 by ballistic heating of the cold trap to 290°C for 3 min. The temperature program started at 40°C (3.5 min hold) and rose 10°C / min to 280°C (2.5 min hold). The column effluent was ionised by electron impact (EI) ionisation at 70 eV. Mass scanning was done from 35 to 400 m/z with a scan time of 0.3 s. The eluted compounds were identified using Xcalibur software (Thermo, Waltham) by comparing the mass spectra with those of authentic reference standards.

For identification of the enantiomer of limonene produced, materials were eluted from Tenax cartridges by ethylacetate, concentrated under nitrogen flow, and analysed on the 7890A gas chromatograph (Agilent technologies; see above) equipped with an enantioselective column with stationary phase Heptakis (6-O-TBMDS-2,3-di-O-methyl)-β-cyclodextrin; 50% in OV1701 w/w) of 25 m and an internal diameter of 0.25 mm. The column was operated with a He flow of 1.2 mL/min. For use with the enantioselective column, the oven temperature was programmed to 50°C for 12 min, followed by a temperature increase of 10°C/min to 170°C, where it is maintained for 2 min. Authentic standards of (-)-limonene and (+)-limonene were used for identification of the enantiomers.

Results

Construction of limonene producing yeast strains

The construction of limonene producing yeast strains was initiated by selection of two limonene synthases with different enantiomer product specificities. The PfLS from *P. frutescens* has been described as a (-)-limonene synthase [Yuba *et al.*, 1996], while the ClLS from *C. limon* has been described as a (+)-limonene synthase [Lucker *et al.*, 2002]. Both limonene synthases were expressed in *E. coli* BL21, and the production of limonene was confirmed by *in vitro* enzyme assays using GPP as a substrate, followed by GC-MS analysis of the products (Supplemental Fig. S2). For expression in yeast, either the full open reading frames (including plastid targeting signal) or the truncated version encoding only the mature enzyme, were introduced in plasmid pESC-HIS-CPR under control of the GAL10 promoter. The plasmids were introduced into the yeast strain AE9 K197G [Fischer *et al.*, 2011] and maintained by auxotrophic selection on histidine. As controls, the strain *Sc*-pESC, containing an empty pESC-HIS, and the strain *Sc*-CPR1, containing an empty pESC-HIS-CPR, were run along. CPR1 was introduced for possible follow up experiments involving cytochrome P450 enzymes. No difference could be observed between *Sc*-pESC and *Sc*-CPR1 in any of the performed experiments.

Collecting limonene produced by yeast cultures

Several options were tested for collecting limonene from the growing cultures for which the different set-ups are shown in Fig. 2. When produced terpenes were collected with SPME or by pentane extraction, which both involve collecting culture samples at a fixed time point, limonene was not detected as a product of the yeast culture. Continuously collecting produced terpenoids by a dodecane overlay or by continuous headspace trapping proved to be more successful. Results from each of the four methods are described below.

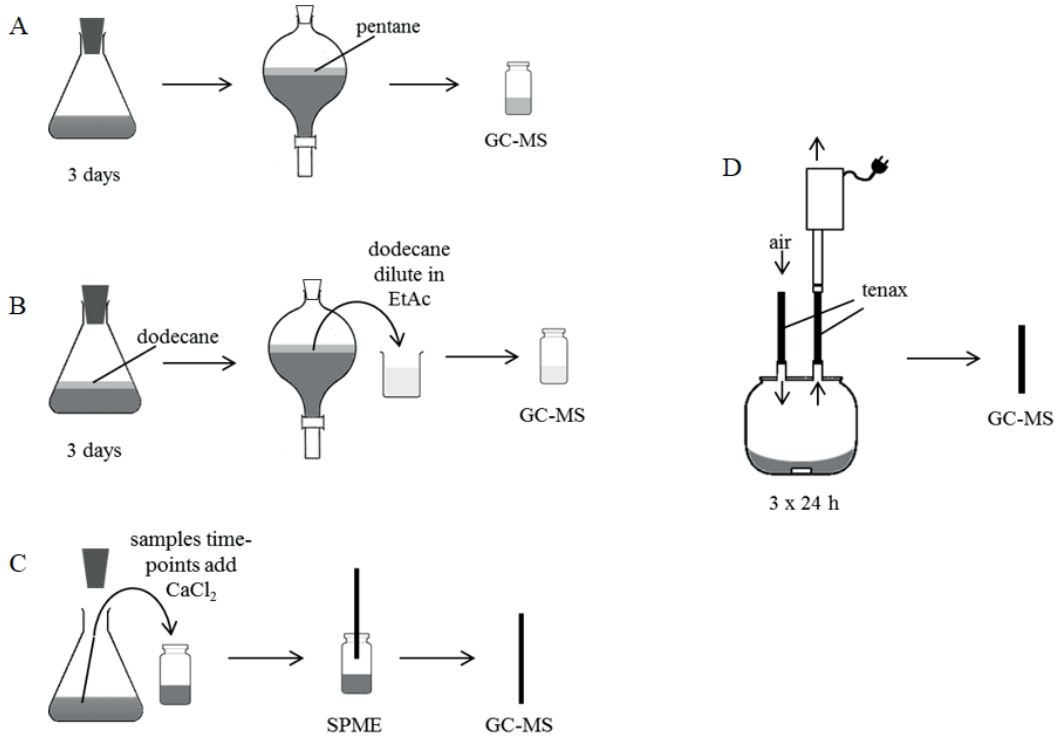

Figure 2 Schematic workflow of tested limonene collection approaches. (A) Pentane extraction. (B) Dodecane overlay. (C) Solid phase micro extraction. (D) Headspace trapping

Pentane extraction

Yeast strains were grown for 3 days in flasks and were subsequently extracted with n-pentane (Fig. 2A). Pentane layers were analyzed by GC-MS. A number of monoterpene alcohols was found, including linalool en geraniol (Supplemental Fig. S3). Retention time and mass spectrum of limonene were established with a standard (Supplemental Fig. S5), but no limonene could be detected.

Solid Phase Micro Extraction

During growth in a shaker flask, culture samples were taken during three days into glass vials (Fig. 2C). Volatiles from the medium were released into the headspace by addition of a high concentration of $CaCl_2$. Volatiles were captured on an SPME fibre and analysed by GC-MS. In all cultures several monoterpene alcohols could be observed at all time-points, including geraniol and linalool, as reported before [Fischer *et al.*, 2011] (Fig. 3, Supplemental Fig. S5). No limonene was observed. Combined, the results above suggest that the bulk of limonene escapes to the headspace before extraction.

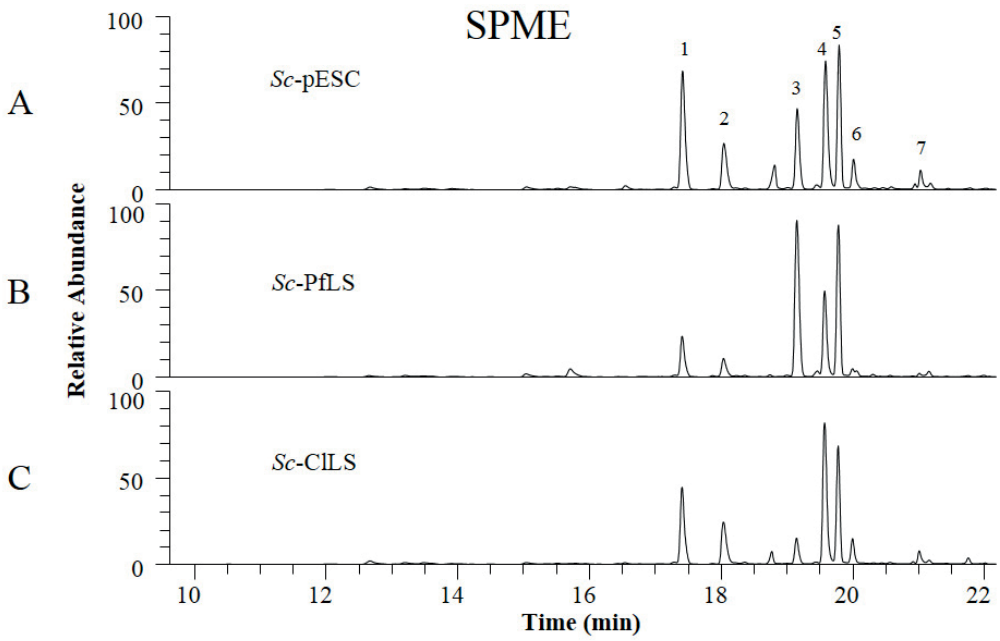

Figure 3 GC-MS chromatograms (total ion count, mass range 45-400 m/z) of solid phase micro extraction at 27 hours (100% = 6e7 mass counts). (A) Sc-pESC. (B) Sc-PfLS. (C) Sc-ClLS. Peaks were identified as (1) linalool, (2) phenylethyl alcohol, (3) camphor, (4) menthol, (5) alkane-like unidentified compound, (6) terpineol, (7) geraniol. Camphor and menthol (peaks 3 and 4) were also present in SPME analysis of culture medium without yeast, indicating that they do not originate from yeast strain activity.

Dodecane overlay

In order to capture volatiles during growth, the strains were grown for 3 days in flasks in the presence of an overlay of n-dodecane [Cankar *et al.*, 2011] (Fig. 2B). Dodecane layers were collected on the third day, diluted in EtAc and analyzed by GC-MS. As dodecane and solvent contaminants (decane, undecane) have similar retention times to monoterpenes, the analysis of the produced monoterpene alcohols and limonene was not straightforward. Still, a small quantity of limonene could be detected in the limonene synthase expressing strains (Supplemental Fig. S4). A quantity of limonene of around 0.028 (± 0.0095) mg/L (+)-limonene and 0.060 (± 0.029) mg/L (-)-limonene was detected in cultures expressing the truncated *Citrus* and *Perilla* synthases respectively. No monoterpene alcohols could be observed in the chromatograms, as these compounds overlap with the excess of dodecane (Supplemental Fig. S4). For cultures expressing the non-truncated limonene synthases, trace amounts of limonene were detected in the dodecane overlay.

Headspace trapping

A set up was built which allowed trapping of exhaust volatiles during growth (Fig. 2D). Cultures were grown in a 500 mL closed cuvette under constant stirring and a continuous airflow through the cuvette headspace. Volatiles were sequestered from the outgoing air through a sampling cartridge containing a volatile absorbent and analyzed by GC-MS (Fig. 4). Sampled air of cultures expressing limonene synthases showed limonene (Rt = 10.3 min) as one of the predominant compounds, in addition to the monoterpene alcohols which occurred in all cultures (Fig. 4). Mass spectra and retention times of produced limonene were matching to the standard (Fig. 4; Supplemental figure S5).

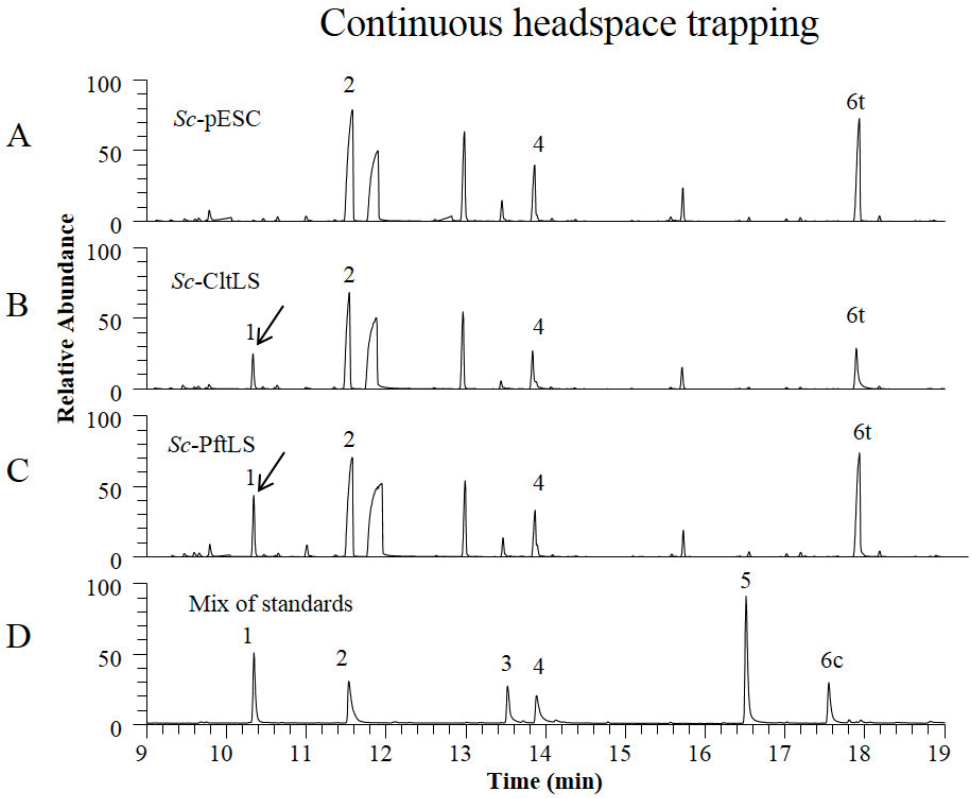

Figure 4 GC-MS chromatograms (total ion count, mass range 35-400 m/z) of continuous headspace trapping 21-45 hours yeast cultures and standard mix. (A) Sc-pESC (100% = 2.4e9 mass counts). (B) Sc-CltLS (100% = 2.4e9 mass counts). (C) Sc-PftLS(100% = 2.4e9 mass counts). (D) Mixture of standards (100% = 8.0e7 mass counts). Indicated peaks represent (1) limonene, (2) linalool, (3) nerol, (4) geraniol, (5) geranyl acetone, (6c) cis-nerolidol, (6t) trans-nerolidol. The arrows indicate the limonene peak in the limonene producing strains Sc-CltLS and Sc-PftLS.

Strong differences in productivity were observed between cultures expressing native and truncated limonene synthases. For the strains expressing the full length LS gene, the *Sc*-PfLS strain produced up to 8 µg limonene/L culture/hr, compared to 0.4 µg limonene/L

culture/h by the *Sc*-ClLS. However, the strains with truncated versions of the limonene synthases (*Sc*-PftLS and *Sc*-CLtLS) displayed higher productivity. The *Sc*-PftLS strain showed a maximal productivity of 24 µg limonene/L culture/hr; for the *Sc*-CltLS strain this was 3.6 µg limonene/L culture/hr. Limonene formation was highest during the first (*Sc*-PftLS) or second day (*Sc*-ClLS, *Sc*-CLtLS, *Sc*-PfLS), but limonene levels reached a plateau value at day three (Fig. 5A). Cumulative, the amount of limonene collected from the headspace over 3 days was 0.33 and 0.014 mg/L for the *Sc*-PfLS and *Sc*-ClLS strain, respectively. For truncated strains, production levels were higher, reaching 0.49 (*Sc*-PftLS) and 0.12 mg/L (*Sc*-CltLS) respectively.

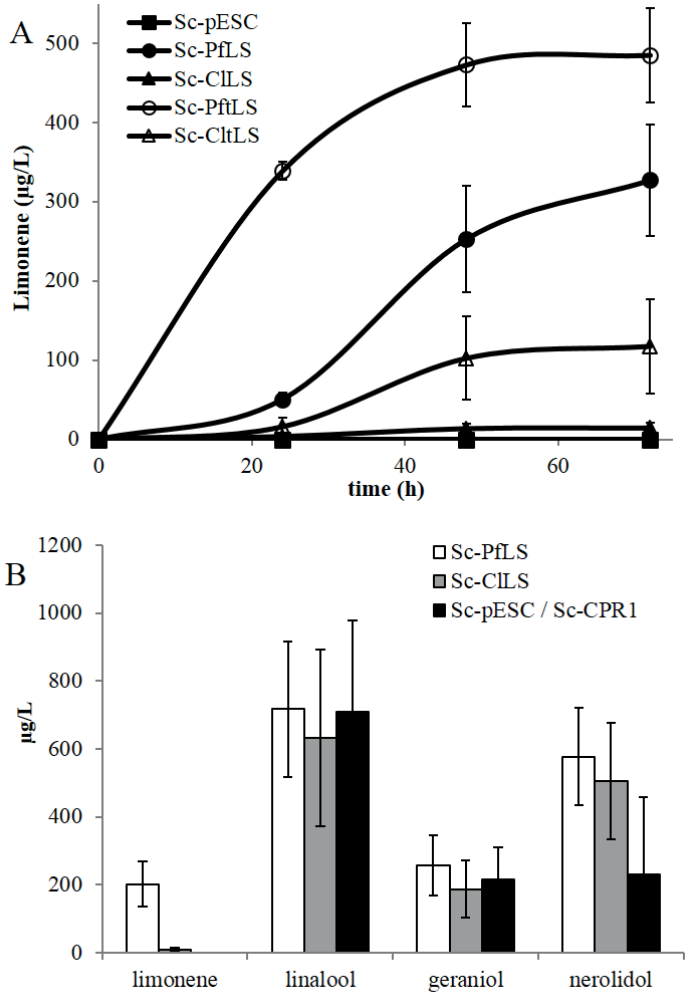

Figure 5 Monoterpene production by yeast strains captured by continuous headspace trapping. (A) Cumulative limonene production by yeast strains during three days. (B) Production of limonene, linalool, geraniol and nerolidol by yeast strains on the second day of fermentation. Indicated are the average values of three independent cultures, and error bars indicate standard deviations.

Limonene formation was compared to formation of the endogenous terpene alcohols at 45h (Fig. 5B). Linalool and geraniol production and formation of the sesquiterpene alcohol nerolidol were not significantly different in the *Sc*-PfLS or *Sc*-ClLS strains relative to the control strain.

The enantioforms produced by the *Sc*-ClLS and *Sc*-PfLS cultures were analysed using GC-MS with an enantioselective column, and confirmed production of (+)-limonene by *Sc*-ClLS and (-)-limonene by *Sc*-PfLS (Fig. 6).

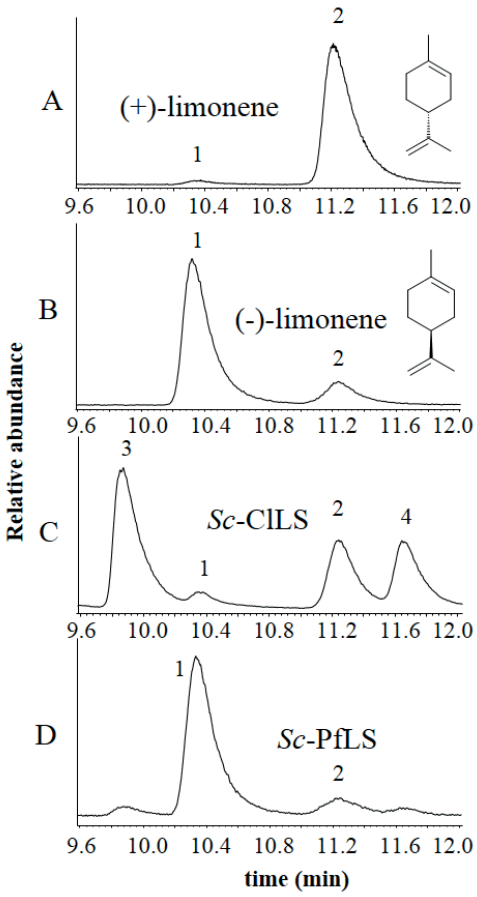

Figure 6 Analysis of chirality of limonene produced by different yeast strains using GC-MS on an enantioselective column. (A) (+)-limonene standard. (B) (-)-limonene standard. (C) products of Sc-ClLS, (D) products of Sc-PfLS. Indicated peaks represent (1) (-)-limonene, (2) (+)-limonene, (3) hexanoic acid and (4) ocimene. Identities of 3 and 4 were suggested by the NIST library.

Discussion

Capturing method may affect limonene yield

In this study we compared ways to capture a highly volatile terpene from limonene-producing yeast cultures. To this end we generated a set of yeast strains producing both enantiomers of the monoterpene olefin limonene. Our studies indicate that the highest yield for capturing of limonene is obtained when produced monoterpene is continuously captured from the headspace during growth, using an absorbent material. No limonene could be captured from the yeast cultures by solvent extraction (Fig. S3) or SPME (Fig. 3), whereas monoterpene alcohols such as linalool were well detected by these methods (Fig. 3). Differences in boiling point between the olefin limonene (176ºC) and the alcohol linalool (198ºC) do exist, but can hardly explain the strong differences in recovery from the culture medium of both compounds. More likely, the behaviour of both compounds can be explained by big differences in partitioning between water and air, which are reflected in Henry's law constant (K_H). For example, at 25ºC, the K_H of linalool is 21 mol/L/atm [Leng *et al.*, 2013], while for limonene the K_H is 500-fold lower (0.048 mol/L/atm), indicating that indeed linalool will mostly stay in the medium, while limonene would mostly evaporate and be lost to the headspace of an aerated shaker flask.

Production of sesquiterpenes from culture broth often deploys a biphasic fermentation, using a dodecane overlay during culturing [Scalcinati *et al.*, 2012]. Such a system could also potentially sequester produced limonene during the production [Anthony *et al.*, 2009]. In our hands, headspace trapping showed a 4 to 8 fold higher titer of limonene (0.12 mg/L (+)-limonene and 0.49 mg/L (-)-limonene) compared to the biphasic system (0.028 mg/L (+)-limonene and 0.060 mg/L (-)-limonene). Possibly, this is the result of the continuous aeration of the cultures for headspace trapping, which may lead to better growth and/or better monoterpene production. Alternatively, the presence of dodecane could inhibit limonene production. Dodecane itself is not known to have strong negative effects on cell viability of yeast. However, contaminants such as decane are present in dodecane (Fig. S4) and may have (mild) toxic properties [Williams *ert al.*, 1998]. A clear advantage of headspace trapping over biphasic fermentation is that limonene is not mixed with compounds of similar polarity and boiling point, thus facilitating down-stream processing. Still, for some applications there is no need for separation, for example if the overlay solvent is chosen in such a way that it can directly be used as a biofuel [Brennan *et al.*, 2012]. Trapping of headspace products from yeast cultures has not yet been applied at industrial scale, and the laboratory set up as explored here (Fig. 2D) may not be suitable for up-scaling. However, devices based on cold-trap condensers could be suitable for trapping of monoterpene olefins on an industrial scale [Booth, 2013].

Previous studies of monoterpene production in yeast have deployed different recovery systems. For instance, production of the monoterpene alcohol linalool has been analyzed by SPME [Rico *et al.*, 2010], while production of geraniol has been analyzed after extraction from culture medium by solid phase extraction using C18 material [Fischer *et al.*, 2011]. Production of cineole in yeast has been monitored by SPME and liquid liquid extractions using ethylacetate and hexane [Ignea *et al.*, 2011]. Very recently, yeast strains producing limonene or sabinene into a dodecane overlay have been reported [Behrendorff *et al.*, 2013; Ignea *et al.*, 2014]. In this study we compared four recovery systems using the same limonene producing strains. Our results indicate that these studies may underestimate the productivity of the constructed strains.

Solvolysis rather than pyrophosphatase activity is responsible for formation of monoterpene alcohols in the *ERG20* mutant

The yeast strain applied in this work carries a mutant FPP synthase, and is capable of sustaining growth (indicating that FPP is still synthesized for ergosterol synthesis), but also produces geranyl diphosphate that is available for synthesis of monoterpenes [Fischer *et al.*, 2011]. In strains carrying no monoterpene synthase, this leads to formation of a number of monoterpene alcohols, such as linalool and geraniol [Chambon *et al.*, 1990; Fischer *et al.*, 2011] (Fig. 3 and 4). These monoterpene alcohols could arise by activity of pyrophosphatases, which may convert GPP into monoterpene alcohols, or could be the result of solvolysis of GPP in the mutant farnesyl diphosphate synthase [Chambon *et al.*, 1990]. When a pyrophosphatase would be a dominant factor in the formation of geraniol, one could speculate that formation of limonene, as observed when expressing limonene synthases, would lead to competition for the GPP pool, and as a result, higher formation of limonene would lead to reduced formation of pyrophosphatase products. However, in none of the experiments reported in this work, a reduction of linalool or geraniol could be observed in the high limonene producing strains. Therefore, the present work supports an important role for solvolysis in the formation of monoterpene alcohols in the strain AE9 K197G. In a recent study, yeast expressing another *ERG20* mutant also showed formation of monoterpene and sesquiterpene alcohols, indicating mutations in FPP synthase will promote solvolysis [Ignea *et al.*, 2014].

Options for boosting volatile terpene production in yeast

Heterologous eukaryotic systems for production of limonene derivatives have so far been hardly explored. In contrast to bacteria, both yeast and plants are capable of functional expression of plant cytochrome P450s, which may be useful for production of oxidized forms of limonene (e.g. [Lucker *et al.*, 2004]). Baker's yeast is a well-known

platform for expression of plant cytochrome P450s, and is used for the production of many food ingredients.

The titer of limonene strongly depends on the availability of GPP. The GPP concentration depends on the presence of GPP synthase activity, but also on the availability of GPP building blocks, IPP and DMAPP. As explained above, we and others have explored the potential of mutant Erg20 enzymes for supplying GPP [Ignea *et al.*, 2014]. Next to using a mutant Erg20 also wild type Erg20 can be deployed to generate GPP. Recently, it was shown that medium composition and pH may trigger GPP formation in yeast, which was demonstrated by formation of limonene upon ClLS expression [Behrendorff *et al.*, 2013], in particular in rich medium. A third option to supply GPP would be to express a true GPP synthase in yeast. For example, the GPP synthase gene from *Picea abies* was coexpressed with the cineole synthase from *Salvia fruticosa* which increased cineole production [Ignea *et al.*, 2011]. Possibly, coexpression of this GPP synthase with monoterpene olefin synthases such as limonene synthase may lead to an improved ratio between the olefin and the monoterpene alcohols, but this remains to be established.

Several strategies have been followed to increase supply of precursors IPP and DMAPP. Behrendorff et al. (2013) showed that limonene can be produced to a titer of 1.5 mg/L limonene using a yeast strain that expresses a truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR) and codon-optimized citrus (+)-limonene synthase. HMGR is the key regulatory point of the yeast mevalonate pathway, and tHMGR is a de-regulated form of that enzyme. Overexpression of the isopentenyl diphosphate isomerase (IDI) that isomerizes IPP and DMAPP units in the precursor pathway has been used to increase sabinene titers in yeast 3-fold [Ignea *et al.*, 2014]. Our AE9 K197G strain provides already a reasonable production of GPP by the mutated FPPS enzyme, but most likely a true GPP synthase in yeast combined with a boosting of IPP production may increase limonene production levels significantly. In *E. coli*, it has been shown that limonene production can be dramatically improved by overexpression of a heterologous mevalonate pathway [Alonso-Gutierrez *et al.*, 2013; Willrodt *et al.*, 2014] and such approach may also be applicable to improve the yield of limonene in yeast [Paddon *et al.*, 2013].

Yeast production of monoterpene olefins like limonene from simple carbohydrates would be a significant contribution to the biotechnological production of flavour molecules, but also for the production of fuels and biopolymers as part of the transition to a biobased economy [Brennan *et al.*, 2012]. Capturing the products from a microbial system has advantages over the plant based system, as limonene production could be more stable and food-grade. At the moment, limonene yields from yeast are not yet on a level to replace plant production [Braddock, 2013] and further strain optimizations are

necessary to increase production [Brennan *et al.*, 2012; Leng *et al.*, 2013], while efficient capturing of the volatile product is essential for optimal harvest of the volatile product.

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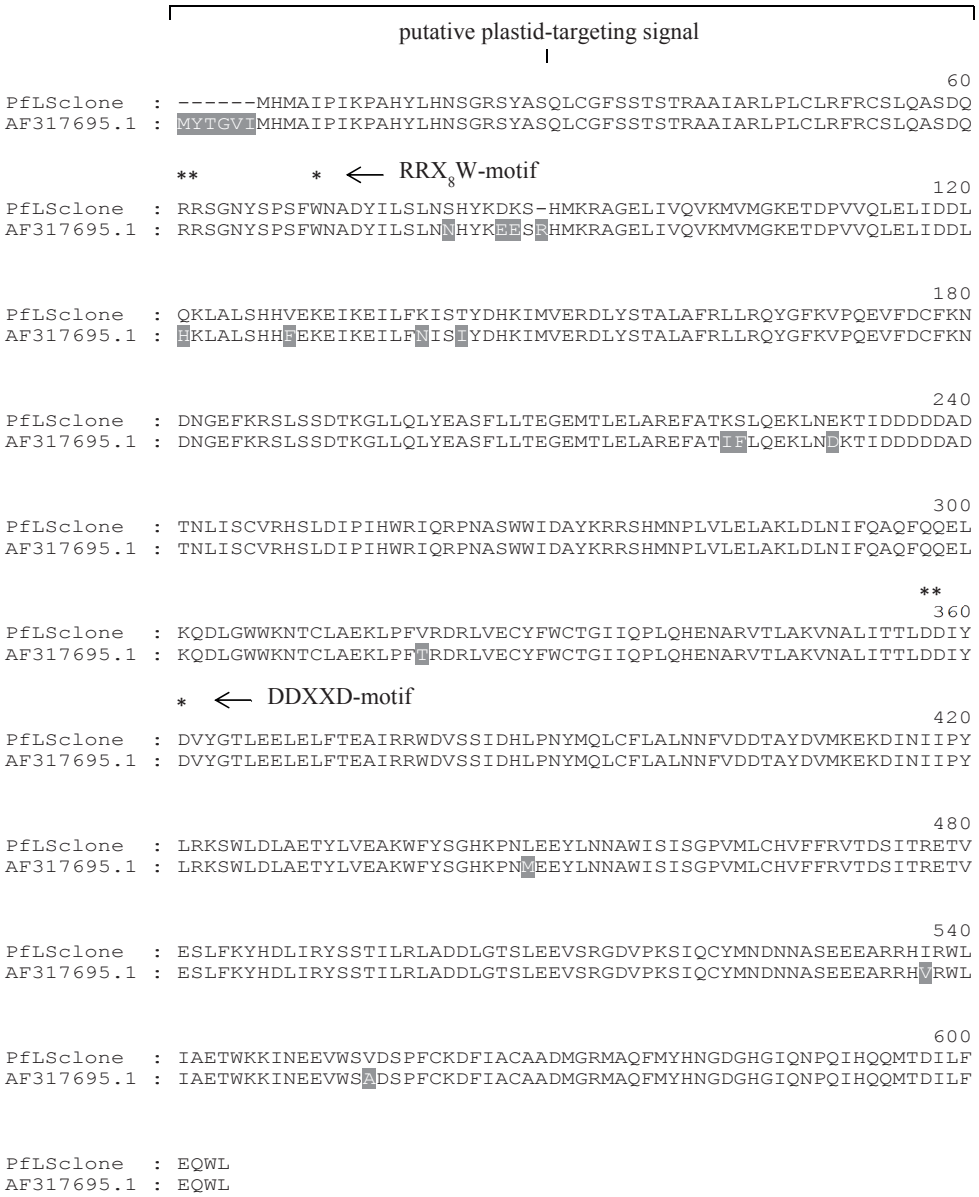
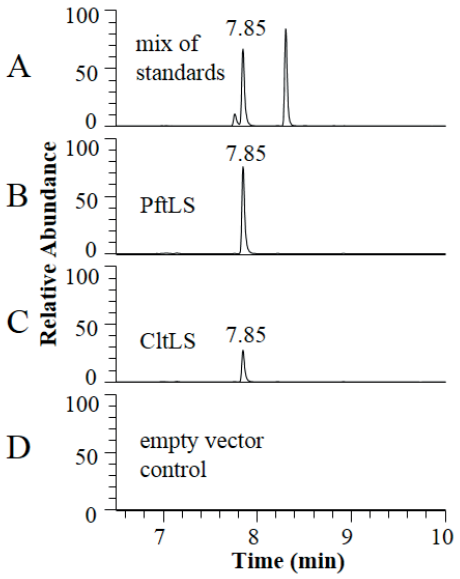
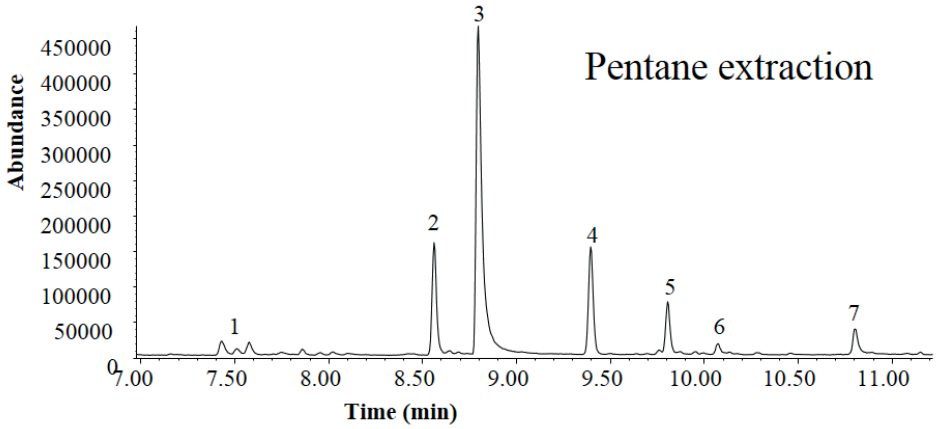
Supplemental Figure S1: Protein sequence alignment of the cloned P. frutescens limonene synthase KM015220 used in this work, and GenBank accession number AF317695 [Yuba et al., 1996]. The sequences share 96% amino acid similarity. The putative plastid targeting signal, RRX8W-motif [Williams et al., 1998] and DDXXD-motif [Bohlmann et al., 1998], all typical for monoterpene synthases, are indicated.



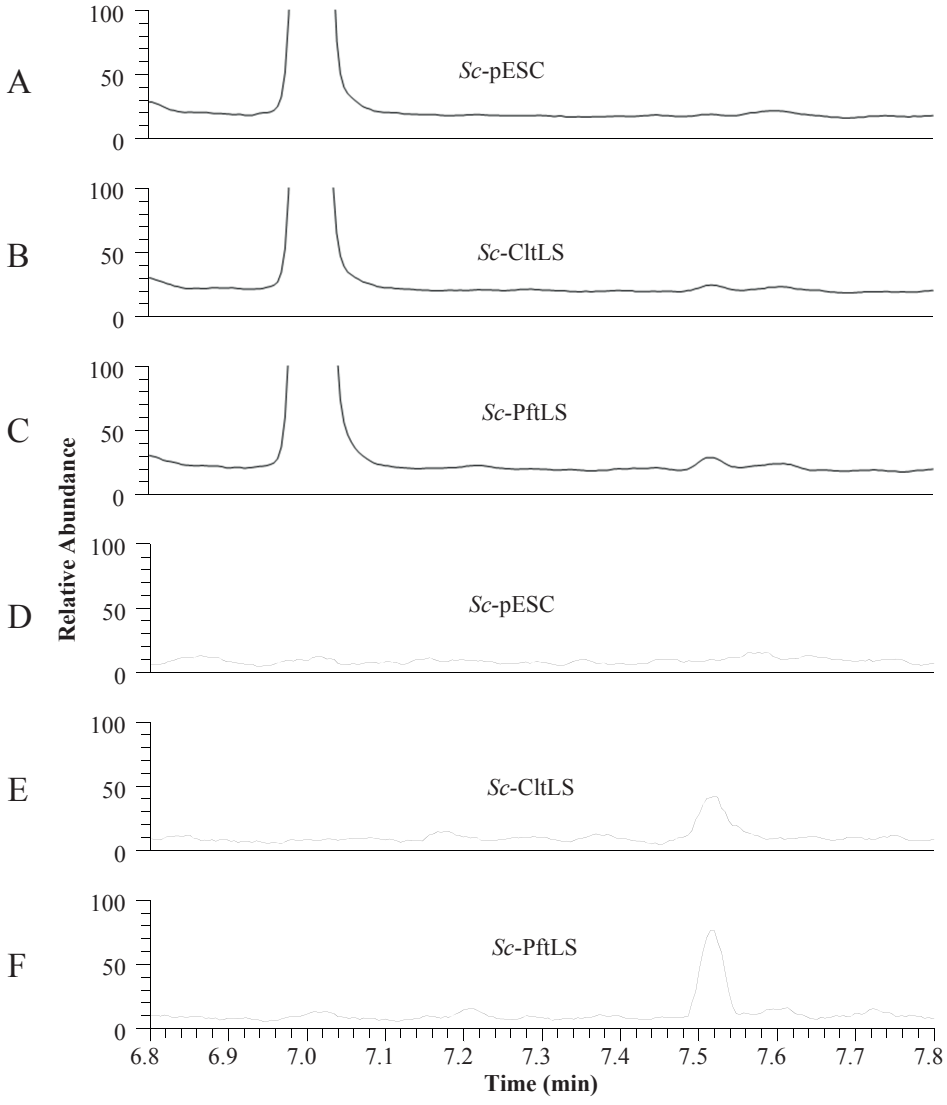
Supplemental Figure S2 GC MS analysis (total ion count, mass range 45-450 m/z) of in vitro assays of limonene synthases. (A) Mix of standards including m-cymene, (-)-limonene and γ-terpinene. Limonene was observed at retention time 7.85 min., (B) Perilla frutescens truncated limonene synthase incubated with GPP; c. Citrus limon truncated limonene synthase incubated with GPP; d. pCDF-duet, empty vector control. (100% = 1e6 mass counts).



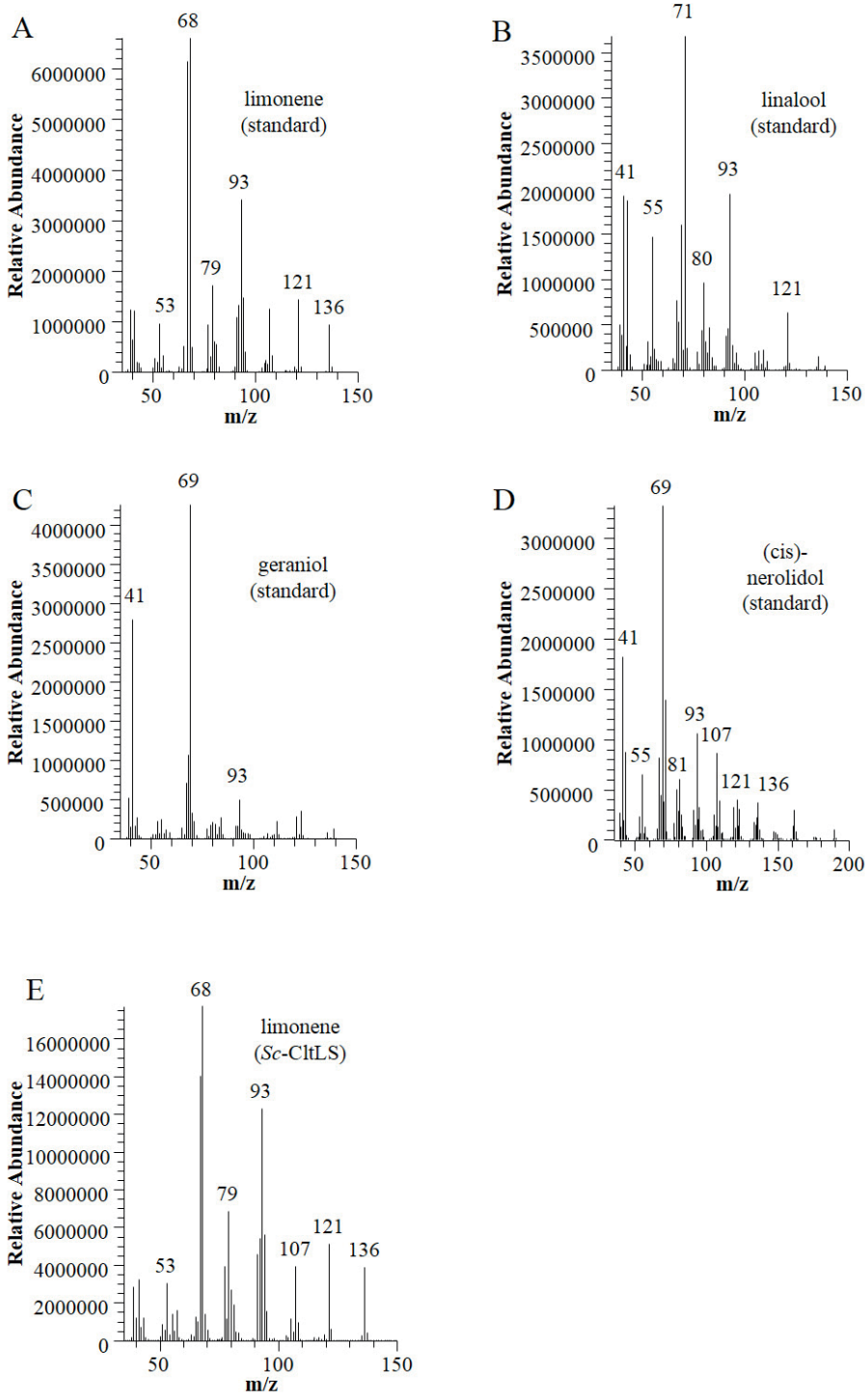
Supplemental Figure S3 GC-MS chromatogram of pentane extract of yeast culture from strain Sc-PfLS. The position of a limonene standard was at position (1). Detected volatiles: linalool (2), phenylethyl alcohol (3), camphor (4), menthol (5), p-ment-1-en-8-ol (6) and geraniol (7). No limonene could be detected. Camphor and menthol (peaks 4 and 5) were also present in pentane extracts of culture medium without yeast, indicating that they do not originate from yeast strain activity.



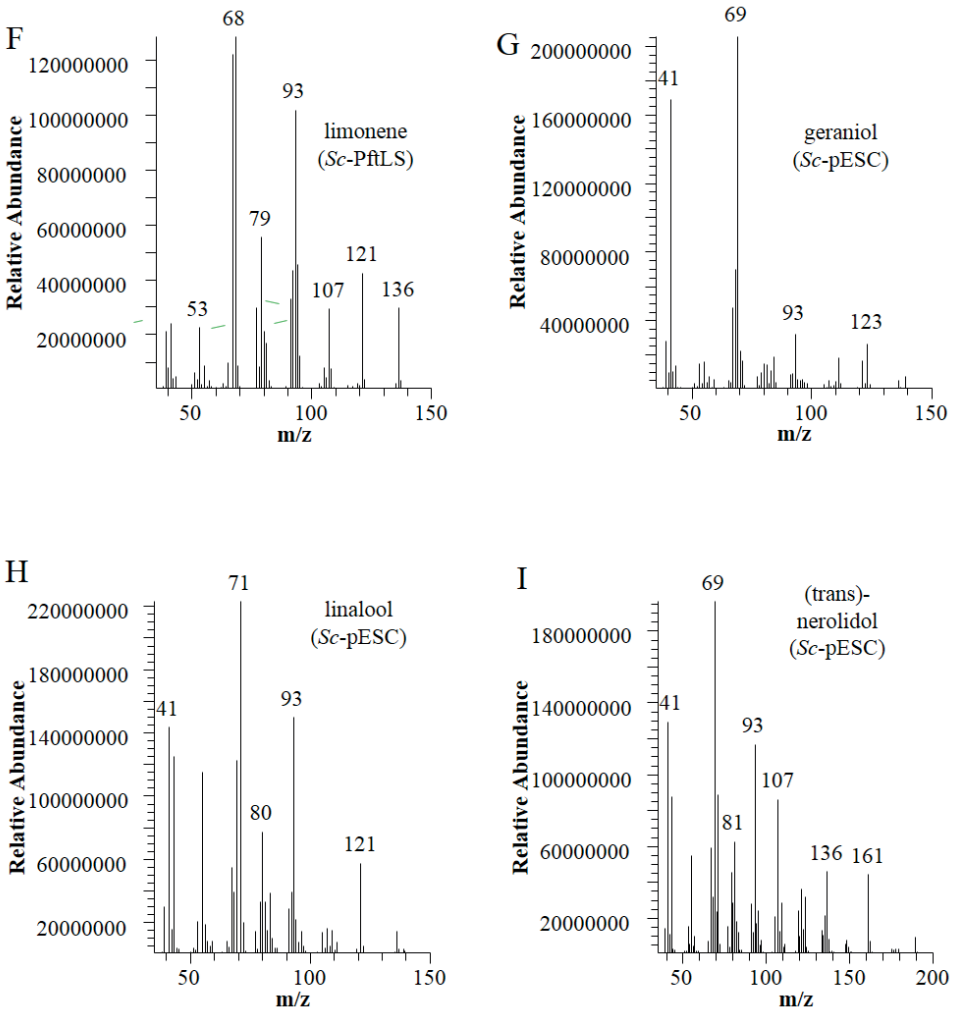
Supplemental Figure S4: GC-MS analysis of dodecane culture overlays after dilution in EtAc. Limonene could be detected (RT 7.51) in the Sc-CltLS (B, E) and Sc-PftLS (C, F) expressing yeast strains and not in the control strain (A, D). (A-C) Total ion count, 100% = 1e4 response units, mass range 45-450 m/z, peak at RT 7.00 represents decane. (D-F) mass range m/z 92-94, 100% = 3e2 response units.



Supplemental Figure S5: Mass spectra of GC-MS peaks from headspace trapping. a. (-)-limonene standard; b. S-linalool standard; c. geraniol standard; d. (cis)-nerolidol standard; e. limonene produced by Sc-CltLS; f. limonene produced by Sc-PftLS; g. linalool poduced by Sc-pESC; h. geraniol produced by Sc-pESC; i. (trans)-nerolidol produced by Sc-pESC



Supplemental Figure S5 (continued)



Chapter 3

Chapter 4
Methyl perillate as a highly functionalized natural starting material for terephthalic acid

ESMER JONGEDIJK, FRITS VAN DER KLIS, ROZEMARIJN DE ZWART, DAAN S. VAN ES AND JULES BEEKWILDER

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Abstract

Renewable commodity chemicals can be generated from plant materials. Often abundant materials such as sugars are used for this purpose. However, these lack appropriate functionalities, and therefore they require extensive chemical modifications before they can be used as commodity chemicals. The plant kingdom is capable of producing an almost endless variety of compounds, including compounds with highly appropriate functionalities, but these are often not available at high quantities. It has been demonstrated that it is possible to produce functionalized plant compounds at a large scale by fermentation in microorganisms. This opens-up the potential to exploit plant compounds that are less abundant, but functionally resemble commodity chemicals more closely. To elaborate this concept, we demonstrate the suitability of a highly functionalized plant compound, methyl perillate, as a precursor for the commodity chemical terephthalic acid.

Keywords: terephthalic acid, monoterpene, methyl perillate, natural functionalization, biobased commodity chemicals

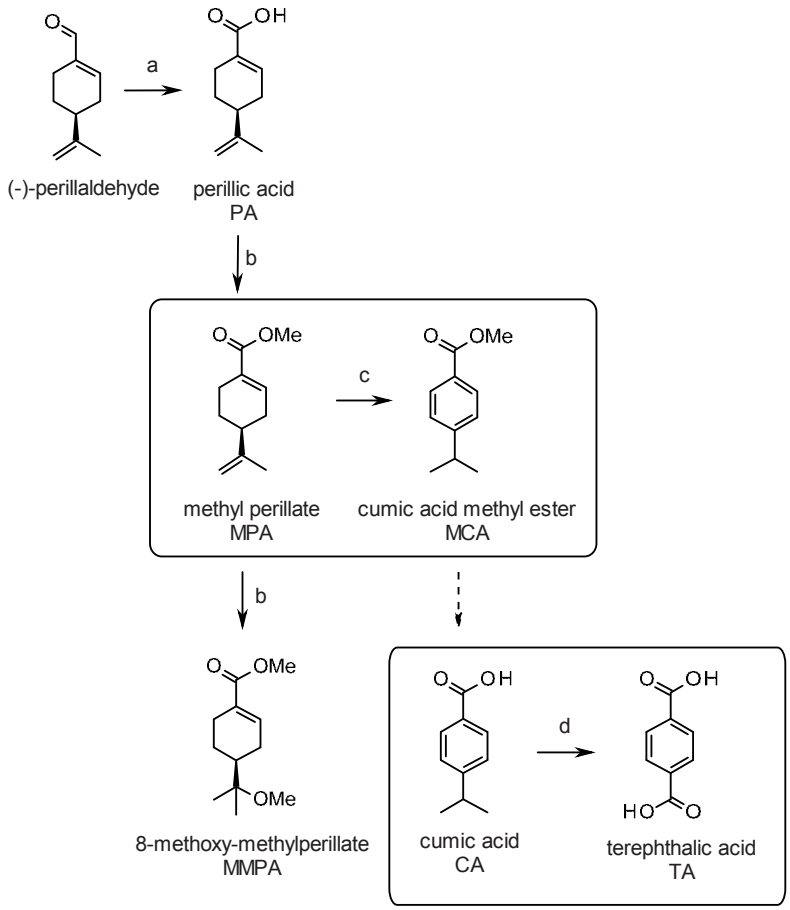
Global material demands inspire research towards bio-based building blocks. For instance, terephthalic acid (TA) is currently produced from petrochemical sources by oxidation of *para*-xylene (pX).[1] Global TA demand is expected to reach 65 million ton in 2018, predominantly for the production of polyethylene terephthalate (PET).[2] One approach for biobased supply of TA is to use sugars or polysaccharides as starting materials, which are abundantly available from biomass[3]. However, the structural similarity between sugars and TA is limited, and therefore a considerable number of harsh synthesis steps are needed for TA production (Table 1).

Table 1 comparison of sugars and methyl perillate as precursors for TA

Compound	Functional groups	Reactions needed	Natural source
sugars	oxygenated ring	solubilization, hydrodeoxygenation, oxidation (100-600^{o}C, 0.1-83 bar)[3]	biomass (sugars)
methyl perillate	oxygenated ring, acid group, functionalized p-position	dehydrogenation, oxidation	essential oil (*Perilla, Salvia*) or fermentation

These steps include solubilizing of sugars, hydrodeoxygenation of the sugar ring, dehydrogenation to produce a mixture of aromatics, separation of pX and oxidation, with temperatures up to 600°C and high pressure.[2] Methyl perillate (MPA), on the other hand, carries an unsaturated 6-ring, is functionalized at the para-position, and has an acid group at the C7 position. MPA is a monoterpenoid which occurs in the plant species *Salvia dorisiana* (fig. S1, table S1). Terpenoids such as artemisinic acid and farnesene are produced with high yields by microbial fermentation[4], using metabolic engineering of microorganisms by introduction of plant metabolic pathways. Though this has not yet been achieved for MPA, its precursors limonene and perillic acid (PA) can be made by microbial systems.[5] Therefore, when compared to glucose (Table 1) and other biobased precursors (Table S2), MPA could be considered as a highly functionalized precursor for TA synthesis.

A mild conversion of MPA to TA was designed (Scheme 1), using dehydrogenation and oxidation reactions, and subsequently tested.



Scheme 1 Synthesis of terephthalic acid from methylperillate as developed in this study

First, MPA was synthesized starting from commercially available (-)-perillaldehyde, using silver oxide as a catalyst, providing PA in reasonable isolated yield (Scheme 1 reaction a) (66% yield, 94% pure) (fig. S2, table S3).[6] The resulting PA was methylated to MPA by esterification in excess methanol with catalytic p-toluenesulfonic acid (Scheme 1 reaction b). During methylation a by-product was formed, 8-methoxy-methyl perillate (MMPA). Short reaction times ensured minimal MMPA formation, and it could be readily separated from MPA (fig. S3, fig. S4). Nearly pure MPA (3.0 g, 48% yield, 96% pure) was used as starting material for the synthesis of terephthalic acid.

Three procedures were considered for dehydrogenation of MPA to methyl cumic acid (MCA). A dehydrogenation procedure using ethylene diamine and metallic sodium has been reported for dehydrogenation of limonene.[7] This procedure consumes metallic sodium and was therefore considered as not fully sustainable. Another procedure described using zeolite NaY as a catalyst yielded a mix of isomers, with only 25% of MCA, this procedure was therefore not considered.[8] Thirdly, a heterogenic catalytic procedure using a solid-supported Pd catalyst for hydrogenation was followed,[9] involving sequential catalytic double bond isomerisation, hydrogenation and dehydrogenation. Acetone was used as a hydrogen acceptor,[10] to steer the reaction towards complete ring dehydrogenation. Initially, limonene was used as a testing substrate. When a Pd/C catalyst was used, formation of dehydrogenated product p-cymene was observed only when the reaction was performed at 150 °C (20h), while at 100 °C (20h), no conversion of limonene could be observed. Changing the support material of the catalyst to alumina (5% Pd/Al_2O_3) resulted in a somewhat reduced yield at 150 °C, but had strongly improved reactivity and selectivity towards the desired product (80%, table S4) at 125°C and 100°C for 20h. At 125°C, the reaction time could be reduced to 1h for full conversion of limonene and comparable, even superior yields of p-cymene (fig. S5, table S5). Also, these shorter reaction times reduced significantly the formation of acetone related by-products diacetone alcohol and mesityl oxide. Thus, a short dehydrogenation procedure for limonene-like substances was developed, which operates efficiently at mild temperature.

Using the developed procedure (125 °C, 1 h, 5% Pd/Al_2O_3), MPA was efficiently converted to MCA (fig. 1, fig. S6). A plausible reaction mechanism involves sequential isomerization and dehydrogenation (scheme S1),[9] in which transfer hydrogenation to acetone pulls the equilibria towards the fully dehydrogenated product MCA. The ratio of MPA to MCA was 85%. The end-product contained similar acetone aldol addition products as detected in the limonene dehydrogenation. Isopropanol (iPrOH) and water are formed by transfer hydrogenation and aldol-addition reactions respectively. However, no hydrolyzed or trans-esterified forms of MPA or MCA were detected in the end-product.

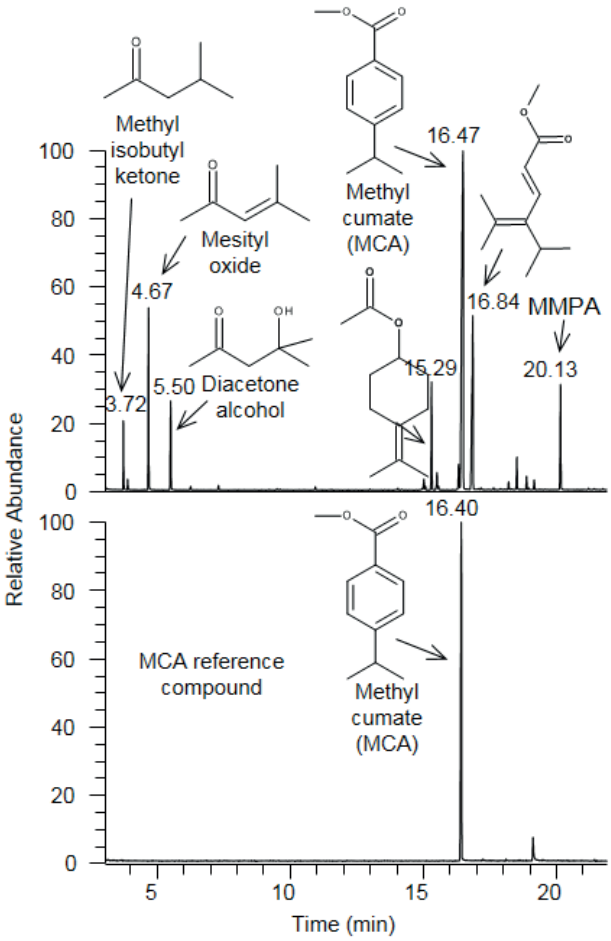


Figure 1 Methyl cumate, the product of dehydrogenation from MP. GC-MS chromatograms of dehydrogenation product and MCA reference compound. Side products related to acetone are detected, the side product of PA methylation, MMPA, is still present, and some other side products are visible, a library-hit of their identity is indicated in the chromatogram.

Use of highly functionalized starting materials such as MPA provide benefits to reduce number of synthetic steps. This is the case for the oxidation to form TA. Previous studies have shown that oxidation of the isopropyl group at position 4 can be readily achieved, but oxidation of the methyl group at position 7 of limonene and its dehydrogenation product p-cymene is more difficult and needs an extra step.[11] In MPA and MCA, the 7 position is already occupied by a carboxyl group. Therefore oxidation to form the end-product TA, can be achieved by an efficient and mild single oxidation step (Scheme 1 reaction d).

Two oxidation methods were tested to convert intermediate MCA to TA. First, oxidation with $KMnO_4$ was tested,[12] but yielded only 12% of TA. Second, a procedure for using oxidation with nitric acid was tested. Aromatic isopropyl groups can be efficiently oxidised to the corresponding carboxylic acid group by nitric acid.[7, 13] Under aqueous nitric acid conditions the methyl ester group of MCA is rapidly hydrolysed, yielding the free cumic acid (CA) in-situ. Oxidation of CA using 65% nitric acid resulted in full conversion, 89% isolated yield (24h reflux, non-optimised, fig. S7). The product contained 70% TA (fig. S7), and a single side-product was identified as 1,1-dinitroethyl benzoic acid by NMR, LC-MS and IR analysis (fig. S7).[14] It is known that 1,1-dinitroethyl benzoic acid is converted to TA as well by heating in 30% nitric acid at 180°C.[14b] This protocol improves earlier described nitric acid oxidation yields starting from benzene (42% yield of TA)[15] and p-cymene (51% yield of p-toluic acid).[16] These results show that it is possible to oxidize MCA to TA in a single step with good yield.

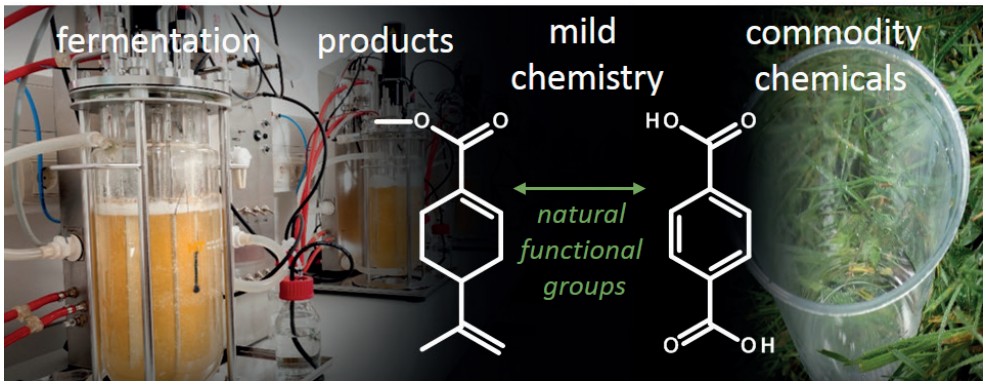


Figure 2 Naturally highly functionalized starting materials from fermentative production allow mild chemistry to produce commodity chemicals

In conclusion, we have demonstrated the applicability of two mild catalytic steps to convert the natural monoterpenoid MPA to the commodity chemical TA. By employing palladium catalyzed dehydrogenation, short and mild conditions can be deployed which offers advantages in terms of sustainability and yield. Subsequently, an oxidation using nitric acid is efficient in producing TA with high yield and high purity. Our work clearly outlines the advantages of selecting highly functionalized molecules as starting materials, to produce commodity materials such as TA (fig. 2). This approach anticipates the ability of the fermentation industry to produce functionalized terpenoid compounds at affordable prices. The fermentative production of complex functionalized molecules such as farnesene, which is positioned as a jet-fuel, indicates that this is a realistic scenario. Therefore the identification of compounds that carry appropriate functionalizations, and the development of sustainable procedures to convert them to biobased building blocks, may have high potential for future application.

Supplemental files to this chapter can be downloaded from:
http://www.wageningenseedlab.nl/thesis/ejongedijk/SI/

Acknowledgements

Source of pictures Figure 2: Wageningen Food & Biobased Research

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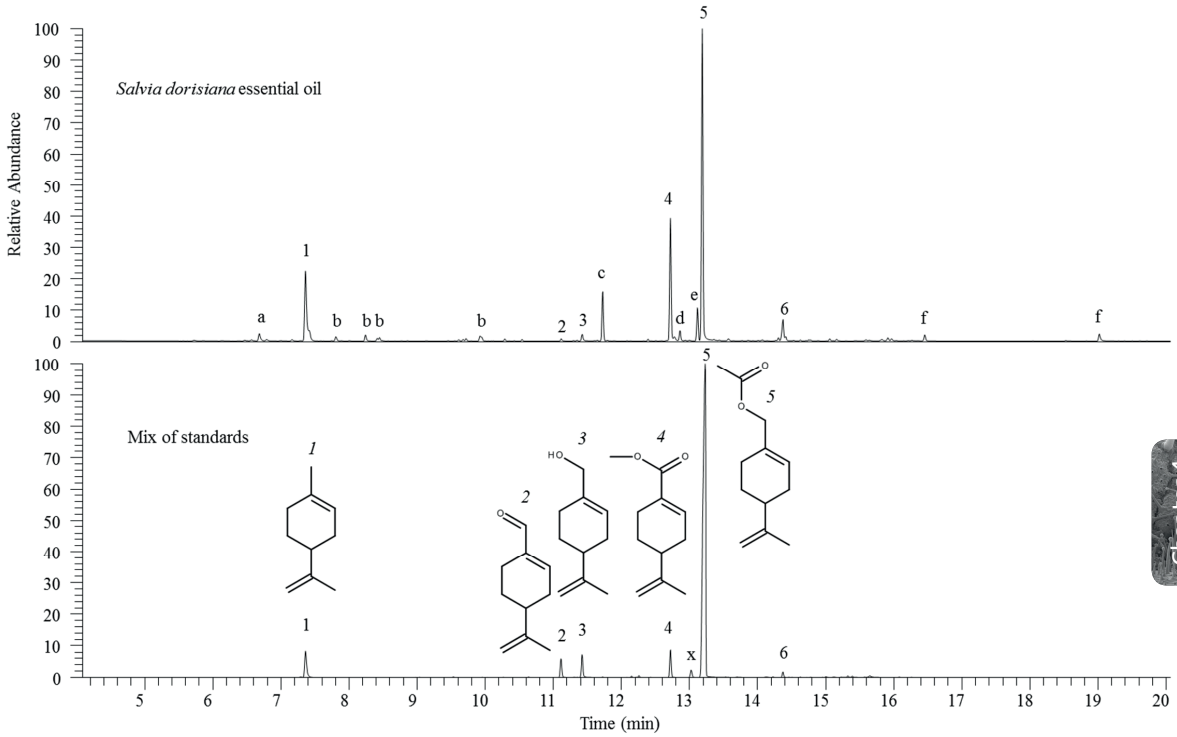
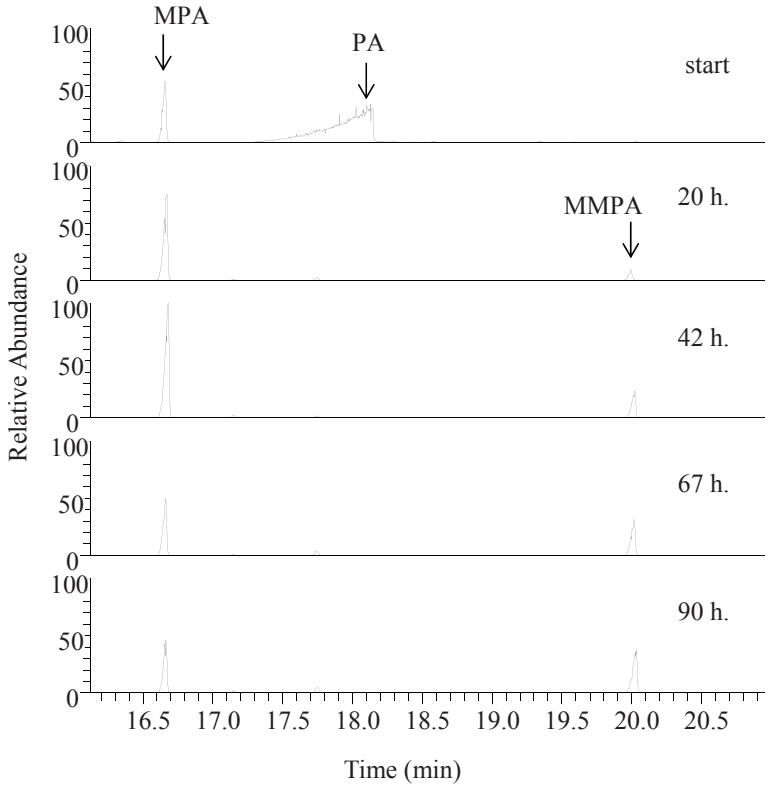
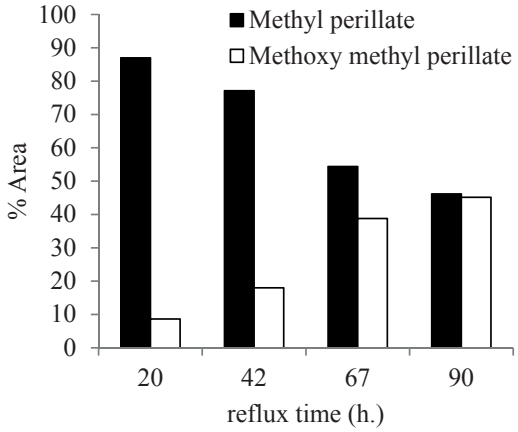
Supplemental Figure S1: GC-MS chromatogram of Salvia dorisiana esssential oil and a mix of standards. Essential oil concentration was 53.2 µg/mL in pentane, with 4.66 µg/mL cis-nerolidol (internal standard). Shown is total ion count (TIC), 100% = 5.13E6. Standard mix consisted of limonene (1) 8.36 µg/mL, perillyl aldehyde (2) 5.36 µg/mL, perillyl alcohol (3) 5.27 µg/mL, methyl perillate (4) 6.68 µg/mL, perilla acetate (5) 4.62 µg/mL and cis-nerolidol (6) 4.66 µg/mL in pentane, shown is TIC, 100%=2.20E7. Identity of compounds a-f and x was determined by library hit: α-myrcene (a), some monoterpenes (b), myrtenyl acetate (c), a monoterpene acetate (d), caryophyllene (e), some sesquiterpenes (f), and in the standard mix there was a small contamination of cuminyl acetate (x).

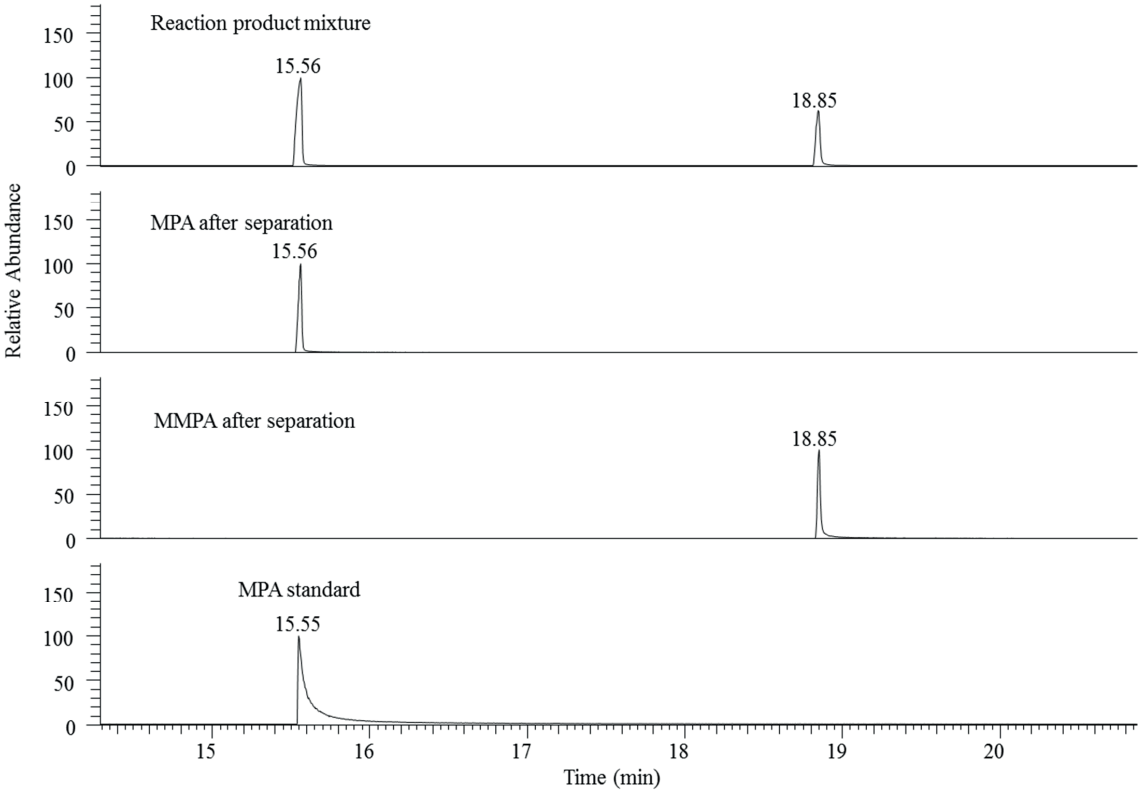


Supplemental Figure S3 GC-MS chromatograms of the methylation reaction of perillic acid (PA) to methylperillate (MPA) at different time-points, reaction samples diluted in chloroform and dried over MgSO4. The reaction yielded 8-methoxy-methyl perillate (MMPA) as a by-product. The longer the reaction time, the more MMPA was formed. At RT 17.75 a peak of another minor by-product is visible, the amount of this by-product increased with time as well. A short reaction time of 21 h ensured complete conversion of PA to MPA and small amount of the by-products, which could be readily separated by column-chromatography.

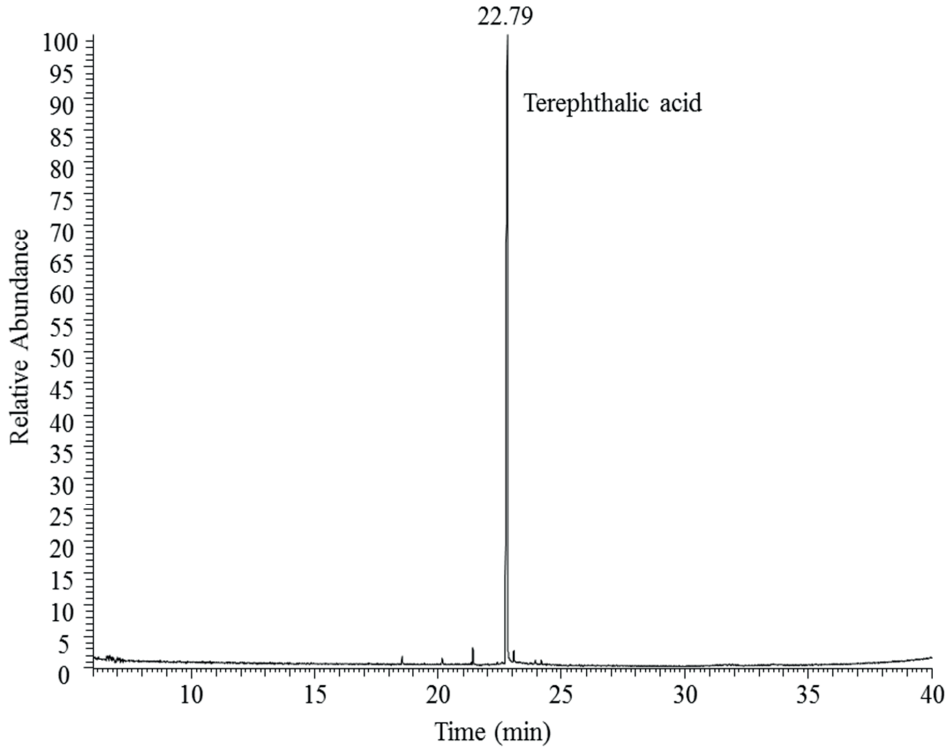




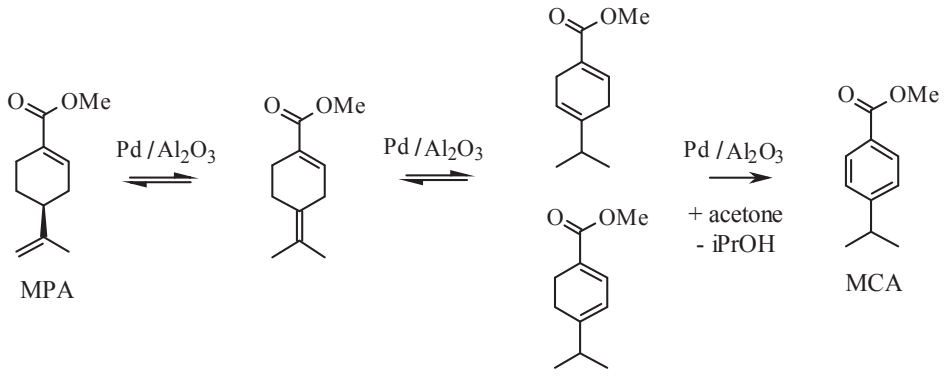
Supplemental Figure S4a: Methylation of perillic acid with p-TSA yielded a mixture of methylperillate (MPA) and methoxymethylperillate (MMPA). The mixture could be readily separated with column chromatography or Kugelrohr distillation. GC-MS chromatograms of the reaction product mixture, MPA and MMPA products after separation and an MPA reference standard



Supplemental Figure S8i Oxidation of methyl cumic acid (MCA) with nitric acid yields terephthalic acid (TA) in one step. GC-MS chromatogram of silylated product



Supplemental Scheme S1: Reaction scheme from MPA to MCA



Supplemental Table S1 Quantification of perillic acid related monoterpenes in Salvia dorisiana hydrodistilled essential oil

Compound	g per kg essential oil
(-)-Limonene	83 (±22)
Perillyl aldehyde	3.7 (±1.0)
Perillyl alcohol	11.9 (±4.8)
Methyl perillate	204 (±83)
Perilla acetate	4.2 (±0.6)

Quantification was done by linear fit to authentic reference compound standard curve. ± standard deviation, n=5. Oil yield was 1.26 g oil / 100 g DW biomass (± 0.94 g, n=7).

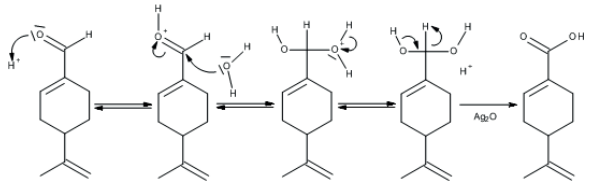
Supplemental Table S2 Reactions of biobased starting materials towards TA

Compound[a]	Functional groups	Reactions needed	Natural source	Fermentative production
sugars	oxygenated ring	solubilization, hydrodeoxy-genation, oxidation (100-600 °C, 0.1-83 bar)	biomass (sugars)	n.a.
lignin and lignocellulose	aromatic ring	Grinding, drying, cooling, solubilizing, pyrolysis, separation, oxidation (50-1000°C)	biomass (lignin)	n.a.
isobutanol (via pX)	oxygenated carbohydrate	dehydration, dimerization, dehydrocyclization, oxidation (175–225°C, 15–30 bar) (existing infrastructure Amoco oxidation)	biomass (sugars)	Yes[1]
fructose (via DMF, pX)	oxygenated ring	dehydration, hydrodeoxygenation, Diels-Alder, dehydration, oxidation (100-300°C, 10-100 bar)	biomass (sugars)	n.a.
muconic acid	oxygenated carbohydrate	microbial synthesis, isomerization, Diels-Alder, catalytic dehydrogenation (150°C)	biomass (sugars)	Yes[2]
FDCA	2 acid groups	dehydration, oxidation, Diels-Alder, dehydration (100-200°C, 5-20 bar)	biomass (sugars)	Yes[3]
limonene, pinene	oxygenated ring, functionalized p-position	dehydrogenation, oxidation, oxidation	essential oil (Citrus)	Yes[4]
malic acid	oxygenated carbohydrate	dimerization/condensation, esterification, Diels–Alder/retro-Diels–Alder/elimination	biomass (sugars)	Yes[5]
methyl perillic acid	oxygenated ring, acid group, functionalized p-position	dehydrogenation, oxidation	essential oil (Perilla, Salvia)	Yes[6]

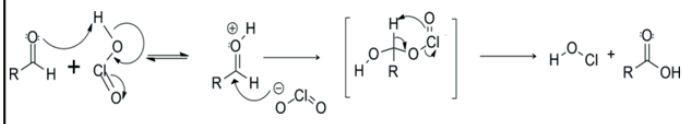
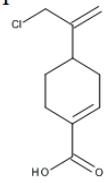
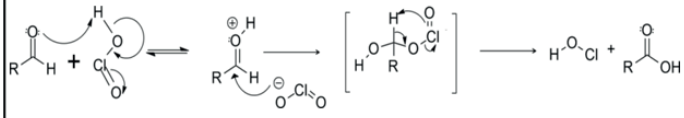
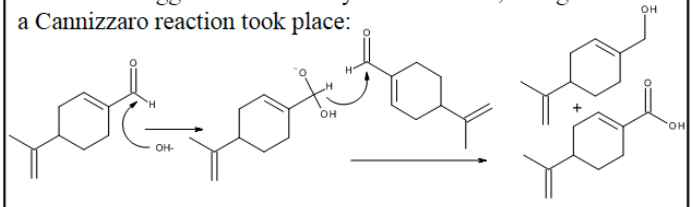
[a] Overview existing TA processes from Collias et al.[7]

1: E. N. Lamsen, S. Atsumi, Front Microbiol 2012, 3, 196.
2: J. W. Frost, K. M. Draths, US patent 5616496, 1997.
3: F. Koopman, N. Wierckx, J. H. de Winde, H. J. Ruijssenaars, Bioresour Technol 2010, 101, 6291-6296.
4: E. Jongedijk, K. Cankar, M. Buchhaupt, J. Schrader, H. Bouwmeester, J. Beekwilder, Appl Microbiol Biot 2016, 100, 2927-2938; E. Jongedijk, K. Cankar, J. Ranzijn, S. van der Krol, H. Bouwmeester, J. Beekwilder, Yeast 2015, 32, 159-171.
5: E. Jongedijk, K. Cankar, M. Buchhaupt, J. Schrader, H. Bouwmeester, J. Beekwilder, Appl Microbiol Biot 2016, 100, 2927-2938; A. E. Mars, J. P. Gorissen, I. van den Beld, G. Eggink, Appl Microbiol Biot 2001, 56, 101-107.

Supplemental Table S3 Different oxidations of (-)-perillaldehyde to PA, or directly to MPA, that were tested. These included Pinnick oxidations (method of oxidation numbers 2 and 3) and oxidations catalyzed by gold/titanium oxide (number 4) and silver oxide (number 1). Highest efficiency was achieved using oxidation with silver oxide. Literature references, reaction details and the obtained result of all oxidation methods are indicated in the table.

Method of oxidation	References	Reaction details	Result
1. Oxidation with Ag2O. Product: PA	Wang et al. (1993) Tetrahedron Vol 49:3 pp 619-638 Hortmann and Ong (1970) J Org Chem Vol 35:12 pp 4290-4292 Kádas et al. (1998) Org Prep Proc Int Vol 30:1 pp 79-85	- Ag2O catalyst made in-situ by AgNO3 + NaOH in water - 55°C, NaOH, filtered + washed with hot water, acidified with HCl (PA precipitates) 	√ 66% yield

Supplemental Table S3 (continued)

2. Pinnick oxidation acetonitrile / water. Product: PA	Dalcanale (1986) J Org Chem Vol 51 pp 567-569	 - Oxidation with HClO2 (formed under acidic conditions from NaClO2) - H2O2 added as HOCl scavenger	+/- yields the chlorinated acid as a by-product 
3. Pinnick oxidation tert-butyl alcohol / 2-methyl-2-butene / water. Product: PA	Bal et al. (1981) Tetrahedron Vol 37 pp 2019-2096 Kitahara et al. (1988) Tetrahedron Vol 44:15 pp 4713-4720 Dong et al. (2013) J Asian Nat Prod Res Vol 15:8 pp 880-884	 - Oxidation with HClO2 (formed under acidic conditions from NaClO2)	+/- low yield ~7%
4. Oxidation on Au/TiO2. Product: PA or MP directly	Marsden et al. (2007) Green Chem Vol 10 pp 168-170 Biella et al. (2003) J Mol Catal A: Chem Vol 197 pp 207-212	To PA: NaOH, water, Au/TiO_2, 5 bar O_2, 80°C The results suggest that the catalyst is not active, it might be that a Cannizzaro reaction took place:  To MP directly: NaOMe (base), MeOH, Au/TiO_2, 5 bar O_2, 80°C	+/- maximum yield ~20%; without catalyst also yield ~20% +/- perillyl aldehyde in end-product (incomplete conversion)

Supplemental Table S4 Reaction conditions tested for dehydrogenation reactions of limonene to p-cymene

Catalyst	Temperature	Yield of p-cymene (mol%)
5% Pd/C	100 °C	0
5% Pd/C	150 °C	80
5% Pd/Al_2O_3	150 °C	56
5% Pd/Al_2O_3	125 °C	69
5% Pd/Al_2O_3	100 °C	75
5% Pd/Al_2O_3	75 °C	0
5% Pd/Al_2O_3	50 °C	0

Reaction time was 17-21 hours.
Limonene : acetone : Pd molar equivalents were 1 : 19 : 0.007.
Catalysts were dry and reduced. Reactions were performed under nitrogen.

Supplemental Table S5 Selectivity of dehydrogenation limonene to p-cymene at different reaction times

Catalyst	Temperature	Time	Yield of p-cymene (mole%)	Ratio p-cymene : diacetone alcohol : mesityl oxide
5% Pd/Al_2O_3	125 °C	1 h	82*	1 : 0.14 : 0.00
5% Pd/Al_2O_3	125 °C	3.5 h	82	1 : 0.13 : 0.00
5% Pd/Al_2O_3	125 °C	20 h	80	1 : 0.15 : 0.18
5% Pd/Al_2O_3	125 °C	27 h	77	1 : 0.19 : 0.21

***optimal conditions, optimized for limonene.**

Supplemental Materials and Methods

Chemicals

(*R*)-(+)-limonene (97%, Sigma Aldrich), perillyl alcohol (96%, Sigma Aldrich), (*S*)-(-)- perillaldehyde (Sigma Aldrich), (*S*)-(-)-perillyl acetate (Wako), (*S*)-(-)-perillic acid (95%, Sigma Aldrich), *cis*-nerolidol (98%, Fluka), pentane (≥99.0%, CHROMASOLV® for HPLC, Sigma Aldrich), cumic acid (4-isopropylbenzoid acid, ≥98%, Sigma Aldrich), magnesium sulphate (dried, 1-2 mol hydration water, Alfa Aesar), ethyl acetate (pure, Acros Organics), hydrochloric acid (37%, reag. Ph. Eur., VWR), *p*-toluenesulfonic acid monohydrate (*p*-TSA, 98.5%, ACS reagent, Sigma Aldrich), methanol (for analysis, Merck), petroleum ether (PE, ACS reagent, boiling range 40-60°C, Acros Organics), trimethyl sulfonium hydroxide solution (TMSH, ~0.25 M in methanol, Sigma Aldrich), sicapent (Merck), silica gel 60 (0.040-0.063 mm, 230-400 mesh, Alfa Aesar), acetone (≥99.8%, Actu-All Chemicals), palladium 5% on alumina powder (reduced, Escat 1241, Strem Chemicals), palladium 5% on activated carbon (reduced, dry powder, Strem Chemicals), platinum on carbon (extent of labelling 10wt% loading, matrix activated carbon support, Aldrich), ruthenium 5% on carbon (Strem Chemicals), chloroform (HPLC grade, stabilized with ethanol, min. 99.9%, Actu-All Chemicals), sodium chloride (VWR Chemicals), celite 545 (Sigma Aldrich), nitric acid (65%, G.R. for analysis, Merck), sodium nitrite (puriss, p.a. ACS >99.0%, Fluka), silylation reagent (BSTFA + TMCS, 99:1, Supelco), DMSO-D_6 (99.5% D, containing 0.03 % v/v trimethylsilane (TMS), Aldrich)

Plant growth, multiplication and measurements

Salvia dorisiana plants were obtained from a local nursery. Standard growth conditions were 12 h day / 12 h night, 20°C / 18°C in the greenhouse.

Leaf extraction

Fresh leaves ≤ 3 cm were snap frozen in liquid nitrogen and ground with pestle and mortar. 139.8 mg of powder was extracted with 6 mL pentane containing 4.66 µg/mL *cis*-nerolidol as internal standard for analysis by GC-MS.

Essential oil distillation

Hydrodistillation was performed on lab-scale according to Stahl [1]. Hundred grams of fresh *Salvia dorisiana* leaves ≤ 3 cm were cut. The leaves were cooked in 1 L demi water in a 2 L round bottom flask for 1 h without organic solvent, and afterwards the pure essential oil layer was removed from the 4°C cooling bulb with a long Pasteur pipette. The essential oil was dried over a $MgSO_4$ column, and the oil yield in w/w% of fresh weight (FW) of the leaves determined, average yield from one distillation 0.13 g (0.13 w/w%).

Steam distillation was performed for larger-scale oil harvesting, in a home-made kettle and cooling system [http://indekoperenketel.nl/]. Tap water (30 L) was heated to 100°C and the steam lead through 16.34 kg of *Salvia dorisiana* prunings during 1 h. Steam with extracted volatiles was then cooled-down gradually to 10-15°C. The water layer of the condensate was continuously removed by a separation funnel during distillation. The water layer was extracted one time with diethyl ether, and this was combined with the oil layer. The solvent was evaporated and the essential oil was dried over $MgSO_4$, yield 10.7 g (0.065 w/w%).

Quantification of monoterpenes

The oil was diluted to 53.2 µg/mL in pentane for analysis by GC-MS. A standard series of limonene, methylperillate, perillyl alcohol, perillyl aldehyde and perillyl acetate was prepared ranging from 0.1-50 µg/mL in pentane. Oil and leaf compounds were quantified using a linear and 2nd order polynomal fitted equation from the standard series.

GC-MS analysis

GC-MS analysis of essential oil was performed on a 7890A gas chromatograph (Agilent) equipped with a mass selective detector (Model 5975C, Agilent) with settings as reported previously [2].

GC-MS analysis of syntheses products was performed on an Interscience TraceGC Ultra GC with AS3000 II autosampler, connected to an Interscience TraceDSQ II XL quadrupole mass selective detector with settings as reported previously [3].

LC-MS analysis

LC-MS was performed on an Accela HPLC tower connected to a LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific), conditions and settings as described previously [4].

NMR analysis

NMR spectra were recorded on a Bruker Avance III spectrometer operating at 400.17 MHz (^{1}H) and 100.62 MHz (^{13}C). Proton NMR chemical shifts are quoted in parts per million (ppm) referenced to the appropriate solvent peak. Carbon NMR was fully decoupled by broad band decoupling.

FT-IR

Fourier transform infrared (FT-IR) spectra were obtained on a Varian Scimitar 1000 FT-IR spectrometer equipped with a Pike MIRacle ATR Diamond/ZnSe single reflection plate and a DTSG-detector. The measurement resolution was set at 4 cm-1, and the spectra were collected in the range 4000-650 cm-1 with 64 co-added scans.

Synthesis of methylperillate

Perillyl aldehyde (50 g) was oxidized to perillic acid using silver oxide, prepared in-situ from silver nitrate and sodium hydroxide as described previously [5]. After oxidation, the combined filtrate and washings were acidified with dilute hydrochloric acid until pH ~ 1.5 and filtered. Pure perillic acid was obtained, 33.65 g (yield 61%). The product was characterized by ^{1}H-NMR and ^{13}C-NMR. The product was characterized by GC-MS after derivatisation with TMSH. ^{1}H-NMR (400.17 MHz, $CDCl_3$): δ 1.41 (2H, m, *J*=4.0Hz, H-5,),1.68 (3H, s, H-10), 1.82 and 1.84 (2H, d, *J*=8.0Hz, H-6), 2,08 (2H, m, *J*=8.0Hz, H-3), 2.28-2.42 (1H, m, *J*=16.0Hz, H-4), 4.56 and 4.70 (2H, s, H-9), 7.07 (1H, s, H-2), 11.05 (1H, s, H-11); ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ 20.69 (C-10), 24.15 (C-6), 26.95 (C-5), 31.26 (C-3), 39.94 (C-4), 109.28 (C-9), 129.48 (C-1), 141.87 (C-2), 148.61 (C-8), 172.82 (C-7).

Perillic acid was esterified with excess methanol, using toluenesulfonic acid as catalyst [6]. Perillic acid (3.00 mmol, 0.50 g) was dissolved in methanol (0.32 mol, 13 mL) and *p*-toluenesulfonic acid (0.15 mmol, 0.029 g), stirred (350 rpm) and heated to reflux (65 °C) for 22 h under N_2. The solution was cooled to room temperature and diluted with 12 mL chloroform, washed twice with saturated sodium bicarbonate (15 mL) and once with brine (15 mL). The organic layer (bottom) was diluted with

chloroform to 20 mL, dried with magnesium sulphate and filtered. The solvent was removed using a rotary evaporator at 40 ˚C. The slightly-yellow oil was identified by GC-MS. A by-product was detected, that was identified as 8-methoxy-methylperillate by NMR and GC-MS. The product mixture was separated on column (2.3 cm diameter, 10 g silica, eluent PE:EtOAc 90:10). Fractions were evaporated and analysed with ^{1}H-NMR, ^{13}C-NMR and GC-MS. Methylperillate yield: 0.259 g (47 mole%). The procedure was scaled up at least 10 times with similar results. ^{1}H-NMR (400.17 MHz, $CDCl_3$): δ 1.43-1.48 (1H, m, *J*=4.0Hz, H-5), 1.75 (3H, s, H-10), 1.82-1.84 (2H, m, *J*=4.0Hz, H-5), 2,08 (2H, m, *J*=4.0Hz, H-6), 2.13-2.18 (2H, m, *J*=8.0Hz, H-3), 2.25-2.40 (1H, m, *J*=4.0Hz, H-4), 3.72 (3H, s, H-11), 4.66 and 4.70 (2H, s, H-9), 6.93 (1H, m, *J*=4.0Hz, H-2); ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ 20.53 (C-10), 24.44 (C-6), 26.92 (C-5), 30.93 (C-3), 39.93 (C-4), 51.30 (C-11), 109.05 (C-9), 129.78 (C-2), 138.90 (C-1), 148.53 (C-8), 167.55 (C-7).

Dehydrogenation to methyl cumate

Dehydrogenation conditions were initially optimized for limonene (Table 2). Methylperillate was dehydrogenated to methyl cumate using a supported palladium catalyst [7]. Purified methylperillate (96% pure), 2.66 mmol, 0.479 g, 0.5 mL, was dissolved in acetone (0.300 mol, 22 mL) in a stirred (830 rpm) 75 mL reactor (MRS5000, Parr instrument company, Illinois, USA) with 5% Pd/Al_2O_3 catalyst (0.2038 g, ~0.096 mmol Pd), flushed 3 times with N_2 and then heated to 125 °C for 1 h under N_2. Subsequently, the reactor was let to cool down to room temperature. Then the solution was filtered over a Celite pad and washed with acetone. The solvent was removed using a rotary evaporator at 40 ˚C. The product was analysed with ^{1}H-NMR, ^{13}C-NMR and GC-MS. Some minor by-products related to acetone were observed, among these were diacetone alcohol, that can be formed by aldol addition of two molecules of acetone, and mesityl oxide, the dehydration product of diacetone alcohol. Yield: 0.721 g, 45% pure (total yield methylcumate from methylperillate 69 mole%). ^{1}H-NMR (400.17 MHz, $CDCl_3$) δ 1.25 (6H, s, H-9 and H-10), 2.92-2.98 (1H, m, *J*=8.0Hz, H-8), 3.89 (3H, 2, H-11), 7.27-7.29 (2H, m, *J*= 8.0Hz, H-3 and H-5), 7.94-7.96 (2H, m, *J*=8.0Hz, H-2 and H-6); ^{13}C-NMR (100.62 MHz, $CDCl_3$) δ 23.63-23.64 (C-9 and C-10), 30.83 (C-8), 34.19 (C-7), 51.85(C-11), 126.40 (C-3 and C-5), 127.74 (C-1), 129.67 (C-2 and C-6), 154.24 (C-4), 167.09 (C-7). NMR characterization matches that reported in literature [8].

Oxidation of cumic acid to terephthalic acid

A 50 mL round bottom flask was equipped with a magnetic stirring bar and a Liebig condenser. The round bottom flask was placed on a stirring plate with an aluminium heating mantle. The flask was charged with water (20 mL), followed by 65% nitric acid (14.4 g) and sodium nitrite (10 mg) to initiate the reaction. The mixture was stirred and gently heated to ~40 ˚C until the mixture became yellow. Next, cumic acid (5.0 g) was added to the stirred nitric acid solution, to give a white suspension. The suspension was heated to reflux, and became clear after ~30 min at reflux. After 24 h reflux, the reaction mixture had turned into a white suspension again. The mixture was allowed to cool down to room temperature, and the white solid was collected from the suspension by filtration (type 3 glass filter). The filter cake was washed with demineralized water, and dried under vacuum to constant weight (40 ˚C, ~50 mbar, in the presence of Sicapent). The product was obtained as a white powder. Yield: 4.5 g (89 mole%). The product was analysed with GC-MS after silylation, and with ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR (400.17 MHz, DMSO-D_6) δ 13.32 (2H, s, H-7 and H-8), 8.03 (4H, s, H-2, H-3, H-5 and H-6); ^{13}C-NMR (100.62 MHz, DMSO-D_6) δ 129.43 (C-2, C-3, C-5, C-6), 134.44 (C-1, C-4), 166.64 (C-7, C-8).

[1] E. Stahl, *Dünnschicht-Chromatographie; ein Laboratoriumshandbuch*, Springer, Berlin, 1962.

[2] E. Jongedijk, K. Cankar, J. Ranzijn, S. van der Krol, H. Bouwmeester, J. Beekwilder, *Yeast* 2015, 32, 159-171.

[3] a) F. van der Klis, J. Le Nôtre, R. Blaauw, J. van Haveren, D. S. van Es, *Eur J Lipid Sci Technol* 2012, 114, 911-918; b) F. van der Klis, J. van Haveren, D. S. van Es, J. H. Bitter, *ChemSusChem* 2017, 10, 1460-1468.

[4] J. J. J. van der Hooft, J. Vervoort, R. J. Bino, R. C. H. de Vos, *Metabolomics* 2012, 8, 691-703.

[5] Q. Wang, S. Y. Fan, H. N. C. Wong, Z. Li, B. M. Fung, R. J. Twieg, H. T. Nguyen, *Tetrahedron* 1993, 49, 619-638.

[6] B. Dayal, J. Speck, E. Bagan, G. S. Tint, G. Salen, *Steroids* 1981, 37, 239-242.

[7] R. J. Grau, P. D. Zgolicz, C. Gutierrez, H. A. Taher, *J Mol Catal A-Chem* 1999, 148, 203-214.

[8] Y. Zhu, H. Yan, L. Lu, D. Liu, G. Rong, J. Mao, *J Org Chem* 2013, 78, 9898-9905.

Chapter 4

Chapter 5
Elucidation of the methylperillate biosynthesis pathway from *Salvia dorisiana* trichomes

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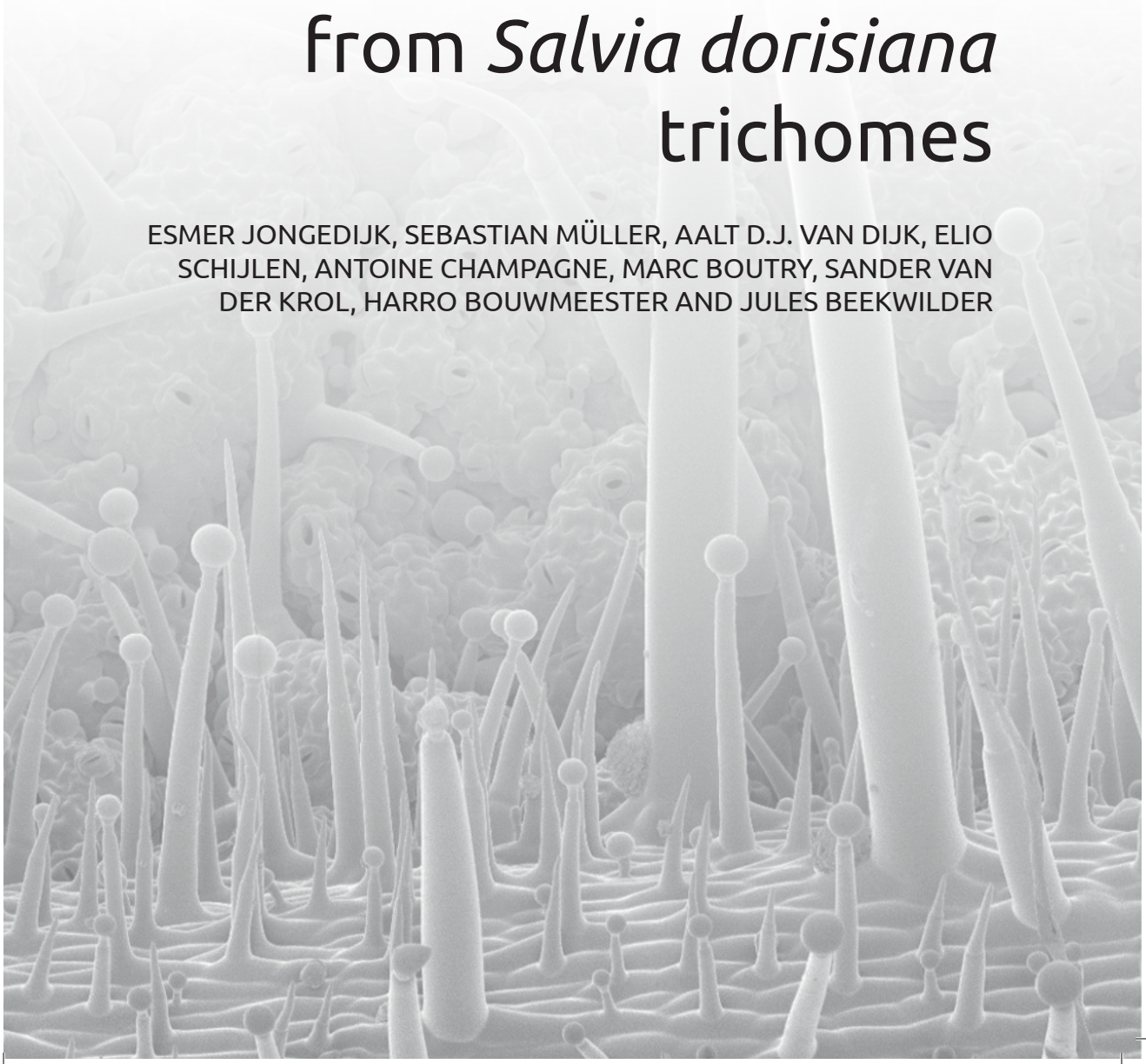


Abstract

Plants produce highly functionalized terpenoids. Presence of for example partially unsaturated rings and carboxyl groups provides handles to use these compounds as feedstock for biobased commodity chemicals. Methylperillate, a monoterpenoid present in *Salvia dorisiana*, is useful for this purpose, as it carries both a ring and a methylated carboxyl group. The biosynthesis of methylperillate is unclear: monoterpene methylation has not been described in the literature so far. In this work we identified type VI-like trichomes from *Salvia dorisiana* as the location of biosynthesis and storage of methylperillate. RNA from purified trichomes was used as a source to identify four genes that constitute the pathway towards methylperillate. This pathway includes a (-)-limonene synthase (*SdLS*), a limonene 7-hydroxylase (*SdL7H*, *CYP71A76*), and a perilla alcohol dehydrogenase (*SdPOHDH*). We also identified for the first time a terpene acid methyltransferase, perillic acid O-methyltransferase (*SdPAOMT*) with homology to salicylic acid OMTs. Transient expression of these four genes, in combination with a GPP synthase to boost precursor formation, results in the reconstitution of the methylperillate pathway in *Nicotiana benthamiana*. This demonstrates the utility of these enzymes for metabolic engineering to produce a feedstock for biobased commodity chemicals.

Introduction

Plants are a source of materials for human use, like wood, rubber and cotton. Since the second half of the 19th century, many of these plant-based materials have been replaced by synthetic materials derived from oil, with new and often superior properties. Now that concerns about the exploitation of fossil resources grow (Owen et al., 2010), new opportunities arise for bio-based substitutes for petrochemical building blocks that can be produced in a sustainable way.

Recently, it was suggested that plant monoterpenoids can be deployed for creating commodity chemical building blocks (Colonna et al., 2011; Jongedijk et al., 2016). Plant species from the Lamiacae family are rich in monoterpenoids, often derived from limonene. For example, the monoterpenoid methylperillate can be converted to the commodity chemical terephthalic acid (Chapter 4). The main application of terephthalic acid is as the building block of PET plastic (Collias et al., 2014). Terephthalic acid consumption is expected to reach 65 million ton in 2018 (Collias et al., 2014). Terephthalic acid is currently produced from oil, through oxidation of para-xylene (Rezaei and Sajadi, 2015).

Methylperillate is naturally present in the plant species *Salvia dorisiana* (Halim and Collins, 1975; Conti et al., 2012). *S. dorisiana* essential oil contains 20.4%

methylperillate, with an oil yield of 1-2 g oil / 100 g DW biomass (Chapter 4). Although at present *S. dorisiana* is not an agricultural crop, production of methylperillate could be of interest as a precursor for biobased materials. Elucidation of the methylperillate biosynthesis pathway would also enable production in heterologous hosts like micro-organisms or other plant species, enabling a sustainable (green) production process. The methylperillate biosynthesis pathway has not been elucidated completely.

Monoterpenoids are C10 compounds, derived from geranyl diphosphate (GPP) that is produced through the MEP pathway in the plastids. Methylperillate is a monoterpenoid with a structure related to the cyclic monoterpene limonene, but carrying a methyl ester group, of which the enzymatic formation has not yet been demonstrated in monoterpenoid biosynthesis (Fig. 1). Biosynthesis of monoterpene alcohols and aldehydes has extensively been studied, for example of menthol and carvone (Lange, 2015). The biosynthesis of acid groups has mainly been

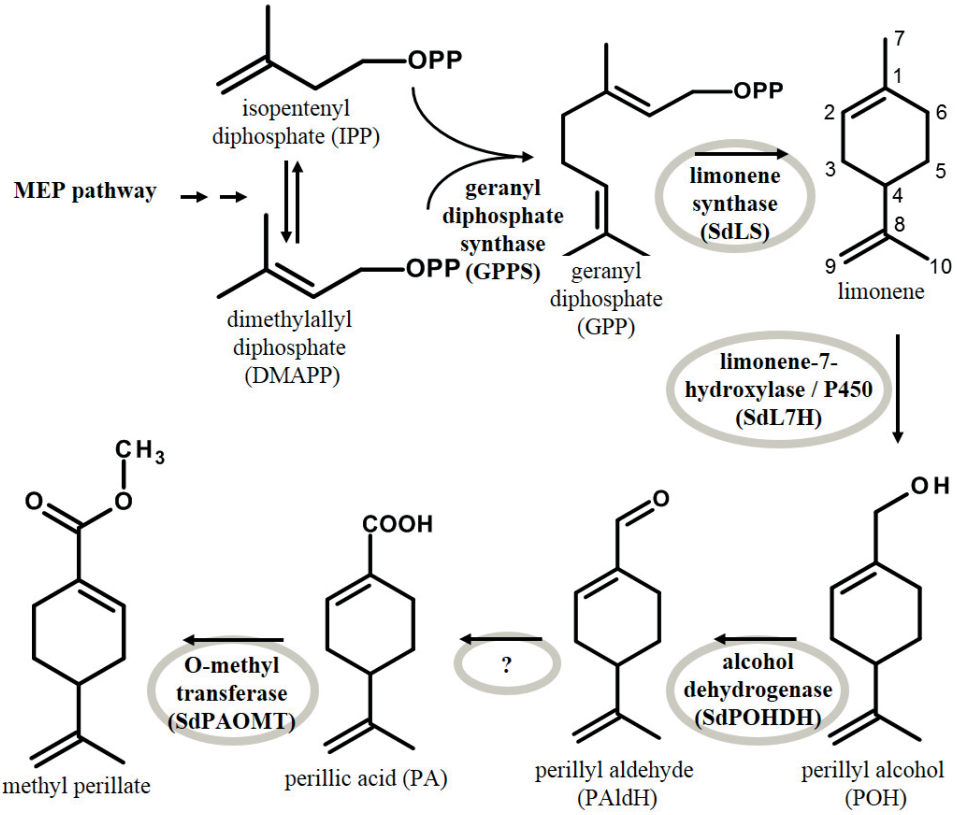


Figure 1 Proposed methyl perillate biosynthesis pathway in *Salvia dorisiana* trichomes.

studied for sesquiterpenes and diterpenes, such as artemisinic acid (Teoh et al., 2006), germacrene A acid (de Kraker et al., 2001; Nguyen et al., 2010; Cankar et al., 2011) and abietic acid (Ro et al., 2005). Also the formation of carboxylgroups in the pathway towards the indole alkaloid strictosidine has been characterized (Miettinen et al., 2014). Knowledge on formation of carboxyl groups in monoterpenoids is much more limited with the exception of the biosynthesis of chrysanthemic acid in pyrethrum that was recently elucidated (Ramirez et al., 2012; Xu et al., 2017). For the monoterpene indole alkaloids, biosynthesis of methyl esters has been reported (Murata et al., 2008). But methylester formation has not yet been demonstrated in monoterpenoid biosynthesis. Therefore elucidating the methylperillate pathway will need characterization of unusual new enzyme classes in monoterpene biosynthesis and the pathway cannot easily be inferred by homology to related pathways in other species.

For an efficient identification of the methylperillate pathway, it is essential to understand its localization in the plant. Biosynthesis of monoterpenes usually takes place in specialized tissues or cells (Lange and Turner, 2013). In Lamiacae species, monoterpene alcohols and ketones are generally produced in specialized trichomes on the leaf surface. Monoterpene acids can also be expected to be synthesized in trichomes. In pyrethrum, the monoterpene chrysanthemol is converted in the trichomes to chrysanthemic acid, allowing the molecule to move from the trichomes to different cell types, where it is then esterified (Ramirez et al., 2012). In *Artemisia annua*, artemisinic acid is stored in glandular trichomes, together with other terpenoids (Bertea et al., 2005). Also formation of strictosidine in *Catharantus roseus* is known to involve several tissues, including internal phloem-associated parenchyma, where acid formation takes place, and epidermal cells, where monoterpene methylation takes place (Murata et al., 2008; Miettinen et al., 2014).

In this study we investigated the biosynthesis of methylperillate in *S. dorisiana*. Biosynthesis occurs in a specific glandular trichome type. Transcript profiling of these trichomes resulted in the identification and characterization of genes involved in the biosynthesis of methylperillate. Thus, we discovered a new concept of cyclic monoterpene biosynthesis in plants (via acid formation and subsequent methylation in the trichomes), which has not previously been described. Heterologous production of methylperillate by transient expression of the entire pathway in *N. benthamiana* was demonstrated, as the first step towards bio-plastic precursor production.

Materials and methods

Plant material and growth

Salvia dorisiana plants were grown in the greenhouse under a 16h photoperiod, 19-21°C, 60% humidity. Plants were multiplied by cutting.

Trichome isolation

Trichomes of apical buds and young leaves until 7 cm were isolated as previously described (Gershenzon et al., 1992), with the following adaptations. XAD-4 (Amberlite®, Acros Organics) and 0.5 mm glass beads (unwashed, 425-600 µm, Sigma) were washed on a Büchner funnel with pump before use: subsequently 95% ethanol, 3M HCl and demi water, 2-3 times each. 15 g buds & leaves were soaked for at least 1h in ice-cold demi water. A bead-beater (Biospec Products, Model 1107900) was filled with the leaves, beads and isolation buffer as described (Gershenzon et al., 1992) and with a rheostat (Powerstat® variable autotransformer) three pulses of 1 min each on 110V were given, while cooling the bead-beater continuously with the ice compartment. The content was passed by a 280-µm nylon mesh (Kabel-Zaandam) and washed two times with washing buffer (isolation buffer without methyl cellulose and PVP). The 280-µm filtrate was passed through a 90-µm mesh and the secretory cells were collected by passing the 90-µm filtrate through 20-µm. The trichomes were washed in the washing buffer and scraped from the mesh. For subsequent RNA extraction or metabolite analysis the trichomes were snap-frozen immediately in liquid N_2 and kept at -80°C up to 1 month, for microscopy the trichomes were dissolved in a bit of washing buffer and kept at 4°C, up to 2 days.

Microscopy

Photographs of leaf surface were made under a ZEISS stemi SV11 binoculars, equipped with a Nikon DS-Fi1 digital sight camera. Pictures of isolated trichomes were made with an inverted microscope (ZEISS Axioskop), at 10-100 times magnification, equipped with the same camera.

Scanning electron microscopy (SEM)

Small pieces of *Salvia dorisiana* leaves, including abaxial and adaxial sides, were attached on a brass Leica sample holder with carbon glue (Leit-C, Neubauer Chemikalien, Germany). The holder was affixed on the cryo-sample loading system (VCT 100, Leica, Vienna, Austria) and simultaneously frozen in liquid nitrogen. The frozen holder was transferred to the cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto the sample stage at -92 °C. For removal of frost contamination on the sample surface the samples were freeze dried for 5 min at -92°C and 1.3x10-6 mbar. After sputter coating with a layer of 20 nm tungsten at the same temperature the sample holder was transferred into the field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, The Netherlands) onto the sample stage at -120°C. The analysis was performed with SE detection at 2 kV and 6.3 pA. SEM pictures were taken at a magnification of 200-3500 times (scale bars are indicated in the respective pictures).

Metabolite quantification

Salvia parts were harvested in three-fold. The fresh weight / dry weight ratio was determined by drying at 105°C for 20h. To measure metabolites, the plant parts were frozen in liquid N_2, ground in a mortar + pestle while still frozen, and 1 mL ethyl acetate, 3 µg/mL γ-terpinen-4-ol as internal standard, was added to 100 mg frozen plant powder, vortexed for 20s, subjected to sonication for 5 min and centrifuged for 5 min, 3400 rpm. The supernatant was analysed on GC-MS as described below. A standard-mix of 3 µg/mL terpinen-4-ol, (R)-(+)-limonene (97%, Sigma Aldrich), perillyl alcohol (96%, Sigma Aldrich), (S)-(-)-perillaldehyde (Sigma Aldrich) and (S)-(-)-perillic acid (95%, Sigma Aldrich) was prepared for identification and semi-quantification. The standard mix was methylated by addition of diazomethane 1:1 and injected as well, to be able to identify and semi-quantify methyl perillate in the samples.

Chloroform dip

Chloroform dipping (Ramirez et al., 2012) was performed. 3 µg / mL (-)-terpinen-4-ol (≥95%, Aldrich) was added to the chloroform as an internal standard. Fresh leaves < 3 cm of known weight were submerged in a time series in glass tubes, 4 leaves in 10 mL $CHCl_3$ for 5 s, in another 10 mL for 10 s and in another 10 mL for 15 s. Leaves were immediately snapfrozen in liquid N_2 after dipping. Intact leaves and leaves after dipping were extracted with chloroform and dried, similar as described under GC-MS of extracts. GC-MS peak areas of the time series were added together. Fresh weight was determined by weighing the whole leaf in case of the content of the chloroform dip (D), and weighing the amount grinded leaf powder in liquid N_2in case of intact leaves (L) and leaves after dipping (DL).

RNA extraction and cDNA preparation

Plant tissues were grinded and RNA was isolated with the RNeasy Plant Mini kit (Qiagen) according to the manufacturer protocol. Trichomes were grinded with a pestle and mortar while still frozen in N_2, and transferred to a precooled Eppendorf-tube in liquid N_2. Buffer RLT was added to the frozen trichomes and RNA was isolated further with the kit. For sequencing, RNA was treated with DNAse1 (Invitrogen). cDNA for cloning was prepared with the SMART RACE cDNA amplification kit (CLONTECH, Palo Alto, CA), using Advantage®2 Reverse Polymerase Mix (CLONTECH, Palo Alto, CA).

PacBio Iso-Seq sequencing

Preparation of a Pacbio Iso-Seq library was performed according to 'Procedure & Checklist - Isoform Sequencing (Iso-Seq™, Pacific Bioscience) using the Clontech SMARTer PCR cDNA Synthesis Kit and Manual Agarose-gel Size Selection'. In short, one microgram total RNA was used for cDNA synthesis using SMARTer PCR cDNA Synthesis Kit (Clontech) followed by dilution with Pacbio elution buffer to 90 uL final

volume. After optimization, a large scale cDNA amplification was done using 80 uL of diluted cDNA, 5'PCR primer II A (Clontech), 1U Kapa HiFi enzyme, KAPA dNTP mix and buffer, cDNA with 18 PCR cycles in eight parallel reactions of 50 uL each. Amplified cDNA was pooled and purified using Ampure PB beads. Three aliquots of 500 ng amplified cDNA were used for size selection on a 0.8% agarose 1XTAE gel stained with SYBR Safe (Invitrogen) with electrophoresis at 70V for 1 hour. Three different size fractions (200 bp to 1 Kb, 1 to 2 Kb, and 2 to 4 Kb) were isolated and purified using MinElute Gel Extraction Kit (Qiagen). Large scale cDNA PCR amplification was performed on each isolated fraction as described previously using adapted elongation times (45, 60 and 120 seconds) and number of cycles (10, 12, 15) for the short, middle and large fraction respectively. Amplified and purified cDNA was used for SMRT bell constuction using DNA template prep kit v3.0 for subsequent DNA damage repair, End repair and adapter ligation. SMRT bells were further used for primer and polymerase (P5) binding according to manufacturer's guidelines (Pacbio). Sequencing was done on a Pacbio RS-II system with Chemistry v3, one cell per well and stage start. The smallest fraction was loaded by diffusion and analysed using 180 min movie length, whereas the others were loaded using magbeads and sequence sequencing was done by a 240 min movie protocol. PacBio isoseq analysis was performed using the PacBio SMRT portal software using standard settings.

Filtered PacBio reads were then assembled into clusters using Cap3 (Huang and Madan, 1999). Getorf (Rice et al., 2000) was applied to the resulting clusters (including the remaining singlets) in order to find regions between stop codons with a minimum size of 600 nt. The resulting predicted open reading frames were clustered using DNACLUST (Ghodsi et al., 2011) using a value of 0.85 for the similarity threshold between the cluster centre and a given sequence in the cluster, and the option allow-left-gaps. In each of the resulting clusters, MUSCLE (Edgar, 2004) was used for sequence alignment and a consensus sequence was obtained using the most often occurring nucleotide at each position.

HiSeq sequencing

Total RNA of plant tissues (leaves < 3 cm, leaves 3-10 cm, leaves > 10 cm, stem, roots and trichomes) was prepared. Of each isolation, one microgram total RNA was used for mRNA isolation and subsequent RNAseq library preparation following TruSeq Stranded mRNA Sample Preparation Protocol (Illumina). In short, mRNA was isolated using oligo dT beads and chemically fragmentized prior to first strand cDNA synthesis using random primers. Strand specificity was achieved by replacing dTTP with dUTP during second strand synthesis and the addition of Actinomycin D to the first strand master mix. Obtained double stranded cDNA fragments were used for 3'Adenylation and adapter ligation using different barcoded adapters. Adapter ligated cDNA was

finally amplified using 15 PCR cycles. Quality control of final libraries was done using an Agilent Tapestation High Sensitivity D1000 Screen Tape assay. Final library quantification was performed using Qubit (Invitrogen). Equimolar amounts of libraries were pooled and diluted towards 6pM concentrations for paired end clustering on one lanes of a flow cell V4 (Illumina). Final sequencing was performed on a HiSeq2500 instrument using a 126,7,126 flow cycle pattern. De-multiplexing of obtained sequences was done using CASAVA 1.8.1. software.

Reads were mapped with CLC Genomics Workbench 8.0 (Qiagen), using the following settings: "Reference type = One reference sequence per transcript; Mismatch cost = 2; Insertion cost = 3; Deletion cost = 3; Length fraction = 0.9; Similarity fraction = 0.9; Global alignment = No; Color space alignment = Yes; Color error cost = 3; Auto-detect paired distances = Yes; Strand specific = Both; Maximum number of hits for a read = 10; Count paired reads as two = No; Expression value = RPKM; Calculate RPKM for genes without transcripts = Yes; Minimum read count fusion gene table = 5".

Co-expression and PFAM analysis

The R function cor was used to calculated Pearson correlation between expression for each of the sequences resulting from the PacBio sequencing with the *SdLS* gene (that had been first characterized), as well as with the metabolite level in each of the tissues. For the latter, the average level of the different metabolites was used. Pearson correlation values and expression values were visualized using plot3D (Soetaert, 2013).

PFAM domains were predicted in each of the sequences resulting from the PacBio sequencing using HMM search (Eddy, 2011). To assess the predictive value of filtering on the two correlations mentioned above and on the expression value, sequences were filtered to have a Pearson correlation of at least 0.6 with *SdLS* and with the metabolite level, and an expression level of at least 1000. Enrichment of PFAM domains in the set of sequences selected in this way compared to the rest of the sequences was assessed using the fisher exact test as implemented in fisher.test (R) followed by multiple testing correction using Benjamin-Hochberg procedure. An FDR cut-off of 0.05 was applied.

P450 proteins were systematically named by David Nelson (Nelson, 2009).

Cloning and (race)PCR (primers, vectors)

Fragments were amplified with Phusion® High-Fidelity DNA Polymerase (NEB) and primers (Table 1), according to manufacturer protocol. All constructs were first transformed to *E. coli* DH5α, isolated with miniprep (REF), and sequenced by Macrogen Europe Amsterdam, and subsequently transformed to their destination strains.

Table 1 Primer sequences used in this study

primer*	reference	sequence
SdLS_NotF		TATGCGGCCGCATGTCTAGTATTATAATGCAATTCGC CATTCCTA
SdLS_PacR		ACTTTAATTAATTACTCATAAGGTTCGAACAGCAAGC TTCT
SdtLS_SalF		ACTGTCGACCGACGAACTGGAGGCTACCA
SdLS_NotR		TGCGGCCGCTTACTCATAAGGTTCGAACAGCAAGCTT CT
SdtPS_AscF		ATGGCGCGCCTGCGAAGATCGGGAAACTACAGTCCAT
SdPS_NotR		ATGCGGCCGCTCAGGCAATAGGGTGGAACAAGCAAT
SdtLiS_AscF		ATGGCGCGCCTGCGACGCTCCGGAAACTACCA
SdLiS_NotR		ATGCGGCCGCTCAATCACATGGTTGAAACAGCAGACT
SdtPCS_AscF		ATGGCGCGCCTGCGACGGTCGGGTAATTACAAGCCTA
SdPCS_NotR		ATGCGGCCGCCTAGACAATAGGTTGAAATATCAAACC TAAAATACGTT
SdtSaS_AscF		ATGGCGCGCCTGAGGAGGTCTGCAAACTACGAAGCT AGT
SdSaS_NotR		ATGCGGCCGCTCAAGGCATCGAAAAGGGCTGAA
SdtEuS_AscF		ATGGCGCGCCTGCGACGAACTGGAGGCTACCAGCCTA CT
SdEuS_NotR		ATGCGGCCGCTTATTCATAAGGTTCGAACAGTAAGCT TCTGAGCTGTT
ClLS_NotF	(Lücker et al., 2002)	TAGCGGCCGCTATGTCTTCTTGCATTAATCCCTCAACC TTGGTTACCT
ClLS_PacR	(Lücker et al., 2002)	ACTTAATTAATCAGCCTTTGGTGCCAGGAGATGC
PfLS_NotF	(Jongedijk et al., 2015)	TAGCGGCCGCTATGTATACCGGTGTAATAATGCATAT GGCGATTCCT
PfLS_PacR	(Jongedijk et al., 2015)	ACTTAATTAATTACAACCATTGCTCGAACAAGATGTC TGTCATCT
SdL7H_GWF		CACCATGGCAGCTCTTCTACTTCTTA
SdL7H_R		TTAATAAGCACGTGGTGTAGTAA
SdADH_GWF		CACCATGGCTGATAACACCATAACTT
SdADH_R		TCAGAACTTGATAATGACCTTGA
SdOMT_BamF		TACGGATCCGATGGAAGTAGTTGAGGTGCTTC
SdOMT_NotR		AATGCGGCCGCTCATCCTCTCCGGATCAAA

***F forward; R reverse; t truncated, removes plastid targeting signal; Sal SalI restriction site added, Asc AscI restriction site added; Not NotI restriction site added; GW cacc tag added for directional cloning in pTOPOdENTRY**

Monoterpene synthase and methyltransferase sequences were cloned in the pCDF-duet vector (NOVAGEN) for in-vitro assay as described before (Table 2) (Jongedijk et al., 2015). Monoterpene synthases were expressed with the plastid targeting signal removed, methyltransferase sequences were expressed full length. When necessary, sequences were amplified to full length by RACE-PCR, according to manufacturer primer design directions and protocol.

Monoterpene synthases, methyltransferases and GPPS sequences were cloned for agro-infiltration in the entry vector ImpactVector pIV1A-1.1 (Table 2) (www.impactvector.com), modified with NotI/PacI restriction sites at the polylinker, and the original PacI site and existing ATG codon removed (ImpacTim). The construct was transferred by LR reaction to the pBIN+ binary destination vector (Vanengelen et al., 1995) according to manufacturer protocol. pBIN+ constructs were used for *Agrobacterium* transformations.

All other sequences were cloned for agro-infiltration in the directional entry vector pENTR™/D-TOPO® (Invitrogen) (Table 2) according to manufacturer protocol. Constructs were transferred to pB7WG2 binary destination vector (Karimi et al., 2002) by LR reaction and used for *Agrobacterium* transformations.

Table 2 Plasmids and strains used in this study

plasmid/strain	antibiotic	features
plasmids		
pCDF-duet	spectinomycin 100 µg / mL	T7 promoter
Impact pIV1A-1.1	gentamycin 20 µg / mL	Rubisco promoter and terminator
ImpacTim	gentamycin 20 µg / mL	Rubisco promoter and terminator, NotI/PacI restriction sites added
pBin+	kanamycin 50 µg / mL	
pTOPOdENTRY	kanamycin 50 µg / mL	
pB7WG2	spectinomycin 100 µg / mL	35S promoter and terminator
pBIN+_PaGPPS	kanamycin 50 µg / mL	Rubisco promoter and terminator, GPP synthase (Dong et al., 2016)
strains		
E.coli BL21-CodonPlus® (DE3)-ril	chloramfenicol 100 µg / mL	(Kleber-Janke and Becker, 2000)
Agrobacterium Agl0	rifampicilin 40 µg / mL	(Lazo et al., 1991)

In-vitro assays

In-vitro enzyme expression was performed as described previously (Jongedijk et al., 2015). Monoterpene synthase assay conditions were as described in that paper. For the methyltransferase assay cell supernatants were stored at -20°C (700µL supernatant with 300 µL 50% glycerol). Methyltransferase assay was performed by mixing 70 µl of cell supernatant/glycerol mix with 50 µL 500 mM Tris pH 7.0, 20 µL 32 mM S-adenosyl-L-methionine (SAM), 10 µL 50 mM substrate (salicylic acid (Acros Organics), perillic acid (95%, Sigma Aldrich), benzoic acid (99.5%, Sigma Aldrich) or jasmonic acid (>95%, OlChemIm) in DMSO), filled up to 500 µL with MQ. The mix was incubated for 1h at room temperature with gentle agitation (20 rpm). Tubes were extracted with 2

mL ethylacetate by vortexing well and sonicating for 5 min, centrifuged for 5 min (3400 rpm), the ethylacetate phase was collected, dried over anhydrous Na_2SO_4 and analyzed by GC-MS. Reference compounds (methyl jasmonate (>95%%, OlChemIm), methyl salicylate (99+%, Aldrich), perillic acid and benzoic acid) were dissolved in methanol, diluted in ethylacetate to the range of 3-12 µg/mL, and for PA and BA methylated by 1:1 addition of diazomethane.

Alignment (clustal-W)

Protein sequence alignments were performed with CLC Genomics Workbench version 8.0.2 (Qiagen Aarhus) software using the alignment algorithm with standard parameters: Gap open cost = 10.0, Gap extension cost = 1.0, End gap cost = As any other, Alignment mode = Very accurate (slow), Redo alignments = No, Use fixpoints = No.

Transient gene expression in *N. benthamiana*

Constructs for agro-infiltration were transformed to *Agrobacterium tumefaciens* strain Agl0 by electroporation. Agro-infiltrations were prepared and performed as described (Cankar et al., 2015) with the following modifications: medium was supplemented with 50 ug/mL kanamycin and 40 ug/mL rifampicilin for pBIN+ based constructs, and with 100 ug/mL spectinomycin and 40 ug/mL rifampicilin for pB7WG2 based constructs.

Compound infiltrations in *N. benthamiana*

Solutions of 400 uM of compound (limonene, perillyl alcohol, perillyl aldehyde, perillic acid) were prepared in 0.2% DMSO. Compounds were co-infiltrated on the 5th day after infiltration of *Agrobacterium* strains, and leaves were harvested 3-5h after compound co-infiltration.

Headspace trapping

Trapping of headspace volatiles was performed as described (Cankar et al., 2015) with following modifications: headspace sampling was performed in a climate room (20±2°C, 56% RH) with LED lighting (adjusted at 100% white, 10% deep red, 100% far red and 5% blue light). Volatiles were trapped by sucking air out of the jar at a rate of 100 mln min-1 (inlet flow at 150mln min-1) for 3-4 hours.

Thermodesorption GC-MS

Trapped headspace volatiles were analyzed using a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Settings as described (Jongedijk et al., 2015), with the following modification: volatiles were injected on the analytical column at split ratio 10.

GC-MS of extracts

Aliquots of 200 mg of frozen, powdered leaf material were extracted with 2 mL ethylacetate. Samples were vortexed 20 sec and centrifuged for 5min at 3400rpm, the

ethylacetate phase was dried over anhydrous Na_2SO_4. GC-MS of extracts was performed on a 7890A gas chromatograph (Agilent) equipped with a mass selective detector (Model 5975C, Agilent) as described previously (Jongedijk et al., 2015).

For identification of the enantiomer of limonene, the gas chromatograph was equipped with an enantioselective column with stationary phase Heptakis (6-O-TBMDS-2,3-di-O-methyl)-β-cyclodextrin; 50% w/w in OV1701) of 25m and i.d. 0.25 mm, with settings as described previously (Jongedijk et al., 2015).

LC-MS

Aliquots of 200 mg of frozen, powdered leaf material were extracted with 0.6 ml of 99.9% MeOH / 0.125% formic acid. After 20 sec. vortex and 5 min. sonication the extracts were centrifuged for 20 min at maximum speed in a table-top centrifuge. LC-MS was performed on an Accela HPLC tower connected to a LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific), conditions and settings as described previously (van der Hooft et al., 2012).

Results

Methylperillate and its precursors localize to *Salvia dorisiana* glandular trichomes

Methyl perillate and its precursors limonene, perillyl alcohol and perillyl aldehyde (Fig. 1) were analyzed in different plant tissues of *Salvia dorisiana* by GC-MS. Leaves of different sizes, stems and roots were compared. Both methylperillate and the other metabolites are overrepresented in young leaves, compared to older leaves and stem and root tissues (Fig. 2). Limonene was exclusively present as the (-)-enantiomer, as shown by GC-MS analysis on an enantioselective column with (-)- and (+)-limonene standards (Fig. S3).

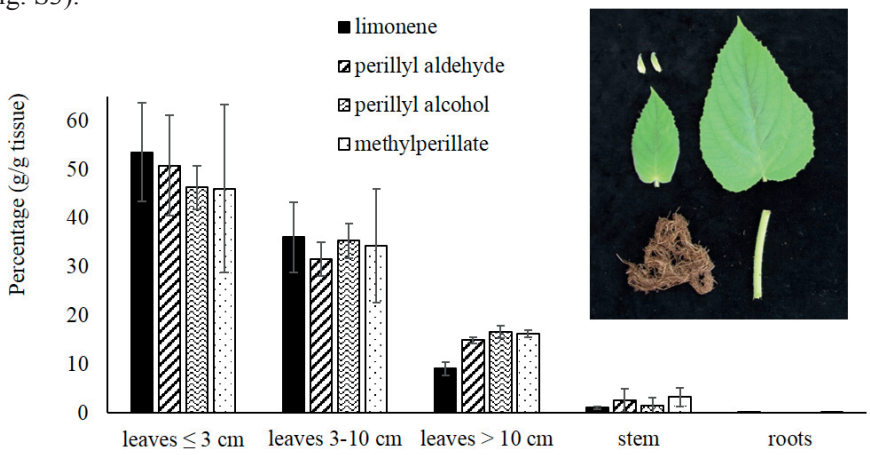
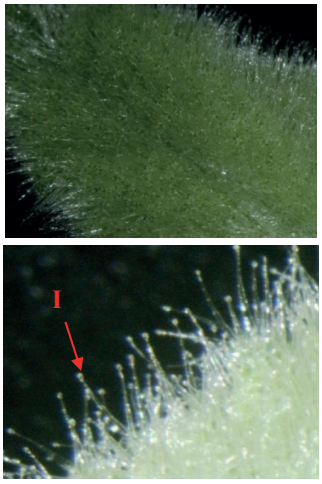


Figure 2 Concentrations of methylperillate and pathway intermediates in *Salvia dorisiana* plant parts. Highest percentages were found in the small leaves. Error bars indicate standard deviations (n=3).

Members of the Lamiacae family accumulate and biosynthesize monoterpenes in the leaf glandular trichomes (Gershenzon et al., 1992; Iijima et al., 2004; Bumblauskiene et al., 2009). Young leaves of *S. dorisiana* are covered with trichomes (Fig. 3a). To extract metabolites present in the trichomes, leaves were briefly dipped in chloroform (Ramirez et al., 2012). GC-MS analysis of the chloroform, and the leaves before and after chloroform treatment showed that methylperillate and its precursors are enriched in the trichomes (Fig. 3b, Fig. S1).

Different trichome types have been described, based on their morphological characteristics (Glas et al., 2012). Trichomes found on *S. dorisiana* leaves display different morphologies when compared to trichomes from other plants (Fig. 3c). While type I and V trichomes correspond well to those identified in tomato, the type II-like trichomes in *S. dorisiana* have a longer stalk (0.5-2.0 mm). Type IV-like trichomes carry shorter stalks compared to tomato (0.05-0.4 mm). Interestingly, the type VI-like glandular trichomes have a very short stalk.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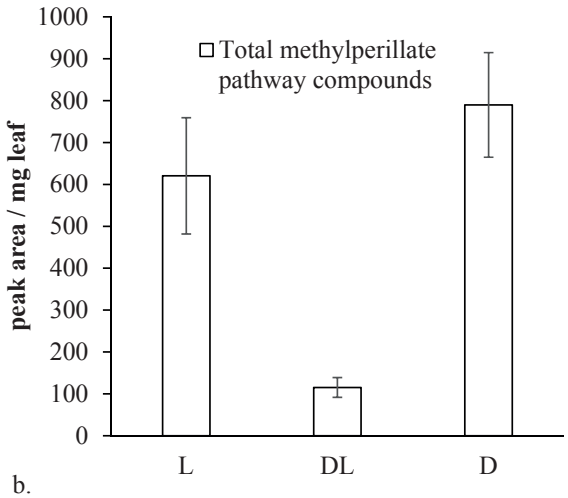
a.


1000
900
800
700
600
500
400
300
200
100
0
peak area / mg leaf
Total methylperillate pathway compounds
L
DL
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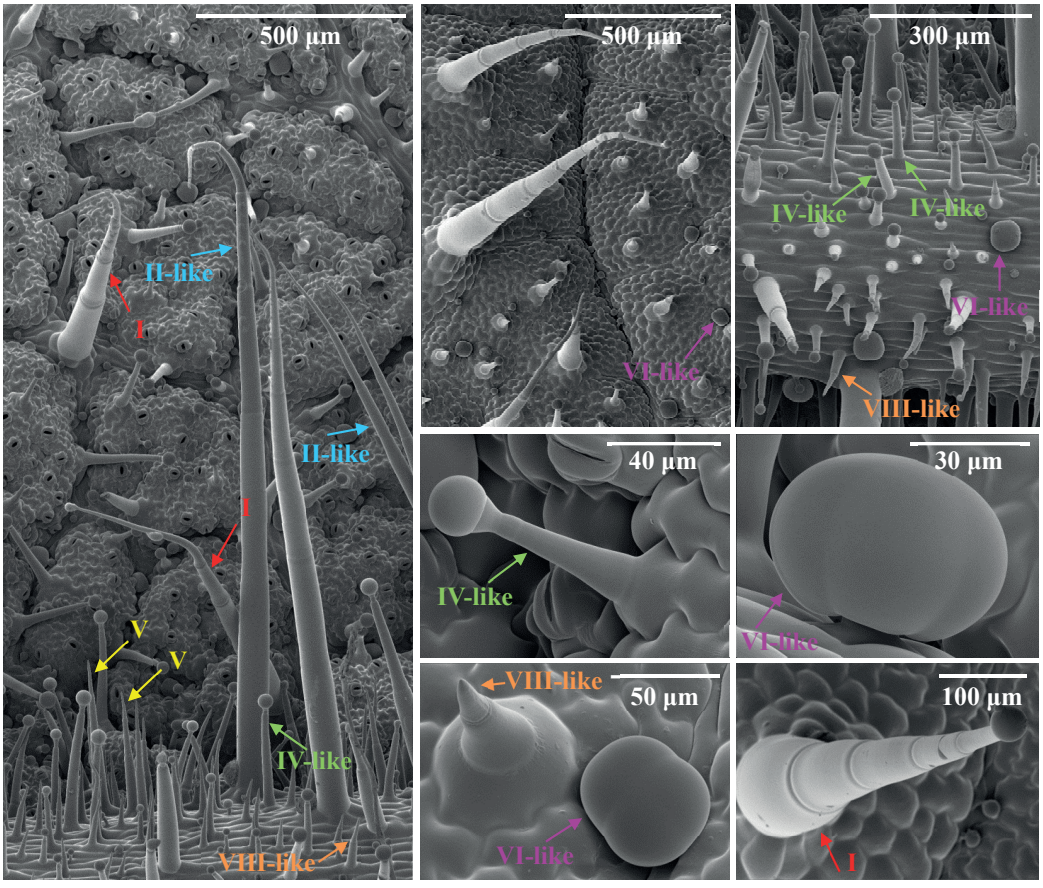
b.


500 µm
II-like
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VI-like
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40 µm
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30 µm
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I

c.

Figure 3 a. Light microscopy of *Salvia dorisiana* leaf surface which is covered in trichomes, b. Methylperillate pathway compounds are enriched in the trichomes. L intact leaves, DL leaves after dipping, D chloroform dip. Integrated peak areas from GC-MS of chloroform dip, c. Cryo-SEM pictures of leaf surface. Arrows indicate different trichome types as has been described for tomato (Glas et al. 2012). Red: trichome type I, blue: type II-like but longer stalk (0.5-2.0 mm), green: type IV-like, shorter stalk occurs as well (0.05-0.4 mm), yellow: type V, pink: type VI-like but a shorter stalk (glandular part very close to leaf surface), orange: type VIII-like, tip cell is not or less leaning.

For comparison, the glandular trichomes of *Mentha* species have also a very short stalk, but the glandular part consist of 8 cells (Turner et al., 2000), instead of four which we observe in *S. dorisiana*. Finally, type VIII-like trichomes in *S. dorisiana* have a tip cell which is not or less leaning, compared to the corresponding tomato trichomes (Fig. 3c). Trichomes from *S. dorisiana* were isolated from fresh leaves (Gershenzon et al., 1992) (Fig. S2a), and analyzed by GC-MS, which confirmed that methylperillate and its intermediates localize to the trichomes (Fig. S2b). *Salvia* trichome types were separated by using a combination of meshes, percoll and sucrose gradients, which resulted in fractions of hairy trichomes (type II-like and IV-like) and a fraction of near-pure glandular (type VI-like) trichomes (Sallets et al., 2014). Metabolite analysis of the separated trichomes shows that the monoterpenes accumulate in the glandular Type VI-like trichomes (Fig. S4).

Comparative expression analysis reveals genes involved in *Salvia dorisiana* trichome metabolism

The observation that methylperillate and its intermediates localize predominantly to the glandular trichomes suggested that biosynthetic genes are present in trichome tissues. Therefore, cDNA from a total trichome fraction was generated (Fig. S2) and sequenced by PacBio IsoSeq sequencing, and transcripts were assembled. In order to select for genes that are predominantly expressed in trichome tissues, short-read HiSeq RNA sequencing was performed of cDNA from trichomes, leaves < 3 cm, leaves 3-10 cm, leaves > 10 cm, stems and roots (Table S1), and reads were mapped on the previously assembled transcripts, to determine expression levels of the different genes.

Selection of candidate genes

The collection of gene sequences from *Salvia* trichomes was filtered on the absolute expression level of transcript in the trichome, and on correlation of its expression profile over the different tissues to the metabolites profile over the different tissues as in Figure 2. To identify candidate genes for enzymes in the pathway towards methylperillate, the highly expressed genes were analysed by PFAM domain analysis (Finn et al., 2016) and additional blast analysis in Genbank (Benson et al., 2013) (Table S2). First, six candidate

monoterpene synthases were extracted from this dataset, cloned and tested, and a limonene synthase was identified (Fig. 5, see below).

To identify subsequent genes in the pathway, an additional sorting of transcripts on co-expression with the limonene synthase was performed. A set of transcripts was selected that had a high absolute expression level in the trichomes (RPKM>1000), a high correlation with presence of pathway metabolites (average of w/FW of pathway metabolites, R>0.6), and a high correlation in co-expression with the limonene synthase (R>0.6) (Fig. 4, Table S2).

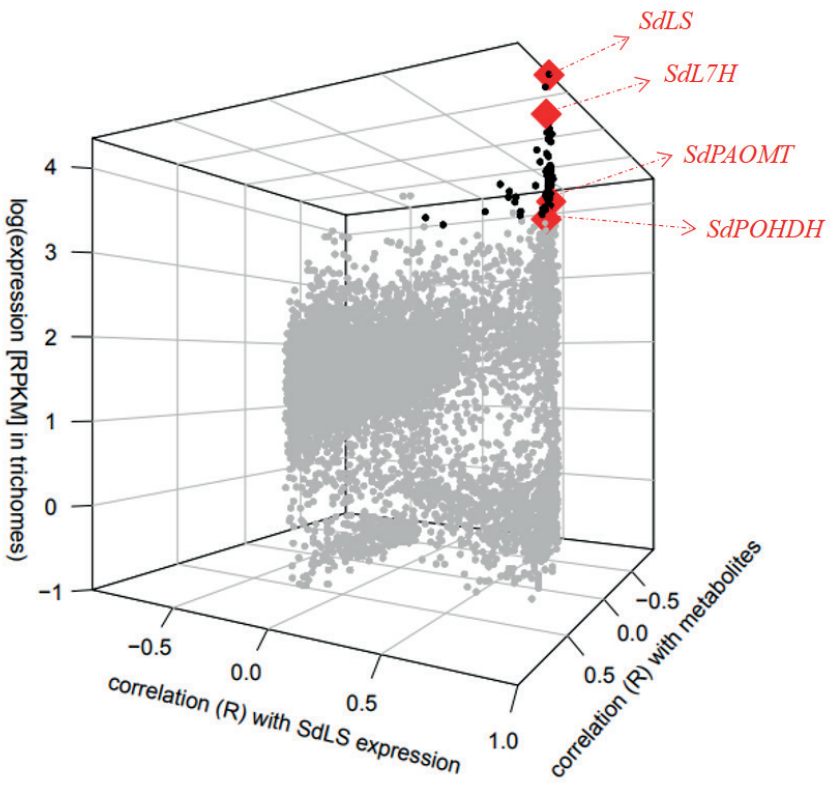


Figure 4 Plot of gene expression patterns in *S. dorisiana* tissues. Expression in trichomes vs. correlation with limonene synthase expression pattern vs. correlation with metabolites presence. Each dot represents a gene candidate. Interesting gene candidates, with high correlation with the monoterpene synthase expression (R>0.6), high correlation with the presence of the pathway metabolites in the tissue (R>0.6), and a high absolute expression in the trichomes (RPKM>1000), were extracted (black dots). Characterized genes that were functional in methylperillate biosynthesis are indicated (red diamonds).

This set of transcripts represents genes encoding enzymes that are active in trichomes and potentially related to monoterpene biosynthesis. In order to see which enzyme classes are represented here, PFAM domains were listed that were significantly enriched in this extracted group of genes, compared to all other genes (Table S3). The resulting PFAM classes include putative terpene synthases, P450s, methyltransferases, dehydrogenases, acetyltransferases, transporters, ATP synthase, NAD(P)H-binding, and some enzyme domains related to fatty acid metabolism.

The set of transcripts was sorted on expression level in the trichomes, in order to select candidates for P450, alcohol dehydrogenase and methyltransferase genes (Figure 1, Table S2).

Characterization of methylperillate biosynthesis enzymes

(1) Bioconversion of methylperillate intermediates in Nicotiana benthamiana

A platform was set-up in *N. benthamiana* to test the activity of the candidate genes. It has been reported that endogenous *N. benthamiana* enzymes may derivatise oxidized monoterpenes to non-volatile glycosides (Dong et al., 2016). To assess if conversion products are formed from the pathway compounds, limonene, perillyl alcohol, perillyl aldehyde and perillic acid were infiltrated in *N. benthamiana,* and leave extracts were analyzed by GC-MS and LC-MS. The analysis shows that limonene is hardly modified by this plant platform, and is emitted into the leave headspace. Other pathway intermediates, including perillyl alcohol, perillyl aldehyde and perillic acid are derivatised to non-volatile products. From these data we selected marker compounds as proxy for the presence of the pathway products. The markers were putatively identified with MS/MS analysis as perillyl alcohol malonyl hexose (POH-M1), perillyl alcohol pentose-hexose (POH-M2), a perillyl aldehyde gluthathione conjugate (PAldH-M1), perillic acid hexose (PA-M1), hydroxylated perillic acid hexose (PA-M2), and perillic acid di-hexose (PA-M3) (Table S4). These markers were used to detect formation of the pathway intermediates in the plant, upon candidate gene expression. From the compound infiltrations it became clear that markers of perillic acid are also detectable upon infiltration of either perillyl aldehyde or perillyl alcohol. This indicates that *N. benthamiana* can convert perillic alcohol and aldehyde to the acid form by endogenous enzymes (Table S5). Interestingly, *N. benthamiana* converts PAldH to PA, but also to POH.

(2) Characterization of limonene synthase

Monoterpene synthase candidate sequences (Fig S5) were cloned. Among these, a monoterpene synthase producing limonene was identified by agro-infiltration in *N. benthamiana*, and analysis of the leaf headspace 5 days after infiltration by GC-MS (Fig

5). For reference, a previously identified limonene synthase from *Perilla frutescens PfLS* (Jongedijk et al., 2015) was infiltrated. Thus, a limonene synthase was identified from *S. dorisiana*, which was called *SdLS*. Five other monoterpene synthase candidates from *Salvia dorisiana* were characterized, including a β-pinene synthase *SdPS*, a linalool synthase *SdLiS*, an α-pinene/camphene synthase *SdPCS*, a sabinene synthase *SdSaS* and a eucalyptol synthase *SdEuS* (Fig S6).

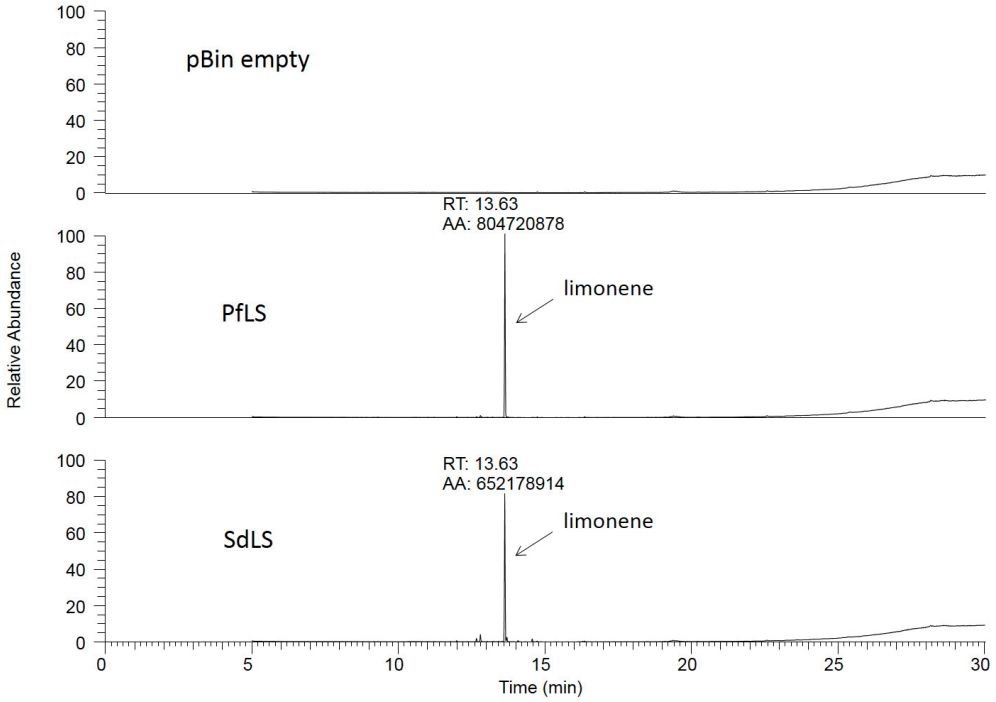


Figure 5 GC-MS TIC chromatograms of *N. benthamiana* headspace after agro-infiltration. pBin empty (empty vector control), PfLS *Perilla frutescens* limonene synthase (positive control), SdLS the newly identified limonene synthase from *Salvia dorisiana*

(3) Characterization of P450

Six cytochrome P450 candidate sequences (Fig. S7) were cloned for characterization by *N. benthamiana* agro-infiltration. A construct expressing *SdLS* was co-infiltrated, to provide the substrate. Two candidate genes produced POH markers in LC-MS analysis. These two genes, *SdCYP71A76v1* and *SdCYP71A76v2*, are nearly identical, except for the two C-terminal amino acids (Fig. S7). *SdCYP71A76v1*, referred to as *SdL7H*, was taken for further characterization (Fig 6). A GC-MS analysis showed that expression of *SdL7H* resulted in a decrease in the amount of limonene in the headspace, indicating that limonene is indeed used as a substrate (Fig 6b). In addition, a small amount of perillyl alcohol was observed in the headspace of the *N. benthamiana* leaves (Fig S8).

Cytochrome P450 enzymes may also convert terpene alcohols to the aldehyde and acid forms (Teoh et al., 2006). In order to see if *SdL7H* can further oxidize POH to PAldH and PA, LC-MS analyses of *SdLS*+*SdL7H* infiltrations were inspected for markers of the presence of PAldH and PA. Since POH can be converted to PAldH and PA by *N. benthamiana* (Table S5), the ratio of POH markers to PAldH and PA markers was determined for *SdLS*+*SdL7H* infiltration, and compared to ratios obtained for experiments where the POH compound was infiltrated without heterologous enzymes. Ratios in *SdLS*+*SdL7H* did not differ from those of the POH infiltrations (Table S6), indicating that SdL7H does not convert perillyl alcohol further to perillyl aldehyde or perillic acid *in planta*.

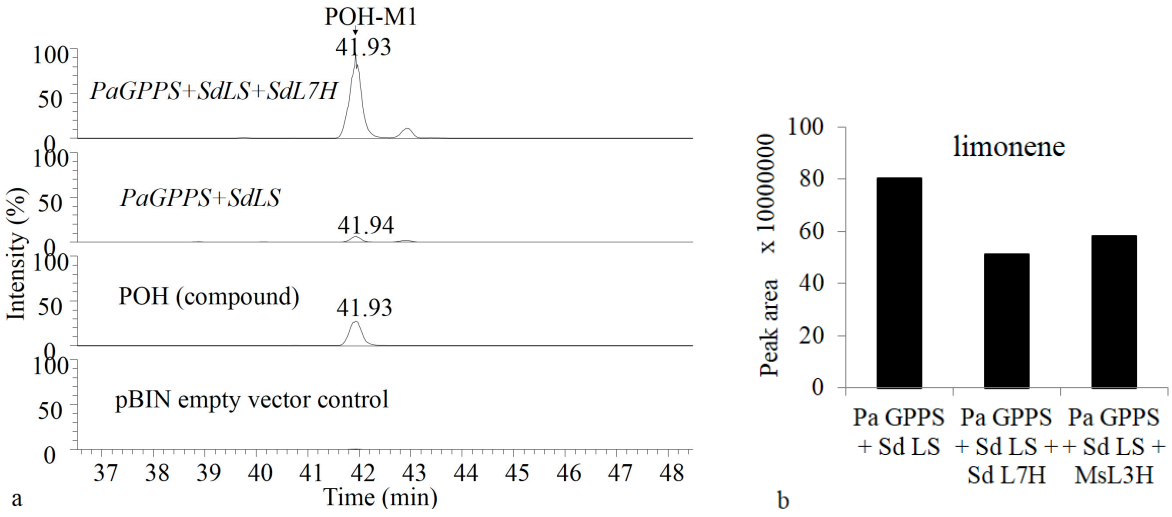


Figure 6 Characterization of *SdL7H*. a. LC-MS chromatograms of *N. benthamiana* agro-infiltrated leaves. *m/z* 355.1760-355.1770 (perillyl alcohol marker POH-M1 at RT 41.93) FTMS - c ESI Full ms [95.00-1300.00] MS NL: 100%= 4.38E6 b. GC-MS peak area of limonene peak in *N. benthamiana* leaf headspace after agro-infiltrations. *SdLS: Salvia dorisiana* limonene synthase, *SdL7H: S. dorisiana* limonene 7-hydroxylase, MsL3H *Mentha spicata* limonene 3-dehydrogenase (isopiperitenol synthase).

(4) Characterization of alcohol dehydrogenase

Five alcohol dehydrogenase sequences (Fig. S9) were cloned and tested by agro-infiltration in *N. benthamiana*, for their ability to convert co-infiltrated POH to PaldH. One dehydrogenase enzyme, referred to as *SdPOHDH* converted perillyl alcohol to perillyl aldehyde as demonstrated by LC-MS analysis of POH- and PAldH-markers (Fig. 7). To investigate whether *SdPOHDH* can also further convert PAldH to PA, the

PA markers were also quantified and compared with their production when PaldH was infiltrated (Table S7). No changes in the ratio between PaldH and PA markers was observed (Table S7), indicating that *SdPOHDH* does not convert PAldH to PA *in planta.*

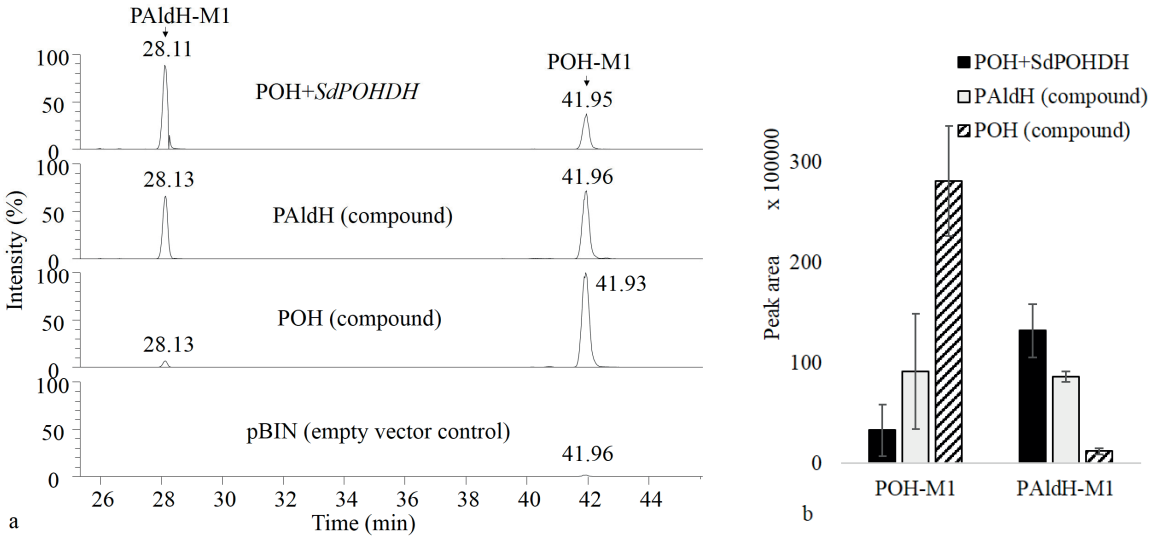


Figure 7 Characterization of POHDH activity. a. LC-MS chromatograms of *N. benthamiana* agro-infiltrated leaves. *m/z* range 355.1760-355.1770 + 458.1960-458.1970 (perillyl aldehyde marker PAldH-M1 at RT 28.11; perillyl alcohol marker POH-M1 at RT 41.93) FTMS - c ESI Full ms [95.00-1300.00] MS NL: 100%= 1.20E6 b. Quantification of peak areas of POH and PAldH markers POH-M1 and PAldH-M1 from chromatograms. POH perillyl alcohol, PAldH perillyl aldehyde, pBIN empty vector control, *SdPOHDH Salvia dorisiana* alcohol dehydrogenase.

(5) Characterization of methyltransferase

Seven methyltransferase (OMT) sequences (Fig. S10) were cloned for characterization *in planta*. and co-infiltrated in *N. benthamiana* with perillic acid. For a candidate gene which was referred to as *SdPAOMT*, methylperillate was detected in the headspace of the agroinfiltrated leaves by GC-MS analysis (Fig 8).

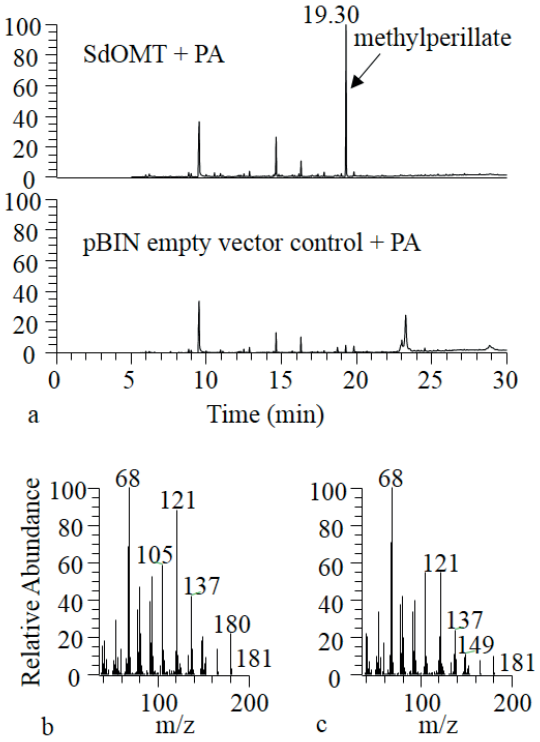


Figure 8 SdPAOMT produces methylperillate *in planta* a. GC-MS chromatograms of *N. benthamiana* leaves headspace. TIC MS 100% = 2.28E9 b. Mass spectrum of SdOMT product, +c Full ms [35.00-400.00], 100% = 2.46E7 c. Library mass spectrum of methylperillate

Reconstitution of the methylperillate biosynthesis pathway in *N. benthamiana*

To establish methylperillate production in *N. benthamiana*, the full methylperillate biosynthesis pathway (Fig 1) was transiently expressed through agro-infiltration. The role of the genes was addressed by combining *Agrobacterium* strains containing the newly characterized genes from *Salvia dorisiana.* Strains were co-infiltrated, each time adding a subsequent enzyme, while the gene load for each of the genes was kept equal. While *N. benthamiana* can provide the necessary precursors from the MEP pathway, it is known that co-expression of an additional ectopic GPP synthase can boost production of monoterpenes in *N. benthamiana* (Dong et al., 2016). Accordingly, co-expression of *Picea abies* GPP synthase (PaGPPS) increased limonene emission by 3-5 times (Fig. S11). Addition of *SdL7H* resulted in the production of perilla alcohol, as observed from the appearance of peaks POH-M1 and POH-M2 in an LC-MS analysis (compare Fig. 9 row 2 to row 3). Subsequent additional expression of *SdPOHDH* strongly decreased the peak area of these products, and resulted in the appearance of PAldH-M1 and PA-M1-

3 (Fig. 9 row 4). GC-MS analysis showed that a significant amount of perilla aldehyde was emitted (Fig. S12). Interestingly, also a small amount of methylperillate was observed in the headspace (Fig. 9 row 4), indicating that *N. benthamiana* expresses an endogenous methyltransferase that can methylate perillic acid. When in addition *SdPAOMT* was co-expressed, the methylperillate emission strongly increased, while the POH-M1-2, PAldH-M1 and PA-M1-3 peaks were strongly reduced (Fig. 9, row 5; Fig. S13). When *SdPAOMT* was co-expressed with *PaGPPS*, *SdLS* and *SdL7H*, but leaving *SdPOHDH* out, methylperillate emission was 6-fold lower (Fig. S13), indicating the importance of *SdPOHDH* for the pathway.

Thus, *de novo* biosynthesis of methylperillate was achieved in *N. benthamiana* by expressing *PaGPPS*, *SdLS*, *SdL7H* and *SdOMT* (Fig. 9).

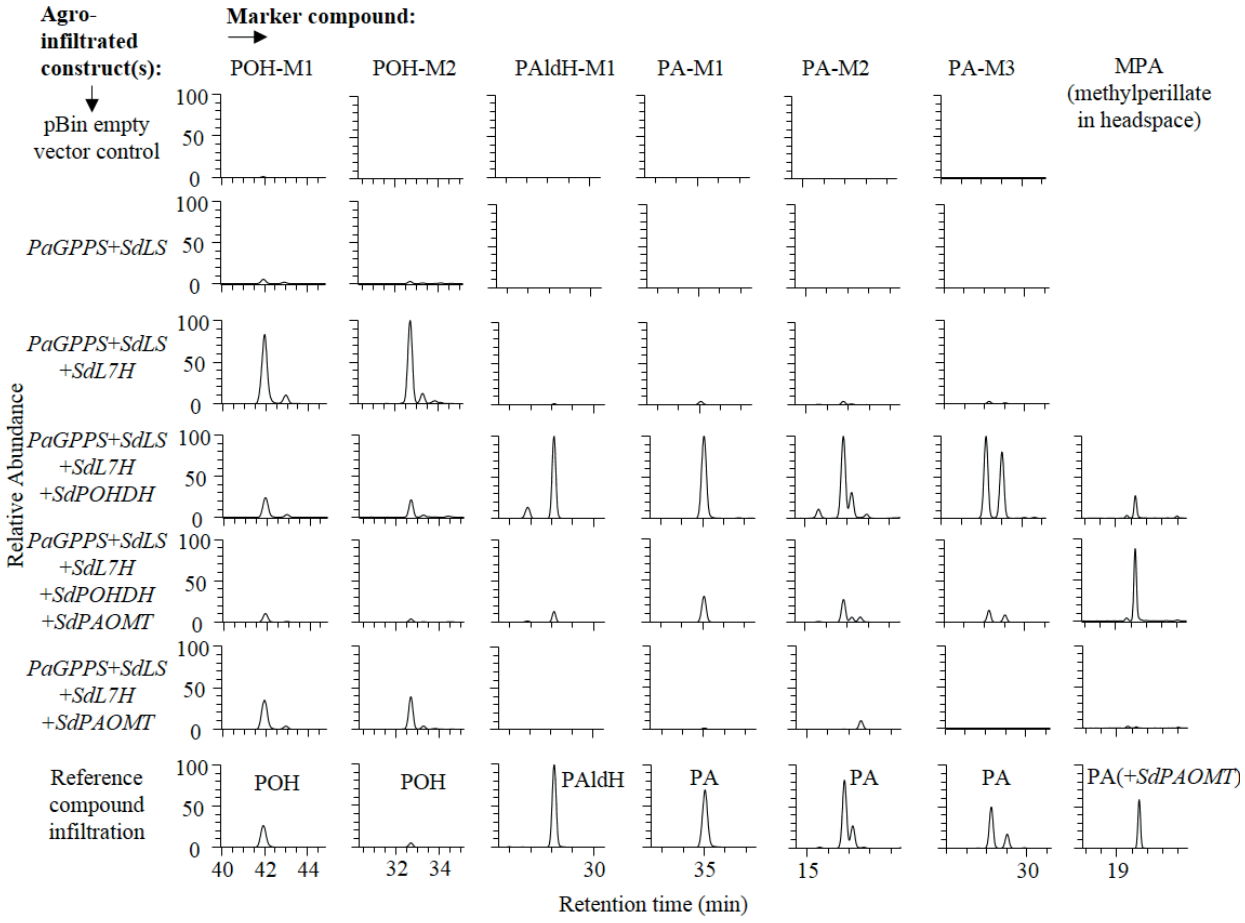


Figure 9 Reconstitution of the methylperillate biosynthesis pathway in *N. benthamiana.* LC-MS chromatograms of marker compounds and GC-MS chromatograms of methylperillate production in leave headspace. Reference compound infiltrations were perillyl alcohol (POH-M1 and M2), perillyl aldehyde (PAldH-M1), perillic acid (PA-M1, M2 and M3) or methylperillate (MPA). Chromatogram characteristics: POH-M1 all NL 4.20E6, *m/z* 355.1760-355.1770. POH-M2 all NL 1.22E6, *m/z* 491.2130-491.2150. PAldH-M1 all but reference compound infiltration NL 6.86E4, reference compound infiltration 6.86E5, all *m/z* 458.1950-458.1980. PA-M1 all but reference compound infiltration NL 1.11E6, reference compound infiltration 6.08E6, all *m/z* 373.1500-373.1530. PA-M2 all NL 4.67E5, *m/z* 389.1450-389.1470. PA-M3 all NL 6.98E5, *m/z* 535.2030-535.2050. Methylperillate all but reference compound infiltration NL 1.20E6, reference compound infiltration 5.23E7, all *m/z*=180-181 MS.

Further characterization of *SdPAOMT*

The protein sequence of *SdPAOMT* was analysed by BLASTP analysis, and was found to be most homologous to methyltransferases from the SABATH family. These proteins generally encode enzymes that are involved in methylation of carboxyl groups of aromatic compounds, but also enzymes acting on nitrogen groups (D'Auria et al., 2003). The most closely related homologues of *SdPAOMT* include a number of enzymes that are involved in methylation of benzoic acid and/or salicylic acid (Fig. 10), for example *AmSAMT*, a gene from snapdragon involved in methylation of salicylic acid in vegetative tissues (Negre et al., 2002).

The ability of SdPAOMT to methylate salicylic acid, benzoic acid and perillic acid was tested in vitro. To this end, the *SdPAOMT* cDNA was subcloned in expression pCDF DUET-1, and SdPAOMT protein was produced in *E. coli* BL21 DE3. Crude extracts of sonicated transformed cells after IPTG induction were tested with benzoic acid, salicylic acid, and perillic acid, and S-adenosyl methionine as methyl-donor. Methylated products were detected for all three substrates (Fig. 11). Methylation of jasmonic acid, on the other hand, could not be observed. This indicated that SdPAOMT is able to methylate both phenolic and monoterpenoid carboxylic acids.

To further investigate the activity of *SdPAOMT in planta*, *N. benthamiana* leaves were infiltrated with *SdPAOMT* or pBIN. After 5 days, PA was infiltrated, and, after incubation for 3h the headspace was trapped for 3h and analysed by GC-MS. Interestingly, infiltration of *SdPAOMT* resulted in emission of well detectable amounts of both MeSA and MeBA, alongside with methylperillate (Fig. S12). This confirms that SdPAOMT methylates both phenolic acids and monoterpene acids, also *in planta*. In the headspace of *Salvia dorisiana* leaves on the other hand, only traces of MeSA were observed, and no MeBA could be detected (Fig. S12), while methylperillate was very dominantly present.

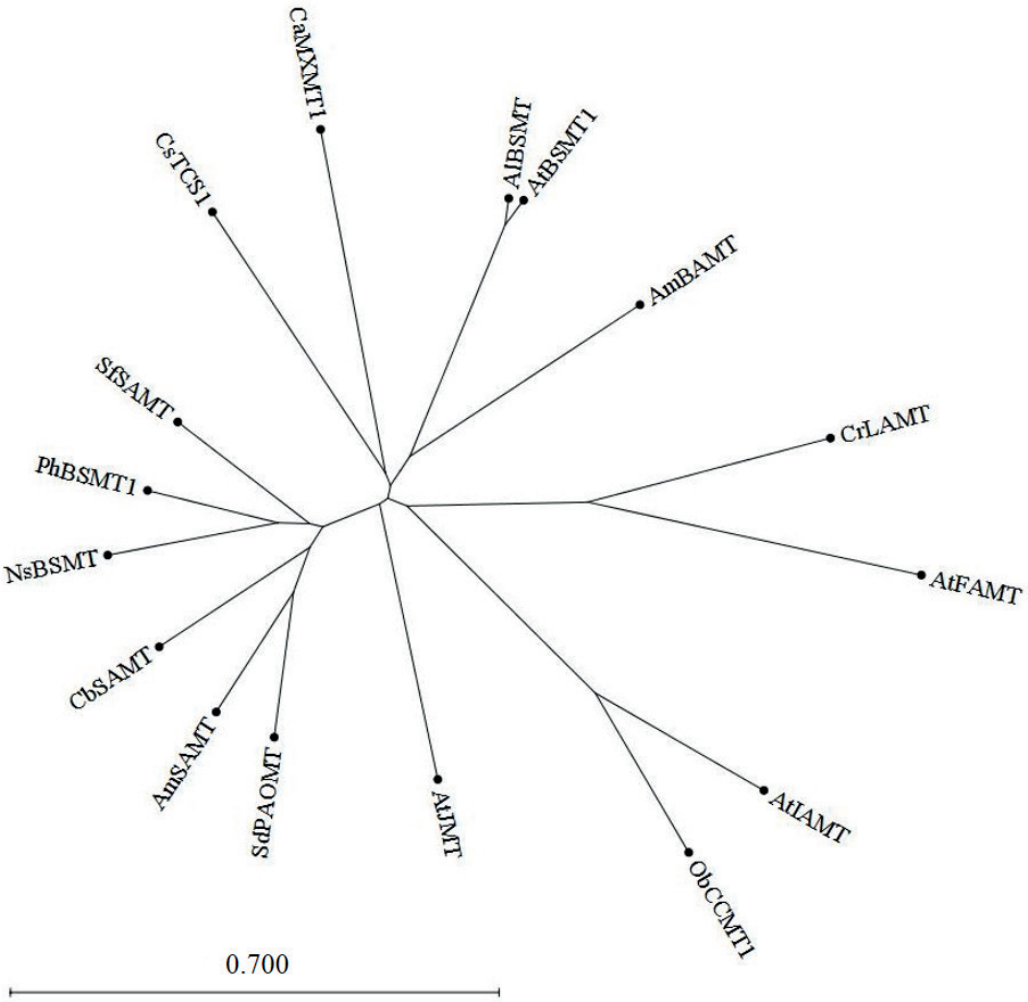
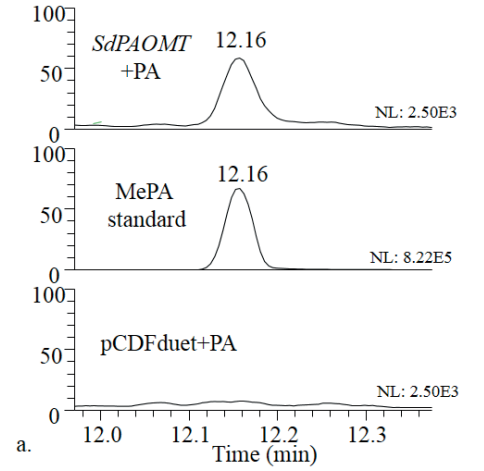
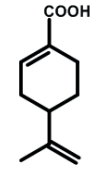
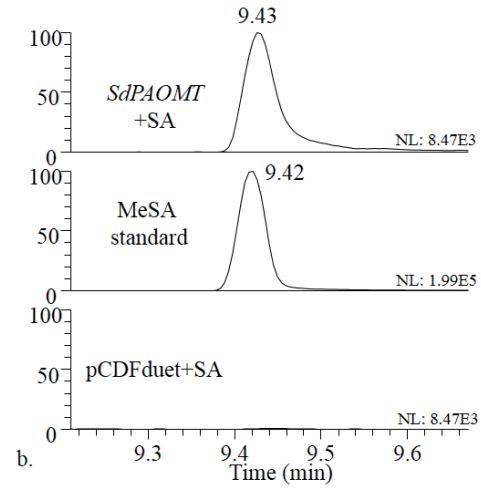


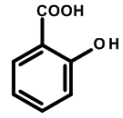
Figure 10 Phylogenetic tree of O-methyltransferase sequences from SABATH family. The newly identified SdPAOMT is from SABATH family, it is in a branch with SA methyltransferases. CrLAMT *Catharanthus roseus* loganic acid methyltransferase, ObCCMT *Ocimum basilicum* cinnamate carboxyl methyltransferase, SfSAMT *Stephanotis floribunda* salicylic acid carboxyl methyltransferase (Pott et al., 2002), AtIAMT *Arabidopsis thaliana* indole-3-acetic acid carboxyl methyltransferase (Zubieta et al., 2003), AtFAMT *Arabidopsis thaliana* Farnesoic acid methyl transferase (Yang et al., 2006), PhBSMT *Petunia hybrida* benzoic acid/salicylic acid carboxyl methyltransferase (Underwood et al., 2005), CbSAMT *Clarkia breweri* salicylic acid methyl transferase (Ross et al., 1999), CaMXMT1 *Coffea arabica* 7-methylxanthine methyltransferase (Ogawa et al., 2001), NsBSMT *Nicotiana suaveolens* benzoic acid/salicylic acid carboxyl methyltransferase (Roeder et al., 2009), AmSAMT *Antirrhinum majus* salicylic acid carboxyl methyltransferase (Negre et al., 2002), AtJMT *Arabidopsis thaliana* jasmonic acid carboxyl methyltransferase (Seo et al., 2001), AmBAMT *Antirrhinum majus* benzoic acid carboxyl methyltransferase (Dudareva et al., 2000), AtBSMT *Arabidopsis thaliana* benzoic acid/salicylic acid carboxyl methyltransferase (Chen et al., 2003), AlBSMT *Arabidopsis lyrata* benzoic acid/salicylic acid carboxyl methyltransferase (Chen et al., 2003), CsTCS1 *Camellia sinensis* caffeine synthase (Kato et al., 2000). Reference methyltransferase sequences were adapted from (Kapteyn et al., 2007).



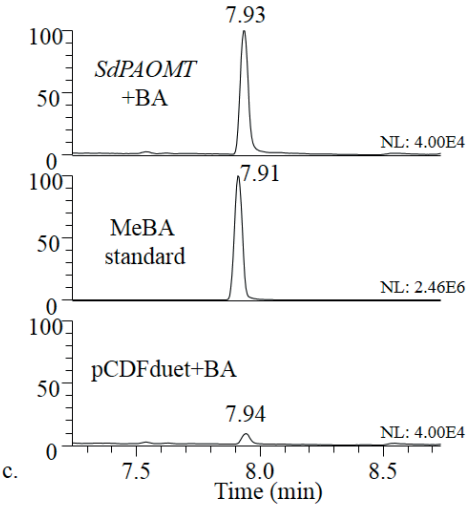


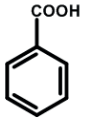
perillic acid (PA)





salicylic acid (SA)





benzoic acid (BA)

Figure 11 GC-MS chromatograms of in-vitro assays *Salvia dorisiana* O-methyltransferase SdOMT with different substrates a. *SdOMT* + perillic acid (PA), *m/z* = 67.50-68.50+120.50-121.50+179.50-180.50 MS b. *SdOMT* + salicylic acid (SA), *m/z* = 151.50-152.50 MS c. *SdOMT* + benzoic acid (BA), *m/z* 76.50-77.50+104.50-105.50+135.50-136.50 MS. pCDFduet empty vector control.

Discussion

In this work, enzymes have been identified that are needed to convert the hardly functionalized monoterpene limonene to methylperillate, which is a compound that can be converted to the commodity chemical terephthalic acid by mild chemistry (Chapter 4). To this end, the biosynthetic pathway towards methylperillate was unravelled in the plant *Salvia dorisiana*. Genes encoding four enzymes from the methylperillate pathway were identified. Apart from well-known terpene synthesizing enzymes such as a terpene synthase, a cytochrome P450 and an alcohol dehydrogenase, a member of an exceptional class of terpene modifying enzymes was shown to be involved, a terpene carboxylic acid O-methyltransferase. The identified genes were used to reconstitute the methylperillate pathway in *N. benthamiana*, demonstrating the ability to produce methylperillate and its precursors. Thus, this work provides a basis for a novel way to produce biomaterials from natural, renewable sources.

SdL7H is an enzyme with novel regio specificity, essential for methylperillate formation

Methylperillate carries an unusual functional group for a monoterpene, a methylated carboxyl group at the para-position, which is essential for its use as a precursor for TA (Chapter 4). Quite a number of plant limonene synthases have been reported (Jongedijk et al., 2016), and we identified a limonene synthase in *S. dorisiana* as a first step towards the identification of the methylperillate pathway. To use limonene as a direct precursor for TA, an enzyme that modifies limonene at its C7 position is essential. The first dedicated enzyme discovered in this work leading to methylperillate is SdL7H, a cytochrome P450 that is capable of functionalizing limonene at the 7 position. After hydroxylation of the 7-position by SdL7H, the resulting primary alcohol group is available for further oxidation to the acid. This type of oxidation has been described for sesquiterpenes like artemisinic alcohol, to form artemisinic acid, and germacrene A alcohol, to form germacrenic acid (Ro et al., 2006; Liu et al., 2011). Several P450s acting on limonene or derived compounds have been identified in *Mentha* species (Bertea et al., 2003; Lucker et al., 2004). However, the secondary alcohols formed by mint P450s at the 3- and 6- position of limonene cannot be taken further towards carboxylgroups, and therefore these enzymes are not applicable for generating a polymer precursor like TA. From *Perilla frutescens*, a cytochrome P450 has been described, that performs a mix of hydroxylations on the C3, 6 and 7 (Mau et al 2010), which is likely involved in the formation of perilla aldehyde in this species, but lacks specificity which would be needed for application in an efficient heterologous production system. The here characterized *SdL7H* hydroxylase is very regiospecific. Like the Perilla and Mentha genes, and all other monoterpene P450s known to date, *SdL7H* is a member of the CYP71 class, but shares very limited sequence similarity to other P450s known to act on limonene (e.g. *Mentha spicata* L6H, 37% identity, *Mentha spicata* L3H, 35%

identity; *Perilla frutescens* L367H, 40% identity). *SdL7H* shares most sequence similarity (69% identity) with menthofuran synthase, a P450 from *Mentha x piperita* (Bertea et al., 2001). Menthofuran synthase acts on the alyllic group of pulegone. Though this enzyme is not active on limonene, it is notable that it does act on a non-ring position of a monoterpene and forms initially a primary alcohol group. This could indicate that a preference for forming primary alcohols may have been the property of the common ancestor of these enzymes.

Perillyl alcohol oxidations towards perillic acid

Using *Salvia* enzymes, de novo production of MPA was established in *N. benthamiana* (Fig. 9). *N. benthamiana* appears to have endogenous POHDH-, PAldHDH- and PAOMT-like activities, albeit at a low level. Therefore, it makes sense to apply specialized enzymes, recruited from *S. dorisiana* for these conversions. This is clearly seen when *SdPOHDH* is added to the system, by which MPA production is enhanced 6-fold. For conversion of perilla aldehyde to PA, no enzyme has yet been isolated from *S. dorisiana*. Several different enzyme classes have been reported to catalyse terpene aldehyde oxidation, such as cytochrome P450 enzymes. For example, the formation of deoxyloganetic acid from iridodial in *C. roseus* is performed by iridoid oxidase, which is a P450 (CYP76A26) (Miettinen et al., 2014). On the other hand, artemisinic aldehyde is converted efficiently to artemisinic acid by a member of the aldehyde dehydrogenase family from *A. annua* (Teoh et al., 2009). Both these enzyme classes were over-represented in the subset of genes from which methylperillate pathway genes were isolated (Table S3). Formation of monoterpene acids has not yet been extensively investigated. In other plants like pyrethrum (*T. cinerariifolium*) and *C. roseus*, acid forms of monoterpenes play a role in transport between cells. However, in *S. dorisiana*, the free acid form could not be detected. Apparently *S. dorisiana* prevents perillic acid to be present in the free form by methylating it efficiently in the trichomes.

Formation of a monoterpene methylester

The methyltransferase *SdPAOMNT* is as far as we know the first terpene methyltransferase discovered. One could speculate that methylation of PA has a biological function in *S. dorisiana*. The enzyme responsible for methylation, SdPAOMT, has high similarity to methyltransferases from the SABATH family, that have been found to act on phenolic compounds such as BA and SA. MeBA plays a role in the fragrance of flowers, for instance in *Clarkia brewerii*. There, the formation of MeBA was suggested to function in the attraction of bumblebees for pollination (Kolosova et al., 2001). Similarly, *SdPAOMT* may play a role in the formation of highly scented compounds such as methylperillate in the foliage of *S. dorisiana*. Methylation of SA was suggested to play a role in the modulation of the activity of SA, which

functions as a defence signal. Methylation of SA causes inactivation and facilitates a more effective long distance transport through the plant due to a higher volatility and membrane permeability (Dempsey et al., 2011). In fact we show that, when fed with BA and SA, SdPAOMT can function as BA-OMT and SA-OMT, respectively. In *Salvia* trichomes, where *SdPAOMT* is extremely highly expressed, availability of these substrates is limited, because trichomes are fully geared towards synthesizing PA. Therefore, one could consider *SdPAOMT* as a neo-functionalised gene that has been recruited from pathways leading to phenolic volatiles, to participate in production of monoterpene methylesters.

SdPAOMT can play an important role in the engineering of monoterpene carboxylic acids in plants. When PA is produced in *N. benthamiana*, a number of glycosides are produced (Table S5). Glycosylation likely leads to detoxification of these compounds, and their sequestration in storage organs such as vacuoles. In *P. frutescens*, PA is known to be stored as glycoside. *SdPAOMT* expression decreases the formation of glycosides in *N. benthamiana*, indicating that *SdPAOMT* efficiently competes with glycosyl transferases from *N. benthamiana*. It remains unclear in what form acid methylation influences toxicity and defence properties of these terpenes.

Outlook

The next step for producing biobased commodity chemicals through this route is to produce methylperillate on a larger scale. This could possibly be achieved by expressing the methylperillate biosynthetic enzymes in microbes. Yeast strains have been reported that produce limonene *de novo*. In general, plant P450s can be expressed rather well in yeast (Arendt et al., 2017), so this could provide a suitable platform for further engineering towards methylperillate production. In *Pseudomonas*, a bacterium with high solvent tolerance, already reasonable amounts of perillic acid were produced by bioconversion of limonene, by means of side-activity of the p-cymene degradation gene cluster (Mars et al., 2001). By elucidating pathways towards methylperillate and other functionalized monoterpenes, engineering of these compounds in microorganisms should now be possible. From this perspective, the elucidation of the pathway is the first step towards biobased production of commodity chemicals. Thus, this work provides a basis for a novel way to produce biomaterials from natural, renewable sources.

Supplemental files to this chapter can be downloaded from:
http://www.wageningenseedlab.nl/thesis/ejongedijk/SI/

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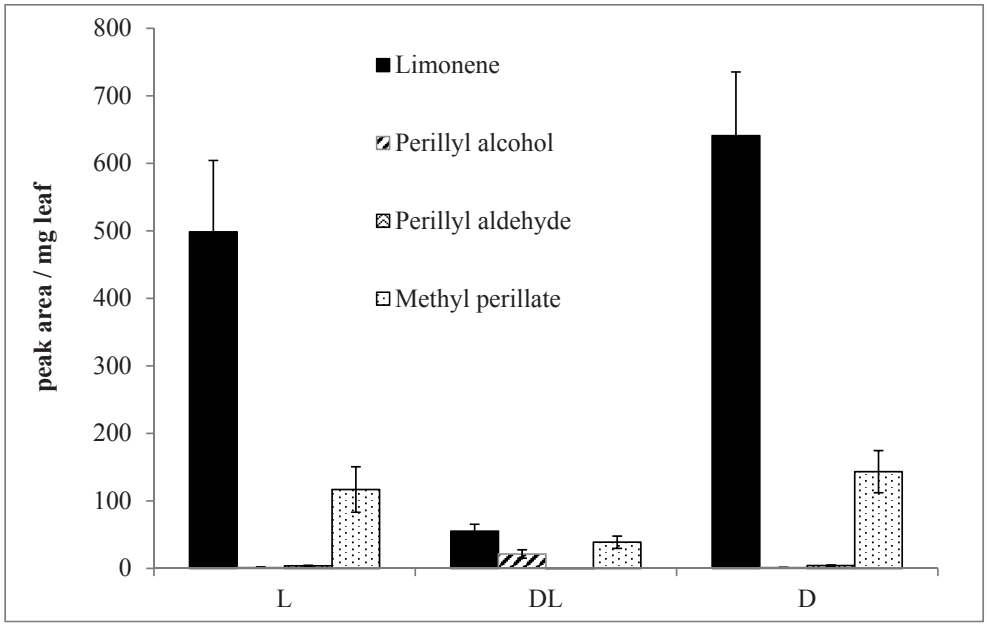
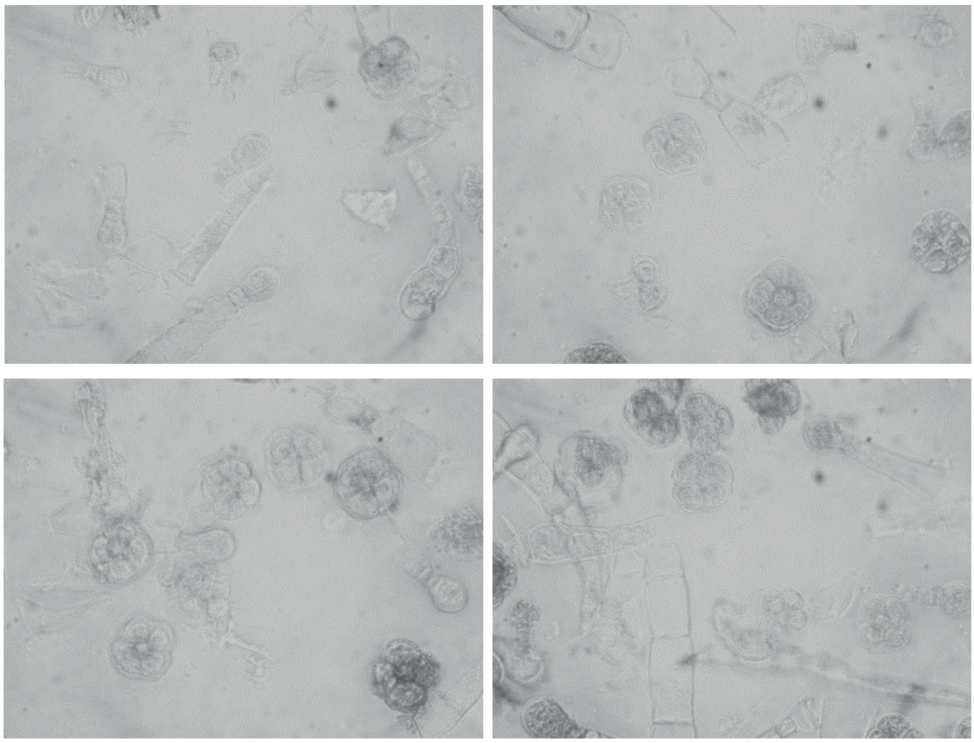
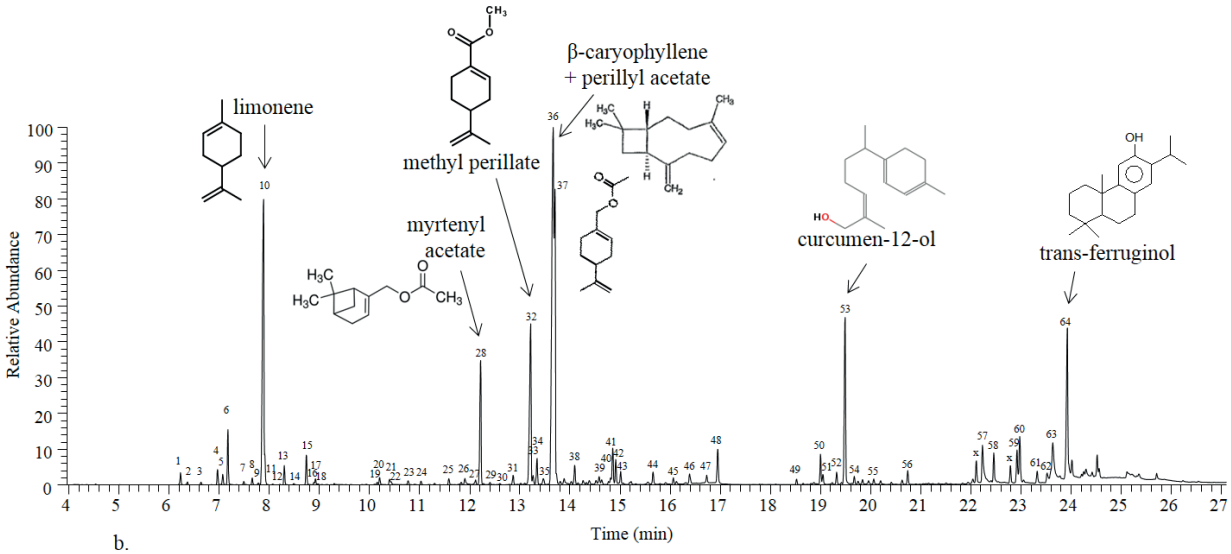
Supplemental Figure S1: Chloroform dip. Bar graph of integrated peak areas from GC-MS. All measured methylperillate related monoterpenes are enriched in the trichomes. L intact leaves, DL leaves after dipping, D chloroform dip.



Supplemental Figure S2 a. Isolated *S. dorisiana* trichomes, b. GC-MS chromatogram of isolated trichomes TIC NL 2.90E7. They contain the volatile methylperillate pathway compounds



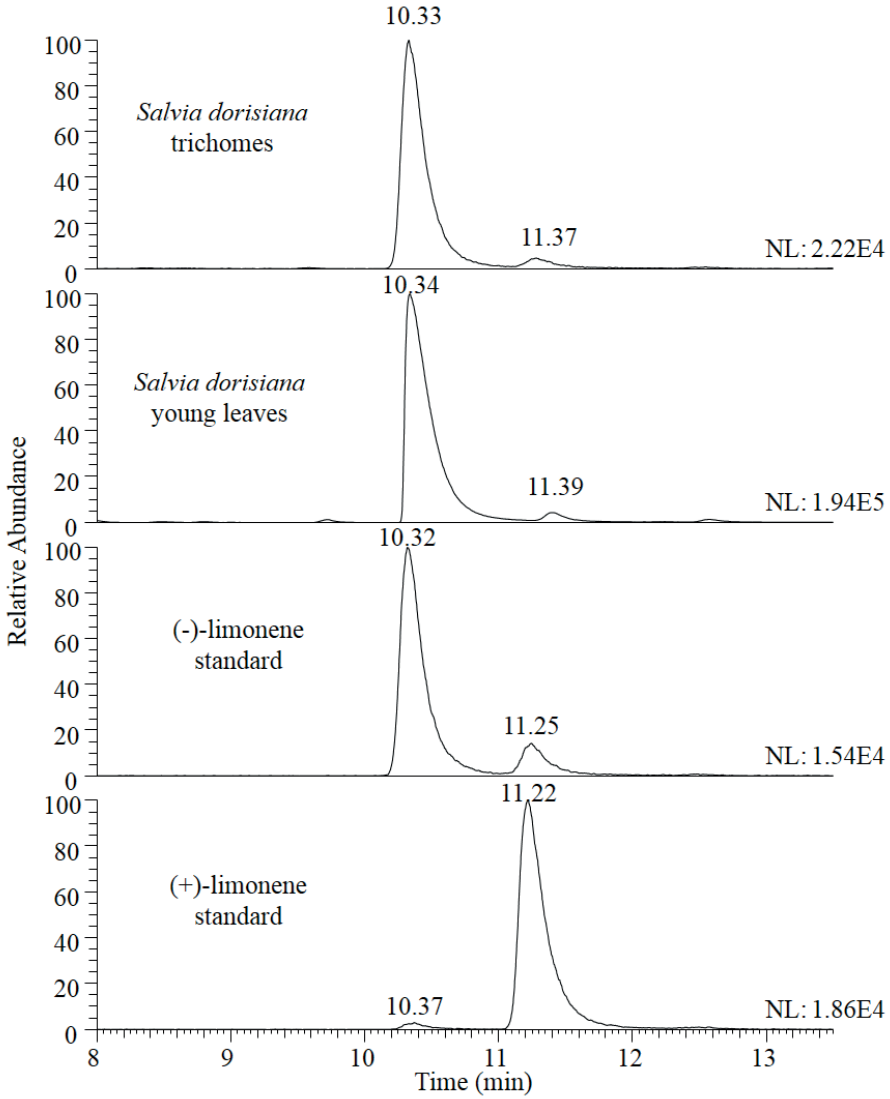
a.



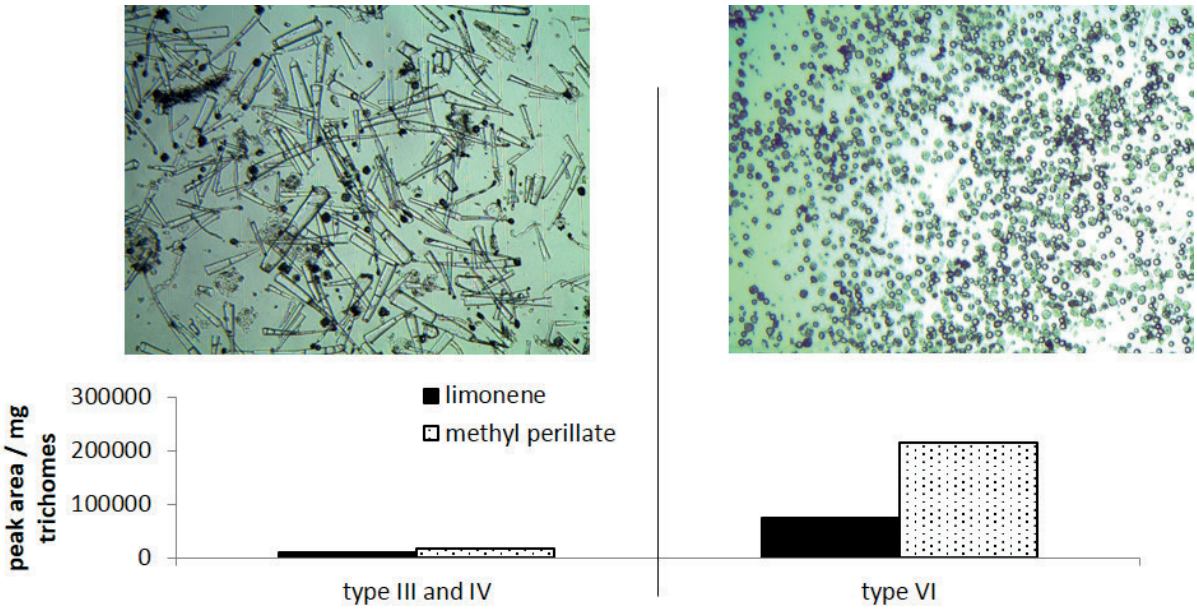
b.

1 α-thujene, 2 α-pinene, 3 camphene, 4 sabinene, 5 β-pinene, 6 myrcene, 7 α-phellandrene, 8 α-terpinene, 9 p-mentha-1,3,8-triene, 10 limonene, 11 trans-B-ocimene, 12 2-methylbutyl butanoate, 13 y-terpinene, 14 trans-4-thujanol, 15 terpinolene, 16 linalool, 17 butanoic acid, 2-methyl-, 2-methylbutyl ester, 18 cis-4-thujanol, 19 isopinocamphone, 20 terpinen-4-ol (internal standard), 21 a-terpineol, 22 8,9-epoxy limonene, 23 B-cyclocitral, 24 p-mentha-1,8-dien-10-ol, 25 perillyl aldehyde, 26 perillyl alcohol, 27 methylgeranate, 28 myrtenyl acetate, 29 bicycloelemene, 30 a-copaene, 31 geraniol acetate, 32 methyl perillate, 33 3-carene, 34 limonene-10-ol, 35 a-gurjunene, 36 B-caryophyllene, 37 perillyl acetate, 38 a-humulene, 39 dicyclogermacrene, 40 d-amorphene, 41 a-thujopsan-2-ol, 42 farnesol, 43 a-bisabolene, 44 caryophyllene oxide, 45 limonene-10-ol, 46 a-longipinene, 47 a-bisabolol, 48 6-A-hydroxy-germacra-1(10),4-dienepropanolyl-1H-inene ate, 49 1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-, 50 trans-bergamota-2,12-dien-14-ol, (E)-, 51 1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-, 52 9(11),15-isopimaradiene, 53 Z-y-curcumen-12-ol, 54 1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-, 55 thujopsene, 56 16-kaurene, 57 sclareol, 58 4,5,6,7-Tetrahydroxy-1,8,8,9-tetramethyl-8,9-dihydrophenaleno[1,2-b]furan-3-one, 59 Podocarpa-1,8,11,13-tetraen-3-one, 14-isopropyl-1,13-dimethoxy-, 60 cis-ferruginol, 61 retinoic acid, 62 carnosol, 63 carnosic acid, 64 trans-ferruginol, x background (column material)

Supplemental Figure S3 GC-MS chromatograms on enantioselective column. *Salvia dorisiana* trichomes and young leaves contain exclusively the (-)-limonene enantiomer. *m/z* 68-69+136-137. RT 10.32-10.37 (-)-limonene, RT 11.22-11.25 (+)-limonene, RT 11.37-11.39 phellandrene peak visible in *Salvia dorisiana* chromatograms. In both (-)- and (+)-limonene standards a minor contamination from the other enantiomer is visible.

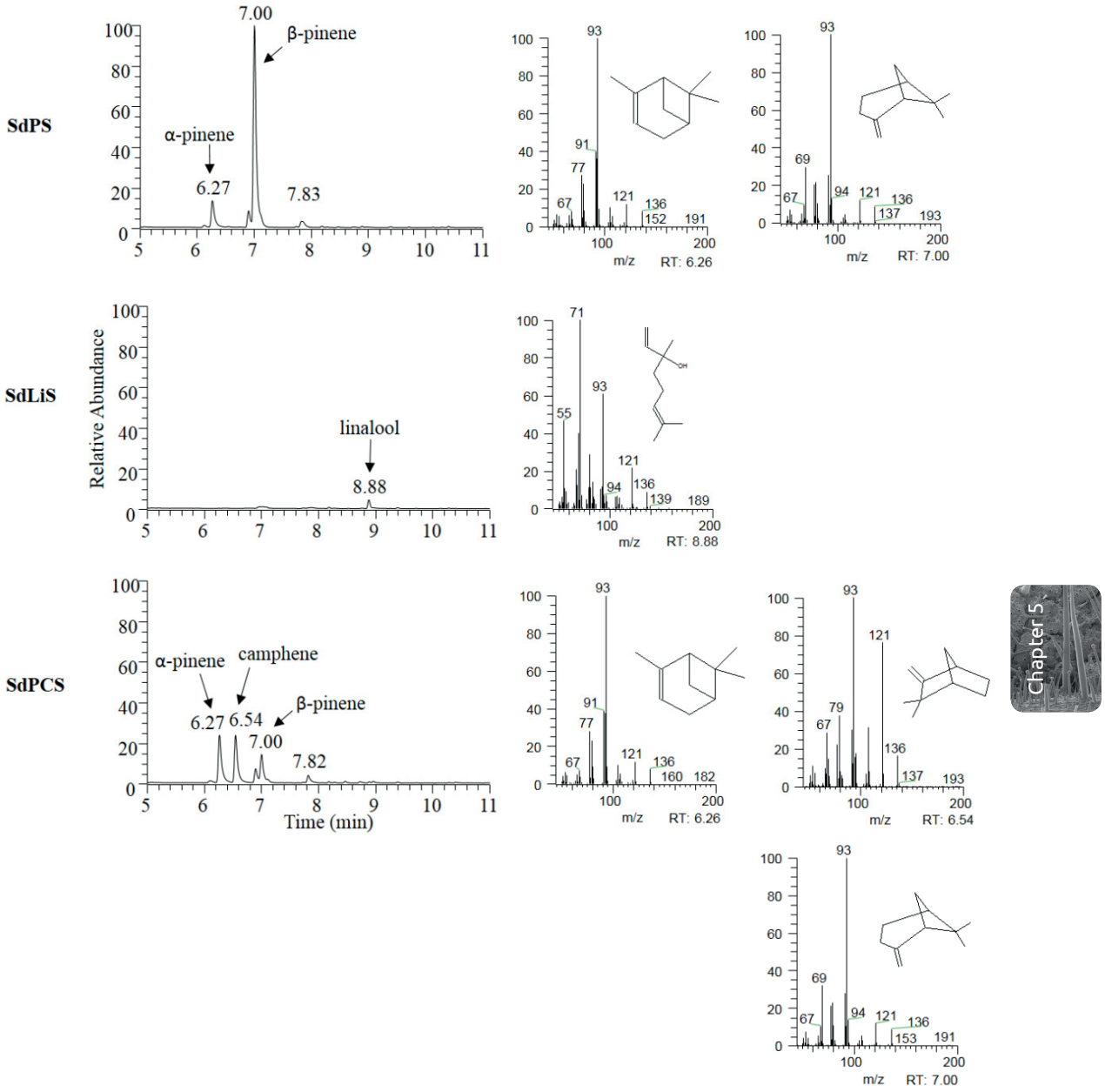


Supplemental Figure S4 Separated *Salvia dorisiana* trichome fractions

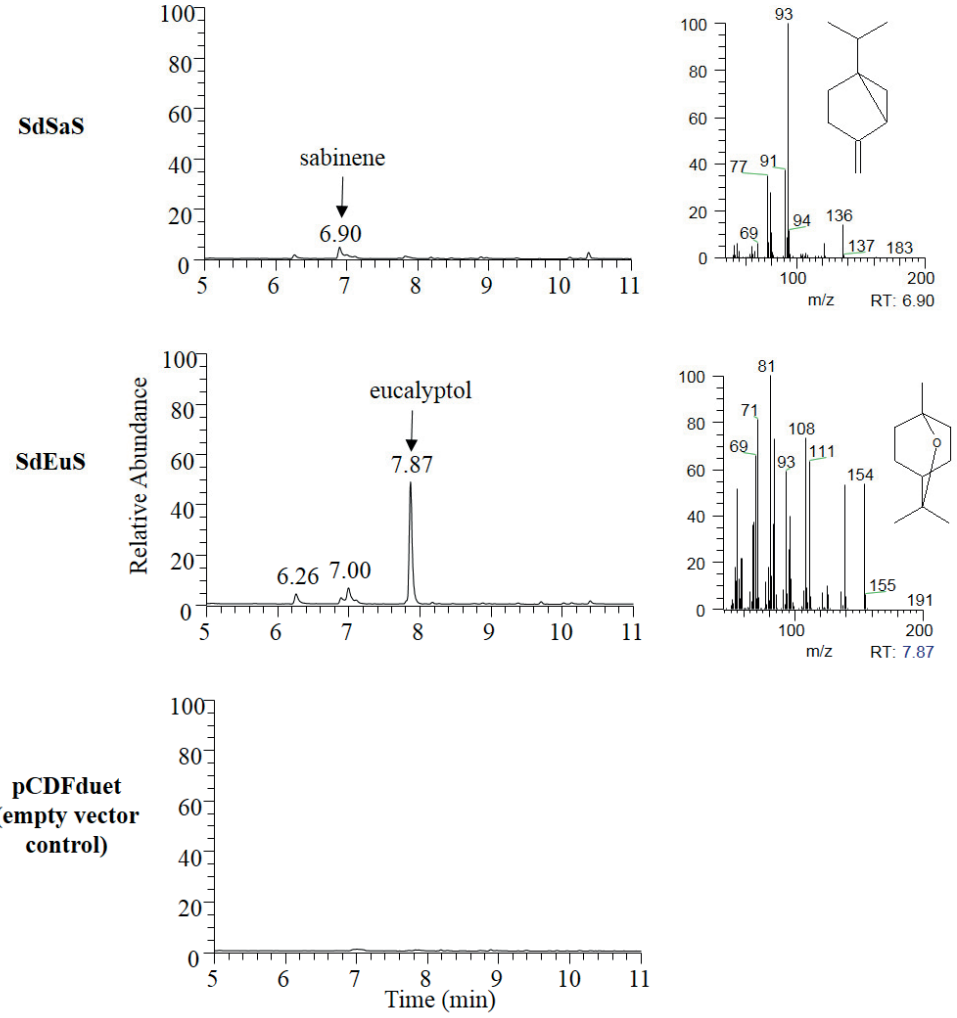


Fresh *Salvia dorisiana* leaves of intermediate size were harvested (1-10 cm), and trichomes were isolated as described previously (Sallets et al. 2014), with following modifications: cutted leaves were shaken for 20 min 115 rpm. Trichomes were separated on size on different meshes, and flushed with washing buffer (Sallets et al. 2014): 350 um, 100 um, washed (100 um fraction was harvested: "type III and IV fraction"), 75 um and 45 um, and washed. The 45 um fraction was further separated by Percoll layers 10-30-60-80%, trichomes were harvested from the 30-60% interface and loaded on a 10-60% continuous sucrose gradient, trichome layer was visible in the light and harvested "type VI fraction".

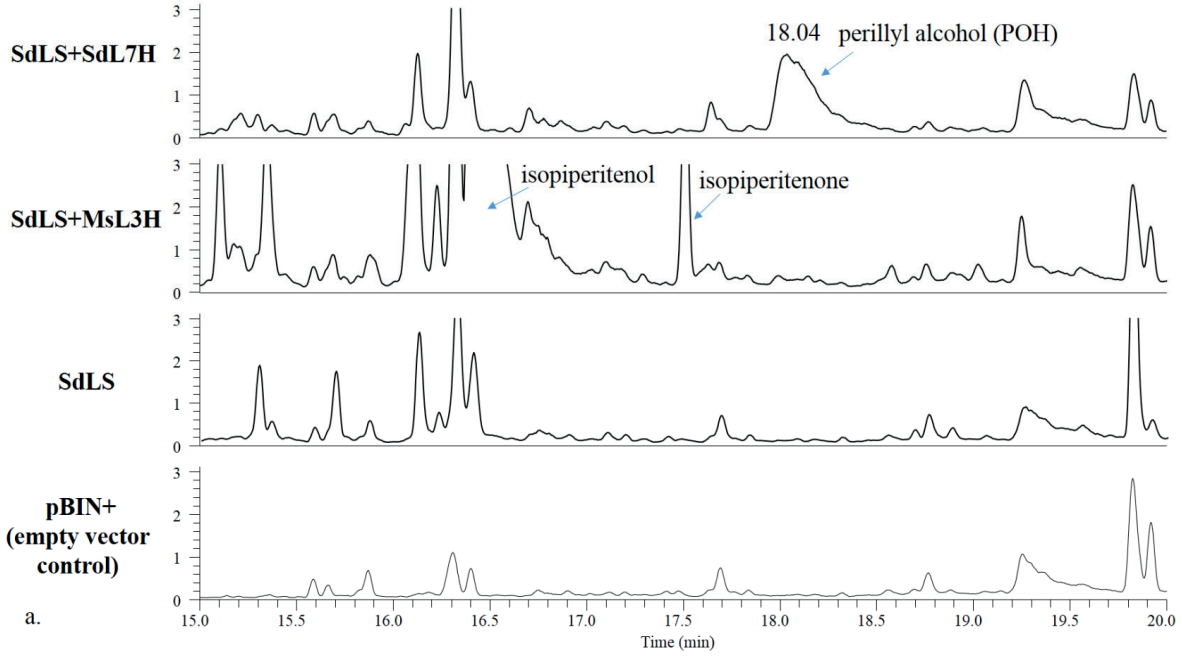
Supplemental Figure S6 In-vitro assay of other *Salvia dorisiana* monoterpene synthases, GC-MS chromatograms. SdPS β-pinene synthase, SdLiS linalool synthase, SdPCS pinene/camphene synthase, SdSaS sabinene synthase, SdEuS eucalyptol synthase, pCDFduet empty vector control. Mass spectra and structures of indicated peaks are depicted on the right of each GC-chromatogram.

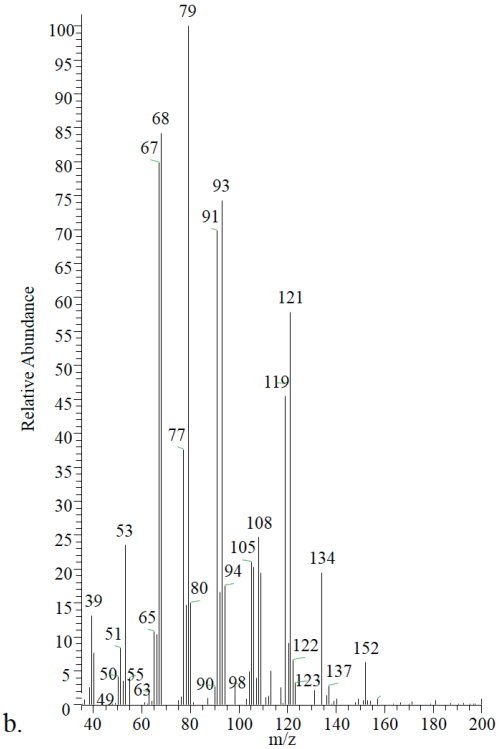


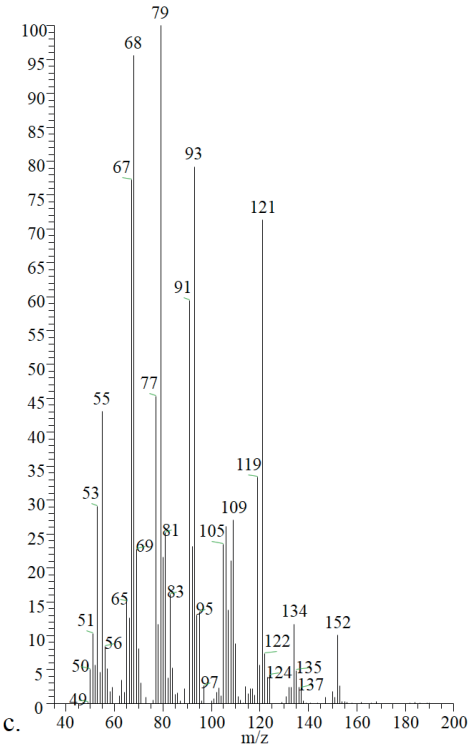
Supplemental Figure S6 (continued)



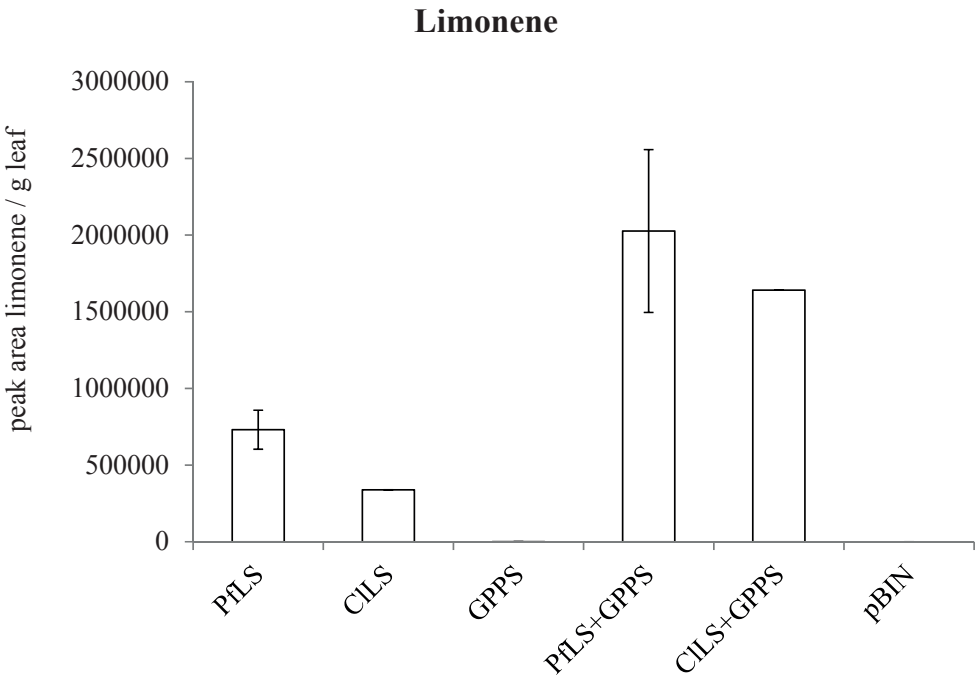
Supplemental Figure S8 a. GC-MS chromatograms of *N. benthamiana* leave headspace after agro-infiltration of constructs, b. Mass spectrum of perillyl alcohol peak in SdLS+SdL7H at RT 18.04 +c Full ms [35.00-400.00], c. Mass spectrum of perillyl alcohol reference compound. NL 5.17E7, *m/z* = 92.5-93.5+120.5-121.5+151.5-152.5 MS.



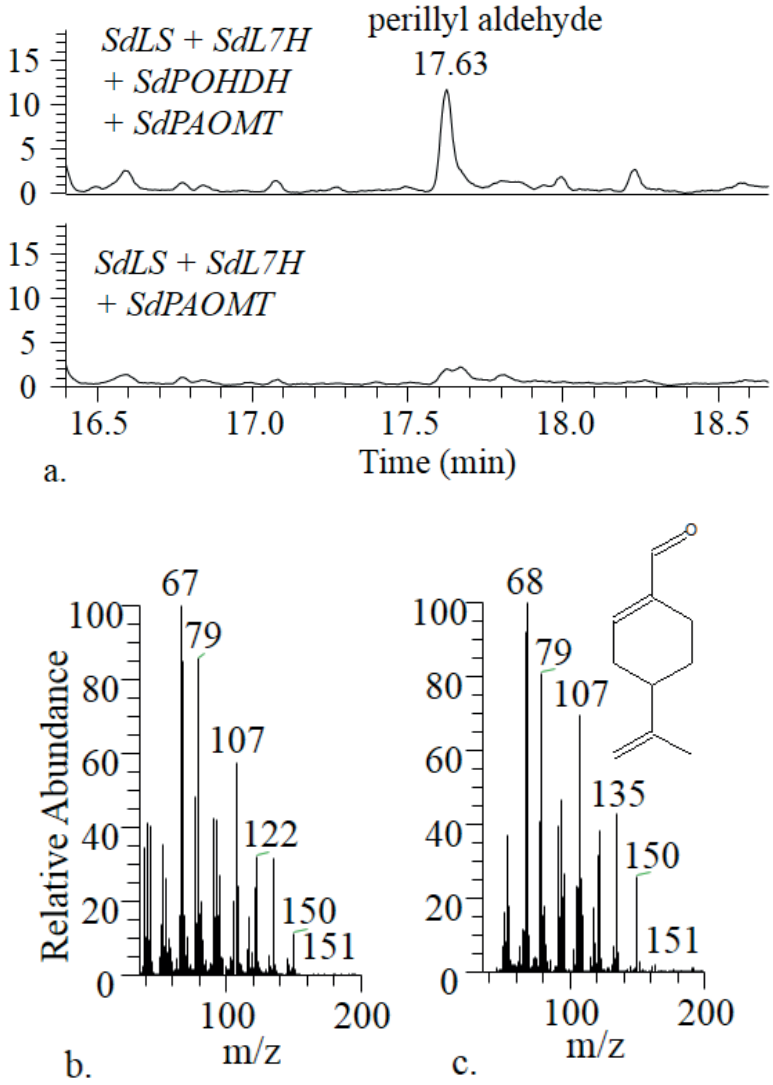




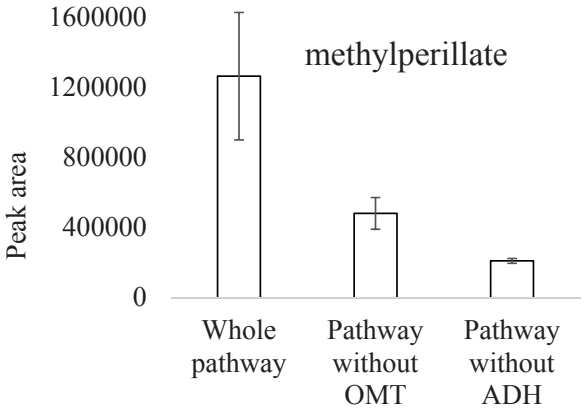
Supplemental Figure S11 Co-expression of *Picea abies* GPP synthase (*GPPS*) with *PfLS* and *ClLS* increases limonene emission by 3-5 times (Fig. S11).



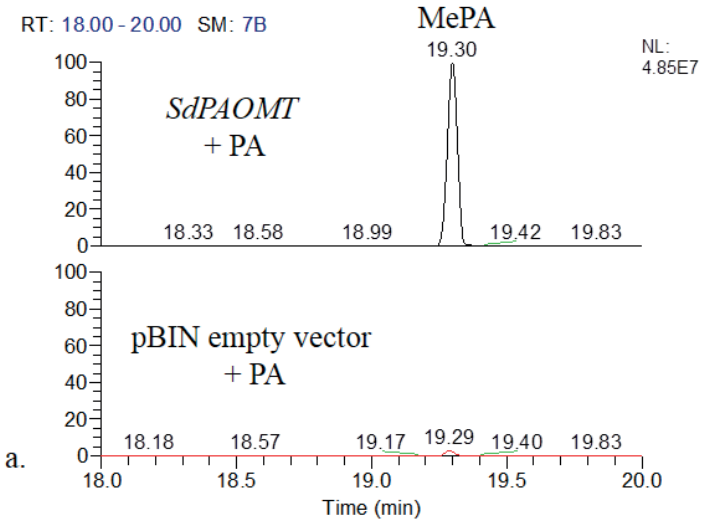
Supplemental Figure S12 Agro-infiltration of methylperillate pathway genes (*SdLS, SdL7H, SdPOHDH and SdPAOMT*) results in production of perillyl aldehyde in the *N. benthamiana* leave headspace, which disappears when *SdPOHDH* is left out. a. GC-MS chromatograms of leave headspace *m/z* 149.5-150.5 NL 2.00E6, b. mass spectrum of perillyl aldehyde peak at RT 17.63, +c Full ms [35.00-400.00] c. mass spectrum of perillyl aldehyde reference compound

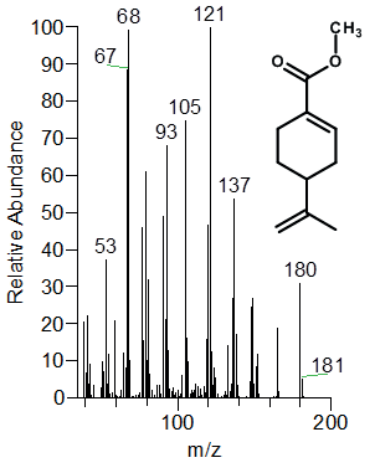


Supplemental Figure S13 Methylperillate in agro-infiltrated *N. benthamiana* leaves headspace. GC-MS peak area methylperillate peak RT 19.26 *m/z* 180. Error bars indicate standard errors, N=3



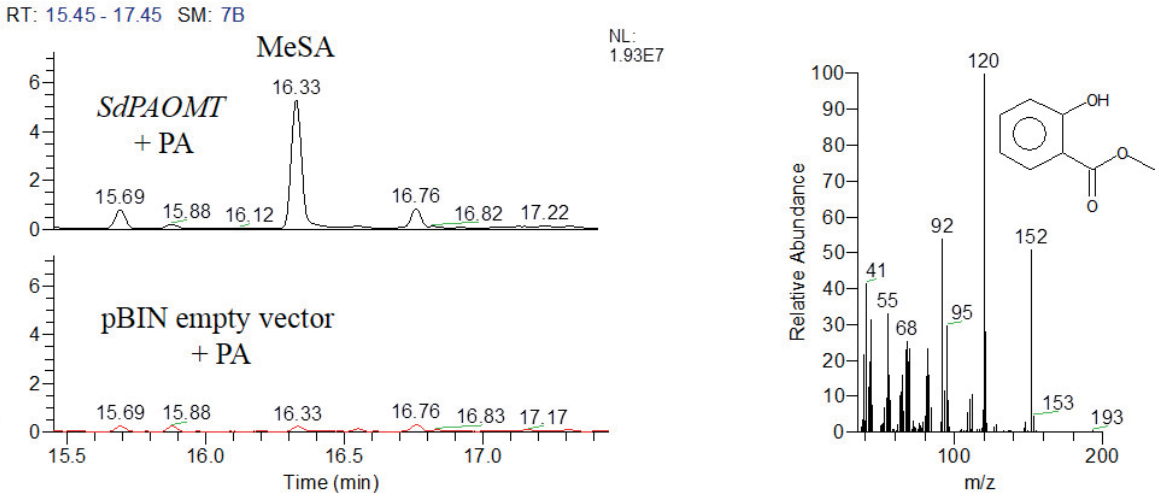
Supplemental Figure S14 Headspace GC-MS analysis of *N. benthamiana* infiltrated with SdPAOMT and *S. dorisiana* leaves. Mass spectra are shown of MePA, MeSA and MeBA peaks. a. *m/z* 180-181 MS, MePA analysis, b. *m/z* 152-153, MeSA analysis, c. *m/z* 136-137, MeBA analysis, d. GC-MS chromatogram of *S. dorisiana* leaf headspace. When specific *m/z* 92-93+120-121+152-153 are extracted from the *S. dorisiana* chromatogram, a small MeSA peak becomes visible at RT 13.06, NL 1.91E6 (in TIC the methylsalicylate peak is covered by a decanal peak at RT 12.95). However, the ratio MeSA: MePA in *S. dorisiana* is much smaller than in *N. benthamiana*, so probably in *S. dorisiana* substrate availability of SA is low.



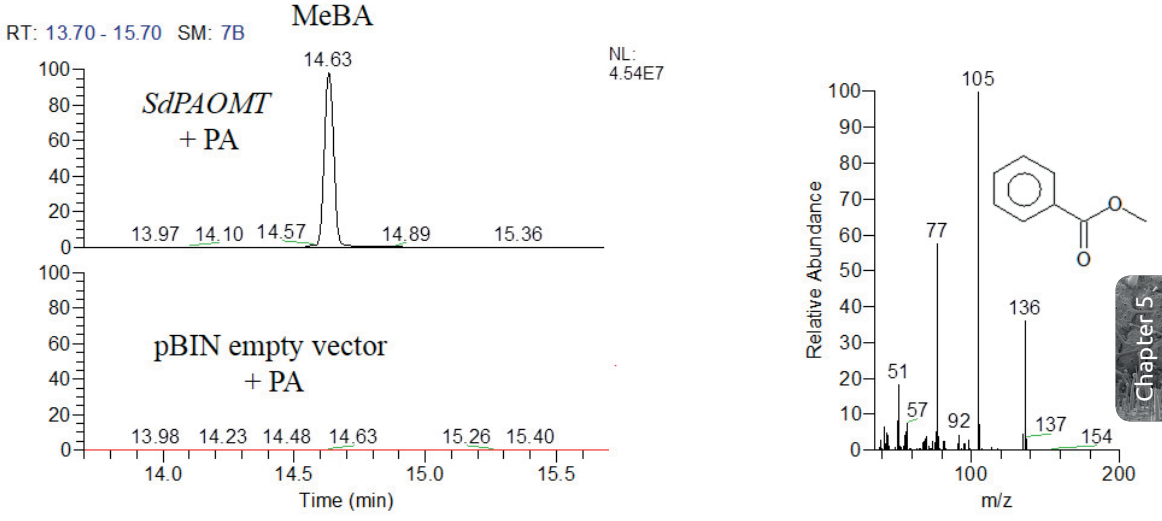


Supplemental Figure S14 (continued)

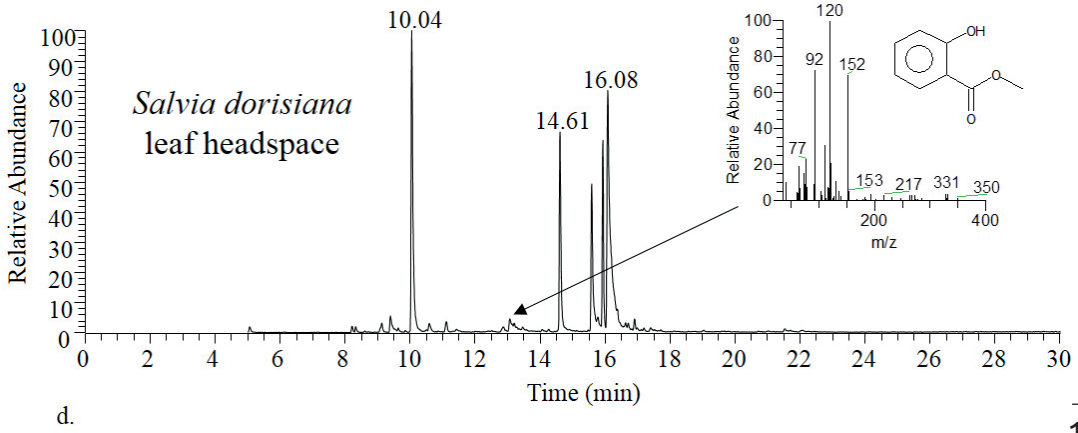
b.



c.



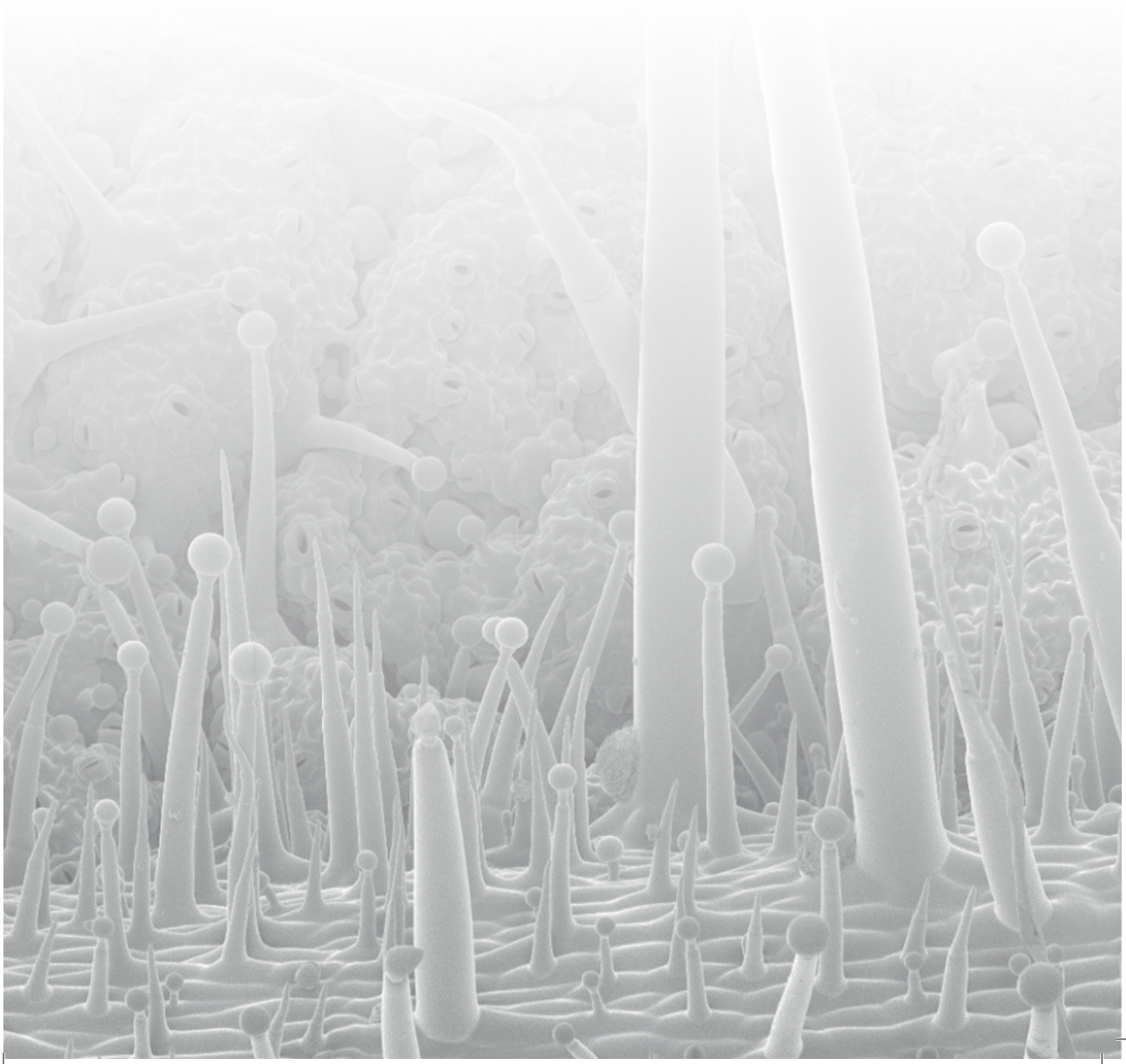
d.



Supplemental Table S4 Marker compounds LC-MS in *N. benthamiana* of perillyl alcohol (POH-M1, POH-M2), perillyl aldehyde (PAldH-M1) and perillic acid (PA-M1, PA-M2, PA-M3), MS/MS fragments and putative identification.

Marker	Retention time (min)	*m/z*	Molecular formula	Accurate mass	MS/MS fragments	Putative identification
POH-M1	41.60	401.1815	C19H29O9	401.1812 [M+FA-H]	355.1763 [M-CO_2-H] 313.1657 [M-malonyl-H]	Perillyl alcohol malonyl hexose
POH-M2	32.29	491.21378	$C_{22}H_{35}O_{12}$	491.21290 [M+FA-H]	445.2087 [M-H] 313.1657 [M-Pent-H] 149.0465 [Pent-H]	Perillyl alcohol pentose-hexose
PAldH-M1	27.73	458.1967	C20H32O7N3S	458.1961 [M-H]	Not measured	Perillyl aldehyde glutathione conjugate
PA-M1	34.68	373.15042	$C_{17}H_{25}O_9$	373.14990 [M+FA-H]	327.14 [M-H] 165.09 [M-Hex-H] 161.05 [Hex-H_2O-H]	Perillic acid hexose
PA-M2	16.41	389.14551	$C_{17}H_{25}O_{10}$	389.14480 [M+FA-H]	343.1397 [M-H] 181.0870 [M-Hex-H]	Hydroxylated perillic acid hexose
PA-M3	27.83	535.20325	$C_{23}H_{35}O_{14}$	535.20270 [M+FA-H]	489.1982 [M-H] 327.1451 [M-Hex-H] 179.0562 [Hex-H]	Perillic acid di-hexose

Chapter 6
General discussion



Chapter 6

In this thesis I worked on metabolic engineering of plant monoterpenes for renewable commodity chemicals. Although I achieved the production of natural feed stocks for oil derived commodity chemicals using plant enzymes and natural production platforms there are still major challenges to make this process commercially viable.

In this discussion chapter I discuss what I contributed and describe what the problems are that need to be solved including some calculations to put the problem in perspective. For the remaining challenges I explore possible solutions in a future perspective.

What is the problem?

Global concerns about the use of fossil feedstocks are growing. Crude oil and coal usage has increased greenhouse gas emissions, but regardless of this problem, oil reserves are also predicted to be finished completely by 2080 (Owen et al., 2010). Therefore, one of the main challenges of the post-fossil feedstock age is to make high-quality materials from renewable resources. Crude oil-derived polymers like PET (polyethylene terephthalate) have highly useful properties, such as low water and gas permeability and high viscosity (Storz and Vorlop, 2013). A significant part of our lifestyle is based on the high quality properties of such materials. Replacing these materials with biobased alternatives should preferably meet or surpass these high quality standards. One option is to look for alternative materials with similar properties. However, one could also try to produce the same materials such as PET but use an alternative, more sustainable source to create its precursor TA (terephthalic acid). In this thesis I demonstrate that this is technically feasible by producing TA from a monoterpene which naturally occurs in the plant *Salvia dorisiana*. The main challenge in this process is to transform abundant biobased resources into the set of commodity chemicals that are useful for producing plastics and other materials, both efficiently and in a commercially competitive way (Hillmyer, 2017). Preferably this is achieved through a sustainable process. Compared to chemical processes a biotechnological process may offer benefits. A biotechnological process can reduce dependence on non-renewable resources, and can improve economics and versatility of products (Gavrilescu and Chisti, 2005).

Biotechnology may also be useful to solve one of the major obstacles of the use of terpenoids as feedstocks: the limited quantities of terpene that can be harvested from natural resources (Kuttan et al., 2011). There is a significant gap between the quantity of terpenoids that can be realistically harvested from a potential source plant species, and the actual demand for the chemical in the industry. Table 1 gives an estimation of the capacity of plant-derived monoterpenes for biobased materials, using the monoterpene yield of the source species *S. dorisiana*, and compares this to current demands for PET plastic. For this, yield parameters of *S. dorisiana*, grown under different conditions, were measured (Supplemental Tables S1-S2). An average yield

from *S. dorisiana* is 4.5 kg essential oil / ha / year. For the sake of calculation, it was assumed that 1% of arable land can be deployed for production of PET precursors, which is an optimistic assumption based on current land requirements for biofuels (FAO, 2008). As established in Chapter 4, around 20% of *S. dorisiana* essential oil consists of methylperillate that can serve as building block for biobased PET plastic (Chapter 4). One can thus estimate that, in order to use *S. dorisiana* as a natural source to meet the global PET demand, current production per hectare needs to increase around 800 times (Table 1). An 800-fold increase in hectares growing *S. dorisiana* plants is not realistic, and this rough estimation therefore shows that natural resources from plants will be limiting. This is in particular the case when the plant species has a relatively low biomass yield per hectare, as is the case for *S. dorisiana* with an estimated biomass yield of about 200 kg DW/Ha (Supplemental Tables S1-S2). Indeed this is small compared to, for instance, the biomass yield of an established production crop like sugarcane, which is around 75,000 kg DW/Ha (Chisti, 2008) suggesting that there is room for improvement. Also the concentration of monoterpenes per gram plant material can likely be improved. For example, for *Stevia rebaudiana*, a species that is exploited for its natural sweeteners called steviosides, yields are reported of 7.5% of the DW terpene steviosides (Abou-Arab et al., 2010), compared to 0.4% (quantified) monoterpenes of DW in *S. dorisiana* (Chapter 4). As discussed later, such differences might relate to the potential storage capacity of plant tissues, which is higher for glycosides (steviosides) than for volatiles (methylperillate).

Table 1 Monoterpene production in *Salvia dorisiana* and PET plastic precursor requirements

Parameters	*Used number*	*Reference*
Essential oil yield	4.5 kg / ha / year	Measured (Supplemental Tables S1-S2)
Oil usable as PET precursor	20 %	Chapter 4
World arable land (2008)	1.5 billion ha	(FAO, 2010)
PET production (2009)	13 billion kg	(Colonna et al., 2011)
Assumed land use for *S. dorisiana* production for bioPET	1 %	(FAO, 2008)
Theoretical conversion rate monoterpenes to PET	1.16	Calculated*
Formula used for calculation of required production increase		
Necessary increase of production = (PET / ha neccessary) / (PET / ha from *S. dorisiana*) = ((PET production / World arable land) / Assumed part land use) / (Essential oil yield * Conversion rate monoterpenes to PET * Part of oil usable = ((13,000,000,000 / 1,526,757,390)/ 0.01) / (4.4565 * 1.16 * 0.2)		
Required production increase		
824 times		

*MW (PET repeating unit) / MW (PA) = 192.2 / 166.1 = 1.16

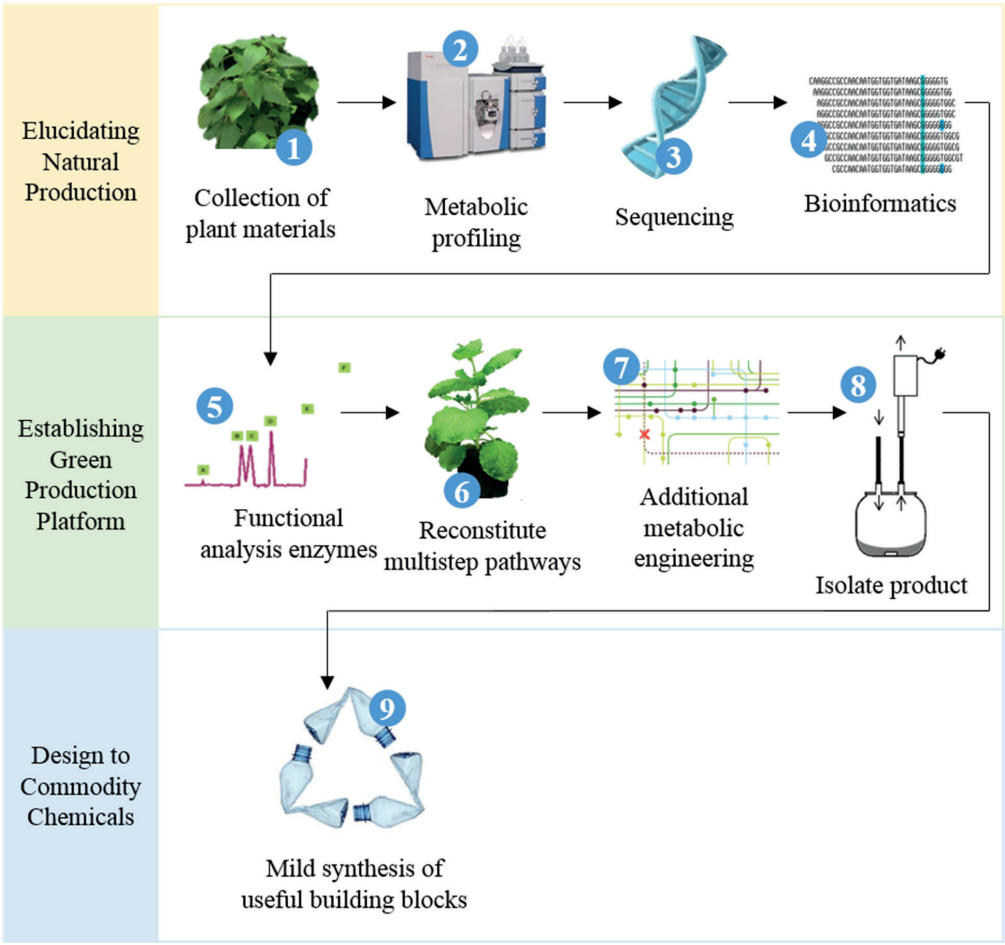
What solutions did I provided in this thesis?

From the calculation (Table 1) it is clear that there is a large gap between monoterpene production in *S. dorisiana* and current plastic demand and thus that *S. dorisiana* is not suitable as a commercially viable source of monoterpenes for the production of PET plastic. In my thesis I provide some solutions on how monoterpenes can be a suitable starting material for biomaterial building blocks, made by sustainable *in-vivo* production in a platform with more favourable properties, based on genetic information from the *S. dorisiana* source (Fig. 1).

In order to exploit monoterpenes as a source for chemical building blocks by means of biotechnological production, three major achievements can be distinguished: (1) elucidating natural production, (2) establishing a green production platform with sufficient capacity and possibility to isolate the product and (3) a smart design for conversion to the commodity chemical compound (Fig. 1). This thesis therefore describes the current status of biotechnological production of limonene (Chapter 2), the production of this compound by metabolic engineering in yeast, dealing with the antimicrobial and volatile properties of limonene by developing a headspace capturing system (Chapter 3), the conversion of the oxidized derivative of limonene,

methylperillate, into the commodity chemical terephthalic acid by means of a synthesis in only two mild steps (Chapter 4), and finally, elucidation of the methylperillate biosynthesis enzymes from *Salvia dorisiana* trichomes and reconstitution of methylperillate biosynthesis in the plant *Nicotiana benthamiana* (Chapter 5).

Figure 1 Workflow from plant metabolites to commodity chemicals



Which challenges are remaining

However, producing monoterpenes in bulk as commodity chemicals also poses challenges for biotechnology: first to identify the most efficient pathway genes but also to design a bio-based production and recovery system that can realistically meet the demand for these compounds. The approach of this thesis depicted in Figure 1 distinguishes nine steps that need to be achieved. Remaining challenges from this approach are discussed here for each of these steps.

(1) identifying target plant from literature data-mining

The first step in the workflow is to identify a plant species that produces a compound with desired functional groups (Fig. 1 Step 1). Recently, tools have been developed to automate literature searches for natural sources of compounds, such as the online literature mining software Anni2.1 (Jelier et al., 2008). We used it to match the compounds from the perillic acid pathway to a concept set of >2500 plants. At that time the software could recognize the compounds perillyl alcohol, perillyl aldehyde and perillic acid, and about half of the 5750 input plant species. It would be good if the database would be updated in order to recognize other interesting compounds from the pathway, such as perillosides and methylperillate. Nevertheless, a plausible plant candidate list was obtained, with *Perilla frutescens* as the first hit. This tool was developed for medical purposes originally, and uses only the PubMed database for output literature, so for the case of our specific research purposes it still had to be complemented with a manual literature search. *S. dorisiana* was not present in this database. Many natural sources of compounds are listed in online databases such as the Dictionary of Natural Products (DNP, 2017), which also includes a chemical structure search tool, which provides the opportunity to find natural products that are similar to the commodity chemical of interest. *S. dorisiana* was obtained as a plant species hit when searching the perillic acid structure in this database. For triterpenes, recently a database was launched "TriForC" containing triterpene molecular structures, characterized biosynthetic enzymes, literature references, and (a limited number of) source plant species (Miettinen et al., 2017). It would be very useful to have such a database for all terpenoids. Automated mining tools are essential for identifying producing plant species in an efficient way, by which the number of plant species to be tested for metabolites in the lab can be reduced.

(2) identifying target tissue and cells for RNA extraction and analysis

Once relevant target plant species have been identified through literature research, representative plant species from the list need to be retrieved, grown and analysed for confirmation of the pathway activity as precise as possible in cells or tissues specialized in producing the compound of interest (Fig. 1 step 2). In *S. dorisiana* specific trichomes

were identified (type VI-like trichomes), which are rich in methyl perillate (Chapter 5). Isolating specialized tissues is important for a comparative expression analysis that will lead to efficient characterization of the relevant genes. A comparative expression analysis can be performed by RNA sequencing of multiple materials. In my case the genes involved were expected to be expressed relatively highly in the glandular trichomes where the compounds are produced, compared to other parts of the plant. But the best choice of samples depends on the biology of the plant species, for example if compounds are upregulated in certain time points, or under certain (a)biotic conditions. For instance, in Norway spruce, monoterpene transcripts were upregulated after jasmonic acid treatment (Martin et al., 2002; Faldt et al., 2003). But in the case of the perillic acid pathway, monoterpene content was unchanged after treatment with the hormones jasmonic acid or salicylic acid (data not shown).

(3 and 4) pathway gene elucidation from RNA-seq assembly and expression profiling

For my thesis work I used mRNA isolated from trichomes (terpene producing tissues), which was sequenced with different sequencing methods. Sequencing methods have recently advanced quickly, however, the translation of this information into useful candidate genes that can be tested is not yet straightforward. The recently developed PacBio method produces long reads, which made it possible to obtain full length transcripts from the sequencing data (Fig. 1 step 3). This saves effort, as the already full length candidates don't need to be analyzed by time-consuming methods such as Rapid Amplification of cDNA Ends (RACE). However, PacBio sequencing produces rather many sequencing errors (Chapter 5). As a consequence, each transcript was present in several variants, of which it remained unclear whether they were real biological isoforms, or sequencing errors. Therefore, it is important to complement this sequencing method with 454 or HiSeq Illumina sequencing to correct mistakes. I performed HiSeq Illumina sequencing on non-normalized cDNA libraries, to also provide gene expression levels. Pfam analysis showed which enzyme classes are active in the producing tissue, from which we could select putative candidate genes based on knowledge of terpene biosynthesis. In our case this combination worked very well, in Chapter 5 we showed a differential expression analysis (Fig. 1 step 4), that yielded four enzymes that together could reconstitute methylperillate production in a heterologous plant species. These four enzymes of interest showed a clear co-expression pattern. Therefore, with this approach we avoided extensive testing of all candidates, which in the case of cytochrome P450s was up to 60 genes (Chapter 5). The only enzyme I could not characterise was an *S. dorisiana* enzyme to convert perillyl aldehyde to perillic acid. I tested the top-2 candidates for aldehyde dehydrogenase, but these were not active on perillyl aldehyde. In order to characterize an enzyme for this conversion in the future, it would be useful

to clone more candidates from the list, look in other pfam classes or perform a classical approach of enzyme assay-guided protein purifications from the trichomes.

For some enzymatic modifications it is not clear which pfam domain is needed. For example, this is the case for terpene alcohol oxidations (Chapter 5). In some reported cases a P450 enzyme converted the terpene olefin to the acid via the alcohol and aldehyde, as for germacrene A acid biosynthesis (Nguyen et al., 2010) and artemisinic acid biosynthesis (Wang et al., 2016). Alternatively, terpene alcohol oxidation can also be performed by alcohol and aldehyde dehydrogenases, for example dehydroartemisinic aldehyde is converted to its acid by an aldehyde dehydrogenase (Nguyen et al., 2011). The pathway of oxidation to the diterpene abietic acid has been reported to be catalysed by enzymes from different pfam classes and in different combinations in different plant species (Ro et al., 2005). In the case of heterologous artemisinic acid production in yeast, one P450 enzyme could convert the olefin into the acid form, but when an additional aldehyde dehydrogenase and alcohol dehydrogenase from the source species *Artemisia annua* were introduced into the yeast strain, the artemisinic acid yield was increased with 18% and 200% respectively (Paddon et al., 2013). In this thesis we successfully reconstituted methylperillate biosynthesis in *N. benthamiana*. In our case, conversion of perillyl alcohol to perillyl aldehyde is performed by an alcohol dehydrogenase, and the aldehyde-to-acid conversion was performed by *N. benthamiana* with unknown enzymes, a better enzyme for the latter conversion could probably still be isolated from *S. dorisiana* as discussed above.

(5 and 6) expression of multistep pathways in plants and microbes

In order to produce high and pure amounts of the compounds of interest, pathway genes can be expressed in a heterologous production host (Fig. 1 steps 5-6). Common criteria for such a host are that it has a commercial infrastructure, that it can be engineered, that it can grow easily to high biomass and that it can produce and store or emit the product efficiently. In this thesis, monoterpenes were produced in two of such hosts, in a microbial production platform, *Saccharomyces cerevisiae* (Chapter 3) and in a plant production platform, *N. benthamiana.* Having explored both platforms, a comparison of properties is made in Table 2.

As energy source for growth and production, plants use (sun)light directly (Table 2). Microbes usually need sugar as their energy source, so when choosing to make use of microbes, still also plant materials need to be produced and refined to deliver that sugar as an energy source, which is in principle less efficient than using plants for production directly (Wang et al., 2015). Other energy sources have been explored to increase the range, for example the monoterpenes sabinene and limonene have been produced from glycerol (Willrodt et al., 2014; Zhang et al., 2014). A promising development that could

combine the advantage of photosynthesis with advantages of microbial production are the use of photosynthetic microorganisms (Melis 2016). Limonene could be produced with photosynthetic cyanobacteria, using CO_2 and light as sources (Davies et al., 2014).

Table 2 Production of monoterpenes in a plant and a microbial platform

	Plant production platform	**Microbial production platform**
Yield	0.9 mg limonene / kg / h	0.007 mg limonene / L / h
Energy source	Light	Sugar
Specificity	High	High
Toxicity for host	Unwanted conversions	Growth hinder
Compartmentalization	Natural subcellular compartmentalization is possible	Complex compartmentalization is challenging
Sequestration of volatile product	Derivatization	Overlay, in-situ headspace trapping
Product extraction	Extraction and liberation procedures necessary	Pure product easily captured or extracted

Both the plant and microbial platforms use enzymes that often have high enantio- and stereo-specificity (Table 2). This is a specific advantage of working with enzymes in comparison to chemical synthesis. For example, the limonene synthases used in my thesis make exclusively (–)- or (+)-limonene. (Semi)-chemical synthesis of limonene, for example from isoprene or turpentine, leads to racemic mixtures (Hanson et al., 1999; Thorp, 2010).

Of course the choice for a production platform for a certain pathway is also of influence on the other steps of the approach. Can the pathway be reconstituted and are the enzymes expressed well, are there possibilities for further engineering and for isolating the product? These aspects are elaborated on in more detail below.

(7) metabolic engineering of plant and microbial platforms

In this thesis, I produced limonene in both plant and microbial platforms with the same plant enzyme PfLS and without major additional engineering (Fig. 1 step 7; Table 2). Yield of limonene from *N. benthamiana* was quantified from the leaf headspace (air around the leaf) to be 0.9 mg limonene / kg leaves in 1 hour. For transient expression in *N. benthamiana*, yield excludes the 5 weeks that are required to grow plants from seed, a period in which nothing is harvested. Microbial yields of limonene calculated

per biomass were a bit lower, 0.007 mg limonene / L culture in 1 hour. However, microbial yields include yeast growth, and limonene can be continuously harvested.

Terpenes in high concentrations often have a toxic effect on plants and microbes (Jongedijk et al., 2016; Molinaro et al., 2016). This can have unwanted effects, when these organisms have to serve as production hosts (Table 2). In plants it has been observed that pathway intermediates and products are converted to non-volatile derivatives, e.g. glycosides. This was a.o. shown for geraniol (Dong et al., 2016) and for perillic acid (Chapter 5). The function of these conversions could be to prevent phytotoxic effects, or it can be a side effect of present background activity of non-selective enzymes. These conversions are usually unwanted. One can play around with this by engineering, or efficient competition with desired enzymes (Chapter 5). In microbes, antimicrobial properties can hinder growth. These effects could be prevented with the trapping system for isolating the product that I developed in Chapter 3. However, it will be even more challenging to engineer further steps with a good flux, as the first volatile compound, limonene, is escaping from the system. Preliminary experiments show that co-expression of limonene synthase PftLS (Jongedijk et al., 2015) with the L7H P450 enzyme (Chapter 5) in yeast does lead to production of perillyl alcohol, but also still limonene is detected. New approaches should be developed and tried out, for example the engineering of fused or co-localized enzymes.

It can be difficult to mimic or circumvent native biosynthesis localizations in heterologous production hosts (Table 2). In the natural plant host, terpene biosynthesis is strongly localized in specialized tissues, cells and subcellular tissues. For example, monoterpene synthases usually localize to the plastids, while P450s localize to the ER (Lange, 2015b). The more distantly related the gene-source species is to the production host, the more difficult to mimic or circumvent localization problems. For example, when expressed in microbes, the subcellular compartmentalization can hinder terpene production. In microbes membrane associations are different than in plants, and subcellular targeting is not well recognized or the target is not present, e.g. yeast has no plastids (Ikram et al., 2015). When expressing ClLS and PfLS in yeast, removing the plastid targeting signal increased production (Chapter 3).

(8) isolating products

Produced volatile terpenes in a heterologous platform will need to be sequestered and extracted (Figure 1, Table 2). The plants that are used for heterologous production might not have or use specialized tissues for storage of terpenes. This is certainly the case when expressing genes under constitutive promoters in *N. benthamiana*. In the absence of a suitable storage capacity in the mesophyll cells, volatiles can be either emitted, or are derivatized if the structure allows it, as was discussed above. In *S. dorisiana*, a large part

of the produced limonene, 25 mg / kg / h is in the headspace, while only 150 mg / kg is stored in the free form in the leaves itself.

Sequestering a volatile anti-microbial compound from a microbial production system is challenging (Fig 1 step 8). The developed in-situ headspace trapping system (Chapter 4) prevents the need of an extraction system for microbial liquid culture.

(9) synthesis of useful building blocks

Plants synthesize a wide range of potential precursors for chemical building blocks. If these compounds can be produced in large amounts in heterologous hosts, we are not limited to glucose and waste biomass as a resource, but also more specialized source molecules from plants (Chapter 4) (Fig. 1 step 9). These specialized chemicals have a higher market price, as it is more difficult to obtain them from other sources. In Figure 2, which is adapted from (Langeveld et al., 2010), it is shown that biochemicals from plant materials usually have a high market price, and are produced in low volumes. They are the top of the pyramid, together with biomaterials, this in comparison to the use of plants for energy production, food and feed The chemical structure of commodity chemicals can be created as close as possible from plant compounds. For this purpose, the wide range of available and future-to-be-elucidated enzyme classes can be used, which can provide methylation, glucosylation, acetylation, regioselective oxidation with P450s, and enantioselective conversions. In this thesis we did a proof of concept by designing a synthesis of the commodity chemical TA (terephthalic acid), the building block of the well-known polyester PET, from monoterpenes. At present it would be difficult to make this route commercially viable, as current prices of limonene are in the 9-10 $/kg range, while prices of TA are around 1 $/kg (Collias et al., 2014; Lange, 2015a).

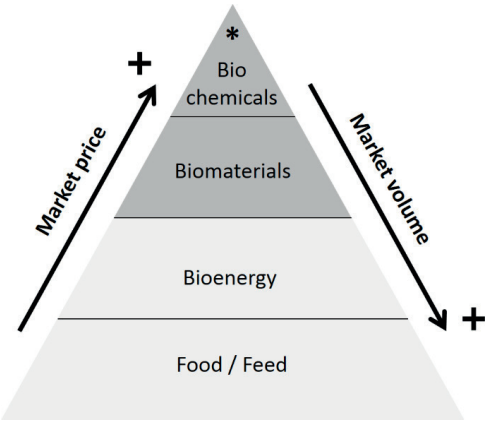


Figure 2 Use of plant materials for human society. Low in the pyramid are high market volumes but with lower prices, high up are high market prices at lower volumes. Terpenes are suitable to function for products higher up in the pyramid due to their specialized properties and lower volumes. Chiral chemicals (*) are at the top, and are especially interesting to be synthesized from terpenes.

Possible solutions to remaining challenges, future perspective

Significant gaps exist in the current status of bio-based production of commodity chemicals from monoterpenes such as methylperillate. Below some of the options to overcome these gaps are discussed.

Completion of the methylperillate biosynthesis pathway (step 4)

It still is to be resolved which enzyme class performs the conversion from perillyl aldehyde to perillic acid, that is observed in *N. benthamiana*, and could possibly be improved by a *S. dorisiana* enzyme. I have several potential candidates for this conversion, carrying pfam domains of P450s or aldehyde dehydrogenases and having the expected co-expression patterns (Chapter 5), and applying the same methods as in the work presented here could also help us to unravel more enzymes, regulatory elements and pathways. Studying the natural trichome and pathway function can give more information about which enzymes we could use for metabolic engineering, for example involved biosynthetic enzymes or transcription factors that could play a role in (cancelling) feedback mechanisms.

Functional improvement of enzymes (step 5)

Once biosynthetic enzymes have been identified, the properties of the enzymes can be further optimized by rational or random mutagenesis. For example product ratios of multiproduct terpene synthases were altered by rational engineering of possible specificity-determining amino acid residues, identified by comparing natural variety in these residues of different terpene synthases (Bian et al., 2017). Another example is the reported improved thermostability of the sesquiterpene presilphiperfolan-8β-ol (PSP, a fungus virulence factor intermediate) synthase, in two rounds of directed evolution introducing two amino-acid substitutions (Lauchli et al., 2013). Enzyme engineering of limonene synthases has been demonstrated as well, increasing conversion velocity, altering the product spectrum or altering product enantioselectivity (Katoh et al., 2004; Srividya et al., 2015). Goal of these studies was mainly to understand the catalytic mechanism, but the knowledge could be used in the future to tune enzyme properties. Simple engineering was performed in this thesis by removal of the plastid targeting signal from limonene synthases for expression in yeast (Jongedijk et al., 2015). Proteins in this thesis were not yet codon-optimized for expression in their production host. However it is known that codon replacement can have a significant impact on gene expression levels and protein folding, so could possibly improve production (Elena et al., 2014). Additional enzyme engineering could be promising in the future to tune enzyme properties in monoterpene biosynthesis pathways, for example to make even more highly enantiopure products.

Futur(e/istic) production platforms (step 6)

Next to the existing plant and microbe production platforms, in the future perhaps other platforms could be exploited for production of terpenes. Some of these new platforms have quite interesting properties but their development in some cases lags behind because of difficult genetic transformation, or practical problems in the culturing like susceptibility for infections. However, it is worth to mention here a few of these interesting hosts. Some plant species are known to sequester high levels of terpenes in parts that are normally not used. An example is chicory, which is known to sequester bitter terpenes in its roots (Cankar et al., 2011). Stable transformation is possible in chicory, so they could serve as a production platform (Vijn et al., 1997; Maroufi, 2015). Also platforms are investigated that combine the advantage of plants with the advantage of a liquid culture system. Microalgae can be cultivated in bioreactors and are studied for their application in for example energy generation or nutrient provision, and to use them for production of pharmaceuticals. They are considered a promising sustainable feedstock (Ruiz et al., 2016). Work is being done on transformability of algae species (Neupert et al., 2012). There are examples of terpenes being extracted from algae (Michalak and Chojnacka, 2015), so this species could be a suitable platform to engineer terpene production. Duckweed are small plant factories that grow in an aquatic way, and thus also not require arable land use. Their clonal growth on liquid is favorable for large scale production. Recently transformation protocols have been developed for this species (Yamamoto et al., 2001). A comparable system to duckweed, but with extended sustainable possibilities, is the aquatic fern *Azolla* (Brouwer et al., 2014). *Azolla* doesn't need any nitrogen fertilizer as it fixes nitrogen by itself by means of symbiotic cyanobacteria. Another promising example of an aquatic plant system is the moss *Physcomitrella*, that can grow in liquid media and can be subjected to metabolic engineering of terpene biosynthesis (Bach et al., 2014). Also artificial suspension-cultured plant cells have been explored for heterologous expression (Plasson et al., 2009). Another cell system, but not plant-derived, are insect cells (Sf9 cells). These are exploited as a production system for recombinant proteins, combined with a baculovirus-derived expression system. They are until now mainly used for their good heterologous expression of P450 enzymes (Pateraki et al., 2015), which can sometimes be difficult to achieve in other hosts.

A sustainable alternative would be to produce non-food products like polymer building blocks in non-consumed plant parts, as a side-product next to the normal production chain, for example in the green parts of tomato and potato. In this case only isolation costs of the compound would have to be covered, and sales would add-up to the selling of the vegetable. Tissue or cell specific promoters can be used to target production of heterologous compounds to certain plant parts, as was demonstrated for overproduction

of anthocyanins in tomato that could be achieved specifically in fruits (Butelli et al., 2008) or not in fruits but in vegetative tissues (Outchkourov et al., 2017).

Increasing production in plants and microbes (step 7)

Plant engineering has the potential to increase production tremendously. Due to developments in plant biotechnology and breeding, yields of commercially interesting plant compounds can be dramatically increased. For example in the case of *Stevia rebaudiana,* breeding efforts increased the content of the natural sweeteners (rebaudiosides) by 25 times (Yadav et al., 2011).

A possibility for engineering is to manipulate compartmentalization. This was done for the sesquiterpene patchoulol, where redirecting compartmentalization (from the cytosol to the plastids) increased production by 40,000 fold in its heterologous host tobacco (Ikram et al., 2015). Also in microbes engineering of compartmentalization seems to be promising, for example triterpenoid production in yeast could be increased by using a strain enriched in ER membranes (Arendt et al., 2017).

One can also play around with natural glycosylations that occur in the plant host. For example by overexpressing glycosyl transferases for increased storage in the vacuole, and later extracting and deglycosylating, or by downregulating them to release the product. There are possibilities to increase flux through the pathway for example by knocking-out competing enzyme activities (Cankar et al., 2015). Recently it has been shown that specific transporters, together with specific stabilizing lipid transfer proteins, can help to transport terpenes from inside the plant cell towards the apoplast (Wang et al., 2016). Perhaps in the future such transporters and lipid transfer proteins could also be engineered for perillic acid or methyl perillate, to help transportat to the apoplast and in that way decrease toxicity and unwanted modifications. An ABC transporter with favourable co-expression pattern was present in the *S. dorisiana* sequences (Chapter 5). This could be a candidate for transporting compounds of the methyl perillate pathway. No lipid transfer proteins were present in our co-expression selection.

In microbes, engineering can increase terpene production significantly, for example artemisinic acid production in yeast was increased from 32 mg/L to 25 g/L by improving strains and conditions (Ro et al., 2006; Paddon et al., 2013). Farnesene produced in yeast is already commercially used as a biobased fuel additive (DeNardo, 2016). The highest yield of limonene from microbial production so far reported is 1.35 g/L from *E. coli* (Willrodt et al., 2014). Depending on the purpose and further engineering possibilities, yields of heterologous products from plants and microbes both could become commercially viable (de Jaeger, 2017; evolva, 2017).

A better insight in optimization of biosynthetic pathways is provided by parameterizing a model of the pathway. Modeling of the m-xylene pathway in the bacterium *Pseudomonas putida* provided prediction of catabolism of the substrate and biomass formation (Koutinas et al., 2011). Biological knowledge about the function of transcriptional regulation and the various molecular components that interact need to be known to be able to model enzymatic pathway, and functions (concentration derivatives over time) have to be assigned to the different genes and regulators. It is necessary to validate solutions of the model by measuring carbon fluxes in the modeled system. A plant genome-scale metabolic reconstruction became recently available for *Arabidopsis.* In the case of the methylperillate pathway, we had limited knowledge about the enzymes involved in the pathway, and about the background systems of *S. cerevisieae* and *N. benthamiana.* It is also still difficult to model spatial and temporal processes, for example tissue, cell and subcellular specificity and peak terpene production in the day in plants. In the future when more knowledge about the metabolism of production hosts and the perillic acid pathway biosynthetic and regulatory enzymes is known, modeling could help to gain further insight in engineering possibilities.

Isolating the product and evaluating production (step 8)

When engineering microbial hosts for production it is an advantage to be able to evaluate production levels quickly, in a high-throughput way. Some investigation has been done in this direction, for example evaluating terpene production levels by coupling those to a colour screening (Behrendorff et al., 2013; Lauchli et al., 2013). To extract volatile products from plants extraction will be necessary in case they are stored in glycosylated form, combined with (enzymatic) deglycosylation procedures. Perhaps if plants emit large amounts of product to the headspace, it would be worthwhile to experiment with headspace trapping on larger scale, in greenhouses or growing containers.

Mild synthesis of building blocks (step 9)

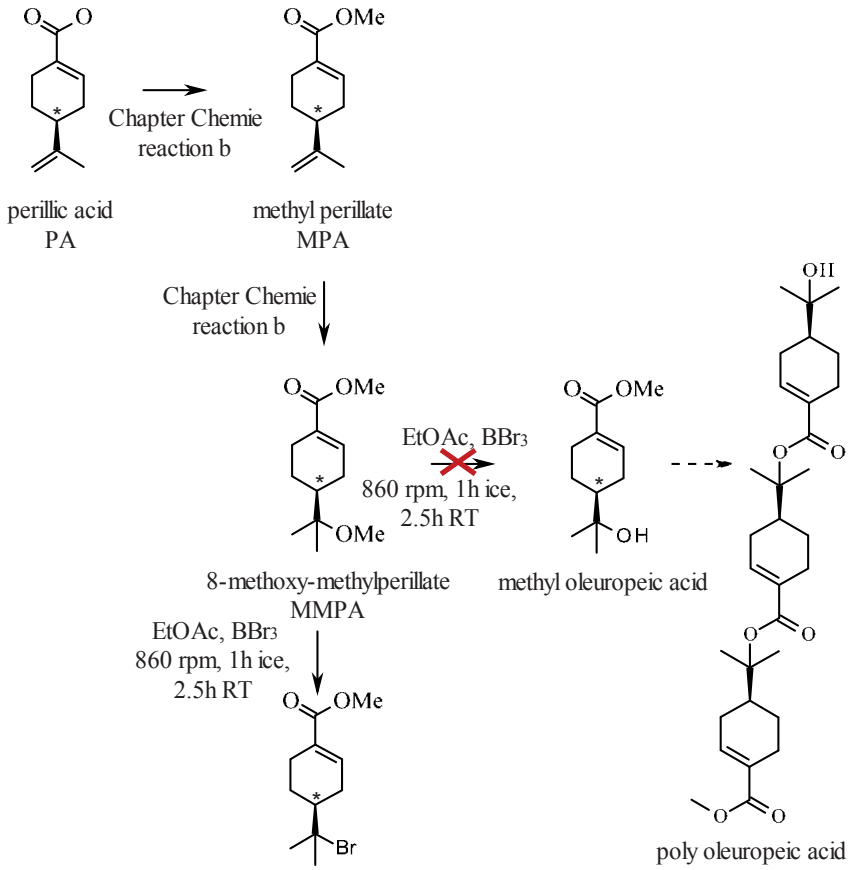
When converting monoterpenes to TA, their unique natural chirality is lost. A larger potential to make use of monoterpenes would be to use them for chiral chemicals and polymers. Commodity chemicals that are pure in conformation and chirality are probably positioned most high up in the pyramid (* in Fig. 2). For comparison, the price of TA is 1 $/kg (Collias et al., 2014), while the price of a building block for chiral liquid crystalline polymers can be thousands of dollars per kg (TCIchemicals, 2017). Also creation of new materials could be explored using the natural precursor pool, with perhaps better material properties, a more sustainable production process or improved recycling possibilities (Hillmyer, 2017).

Chiral compounds are interesting for flavor and fragrance and medicinal applications, but also for the construction of polymers, synthetic reagents, catalysts and starting

material for organic synthesis (Brill et al., 2017). Chiral polymers are applied as a catalyst in asymmetric synthesis or for the separation of racemic compounds (Itsuno, 2005). Combinations of chiral monomers can influence polymer properties. For example in polylactic acid (PLA), a promising biodegradable renewable polymer, properties are influenced by the ratio of (-) and (+) lactic acid monomers that is used (Van Wouwe et al., 2016). This study indicates that it is favourable to synthesize both pure enantiomeric acids, to be able to compose the polymerization feed as desired. Enantioselectivity is not so straightforward to obtain from fossil crude oil in a chemical way, and often maximum yield of a single enantiomer is 50% (Kataoka et al., 2016). Terpenes have often a chiral centre and in many cases are already preferentially biosynthesized by nature in one enantiomeric conformation. Cyclic monoterpenes have a favorable structure, can be specifically produced in the (+) or (–)-form, and can be oxidized in specific positions. In this thesis in the example of limonene, it was produced in exclusively the (+)-form by ClLS, and in the (-)-form by PfLS and SdLS (Chapter 4, Chapter 5), and it can be oxidized on C3, C6, C7 and mixed positions (Chapter 5) (Bertea et al., 2003; Lucker et al., 2004; Mau et al., 2010). It remains to be investigated how chirality behaves through the different steps of the methylperillate biosynthesis pathways, and whether selectivity in different steps is caused by production of only one enantiomer by a biosynthetic enzyme, or by acceptance of one specific enantiomeric substrate by the next biosynthetic enzyme in the row. Terpene synthases have been reported that produce mainly one enantiomer (Jongedijk et al., 2016), and also P450 enzymes have been described to hydroxylate chiral terpenes while keeping the specific enantiomer (Lucker et al., 2004). The *S. dorisiana* limonene synthase produced mainly (-)-limonene, but also a minor fraction of (+)-limonene. It would be interesting to measure enantiopurity of other intermediates in the *S. dorisiana* plants (besides limonene), and characterize the biosynthetic enzymes for their enantioselectivity for substrates and products. And to see if these properties can be influenced and if necessary improved by enzyme engineering, in order to apply them for applications where enantio-purity is important.

Enantiopure limonene has interesting properties for chiral polymers (Firdaus et al., 2011). Perhaps new polymers could be designed from chiral monoterpenes with interesting new material properties and/or improved sustainability. I did a trial experiment, shown in Figure 3, to process methylperillate to a chiral polymer polymethylperillate, which could possibly have more specific properties, like liquid crystallinity (mesogenicity) combined with optical rotation. This could be of use for specific applications, for example in LCD screens (Chien and Liu, 2015). It would be worthwhile focussing in the future on the development of chiral commodity chemicals and materials from monoterpenes.

Using chirality: methylperillate to poly oleuropeic acid.
Methylperillate is a chiral monoterpene that can be used for the synthesis of chiral polymers, possibly with liquid crystalline properties. The building block (-)-methyl oleuropeic acid could be synthesized from (-)- methyl perillate, and subsequently polymerized to a head-to-tail chiral homopolymer, poly oleuropeic acid (Scheme 1). In this thesis work, time was insufficient to extensively develop the required chemical reactions. Oleuropeic acid (OA, 8-hydroxy-p-menth-1-en-7-oic acid) itself is a natural product that could perhaps in the future serve as a starting molecule for these reactions. It is a.o. a constituent of olive, *Olea europaea* (Mechoulam 1962), eucalyptus, *Eucalyptus globulus* (Tian et al 2009), and some other species (Goodger and Woodrow, 2011). In eucalyptus it occurs purely in the (+)-form (Tian et al 2009), while in olive it occurs purely in the (-)-form (DNP, 2017).



Scheme 1 Chiral polymer synthesis from methyl perillate

However, some preliminary experiments were performed. Prolonged reaction time during esterification of perillic acid to methylperillate (MPA) resulted in the formation of methoxy methyl perillate (MMPA) by etherification at the C8 (Chapter 4). This compound could be readily separated from MPA (Chapter 4). In an attempt to convert MMPA to (-)-methyl oleuropeic acid (MOA), the method of (Shimomura et al 1978) was used, employing BBr_3 in ethyl acetate. Unfortunately, the isolated product turned out to be the bromide, instead of the required tertiary alcohol (Scheme 1). Due to lack of time and MMPA, further experiments to obtain MOA were not possible within the current project.

Figure 3 Chiral polymer synthesis from methyl perillate

The capacity to transform plant compounds to chiral materials is under-utilized. Chiral terpenes, carbohydrates and amino acids form an abundant collection of chiral building blocks, the "chiral pool", that can be explored to synthesize all kind of materials (Brill et al., 2017). A search in DNP for compounds with optical rotation tells that there are over 100,000 chiral natural products already identified from nature (DNP, 2017). It is clear that nature contains a wide range of chiral chemical diversity, that is promising for economical and sustainable purposes.

Future perspectives from other angles

In my thesis I focussed mainly on exploring ways to make sustainable plant based material precursors available. The gap between availability of terpenes and human demands for commodity chemicals of course can become smaller from two directions, not only by increasing material availability, but also by decreasing demands. Demands for 'fresh' (fossile or natural) sources for materials decreases, when already synthesized materials are recycled after use. Especially degradation of very large quantities of defined industrial polymer waste is economically profitable (Sinha et al., 2008). PET plastic can be readily recycled. Microbial strains are explored for the purpose of recycling PET plastic. Recently a novel bacterium strain was isolated from PET plastic waste, that is able to use PET as its major energy and carbon source (Yoshida et al., 2016). Also demands decrease if alternative polymers could be developed with similar properties, such as PEF (Eerhart et al., 2012). Furthermore consumer choices could be of large influence to decrease plastic use (Yoshida et al., 2016). From an economical perspective, alternative sources to oil become more and more feasible by time, as oil is not endless and on the long term prices can only go up (Owen et al., 2010).

Conclusions

Plant terpenes could provide a sustainable feedstock for many practical applications but then their availability should improve. Currently, a major challenge is that natural production of terpenes is in low quantities in specialized tissues, while for commodity chemicals large amounts are required against a relatively low price.

In my thesis work I explored applications for terpenes from the methylperillate pathway and methods to produce them. I developed methods (Fig. 1) that allowed quite efficient compound identification, enzyme isolation and the construction of new pathways. I studied the production of volatile and anti-microbial products in microbes, produced products with high stereo- and enantio-specificity, and produced a polymer building block from a natural product with minimal chemical modifications.

It is evident that in order to be able to close the gap between the low natural availability of suitable and sustainable precursors and large global demands for feed stocks, an

interdisciplinary approach is necessary. Provided that we face the challenges in the available green production systems, we could make new compounds of interest with economically profitable yields. To reduce the efforts and non-sustainable chemical conversions needed, it is important to explore nature for the most suitable starting material, using special properties already present like chirality and functional groups. Using special properties can increase the price of the compounds produced, which allows higher development and production costs.

Our methods could be generally applied to use plant compounds as a source for human needs. In the future it is important, to increase production, to find solutions for volatility and toxicity in different production platforms and to make more specialized materials with improved properties. This work made an initial but important step to produce commodity chemicals from plant secondary metabolites as alternative starting material, in order to decrease dependence on oil and improve sustainability of production processes.

Supplemental files to this chapter can be downloaded from:
http://www.wageningenseedlab.nl/thesis/ejongedijk/SI/

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Supplemental Table S1 Yield parameters of *Salvia dorisiana* plants

Surface area per plant	825 cm^2 = 825E-8 Ha
Amount of plants per Ha (= 1 / surface area in Ha)	121258
Fresh weight per plant	18.73 g
Essential oil yield	1.25654 mL / kg FW
Essential oil density (ρ)	0.976 g / mL
Essential oil yield	2.78 kg essential oil per Ha
Average harvesting times per year	1.6 times[1]
Formula for calculation	$\frac{mL\ essential\ oil}{kg\ FW} \times \rho\ essential\ oil \times \frac{kg\ FW}{plant} \times \frac{plants}{hectare} \times \frac{\#\ harvests}{year}$ $= essential\ oil\ yield\ (L/Ha/Y)$
Essential oil yield per year	4.46 kg essential oil / Ha / Y

For other Salvia varieties, essential yields have been reported in the field from 16 – 352 L / Ha / Y10 [1]. The Salvia data from Mossi et al. were obtained from plants grown in the field for 4-5 years, harvested 1-2 times per year, while in our study the plants were grown for 56 days only. This difference might explain the higher biomass yields per plant in real agricultural conditions, and thus the higher oil yield.

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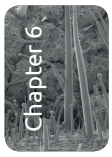
Chapter 6

Summary

This thesis aimed to investigate how plant monoterpenes can be used to produce biobased plastics. Monoterpenes are volatile compounds, produced by plants to defend themselves against insects and pathogens or to attract pollinators. Many monoterpenes have a characteristic odour, and are used by humans in all kinds of products for their nice smell or taste. For example the monoterpene (+)-limonene has a fresh citrus odour, and is used in cosmetics and sodas. Recently, however, it was demonstrated that the chemical structure of some monoterpenes may also be suitable to serve as a feed stock for the synthesis of commodity chemicals and biomaterials.

Plants produce monoterpenes in specialized structures, such as glandular trichomes. Trichomes are gland-like structures on the leaf surface that serve as small biochemical factories. Plants produce and store monoterpenes and other volatile compounds in these trichomes. However, the amount of monoterpenes in plants is often not large enough for bulk applications. Therefore, I set out to investigate which genes plants use to produce monoterpenes, and if I can express these genes in a better production platform, in order to produce larger amounts of monoterpenes.

Monoterpenes consist of 10 carbon atoms and are synthesized from the precursor geranyl diphosphate (GPP) in the plastids of the plant cell. After synthesis of the monoterpene backbone, usually several structural modifications, for example oxidation, take place, by other enzymes in the cell. Chapter 1 of this thesis introduces what monoterpenes are, how they are synthesized in plants and how they can be produced by metabolic engineering in heterologous hosts like micro-organisms for human applications.

One of the best studied monoterpenes is limonene. Chapter 2 reviews the existing and potential applications of limonene as well as the state of the art in its microbial production. The chapter describes which genes have been used for the biosynthesis of limonene, as well as the strategies that have been employed to enhance the production in micro-organisms.

Chapter 3 describes our production of limonene using the micro-organism *Saccharomyces cerevisieae* (yeast). For this purpose, a mutated yeast strain was used, which produces a small amount of GPP as precursor for limonene biosynthesis. Limonene has a chiral centre, which means it can exist in two enantiomers, (+) or (-), which are mirror images. I showed that it is possible to produce both forms in yeast by introducing limonene synthase genes from different plant species. It turned out that it is not straightforward to harvest limonene from yeast cultures, as it is very volatile and does not mix well with the culture broth. Therefore, a system was developed to trap

limonene from the yeast culture headspace during production. Compared to other limonene harvesting systems, this resulted in a better yield.

Chapter 4 describes how a natural derivative of limonene, methylperillate, can be converted to plastic. Methylperillate has a suitable structure to be converted into a polymer building block. To demonstrate this, methylperillate was converted to the bulk chemical terephthalic acid, which is the building block of polyethylene terephthalate (PET). Due to the high structural similarity between methylperillate and terephthalic acid, a short chemical synthesis route consisting of two steps could be developed.

For the large scale application of methylperillate for biobased commodity chemicals, it would be useful to produce methylperillate in micro-organisms. Methylperillate has the same backbone as limonene, but with a methylated carboxyl group at the C7-position. At the onset of this thesis not much was known about the enzymes involved in the biosynthesis of such methylated carboxyl groups. In Chapter 5, I characterise a biosynthetic pathway to methylperillate. After screening several plant species we found that *Salvia dorisiana*, a sage species, can produce methylperillate in the glandular trichomes on its leaves. Trichomes were isolated from the leaves and used as the source, using genomics techniques, for the isolation of four genes, which I showed are involved in the biosynthesis of methylperillate. Production of methylperillate was established in the tobacco-like model plant, *Nicotiana benthamiana,* using these four *Salvia* genes. In the future these genes could also be used in yeast or other microbes to produce methylperillate in fermenters.

In Chapter 6 the research results of this thesis are discussed. A perspective is provided on producing bioplastics from compounds like methylperillate. Questions addressed in this chapter include how much monoterpenes should be produced to realistically use them for the production of biomaterials, and which possible solutions can be foreseen to produce monoterpenes on a larger scale. One future scenario is to focus on the use of monoterpenes for more specific, high-value applications by taking advantage of their natural chirality.

All in all, this research is an important first step to use specific molecules from plants as an alternative source for biomaterials. Potentially, this will decrease dependence on fossil oil, and improve sustainability of production processes.

Samenvatting

Dit proefschrift heeft als doel om te onderzoeken hoe monoterpenen uit planten gebruikt kunnen worden om bioplastics te produceren. Monoterpenen zijn vluchtige stoffen, die planten aanmaken om zich te verdedigen tegen insecten en ziekteverwekkers of om bestuivers aan te trekken. Veel monoterpenen hebben een karakteristieke geur, en worden door mensen gebruikt in allerlei producten voor hun geur of smaak. Bijvoorbeeld het monoterpeen (+)-limoneen heeft een frisse Citrus geur, en wordt gebruikt in cosmetica en frisdranken. Onlangs is ontdekt dat de chemische structuur van monoterpenen ze ook geschikt maakt als grondstof voor de synthese van fijnchemicaliën en biomaterialen.

Planten produceren monoterpenen meestal in gespecialiseerde orgaantjes, zoals glandulaire trichomen. Dit zijn klierachtige structuren op het bladoppervlak die als kleine biochemische fabriekjes dienen. Planten produceren monoterpenen en andere vluchtige stoffen in deze trichomen, en slaan ze daar op. Echter, de hoeveelheid monoterpenen die de plant op deze manier kan produceren is vaak niet groot genoeg voor grootschalige toepassing. Daarom heb ik onderzocht welke genen planten gebruiken om monoterpenen te maken, en of ik die genen in een beter productieplatform tot expressie kan brengen, om zo grotere hoeveelheden monoterpenen te produceren.

Monoterpenen bestaan uit tien koolstofatomen, en worden gemaakt vanuit de bouwstof geranyl difosfaat (GPP) in de plastiden van de plantencel. Na het maken van het molecuulskelet van de monoterpeen vinden er vaak nog allerlei structurele modificaties, bijvoorbeeld oxidatie, plaats, door andere enzymen. Hoofdstuk 1 van dit proefschrift introduceert wat monoterpenen zijn, hoe ze gemaakt worden in planten en hoe ze geproduceerd kunnen worden door middel van metabole engineering in een productieplatform, zoals micro-organismes of planten, voor gebruik door de mens.

Een van de meest bestudeerde monoterpenen is limoneen. Hoofdstuk 2 beschouwt bestaande en potentiele toepassingen van limoneen, en de recente ontwikkelingen van de microbiële productie ervan. Het hoofdstuk behandelt de genen die nodig zijn voor de biosynthese van limoneen, en de strategieën die zijn gebruikt om limoneenproductie in micro-organismen te laten plaatsvinden.

Hoofdstuk 3 beschrijft hoe ik limoneen heb geproduceerd, waarbij ik gebruik heb gemaakt van het micro-organisme *Saccharomyces cerevisieae* (gist). Hiervoor heb ik een gemuteerde giststam gebruikt, die een beetje GPP maakt dat nodig is als bouwstof voor de biosynthese van limoneen. Limoneen heeft een chiraal centrum, wat betekent dat er twee vormen van limoneen zijn, (+)- of (-)-limoneen, die elkaars spiegelbeeld zijn. Ik heb laten zien dat het mogelijk is om beide spiegelbeelden in gist te maken door limoneensynthase genen van verschillende plantensoorten te introduceren. Het oogsten van limoneen uit de gistculturen bleek niet heel makkelijk, omdat het erg vluchtig is en

niet goed mengt met de gistcultuur. Daarom heb ik een systeem ontwikkeld om limoneen af te vangen uit de lucht boven de gistcultuur tijdens de productie. Dit leverde een betere opbrengst op vergeleken met andere systemen om limoneen te oogsten.

Hoofdstuk 4 beschrijft hoe een natuurlijke afgeleide van limoneen, methylperillaat, omgezet kan worden naar bioplastic. Methylperillaat leek qua structuur geschikt als grondstof voor polymeren. Om te demonstreren of dat echt zo is heb ik de omzetting ontwikkeld van methylperillaat in tereftaalzuur, de bouwsteen van polyethyleentereftalaat (PET). Vanwege de grote structurele overeenkomsten tussen methylperillaat en tereftaalzuur kon een korte chemische synthese van twee stappen worden ontwikkeld.

Voor grootschalige toepassing van methylperillaat voor biomaterialen of fijnchemicaliën zou het nuttig zijn om methylperillaat in micro-organismen te maken. Methylperillaat heeft hetzelfde molecuulskelet als limoneen, maar dan met een gemethyleerde carboxylgroep op de C7-positie. Bij aanvang van mijn onderzoek was nog niet veel bekend over de enzymen die betrokken zijn bij de aanmaak van zulke gemethyleerde carboxylgroepen. In hoofdstuk 5 ging ik daarom op zoek naar een biosyntheseroute voor methylperillaat. Na het screenen van verschillende plantensoorten heb ik ontdekt dat *Salvia dorisiana*, een saliesoort, methylperillaat kan maken in de glandulaire trichomen op de bladeren. Deze trichomen heb ik geïsoleerd van de rest van het blad en gebruikt als bron, via 'genomics' technieken, voor de isolatie van vier genen, waarvan ik heb laten zien dat ze onderdeel zijn van de biosynthese van methylperillaat. Vervolgens heb ik met behulp van deze genen methylperillaat gemaakt in de tabak-achtige modelplant *Nicotiana benthamiana*. In de toekomst kunnen deze genen ook gebruikt worden in gist of andere micro-organismen om methylperillaat te maken in fermentors.

In hoofdstuk 6 worden de onderzoeksresultaten van dit proefschrift bediscussieerd. En schets ik het perspectief voor het produceren van bioplastics uit stoffen zoals methylperillaat. Ik bediscussieer onder andere hoeveel hoger de productie van monoterpenen in planten zou moeten worden om ze realistisch te kunnen beschouwen voor de productie van biomaterialen, en welke oplossingen te verwachten zijn om monoterpenen op grotere schaal te kunnen produceren. Een van de toekomstperspectieven is om te focussen op het gebruik van monoterpenen voor meer specifieke, hoogwaardige toepassingen door gebruik te maken van hun natuurlijke chiraliteit.

Alles bij elkaar is dit onderzoek een belangrijke stap op weg naar de productie en het gebruik van specifieke moleculen uit planten als alternatief startmateriaal voor biomaterialen. Mogelijkerwijs kan dit de afhankelijkheid van aardolie verminderen, en de duurzaamheid van productieprocessen verbeteren.

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