Transcription factors regulating terpene synthases in tomato trichomes

Spyropoulou, E.

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Chapter 2

Discovery and characterization of mono- and sesquiterpene synthases from *Solanum lycopersicum*

Eleni A. Spyropoulou, Petra M. Bleeker, Michel A. Haring and Robert C. Schuurink

Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
ABSTRACT

Terpenes constitute a large class of chemical compounds produced by almost all living organisms. In plants they have various functions, ranging from attracting pollinators to acting as defense compounds against pests. The recent release of the genomic sequence of tomato (*Solanum lycopersicum*) enabled the identification of (all) terpene synthases (TPS) involved in the production of these volatile compounds. Here we present the characterization of three novel sesquiterpene synthases and three novel monoterpene synthases from *S. lycopersicum* cv Moneymaker that are expressed in various organs. Two TPS genes, expressed predominately in trichomes, were induced by treatment with jasmonic acid. The products of these six terpene synthases were also analyzed *in vitro*. Our data contribute to the deciphering of the tomato terpenome.

INTRODUCTION

Terpenoids constitute a large class of chemical compounds produced by most, if not all, living organisms. Over 23,000 different terpenoid compounds have been characterized (Sacchettini and Poulter, 1997). Plants produce terpenoids that function in primary metabolism such as phytohormones (abscisic acid, gibberellins, cytokinins and brassinosteroids), are part of photosynthetic pigments (phytol and carotenoids), electron carriers (ubiquinone) or constitute structural components of membranes (phytosterol). However, the majority of plant terpenoids are secondary, or specialized metabolites, present only in a subset of plant lineages. They can be active as direct defensive compounds, such as phytoalexins that accumulate upon
pathogen infection (Akram et al., 2008). In addition, volatile terpenoids can function as indirect defensive compounds by attracting predators or parasitoids of the attacking insect (Walling, 2000). The emission of different terpenoids is induced by insect herbivory (Kant et al., 2004; Olson et al., 2008; Navia-Gine et al., 2009).

Two distinct pathways leading to the universal terpene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) operate in plants. The mevalonate (MVA) pathway produces IPP in the cytosol, which can be converted to DMAPP by IPP isomerase. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway produces plastidial IPP and DMAPP. Head-to-tail elongation of IPP with DMAPP catalyzed by geranyl diphosphate (GPP) synthase or by neryl diphosphate (NPP) synthase lead to the formation of GPP and NPP (the cis-isomer of GPP; Croteau and Karp, 1979), respectively, which are the precursors for monoterpenes. (E,E)-Farnesyl diphosphate (FPP), and (Z,Z)-Farnesyl diphosphate (Kellog and Poulter, 1997) serve as precursors for sesquiterpenes, and are synthesized by (Z,Z)- and (E,E)-FPP synthases, respectively. (E,E,E)-Geranylgeranyl diphosphate (GGPP) synthase catalyzes formation of GGPP, the diterpene precursor. The MEP pathway provides precursors for the synthesis of monoterpenes and diterpenes in plastids, whereas sesquiterpenes are derived from precursors of the MVA pathway in the cytosol. However, exchange of precursor between cytosol and plastids has been reported. Snapdragon flowers can synthesize sesquiterpenes from plastidial isoprenes, indicating transport of IPP from the plastids to the cytosol (Dudareva et al., 2005). More recently it was shown that several sesquiterpenes from wild tomato (Solanum habrochaites) are synthesized in the plastids from plastidial (Z,Z)-FPP (Sallaud et al., 2009).

The prenyl diphosphates are converted to terpenes by the action of terpene synthases (TPSs), a group of structurally and evolutionarily related enzymes (Chen et al., 2011). Induced terpenoid synthesis is often correlated with induced expression of terpene synthases (Herde et al., 2008; Navia-Gine et al., 2009; Zulak et al., 2009). Besides regulation at the level of terpene synthases, induction of
precursor biosynthetic genes has also been reported (Kant et al., 2004; Ament et al., 2004). In vitro, sesquiterpene synthases can often produce monoterpenes when provided with GPP as substrate, and monoterpane synthases can produce sesquiterpenes when provided with FPP. Therefore, subcellular targeting of terpene synthases determines which substrate the terpene synthase encounters and thus which product is made in vivo. For instance, two nearly identical terpene synthases from snapdragon both catalyze the conversion of GPP to linalool and of FPP to nerolidol in vitro. However, only one of these linalool/nerolidol synthases has a transit peptide, localizing the protein to the plastids (Nagegowda et al., 2008).

To be effective against pests and diseases, defensive terpenoid compounds are often produced at the surface of the plant. Trichomes, which are specialized secretory structures on the surface of leaves and stems, contain high levels of terpenes in many species (Chatzivasileiadis et al., 1999; Besser et al., 2009), as well as other secondary metabolites (Fridman et al., 2005; Ben-Israel et al., 2009). Furthermore, many investigations have shown that these compounds, including terpenes, are usually synthesized de novo in the trichomes (Olsson et al., 2009; Maes et al., 2011).

New terpene synthases have been so far discovered by homology-based cloning (van Der Hoeven et al., 2000; van Schie et al., 2007; Portnoy et al., 2008; Jones et al., 2008), by random sequencing of cDNAs (van Der Hoeven et al., 2000; Wang et al., 2008) followed by bioinformatic searches of the resulting EST databases (Keeling et al., 2011) or by genome mining (Aubourg et al., 2002; Martin et al., 2010). However, terpene synthases that are less abundant in a particular organ or structure, or have low sequence similarity to known terpene synthases might not be identified by these methods. Lately the use of massive parallel pyrosequencing of transcripts, termed “RNA-seq”, is becoming more widely used (Wilhelm and Landry, 2009; Schilmiller et al., 2010), since its major advantage above the construction of EST databases is that quantitative expression levels of transcripts are better estimated due to the larger set of data. High-throughput sequencing has also been used for sequencing whole genomes of organisms. The determination of
the sequence of the cultivated tomato genome made it possible to characterize the terpene synthase family (Falara et al., 2011). In combination with RNA-seq approaches (Bleeker et al., 2011) and the available volume of information about TPSs obtained so far by various other techniques, allowed us to construct a more complete picture about the biosynthesis of this very interesting class of compounds. Here we describe the identification and characterization of three novel sesquiterpene synthases and three novel monoterpene synthases from Solanum lycopersicum. Functional expression in E.coli provided information on the proteins encoded by these cDNAs. Determination of tissue-specific expression of the cultivated tomato terpene genes showed that the expression of most of them was highest in tissue containing trichomes. Furthermore, one sesquiterpene and one monoterpene synthase were induced by jasmonic acid treatment, suggesting their involvement in herbivore-induced terpenoid emission.

MATERIALS AND METHODS

Hormone Treatment, RNA Isolation and cDNA synthesis

Tomato plants (Solanum lycopersicum cv Moneymaker C32) were grown in soil in a greenhouse with day/night temperatures of 23°C/18°C and a 16/8 h light/dark regime for 4 weeks. Jasmonic acid (JA; Duchefa, NL) was applied to plants by spraying 1mM solution made with tap water containing 0.05% SilwetL-77 (GE Silicones, VA, USA). Control plants were sprayed with tap water containing 0.05% SilwetL-77. Trichomes were collected 24h later at the bottom of a 50ml tube by vortexing stem pieces frozen in liquid nitrogen and subsequent removal of the stem pieces. All other tissues and organs analyzed were also frozen in liquid N2 and then
the material was ground and total RNA was isolated using Trizol (Invitrogen, Paisley, UK). DNA was removed with DNase (Ambion, Huntingdon, UK) according to the manufacturer’s instructions and cDNA was synthesized from 1.5μg RNA using M-MuLV H’ Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany).

**Quantitative Real Time PCR**

For Q-RT-PCR cDNA equivalent to 100ng total RNA was used as template in 20μl volume and reactions were performed in an ABI 7500 Real-Time PCR System (Applied Biosystems) using an Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Paisley, UK) with the following cycling program: 2min 50°C, 7min 95°C, 45 cycles of 15sec at 95°C and 1min at 60°C, followed by a melting curve analysis. Primer pairs were tested for amplification kinetics and linearity with a standard cDNA dilution curve and new primers were designed if necessary. Expression levels were normalized using *ACTIN* (SGN-U579547) mRNA levels. Effect of JA in gene expression was analyzed in three biological replicates by T-test using PASW Statistics 17.0 (http://www.spss.com). The homogeneity of variance was tested by Levene’s test and values were log transformed before the analysis if necessary.

**Production of recombinant proteins**

*SITPS3, SITPS7* and *SITPS8* open reading frames (ORF) were cloned from a mix of trichome, leaf, stem and root cDNA in pGEM-T easy (Promega, Madison, USA) and verified by sequencing. They were subsequently digested and re-cloned in pET32a (Novagen) downstream of the histidine (His) tag between restriction sites EcoRV (at the ATG site) and SacI (after the stop codon) for *SITPS3* and in pGEX
KG (Guan and Dixon, 1991) downstream of glutathione S-transferase (GST) between restriction sites NcoI (at the ATG site) and SacI (after the stop codon) for SITPS7. SITPS8 was directionally cloned in pET200-TOPO downstream of the histidine (His) tag using the pET TOPO expression kit from Invitrogen (Paisley, UK). All constructs were maintained in E. coli TOP10 cells. For the expression analysis the constructs were transformed freshly into E. coli C41 (D3) cells (Duman-Seignovert et al., 2004). Cells were grown in Terrific Broth with appropriate antibiotics to OD<sub>600</sub> of 0.6 from a pre-culture inoculated from a single colony, incubated at 4°C for 30min and then induced with 1mM isopropyl-β-D-thiogalactoside (IPTG) for 16h at 16°C, shaking at 200rpm. Cells were harvested by centrifugation at 4500rpm for 15min at 4°C. The supernatant was removed and the pellets resuspended in 2-4ml of STS buffer (25mM Hepes-HCl pH 7.2, 10mM MgCl₂, 10% (v/v) glycerol) with added lysozyme (1mg/ml) and protease inhibitors (Complete, Roche, Basel, Switzerland). Cells were incubated on ice for 30min and subsequently sonicated (45sec, output control 3, duty cycle 30). Lysates were centrifuged at 4°C for 25min at 12,000rpm. The supernatants containing the bacterial crude extracts with recombinant proteins were aliquoted, snap frozen in liquid N₂ and stored at -80°C. Cloning of the sesquiterpene synthases and recombinant protein production has been described in Bleeker et al. (2011).

**Functional expression analysis**

Activity assays were performed for the monoterpene synthases in 20ml glass vials in a total volume of 1.5ml containing STS buffer (25mM Hepes-HCl pH 7.2, 10mM MgCl₂, 10% (v/v) glycerol) including 5mM DL-Dithiothreitol (DTT), 150μl of crude protein extract and 1mM of precursors as substrate (GPP; geranyl diphosphate, NPP; neryl diphosphate, (E,E)-FPP; (E,E)-farnesyl diphosphate, (Z,Z)-FPP; 2z-6z-farnesyl diphosphate or GGPP; geranylgeranyl diphosphate;
Echelon Biosciences Incorporated, Salt Lake City, USA). As negative control no precursor was used. For the sesquiterpene synthases activity assays were performed in 20ml glass vials in a total volume of 500µl containing 50mM HEPES pH7.2, 100mM KCl, 7.5mM MgCl₂, 20µM MgCl₂, 5% (v/v) glycerol, 5mM DTT with 50µl protein extract and either 2mM \((E,E)\)-FPP, \((Z,Z)\)-FPP, GPP or GGPP as substrate. Vials were immediately closed with a Teflon lined crimp cap and incubated at 30°C for 1h shaking at 150rpm. Enzyme products were sampled with a Solid Phase Micro Extraction (SPME) fiber for 10min after the vial had been agitated and heated to 50°C. The fiber was desorbed for 1min in an Optic injector port (ATAS GL Int., Zoeterwoude, NL), which was kept at 250°C. Compounds were separated on a DB-5 column (10m x 180µm, 0.18µm film thickness; Hewlett Packard) in an 6890 N gas chromatograph (Agilent, Amstelveen, NL) with a program set to 40°C for 1.5min, ramp to 250°C at 30°C per minute and 250°C for an additional 2.5min. Helium was used as a carrier gas, the column flow was set to 3ml per minute for 2min and to 1.5ml per minute thereafter. Mass spectra were generated with the ion source set to -70V at 200°C and collected with a Time-of-Flight MS (Leco, Pegasus III, St. Joseph, MI, USA) at 1850V with an acquisition rate of 20 scans per second. Because some terpenes, such as germacrenes, are prone to thermal conversion (Colby et al., 1998; Faraldos et al., 2007) the enzyme assays were also extracted with pentane which was injected at 50°C in the injection port. For this, 500µl lysate was assayed in the presence of 5mM DTT and either \((E,E)\)-FPP, \((Z,Z)\)-FPP, GPP or GGPP as a substrate, overlaid with 2ml pentane (Sigma). After one hour of incubation at 30°C the pentane layer was transferred to a 2ml glass vial and concentrated on ice with nitrogen gas, to a final volume of 50µl. Terpenoids were analyzed by injection of 2µl into the Optic injection port (ATAS GL Int.) at 50°C, subsequently heated to 275°C at a rate of 4°C/s followed by gas-chromatography and mass-spectrometry as described by Bleeker et al. (2009). Boiled protein extract could not convert any of the precursors and was taken along as a negative control. Terpene products were identified using standards
when possible or by comparing mass spectra and Kovats Indices (Adams, 2002). The enzymatic assays for SITPS31 from cultivar M82 and the analyses of their products were performed as described in Falara et al. (2011).

**List of primers used**

<table>
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<tr>
<th>Primer name</th>
<th>Sequence 5' -&gt; 3'</th>
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<tr>
<td>SITPS3_QR</td>
<td>CAAACTAATTTTTGGCTCCTCCATAG</td>
</tr>
<tr>
<td>SITPS7_QF</td>
<td>GATTCTATTTAAACGGCATGGAACATC</td>
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**RESULTS**

**Phylogenetic analysis of the tomato terpene synthase genes**

The nearly completed genomic sequence of *S.lycopersicum* has been recently analyzed and a total of 44 terpene synthase (TPS) genes were identified, out of which 29 appear to have uncompromised open reading frames (Falara et al., 2011). A recent classification of TPS genes divides them into seven clades: TPS-a, TPS-b, TPS-c, TPS-d (gymnosperm-specific), TPS-e/f, TPS-g and TPS-h (*Selaginella moellendorffii*-specific; Chen et al., 2011). In Figure 1 the phylogenetic tree of
these 29 TPS is presented, grouped in the different clades. Tomato clade TPS-a genes encode mostly sesquiterpene synthases, whereas TPS-b, TPS-e/f and TPS-g genes typically encode monoterpene synthases. Clade TPS-c contains a diterpene synthase (TPS40; CPS1) and a second gene (TPS41) whose function is still unknown (Falara et al., 2011).

Characterization of TPS-a clade sesquiterpene synthases from S. lycopersicum cv. Moneymaker

Analysis of the genomic sequence of S. lycopersicum showed that the majority of TPS genes in the tomato genome encode sesquiterpene synthases and most of these
putative sesquiterpene synthases belong to the TPS-a clade. In this clade there are seven functional genes: SlTPS9 (previously shown to encode germacrene C synthase (van Der Hoeven *et al.*, 2000), SlTPS12 (previously shown to encode β-caryophyllene/α-humulene synthase; Schilmiller *et al.*, 2010), SlTPS14, SlTPS17, SlTPS31 (TPS31 was formerly known as LeVS2 (GenBank: AAG09949.1), but its function was not established), SlTPS32 and SlTPS36.

To investigate in which tissues the active *S. lycopersicum* sesquiterpene synthases SlTPS9, SlTPS12, SlTPS14, SlTPS17 and SlTPS31 were transcribed (SlTPS32 and SlTPS36 have been described in Falara *et al.*, 2011), we dissected mature *S. lycopersicum* plants for RNA isolation and subsequent quantitative RT-PCR. Expression of all terpene synthases was lowest in fruits (Fig.2). SlTPS9 and SlTPS17 displayed comparable expression patterns, with highest expression in stem trichomes (Fig.2a and 2d). SlTPS12 expression was highest in leaves (Fig.2b), probably in the leaf trichomes as shown for SlTPS12 in the cultivar M82 (Schilmiller *et al.*, 2010). SlTPS14 was expressed mostly in the roots (Fig.2c). Expression of SlTPS31 was very low overall, but highest in stem trichomes and leaves (Fig.2e), possibly in their trichomes.

Since jasmonic acid (JA) treatment resulted in the induction of SlMTS1 (SlTPS5) in stem trichomes (van Schie *et al.*, 2007), we tested whether expression of terpene synthases SlTPS9, SlTPS12, SlTPS17 or SlTPS31 that are expressed in trichomes, was induced by JA treatment. Moneymaker plants that were 4 weeks old were treated with 1mM JA or sprayed with control solution, RNA was isolated after 24 hours, and the levels of transcripts were measured by Q-RT-PCR (Fig.2). Only the expression of SlTPS31 was significantly induced by JA treatment approximately 3-fold, comparable to the positive control SlMTS1 (data not shown). Interestingly, expression of SlTPS17 appeared to be reduced roughly 2-fold in JA-treated plants (p = 0.059).
Figure 2. Tissue specific expression and JA induction of *SlTPS*s. Relative transcript levels for (a) *SlTPS9*, (b) *SlTPS12*, (c) *SlTPS14*, (d) *SlTPS17* and (e) *SlTPS31* as determined by Q-RT-PCR. Mean values (+SE) of 3 biological replicas are shown, normalized for *Actin* expression. *L*: leaf, *WS*: whole stem, *BS*: bald stem, *T*: stem trichomes, *R*: root, *Fr*: fruit, *Fl*: flower, *C*: control and *JA*: jasmonic acid induced stem trichomes. Asterisks indicate significant difference (*p < 0.05*) according to T-test. *ns*: not significant.
Proteins isolated from *E.coli* cells expressing the putative sesquiterpene synthases were assayed for their ability to convert (E,E)-FPP into sesquiterpenes. The protein encoded by the Moneymaker allele of SITPS9 produced germacrene C, and minor amounts of germacrene A, B and D (Fig.3a), similar to the protein encoded by this locus in cultivar VFNT Cherry tomato. Recombinant SITPS12 protein had β-caryophyllene/α-humulene synthase activity (Fig.3b), similar to the protein encoded by CAHS in cultivar M82. SITPS17 produced mostly valencene from (E,E)-FPP and also an unidentified sesquiterpene, besides azulene and α- and β-farnesenes (Fig.3c). SITPS31 predominantly made viridiflorene from (E,E)-FPP (Fig.3d).

**Figure 3.** Enzymatic activity of recombinant SITPSs assayed with (E,E)-FPP. GC-MS chromatograms of products produced by (a) SITPS9, (b) SITPS12, (c) SITPS17 and (d) SITPS31 ectopically expressed in *E.coli*, assayed with (E,E)-FPP. SITPS9 products were extracted in pentane, SITPS12, 17 and 31 products were measured by Solid Phase Microextraction (SPME) sampling. Sesquiterpene peaks: 1 germacrene D; 2 germacrene A; 3 germacrene C 4 germacrene B; 5 β-caryophyllene; 6 α-humulene; 7 (E)-β-farnesene; 8 γ-gurjunene; 9 valencene; 10 (E,E)-α-farnesene; 11 viridiflorene; asterisk unidentified. The chromatogram shows the detector response for the terpene-specific ion mass 93 for SITPS9-12-17 and total ion current for SITPS31.
Remarkably, all recombinant proteins that could use \((E,E)\)-FPP as substrate could also use \((Z,Z)\)-FPP as substrate (Fig.4). SITPS9 made mostly germacrene C from both \((E,E)\)-FPP as well as \((Z,Z)\)-FPP. SITPS12 made curcumene and \(\beta\)-bisabolene from \((Z,Z)\)-FPP and SITPS17 made various bisabolenes. SITPS14 could use either \((E,E)\)-FPP or \((Z,Z)\)-FPP equally well to make mostly \((Z)\)-\(\gamma\)-bisabolene or \(\alpha\)-bisabolene, respectively (Falara et al., 2011).

![Figure 4. Enzymatic activity of recombinant SITPS9, 12 and 17 assayed with \((Z,Z)\)-FPP. GC-MS chromatograms of sesquiterpenes formed by \(S.\ lycopersicum\) (Moneymaker) terpene synthases ectopically expressed in \(E.\ coli\), assayed with \((Z,Z)\)-FPP. SITPS9 products were extracted in pentane, SITPS12 and SITPS17 products were measured by Solid Phase Microextraction (SPME) sampling. (a) SITPS9 (b) SITPS12 and (c) SITPS17. Sesquiterpene peaks: 1 \(\alpha\)-humulene; 2 germacrene A; 3 \(\beta\)-bisabolene; 4 germacrene C; 5 germacrene B; 6 \(\gamma\)-curcumene; 7 \((Z)\)-\(\gamma\)-bisabolene; 8 \((Z)\)-\(\alpha\)-bergamotene; 9 \((E)\)-\(\gamma\)-bisabolene; 10 \((E)\)-\(\alpha\)-bergamotene; asterisk unidentified. The chromatogram shows the detector response for the terpene-specific ion mass 93.](image)

The sesquiterpene synthases were assayed for monoterpene synthase activity with GPP as substrate and could all convert this precursor to a range of most simple monoterpenes (Fig.5). SITPS9 and SITPS12 produced mostly \(\beta\)-myrcene, limonene
and low amounts of terpinolene. In the assays with SlTPS17 additionally the monoterpenes (Z)-β-ocimene, (E)-β-ocimene and linalool were produced (Fig.5). No products were formed with GGPP as substrate (data not shown).

**Figure 5.** Enzymatic activity of recombinant SlTPS9, 12 and 17 assayed with GPP. GC-MS chromatograms of monoterpenes formed by *S. lycopersicum* (Moneymaker) terpene synthases ectopically expressed in *E. coli*, assayed with GPP. Products were measured by Solid Phase Microextraction (SPME) sampling. (a) SlTPS9 (b) SlTPS12 and (c) SlTPS17. Monoterpene peaks: 1 α-pinene; 2 β-myrcene; 3 δ-2-carene; 4 limonene; 5 α-terpinene; 6 terpinolene; 7 (E)-β-ocimene; 8 (Z)-β-ocimene; 9 γ-terpinene; 10 linalool; asterisk unidentified. The chromatogram shows the detector response for the terpene-specific ion mass 93.

**Characterization of TPS-b clade monoterpane synthases from *S. lycopersicum* cv. Moneymaker**

Homology searches of *SlMTS1* on chromosome 1 of *Solanum lycopersicum* (http://solgenomics.net/) led to the discovery of seven additional monoterpene synthases (*SlTPS1, 2, 3, 6, 7, 8 and 22*) that all belong to clade b of the terpene synthases (TPS). In total, there have been 13 monoterpane synthases identified in TPS-b (*Falara et al.*, 2011). *SlMTS1* and *SlMTS2* (renamed *SlTPS5* and *SlTPS4*)
have been previously characterized (van Schie et al., 2007). Eight more were non-functional or mutated (Falara et al., 2011), leaving \textit{SlTPS3}, \textit{SlTPS7} and \textit{SlTPS8} on chromosome 1 to be further characterized.

Q-RT-PCR was performed on cDNA from different \textit{S. lycopersicum} cv. Moneymaker tissues in order to establish the expression pattern of these monoterpenene synthases. \textit{SlTPS3} was predominately expressed in isolated stem trichomes and was also significantly induced by JA (Fig.6a). \textit{SlTPS7} was present in all the tissues examined except fruits, with highest expression in flowers and leaves and was not significantly induced by JA (Fig.6b). Finally, \textit{SlTPS8} was predominately expressed in roots and was not induced by JA in the trichomes (Fig.6c).

\textbf{Figure 6.} \textit{Tissue specific expression and JA induction of SlTPSs.} Relative transcript levels for (a) \textit{SlTPS3}, (b) \textit{SlTPS7} and (c) \textit{SlTPS8} as determined by Q-RT-PCR. Mean values (+SE) of 3 biological replicas are shown, normalized for \textit{Actin} expression. \textit{L;} leaf, \textit{WS;} whole stem, \textit{BS;} bald stem, \textit{T;} stem trichomes, \textit{R;} root, \textit{Fr;} fruit, \textit{Fl;} flower, \textit{C;} control and \textit{JA;} jasmonic acid induced stem trichomes. Asterisks indicate significant difference (* \(p < 0.05\)) according to T-test. \textit{ns;} not significant.
The enzymatic activity of the proteins was investigated in crude *E.coli* extracts. Recombinant SITPS3 catalyzed the formation of β-myrcene, terpinolene and limonene as well as camphene, geranial and linalool when using GPP as substrate. The same products were produced when using NPP as substrate except geranial and linalool (Fig.7a). SITPS7 recombinant protein produced β-myrcene and limonene as well as (dihydro) carveol and linalyl-acetate (the acetate ester conversion of linalool) from GPP. When provided NPP as substrate, SITPS7 produced the same compounds as with GPP including two unidentified terpenes (Fig.7b). Finally, the full-length *SITPS8* cDNA was also expressed in *E.coli* and the resulting protein was found to produce 1,8-cineole, as well as β-myrcene and limonene from GPP and NPP (Fig.7c).

**Figure 7.** Enzymatic activity of recombinant SITPSs. GC-MS chromatograms of products produced by (a) SITPS3, (b) SITPS7 and (c) SITPS8 ectopically expressed in *E.coli*,
assayed with GPP and NPP and measured by Solid Phase Microextraction (SPME) sampling. Monoterpene peaks: 1 camphene; 2 β-myrcene; 3 limonene; 4 terpinolene; 5 geranial; 6 linalool; 7 (dihydro) carveol; 8 linalyl-acetate; 9 1,8-cineole; asterisk unidentified. The chromatogram shows the detector response for the terpene-specific ion mass 93.

Both (E,E)-FPP and (Z,Z)-FPP could be used as substrate by SITPS7, which produced (E)-α-bergamotene, (E)-β-farnesene and (E,E)-α-farnesene with the canonical FPP and (Z,Z)-α-farnesene with zFPP (Fig.8a). SITPS8 could only use (Z,Z)-FPP as substrate, producing (Z)-α-bergamotene, γ-curcumene and α-bisabolene (Fig.8b). SITPS3 did not form any products when assayed with (E,E)- and (Z,Z)-FPP and none of the monoterpene synthases tested could use GGPP as substrate (data not shown).

Figure 8. Enzymatic activity of recombinant SITPSs. GC-MS chromatograms of products produced by (a) SITPS7 and (b) SITPS8 ectopically expressed in E.coli, assayed with zFPP or FPP and measured by Solid Phase Microextraction (SPME) sampling. Sesquiterpene peaks: 1 (Z,Z)-α-farnesene; 2 (E)-α -bergamotene; 3 (E)-β-farnesene; 4 (E,E)-α-farnesene; 5 (Z)-α-bergamotene; 6 γ-curcumene; 7 α-bisabolene. The chromatogram shows the detector response for the terpene-specific ion mass 93.
An overview of all the products produced by the TPSs when assayed with the various precursors is given in Table 1.

**Table 1.** TPS products of *in vitro* enzymatic assays. Terpene products of the various TPSs assayed with GPP, NPP, FPP or zFPP. GGPP was assayed for all TPSs except SITPS31 and could not be used as substrate by any of the enzymes. ND; not detected, NA; not assayed.

<table>
<thead>
<tr>
<th>TPS nr</th>
<th>GPP</th>
<th>NPP</th>
<th>FPP</th>
<th>zFPP</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>camphene β-myrcene limonene terpinolene geranial linalool</td>
<td>camphene β-myrcene limonene terpinolene</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
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<td>β-myrcene (dihydro)carveol linalyl-acetate</td>
<td>(E)-α-bergamotene (E)-β-farnesene (E,E)-α-farnesene</td>
<td>(Z,Z)-α-farnesene</td>
</tr>
<tr>
<td>8</td>
<td>β-myrcene limonene 1,8-cineol</td>
<td>β-myrcene limonene 1,8-cineol</td>
<td>ND</td>
<td>(Z)-α-bergamotene γ-curcumene α-bisabolene</td>
</tr>
<tr>
<td>9</td>
<td>α-pinene β-myrcene δ-2-carene limonene α-terpinene terpinolene</td>
<td>NA</td>
<td>germacrene D germacrene A germacrene C germacrene B</td>
<td>α-humulene β-bisabolene germacrene A germacrene C germacrene B</td>
</tr>
<tr>
<td>12</td>
<td>β-myrcene limonene (E)-β-ocimene terpinolene</td>
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<td>β-caryophyllene α-humulene</td>
<td>β-bisabolene γ-curcumene (Z)-γ-bisabolene</td>
</tr>
<tr>
<td>17</td>
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<td>NA</td>
<td>(E)-β-farnesene γ-gurjunene valencene (E,E)-α-farnesene</td>
<td>β-bisabolene (Z)-γ-bisabolene (Z)-α-bergamotene (E)-γ-bisabolene</td>
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<td>31</td>
<td>NA</td>
<td>NA</td>
<td>viridiflorene</td>
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**DISCUSSION**

The presence and biosynthesis of monoterpenes and sesquiterpenes in *S. lycopersicum* has been previously investigated (Colby *et al*., 1998; van Der Hoeven *et al*., 2000; van Schie *et al*., 2007; Bleeker *et al*., 2009; Schilmiller *et al*., 2010). Van Schie *et al.* (2007) used a degenerate primer approach to identify two
monoterpene synthases (*LeMTS1* and *LeMTS2*, renamed *SlTPS5* and *SlTPS4*). *SlTPS5* catalyzed the formation only of linalool from GPP and (E)-nerolidol from FPP, whereas *SlTPS4* generated several monoterpene products from GPP and had no sesquiterpene synthase activity. Colby *et al.* (1998) isolated a cDNA from the cultivar VNFT Cherry and showed that it encodes germacrene C synthase and van der Hoeven *et al.* (2000) showed that synthesis of germacrene C, β-caryophyllene and α-humulene was controlled by a locus on chromosome 6. Using the VFNT cDNA of germacrene C as a probe to screen cDNA libraries, van der Hoeven *et al.* (2000) also isolated two cDNAs, designated SSTLE1 and SSTLE2, that were very similar to VNFT germacrene C. Based on the sequences of both cDNAs, we can conclude that they are different alleles (or one of them may be a cloning artifact) of *SlTPS9*, which we now know to be located on chromosome 6 (Falara *et al.*, 2011).

Subsequently, Schilmiller *et al.* (2010), using a proteomic approach, identified a protein sequence in *S.lycopersicum* cv. M82 trichomes that catalyzed the formation of β-caryophyllene and α-humulene, and was therefore designated as CAHS (β-caryophyllene/α-humulene synthase). Our data show that *SlTPS12* encodes CASH.

With the recent mining of the tomato genome more TPSs were identified, some of which have been characterized here. The primarily root-expressed *SlTPS14* (Fig.2c) was shown to encode a bisabolene synthase (Falara *et al.*, 2011). Bisabolene has been reported before to be the product of root (-specific) sesquiterpene synthases (Ro *et al.*, 2006; Köllner *et al.*, 2008; Valdez-Calderon *et al.*, 2011). In *Arabidopsis*, *AtTPS12* and *AtTPS13* form mainly (Z)-γ-bisabolene and are shown to be wound-inducible, suggesting an involvement in the protection of the *Arabidopsis* roots from bacterial and/or fungal infections. However, (Z)-γ-bisabolene could not be detected in *Arabidopsis* tissues or headspace and it was suggested that it could be escaping detection due to minute amounts or further metabolized (Ro *et al.*, 2006). In maize, the β-bisabolene synthases *ZmTPS6* and *ZmTPS11* are shown to be expressed both in roots and leaves and induced in the roots by herbivore damage in the leaves. Furthermore, β-bisabolene is only
detected in the roots, suggesting a long-distance signaling cascade (Köllner et al., 2008). In *S. lycopersicum* bisabolene has not been detected, at least in the headspace or leaf extracts (Bleeker et al., 2009). Here, we have only checked induction of TPS expression by JA (a hormone that induces defenses similar to those of wounding) in stem trichomes, where the bisabolene synthase *SlTPS14* is not expressed, but it could be conceived that in tomato it has a similar function as in *Arabidopsis* or maize. *SlTPS17* gave multiple products, but mainly valencene (Fig.3c), which is typically a flavor and aroma compound of citrus plants (Sharon-Asa et al., 2003). *SlTPS17* is almost exclusively expressed in the trichomes and was down-regulated by JA, although not at a statistically significant level (p=0.059; Fig.2d). Even though nothing has been reported about an effect of valencene in the tomato defense system, it has been shown that transgenic *Citrus sinensis* fruit peel with a reduced accumulation of limonene was less attractive to the citrus pest medfly compared to control fruit (Rodriguez et al., 2011). It is not improbable that terpene biosynthesis is down-regulated as part of the plant’s response to pests and pathogens. Finally, *SlTPS31*, which is a very lowly expressed gene in all the tissues (Fig.2e), makes viridiflorene as a single product (Fig.3d). Viridiflorene has been described before as part of the essential oil mixture from various plant species like *Atriplex*, exhibiting, as a mix, antioxidant and cytotoxic activity (Ho et al., 2010; Rodriguez et al., 2010; Beattie et al., 2011). *SlTPS31* was up-regulated in stem trichomes by treatment with JA, indicating a potential role in the plant’s induced defenses.

Interestingly, the sesquiterpene synthases tested here (*SlTPS9, 12 and 17*) accepted both *(E,E)-FPP* (Fig.3) and *(Z,Z)-FPP* (Fig.4) as substrates. Our observations of sesquiterpene synthases reacting with substrates other than the canonical *(E,E)-FPP* adds additional weight to previous intimations of the flexibility of these enzymes. For example, Jones et al. (2011) reported a cytosolic sesquiterpene synthase from sandalwood able to use both *(E,E)- and *(Z,Z)-isomers of FPP to produce similar compounds. Such observations from multiple species suggest that these properties...
of the enzyme are not in vitro artifacts, but might have in vivo relevance. However, whether (Z,Z)-FPP is available in the cytosol of the Solanum trichomes is not yet known, although evidence has been presented that S.habrochaites accession LA1777 produces (Z,Z)-FPP in the plastids (Sallaud et al., 2009). The sesquiterpene synthases described here were also able to convert GPP to (mono) terpene products (Fig.5) indicating a level of plasticity of the active pocket of the protein. While some sesquiterpene synthases are restricted to the use of a single substrate even with regard to precursors of the same size (E,E)-FPP or (Z,Z)-FPP; Besser et al., 2009), there are examples of terpene synthases that can accommodate both GPP as well as (E,E)-FPP in a productive manner (van Schie et al., 2007) or even GGPP as a third possible substrate (Arimura et al., 2008; Martin et al., 2010). It has been proposed that trace amounts of GPP are present in the cytosol, and minor amounts of (E,E)-FPP are available to plastid-localised terpene synthases (Aharoni et al., 2006; Wu et al., 2006). Hence, the active site plasticity of some TPS to accommodate isoprenyl diphosphates of different chain length may be biologically relevant. The newly identified monoterpene synthase SITPS3 seems to be less promiscuous enzyme, as it can only accept GPP or NPP, the (Z)-isomer of GPP, as substrates. SITPS3, expressed primarily in the trichomes, was significantly induced by treatment with JA. In vitro, recombinant protein produced β-myrcene, limonene, terpinolene, camphene, geranial and linalool. Although in tomato emission of β-myrcene and limonene have not been correlated with repellence to whiteflies (Bleeker et al., 2009) or spider mites (Ament et al., 2004), for example in Arabidopsis, myrcene is one of the (minor) constituents of AtTPS03 recombinant protein product-mix and it is shown that AtTPS03 is up-regulated in leaves as a response to mechanical wounding and treatment with JA (Fäldt et al., 2003). It is therefore possible that terpinolene or camphene, or perhaps more likely a blend of all the produced compounds, plays a role in the plant’s defenses. In Cupressus lusitanica (white cedar) cell cultures elicited with a yeast extract, β-thujaplicin was
one of the major components of the ether extract of the cells. It was shown that the time course for terpinolene synthase activity coincided with β-thujaplicin biosynthesis, indicating that most of terpinolene is metabolized to β-thujaplicin rather than emitted (De Alwis et al., 2009). Hence it is also conceivable that the produced compounds of SITPS3 (or other TPSs) are further metabolized in planta, and it is these final products that exhibit a role in the plant’s defense. SITPS7, which is expressed in various tissues, catalyzes the formation of β-myrcene and limonene from GPP as shown before (Falara et al., 2011), however in this assay it also catalyzed the formation of (dihydro) carveol and linalyl-acetate. (Dihydro) carveol or its simpler counterpart, carveol, was most likely formed as a product of limonene hydroxylation (McCaskill and Croteau, 1997) and linalyl-acetate could be conversion of the precursors by the crude extracts. Purified protein was not tested. Interestingly, SITPS7 could also use (E,E)- and (Z,Z)-FPP as substrates, producing an array of farnesenes as well as (E)-α-bergamotene. SITPS8 is expressed mainly in the roots and produces 1,8-cineole, β-myrcene and limonene from GPP and NPP. 1,8-cineole is a compound reported before to be either formed directly by a root (-specific) terpene synthase (Chen et al., 2004) or be part of essential oil mixtures from roots (Shafaghat et al., 2006). In Arabidopsis it was shown that 1,8-cineole was released from the roots after bacterial infection or damage by a root-feeding insect (Steeghs et al., 2004). Here we focused on induction of TPSs in stem trichomes, where SITPS8 shows minimal expression, and treatment with JA did not result in up-regulation of this gene in this tissue (Fig.6c), however it is possible that induction occurs in the roots. SITPS8 could produce also sesquiterpene products when assayed with (Z,Z)-FPP.

Only some of the terpene products of the analyzed TPSs have been previously detected in the headspace of undamaged Moneymaker plants (Bleeker et al., 2009). Camphene, 1,8-cineole, γ-gurjunene, valencene and viridiflorene have not been previously reported to be emitted by Moneymaker plants, although for example α-
gurjunene has been measured from the emissions of *S. lycopersicum* cv. Castlemart undamaged plants (Ament et al., 2004). Often the presence of many, but not all of the terpenes observed in the respective glands can be explained by detection of terpene synthase activity in the same tissue. Since some terpenes are made by more than one terpene synthases in the same species, or conversely, since one terpene synthase can make more than one product even from a single precursor, it is difficult to determine the direct contribution of each of them to the observed mixture even when the expression levels of individual terpene synthases are examined in detail, and it is also challenging to estimate the conditions and timing under which each product is synthesized and can be detected *in planta*.

When terpene biosynthesis is seen as a whole, it is noteworthy that despite the fact that monoterpenes appear to dominate *S. lycopersicum* volatile emissions (Buttery et al., 1987; Ament et al., 2004; Bleeker et al., 2009), the majority of TPS genes mined from the tomato genome are sesquiterpene synthases (Falara et al., 2011). In the *S. lycopersicum* cv. Moneymaker stem trichome transcriptome (see Chapter 5) several SlTPSs can be identified, most of which encode sesquiterpene synthases (Table 2). However, the majority of terpenes present in Moneymaker trichomes are monoterpenes (Table 3). Although transcript abundance need not be translated into protein abundance, another explanation for the low sesquiterpene content of *S. lycopersicum* might be found in low precursor biosynthesis. Quantitative RT-PCR on cDNA derived from stem trichomes of *S. lycopersicum* and *S. habrochaites* LA1777, which produces copious amounts of sesquiterpenes, showed that expression of HMG-CoA reductase, a key enzyme in the MEP pathway, is approximately 7-fold higher in *S. habrochaites* (Besser et al., 2009).

<table>
<thead>
<tr>
<th>TPS nr</th>
<th>Contig nr</th>
<th>Transcript length (bases)</th>
<th>Reads trichome</th>
<th>JA induction (fold)</th>
<th>Main product(s) of in vitro enzymatic assay</th>
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<td>2099</td>
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<td>7</td>
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<td>2186</td>
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<td>1</td>
<td>3</td>
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<td>8</td>
<td>10432</td>
<td>264</td>
<td>442</td>
<td>-</td>
<td>see Table 1</td>
</tr>
<tr>
<td>9 (SST1)</td>
<td>3548</td>
<td>2011</td>
<td>13286</td>
<td>-</td>
<td>germacreneC&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>12</td>
<td>17073</td>
<td>407</td>
<td>15</td>
<td>-</td>
<td>β-caryophyllene/ α-humulene&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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<td>16</td>
<td>10687</td>
<td>1868</td>
<td>95</td>
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<td>10174</td>
<td>1190</td>
<td>20</td>
<td>-</td>
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<td>14448</td>
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<td>211</td>
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<tr>
<td>20 (PHS1)</td>
<td>10263</td>
<td>1148</td>
<td>602</td>
<td>-</td>
<td>β-phellandrene&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>23265</td>
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<td>ent-kaurene&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>4</td>
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<td>3672</td>
<td>2368</td>
<td>375</td>
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Table 3. Sesqui- and mono-terpenes present in trichomes of *S.lycopersicum* cv. Moneymaker (compiled from Bleeker *et al*., 2009).

<table>
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<th>Sesquiterpenes</th>
<th>Monoterpenes</th>
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</thead>
<tbody>
<tr>
<td>azulene</td>
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</tr>
<tr>
<td>α-copaene</td>
<td>2-carene</td>
</tr>
<tr>
<td>caryophyllene</td>
<td>4-carene</td>
</tr>
<tr>
<td>γ-elemene &lt;sup&gt;*&lt;/sup&gt;</td>
<td>linalool</td>
</tr>
<tr>
<td>α-humulene</td>
<td>limonene</td>
</tr>
<tr>
<td>germacrene C</td>
<td>α-phellandrene</td>
</tr>
<tr>
<td>germacrene D</td>
<td>β-phellandrene</td>
</tr>
<tr>
<td>cuparene</td>
<td>α-terpinene</td>
</tr>
<tr>
<td></td>
<td>γ-terpinene</td>
</tr>
<tr>
<td></td>
<td>terpinolene</td>
</tr>
<tr>
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<td>p-cymene</td>
</tr>
<tr>
<td></td>
<td>pinene</td>
</tr>
<tr>
<td></td>
<td>(2)-β-ocimene</td>
</tr>
<tr>
<td></td>
<td>(E)-β-ocimene</td>
</tr>
</tbody>
</table>

<sup>*</sup>could be produced by conversion of germacrene

Jasmonic acid (JA) is a hormone known to be involved in plant defenses (Ballare, 2011). In cultivated tomato, it has been shown that expression of *SIMTS1* (*SITPS5*; van Schie *et al*., 2007) and emission of TMTT, a homoterpane derived from geranyllinalool (Ament *et al*., 2006), increase approximately 2-fold upon treatment with JA. Here we have tested the effect of JA by Q-RT-PCR on all the analyzed terpene synthases that showed expression in trichomes. Only two terpene synthases (*SITPS3* and *SITPS31*) were induced by the treatment and one (*SITPS17*) seemed to be down-regulated by JA, although not at a statistically significant level (discussed
earlier). Furthermore, we were able to obtain an overview of the trichome-expressed SlTPSs and their regulation or not by JA (Table 2). The data were acquired by expression profiling (Illumina GA II) of JA-treated and control stem trichomes of 4-week-old Moneymaker plants and are described more analytically in Chapter 5. Interestingly, out of the 14 terpene synthases found in the stem trichome transcriptome only five are induced, out of which four produce monoterpenes.

In conclusion, the release of the tomato genomic sequence allowed the identification of (all) terpene synthases and the characterization of the respective genes and proteins. Here we focused on a subset of SlTPS that we cloned from cultivar Moneymaker, focusing mostly on those found in the trichomes. We analyzed the gene expression patterns, investigated whether they are induced by JA and made recombinant proteins to test the enzymatic activity of the TPSs in vitro. We were able to correlate the expression of the synthases with the products formed in the trichomes for a few terpenes and we showed that many could also use precursors other than the canonical ones to make various products. It goes without saying that as a community we are only starting to understand the tremendous complexity and variety of the tomato terpene synthase family.

ACKNOWLEDGEMENTS

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