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Identification and characterization of two bisabolene synthases from linear glandular trichomes of sunflower (*Helianthus annuus* L., Asteraceae)

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ABSTRACT

Sunflower is known to produce a variety of bisabolene-type sesquiterpenes and accumulates these substances in trichomes of leaves, stems and flowering parts. A bioinformatics approach was used to identify the enzyme responsible for the initial step in the biosynthesis of these compounds from its precursor farnesyl pyrophosphate. Based on sequence similarity with a known bisabolene synthases from *Arabidopsis thaliana* AtTPS12, candidate genes of *Helianthus* were searched in EST-database and used to design specific primers. PCR experiments identified two candidates in the RNA pool of linear glandular trichomes of sunflower. Their sequences contained the typical motifs of sesquiterpene synthases and their expression in yeast functionally characterized them as bisabolene synthases. Spectroscopic analysis identified the stereochemistry of the product of both enzymes as (*Z*)-γ-bisabolene. The origin of the two sunflower bisabolene synthase genes from the transcripts of linear trichomes indicates that they may be involved in the synthesis of sesquiterpenes produced in these trichomes. Comparison of the amino acid sequences of the sunflower bisabolene synthases showed high similarity with sesquiterpene synthases from other Asteracean species and indicated putative evolutionary origin from a β-farnesene synthase.

1. Introduction

Terpenoids, with more than 40,000 known substances, represent the largest group of plant metabolites and have an important function in plant defense (Kumari et al., 2013). They play an ecological role as components of resins, milky latexes and oils. Other members of this class are used as pigments or fragrances and thus are involved in attracting insects to disperse pollen (Heldt, 2003). For humans, terpenoids have a considerable economical, pharmacological and agricultural value (Kessler and Baldwin, 2001; Vranova et al., 2012), and recent studies have found their uses as advanced bio-fuels (Peralta-Yahya et al., 2011). Despite their enormous diversity in structures and functions, all terpenoids are formed from the same basic five-carbon unit, hence being formally understood as oligomers of the hydrocarbon IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethyl allyl diphosphate) (Ružička, 1953).

C_{15} terpenoids, namely sesquiterpenes, with their several thousand representatives and more than 300 different skeletons represent the largest group of terpenes (Connolly and Hill, 1991; Breitmaier, 2005). Insecticidal, anti-microbial, anti-tumor and anti-inflammatory properties belong to their wide range of bioactivities (Li-Weber et al., 2002; Majdi et al., 2011; Trusheva et al., 2010).

For the biosynthesis of sesquiterpenes, sesquiterpene synthases (STS) are responsible, which use the acyclic farnesyl pyrophosphate (FPP) as a substrate (Cane, 1990). STS have a typical size of 550–580 amino acids and show highly conserved sequence motifs (Degenhardt et al., 2009). Among plant STSs, the amino acid sequence accordance is very high, as was shown by a comparison of the genomic organization of terpene synthase (TPS) genes from different plant families (Trapp and Croteau, 2001). Through different cyclisation reactions of the substrate FPP, several distinct carbo-cations are formed and like that an exceptional variety of sesquiterpenes arise (Cane and Iyengar, 1979; Cane et al., 1981; Cane and Yang, 1994; Cane and Tandon, 1995; Alchanati et al., 1998; Heldt, 2003; Degenhardt et al., 2009).

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After a 1,6-cyclization of an nerolidyl cation, a bisabolyl cation is formed. This can be further converted by a deprotonation to produce (*E*)-α-bisabolene, β-bisabolene or γ-bisabolene (Davis and Croteau, 2000). These basic bisabolene-type structures can be further modified by cytochrome P450 monooxygenases to form a diverse array of bisabolenes, which are widely distributed in the plant kingdom and are found in many essential oils. Such essential oils are often produced and stored in specialized plant tissues like glandular trichomes, and thus these tissues bear a high potential to study the biosynthesis of these natural compounds (Agren and Schemske, 1994; Ascensao and Pais, 1998; Duke et al., 2000).

Sunflower possesses two types of glandular trichomes, the LGT (linear glandular trichomes) and the CGT (capitate glandular trichomes) (Spring et al., 1987, 1992; Aschenbrenner et al., 2013; Aschenbrenner, 2014). In addition to their morphological distinctiveness, these two types of trichome also differ in their localization on the plant, development and metabolic composition. The CGT of sunflower are well known for the biosynthesis of sesquiterpene lactones, sometimes in combination with flavonoids (Spring and Bienert, 1987; Spring and Schilling, 1989; Göpfert et al., 2006). On the other hand, the LGT were found to accumulate bisabolene-type sesquiterpenes (Spring et al., 1992), namely the glandulones A, B and C (Fig. 1). This was the first identification of such compounds within the Helianthinae, later on followed by the purification of so-called heliannanes, another bisabolene-type of sesquiterpenes with potential allelochemical properties in sunflower (Fig. 1; Macias et al., 1993, 1994, 1999, 2002) and additional compounds of this type were recently shown to be stored in LGT (Spring et al., 2015). For the biosynthesis of heliannuols, Macias et al. (1994) proposed the oxidative conversion of γ-bisabolene into a quinone structure before subsequent reactions lead to the formation of seven- or eight-membered ring typical for this type of sesquiterpene. The tissue specific occurrence of heliannuols remained unknown until their localization in LGT was shown recently (Aschenbrenner, 2014; Spring et al., 2015). These substances appear to be a very specific group of sunflower compounds, and they showed some interesting bioactivities (Spring et al., 1992; Macias et al., 1999).

With respect to the biosynthesis of bisabolene-type sesquiterpenes, some studies on *Arabidopsis thaliana* or *Abies grandis* have been carried out (Ro et al., 2006b; Bohlmann et al., 1998a), but no information is yet available on bisabolene synthases in LGT of sunflower or any other Asteraceae. Therefore, the aim of the present study was to screen transcripts of sunflower LGT for the presence of putative bisabolene synthases. For this purpose, candidate genes from an RNA-pool of LGT were selected by sequence homologies through a database search (GenBank, NCBI). In a second step, the functional characterization of the candidate genes was carried out using a yeast strain engineered to abundantly produce STS substrate, FPP (Ro et al., 2006a, 2008).

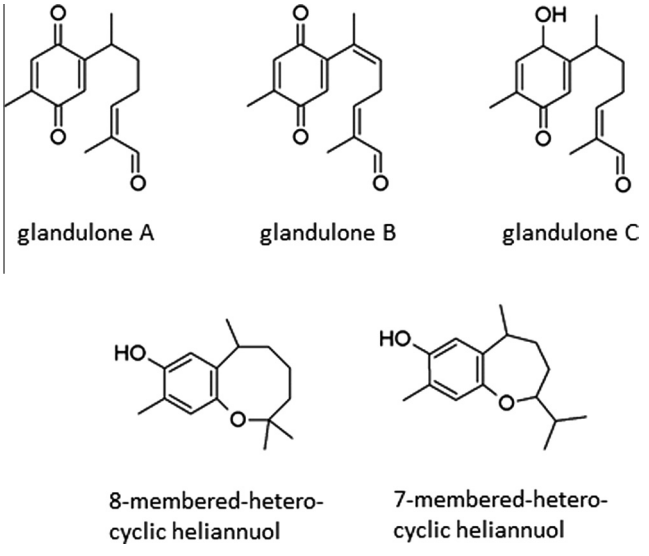

Fig. 1. Bisabolene-type sesquiterpenes reported from sunflower *Helianthus annuus* (Spring et al., 1992; Macias et al., 1999).

2. Results

2.1. Isolation of putative bisabolene synthases

To identify gene candidates for possible bisabolene synthase activity, a homology screening (BLAST analysis) was performed on the public *Helianthus* EST (expressed sequence tag) database (www.ncbi.nlm.nih.gove/genbank/). As a query, the previously identified *Arabidopsis* bisabolene synthase (*AtTPS12*, NM_117401.2) was used (Ro et al., 2006b).

From the EST sequences which show high sequence similarity to AtTPS12 and other STSs, primers were designed and used for a homology-based PCR screening in the cDNA pool of sunflower LGT. For this purpose LGT were harvested manually under a dissecting microscope in order to avoid contamination with cells from other plant tissues. A fragment of a candidate gene (named as HaTPS12) was amplified, and sequence comparison by database research (GenBank, NCBI) revealed sequence similarity with known sesquiterpene synthases (STSs). By means of rapid-amplification of cDNA ends (RACE)-experiments, the full open reading frame (ORF) of 1698-bp of the putative bisabolene synthase was established, encoding 565 amino acids. PCR-amplification of the complete ORF was performed, and the amplified products were cloned in *E. coli* for subsequent sequencing. Sequencing of several clones revealed the presence of two potential isoforms (named HaTPS12_K7 and HaTPS12_K11) of the HaTPS12 with the same length, but differing in five amino acids (Fig. 2). The calculated molecular weight was 66.49 kDa for HaTPS12_K7 and 66.53 kDa for HaTPS12_K11, which is within the typical mass range of plant STSs (Wallaart et al., 2001; Back and Chappell, 1995). Both genes encoded for conserved motifs, such as DDxxD and RxR (boxed in Fig. 2), which are typical for the STS family (Bohlmann et al., 1998b).

2.2. Functional screening of HaTPS12 cDNAs in engineered yeast

To evaluate the biochemical activities of the target genes, the complete ORFs of HaTPS12_K7 and K11 were heterologously expressed in the EPY300 yeast strain, which was previously engineered to synthesize a copious amount of FPP from simple carbon source, using the high-copy plasmid pESC-Leu2d (Ro et al., 2006a, 2008). The volatile products of the transformed yeast cultures were analyzed by GC–MS (Fig. 3). The spectroscopic data of the two products obtained with the pESC-Leu2d::HaTPS12 constructs (K7 and K11) indicated that both compounds showed the structure of γ-bisabolene (Fig. 3; Peak A, retention time (Rt): 11.45 min; retention index (RI): 1511), but the enzyme isoform K7 synthesized about a threefold higher quantity. High accumulation of FPP (Fig. 3; Peak B, Rt: 11, 9 min; RI: 1549) in both yeast cultures indicated an incomplete substrate conversion. To improve the activity of the enzymes and to increase the amount of the compound, the two isoforms K7 and K11 were expressed as thioredoxin-fused proteins in EPY300 as described by Göpfert et al. (2009). This fusion-protein strategy tripled the γ-bisabolene production, and the FPP accumulation was reduced significantly (Fig. 4). The difference in

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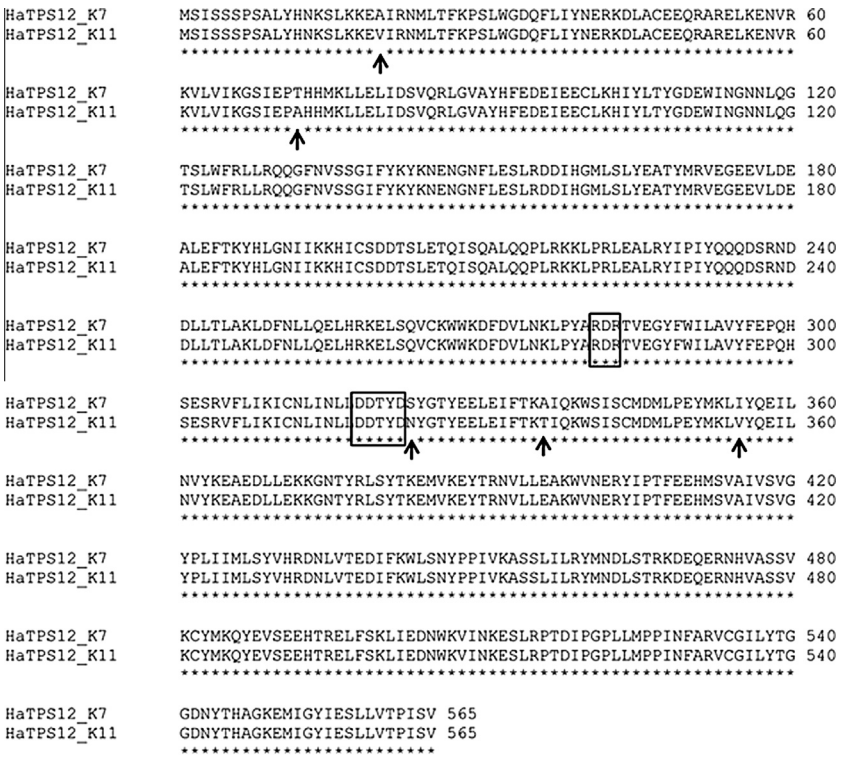

Fig. 2. Alignment of the deduced amino acid sequences of bisabolene synthase genes HaTPS12_K7 and HaTPS12_K11 from linear glandular trichomes of sunflower. Boxes: typical amino acid sequence motives of sesquiterpene synthases (RxR and DDxxD motive). Arrows: amino acid differences between the two enzyme isoforms.

productivity between K7 and K11 was not affected by the N-terminal thioredoxin fusion.

To verify the activity differences, five independent transformants of each isoform in both vectors (pESC-Leu2d and pESC-Leu2dTrx) were tested for their *in vivo* bisabolene-synthetic activity, and the γ-bisabolene amounts were quantified with GC-FID (Fig. 5). HaTPS12_K7 produced an average of 33 mg/L bisabolene and HaTPS12_K11 12 mg/L. With the N-terminal fusion of thioredoxin, the product titre of K7 increased by 5-fold (166 mg/L) while that of K11 increased by 3.5-fold (42 mg/L). With this thioredoxin fusion, the bisabolene quantity of HaTPS12_K7 increased significantly, and a marked difference in the product amounts between HaTPS12_K7Trx and HaTPS12_K11Trx was observed.

2.3. Characterization of the HaTPS12_1 product

Using a GC–MS analysis, the identification of the product of the two STS HaTPS12_K7 and K11 succeeded with the mass fragmentation pattern and the retention index through literature references (Huber et al., 2005). Since the ^{1}H NMR shift values did not allow differentiating between the *cis*- and *trans*-form of γ-bisabolene, the elucidation of the stereochemistry required additional spectroscopic experiments. Using the EPY300 yeast strain expressing *HaTPS12_K7*Trx, a sufficient amount of bisabolene was purified for NMR analyses. The identification as (*Z*)-γ-bisabolene was achieved by 2-D ^{1}H NMR experiments using long range COSY and ROESY measurements (Fig. 6) in the following way: The assignment of the ^{1}H chemical shift values of the methyl groups C-12 (δ 1.60 ppm), C-13 (δ 1.66 ppm) and C-15 (δ 1.67 ppm) were deduced from the couplings with the olefinic protons, H-10 (5.12 ppm) and H-2 (5.36 ppm), respectively. Additional coupling of H-2 allowed the identification of H-1 (2.76 ppm) which was confirmed by an additional long-range coupling of H-1 with the protons of the methyl group at C-15. In the ROESY experiment, H-1 revealed a NOE with H-8 (1.95–2.05 ppm), and H-5 (2.31 ppm) showed a NOE with the protons of the C-14 methyl group. Both NOE correlations unambiguously establish the *cis*-form of γ-bisabolene. Therefore, these two sunflower STSs were firmly identified as (*Z*)-γ-bisabolene synthase.

2.4. Phylogenetic analysis

Plant TPSs are well-known for their catalytic plasticity often resulting in converged evolution (i.e., the same terpene formation from TPSs of evolutionarily different lineages; Bohlmann et al., 1998b). In the BLAST search using two HaTPS12_K7/K11 as queries, the enzymes identified to have the highest similarity were β-farnesene synthases from *Artemisia annua* and chamomile (~60% amino acid identity) (Picaud et al., 2005; Son et al., 2014). Recently, it was hypothesized and demonstrated that a simple terpene, such as linear β-farnesene, has diverged to more complex cyclic terpenes, such as bisabolol, bisabolene, and amorpha-4,11-diene in Asteraceae, where sunflower belongs (Salmon et al., 2015). Inspired by the BLAST search results, we questioned the possibility that (*Z*)-γ-bisabolene activity of HaTPS12_K7/K11 was emerged from β-farnesene synthase. In order to probe this, a phylogenetic tree was reconstructed using 27 STS enzymes from diverse plants (Fig. 7A). In this analysis, statistical data strongly supported that HaTPS12_K7/K11 share the same lineage with amorphadiene synthase from *A. annua*, α-bisabolol synthases from *A. annua* and chamomile, and β-farnesene synthases from *A. annua* and chamomile. All three plant species (*A. annua*, chamomile and sunflower) are from Asteraceae family. On the other hand, functionally identical Arabidopsis (*Z*)-γ-bisabolene synthases were not closely related with HaTPS12_K7/K11, demonstrating an independent converged evolution of (*Z*)-γ-bisabolene synthases in sunflower and *Arabidopsis*. Intriguingly, recent molecular breeding studies of *A. annua* β-farnesene showed that positioning aromatic residues

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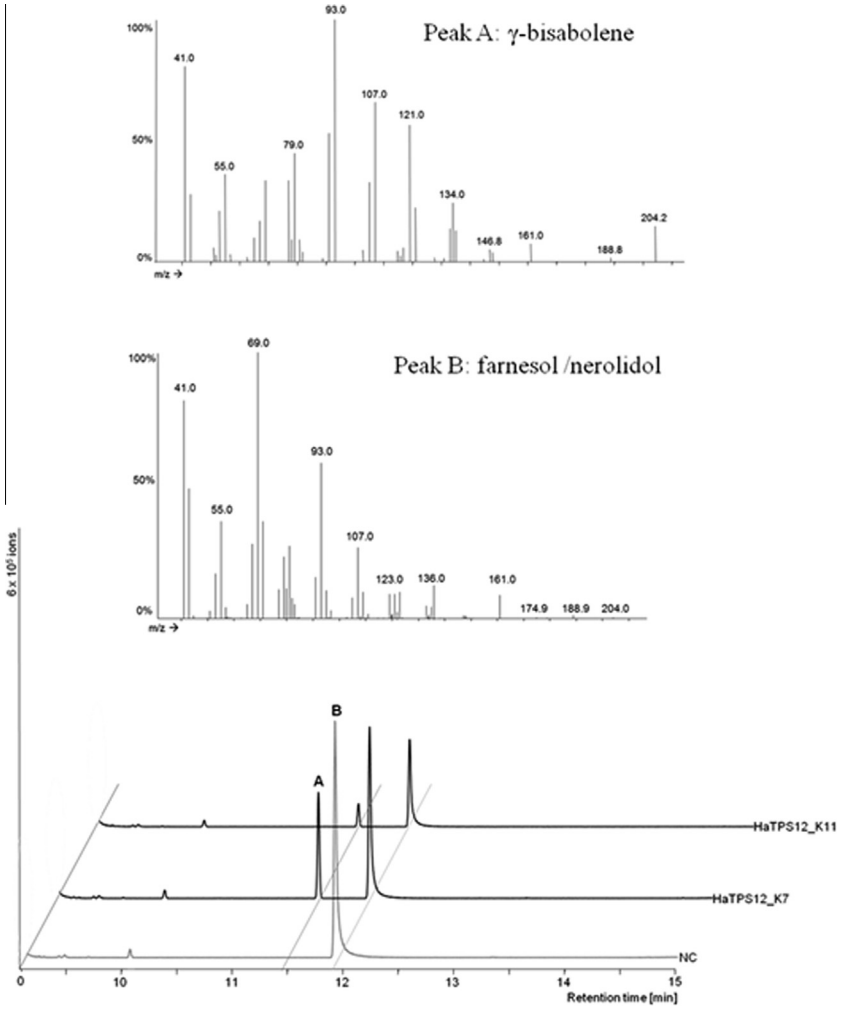

Fig. 3. GC–MS analysis of sesquiterpene products of the *in vivo* expression of HaTPS12_K7 and HaTPS12_K11 in *S. cerevisiae* EPY300. The GC diagrams show metabolite profiles of extracts from yeast cultures transformed with the candidate genes in the high-level expression plasmid pESCLeu2d in compared to a yeast train transformed with the empty vector (NC, negative control). Mass spectra of the identified peak A (γ-bisabolene) and B (farnesyl/nerolidol) are shown.

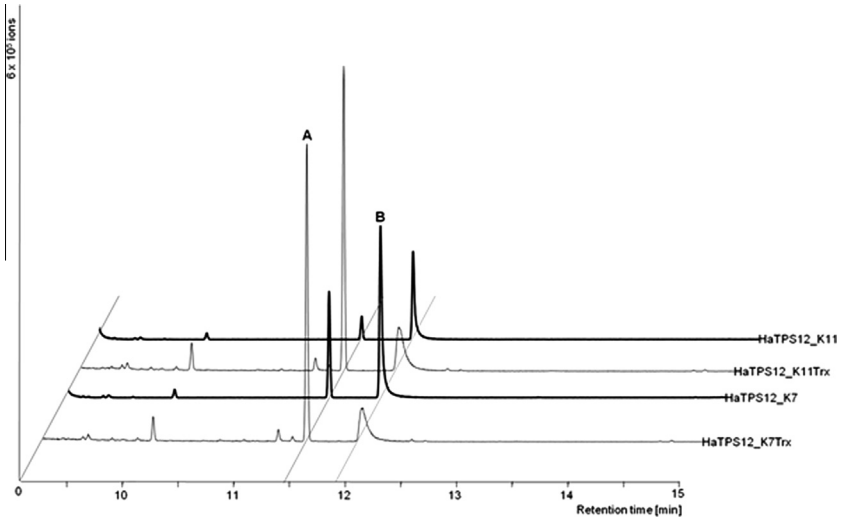

Fig. 4. GC analysis of sesquiterpene products from *in vivo* expression of HaTPS12_K7Trx and HaTPS12_K11Trx in *S. cerevisiae* EPY300 compared to HaTPS12_K7 and HaTPS12_K11.A (γ-bisabolene), B (farnesyl/nerolidol).

(tyrosine, phenylalanine, and tryptophan) on the 402th residue of *A. annua* β-farnesene (or on the equivalent residue in related homologs) interferes with cyclization, thereby producing acyclic terpene β-farnesene, whereas small aliphatic residues (leucine and valine) allow cyclization reactions (Salmon et al., 2015). Consistent to this finding, the residues of HaTPS12_K7/K11, respective

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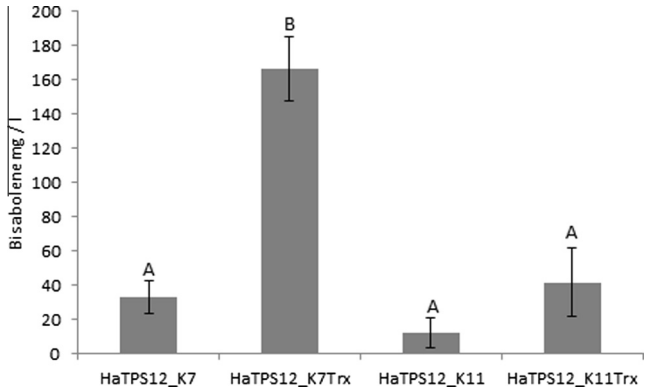

Fig. 5. Quantification of *cis*-γ-bisabolene produced in yeast expression experiments with HaTPS12_K7 und HaTPS12_K11 and the corresponding N-terminal thioredoxin fusion (Trx) constructs. The values represent means and standard deviations of $n = 5$ independent experiments; different letters indicate statistical significance at the level of $p > 0.05$.

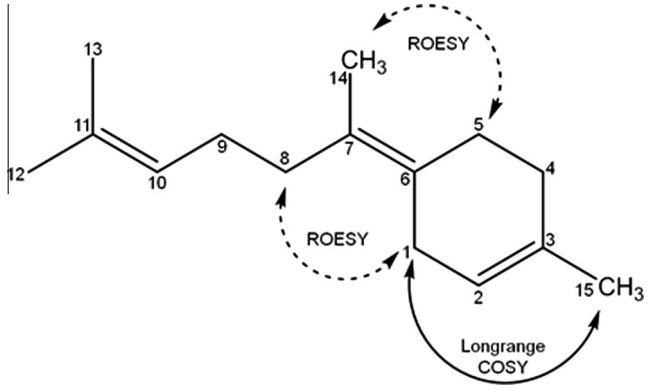

Fig. 6. Observed longrange coupling (solid arrow) and nuclear overhauser effects (dotted arrow) in COSY and ROESY ^{1}H NMR 2D experiments with the purified enzyme product cis-γ-bisabolene.

to the Y402 residue of β-farnesene synthase, are occupied by a small aliphatic residue, valine (Fig. 7B). In conclusion, these observations suggested that the two sunflower (*Z*)-γ-bisabolene synthases have arisen by subtle mutations from β-farnesene synthase.

3. Discussion

Previous identification of glandulones (Spring et al., 1992) and heliannuols (Macias et al., 1993, 1994, 1999, 2002) in sunflower predicted the existence of a bisabolene synthase which catalyzes the complex transformation of FPP into the bisabolene skeleton (Degenhardt et al., 2009). Bisabolene serves as a skeletal backbone for subsequent structural rearrangements and hydroxylations leading to a diverse array of volatile and non-volatile sesquiterpenes in this plant species as previously suggested by Macias et al. (1994). Since no bisabolene synthases were yet described from Asteraceae, a plant family rich for terpenoid allelochemicals, our approach to retrieve candidate genes in sunflower was based on searching genes with sequence homologies to a previously characterized bisabolene synthase from *Arabidopsis* (Ro et al., 2006b) in *Helianthus* EST databases (Lai et al., 2005). Moreover, the allocation of bisabolene-derived sesquiterpenes in LGT of sunflower (Spring et al., 1992, 2015; Aschenbrenner, 2014) suggested the presence of such candidate genes in the RNA pool of these trichomes. The fidelity of the two identified cDNAs could be confirmed by comparison with data of the sunflower genome project (http://www.sunflowergenome.org 15.03.2014; kindly provided by L. Rieseberg, University of British Columbia, Vancouver, Canada) and allowed us to locate both genes on linkage group 17. Their calculated amino acid sequences showed the typical lengths for STS (550–580 amino acids). Both candidates showed the typical STS sequence motifs among which the aspartate rich DDxxD region is the absolutely conserved one and appears in all previously described TPSs, as well as in isoprenyl pyrophosphate synthases and microbial TPSs. About 35 amino acids upstream of this region occurs the RxR motif, which is involved in the complex formation with diphosphate by the ionization of the substrate to prevent nucleophilic attack of carbocation intermediates (Starks et al., 1997). Based on these features, the two candidate cDNAs from sunflower LGT showed a clear relationship to the class I TPS (Wendt and Schulz, 1998). The class I TPS reaction is initiated by Mg^{2+}-dependent cleavage of diphosphate group while the class II TPS reaction is initiated by a protonation on allylic groups of terpene backbones. All STS reactions belong to the class I-type, except for the drimenol synthase from valerian plant that appears to use both class I and II-type reactions in a single polypeptide (Kwon et al., 2014).

The two candidate STSs were further characterized by expressing them in the genetically modified EPY300 yeast strain which had proven suitability for the *in vivo* characterization of putative STSs in previous studies (Ro et al., 2006a; Göpfert et al., 2009; Nguyen et al., 2012). Both candidates, HaTPS12_K7 and HaTPS12_K11, formed γ-bisabolene as a single product in the yeast expression system. This is not common for TPSs in general. They often convert substrates into multiple products, thus contributing to the high diversity of terpenes compared to a relative low number of enzymes (Keeling et al., 2008; Martin et al., 2004; Steele et al., 1998; Tholl et al., 2005). In addition to its main product, almost half of all characterized mono- and sesqui-terpene synthases form also significant amounts of by-products (defined as at least 10% of the total amount of product), when the proteins were expressed *in vitro* (Degenhardt et al., 2009). However, for bisabolene synthases, the formation of a single product has previously been reported (Bohlmann et al., 1998a).

The elucidation of the stereochemistry of the produced γ-bisabolene (peak A in Figs. 3 and 4) could not be solved on the basis of the mass fragmentation pattern or retention index, but required NMR measurements. To increase the amount of the bisabolene product for 2D-NMR experiments, both enzymes were modified by adding thioredoxin at the N-terminal end. As shown originally for *E. coli* expression systems (La Vallie et al., 1993), this fusion can also increase the solubility and proper folding of a target protein in yeast and thus positively influence the enzyme activity (Göpfert et al., 2009). Long-range 2D-COSY and ROESY experiments unraveled the *cis*-configuration of the enzyme product, thus characterizing HaTPS12_K7 and HaTPS12_K11 as two (*Z*)-γ-bisabolene synthase genes. A catalysis of such stereospecific products is typical for TPSs. Through a temporary stabilization of the carbo-cation, it may come to that enzyme-specific isomerization. Furthermore terpenoids are known to have stereospecific biological activities (Bohlmann and Keeling, 2008).

The two enzyme isoforms differed only in 5 amino acids, but the protein HaTPS12_K11 formed less γ-bisabolene in the yeast expression experiments than HaTPS12_K7. Such efficiency differences are known from other bisabolene synthases tested in yeast (Peralta-Yahya et al., 2011). In our case, the difference could be due to the fact that the amino acid substitution at position 323 directly behind the DDxxD motif. Here, HaTPS12_K7 is bearing a serine and HaTPS12_K11 an asparagine. The DDxxD motif is located immediately at the beginning of the catalytic center and plays an important role in the positioning of the substrate. A mutation in this area has been described as frequently responsible for the catalytic activity and thus decreases the formation of products

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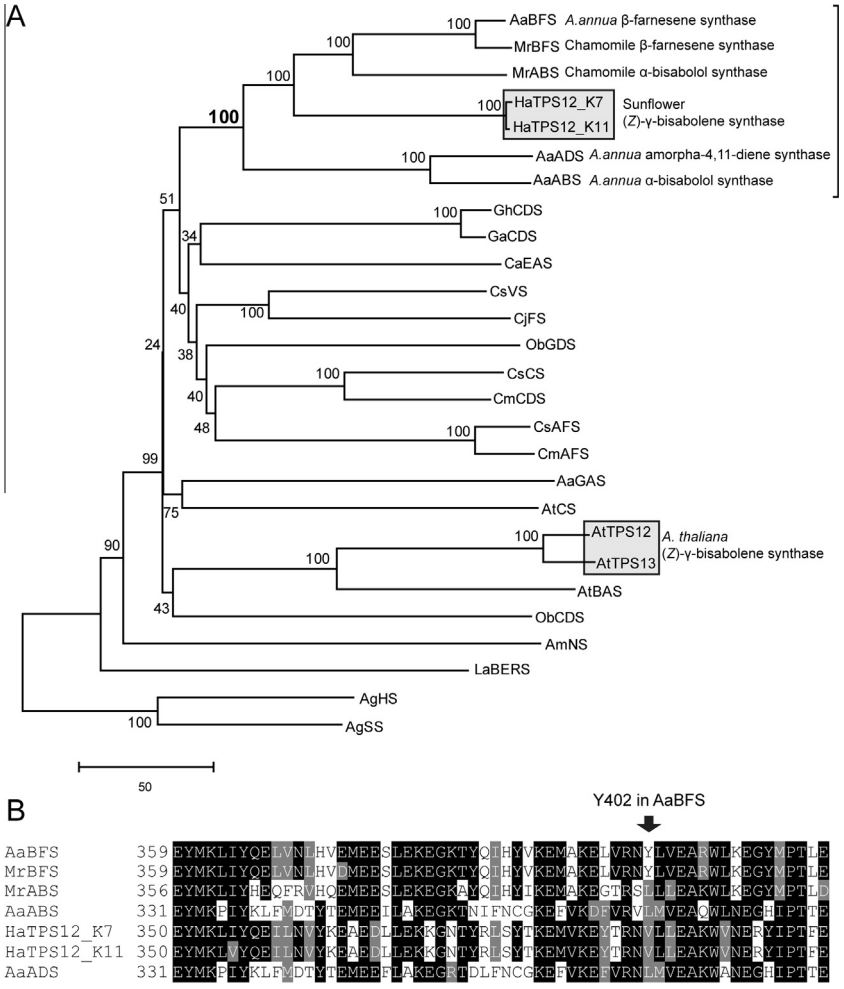

Fig. 7. Phylogenetic analysis and partial sequence comparison. (A) Phylogenetic tree based on 27 plant STSs. (*Z*)-γ-bisabolol synthases from sunflower and *Arabidopsis thaliana* are boxed, and bootstrap values are given in each node. Gymnosperm *Abies grandis* STSs were used to serve as a root. Sequences used (but not described in the Figure) are: GhCDS, δ-cadinene synthase [*Gossypium hirsutum*]; GaCDS δ-cadinene synthase [*Gossypium arboreum*]; CsAFS, α-farnesene synthase [*Cucumis sativus*]; CsCS, δ-caryophyllene synthase [*Cucumis sativus*]; CsVS, valencene synthase [*Citrus sinensis*]; CjFS, δ-farnesene synthase [*Citrus junos*]; ObGDS, germacrene D synthase [*Ocimum basilicum*]; CmCDS, δ-cadinene synthase [*Cucumis melo*]; CmAFS, α-farnesene synthase [*Cucumis melo*]; CaEAS, 5-*epi*-aristolochene synthase [*Capsicum annuum*]; AaGAS, germacrene A synthase [*Artemisia annua*]; AtCS, δ-caryophyllene synthase [*Arabidopsis thaliana*]; AtATP12 (*Z*)-γ-bisabolene synthase 1 [*Arabidopsis thaliana*]; AtTPS13 (*Z*)-γ-bisabolene synthase 2 [*Arabidopsis thaliana*]; AtBAS α-barbatene synthase [*Arabidopsis thaliana*]; ObCDS γ-cadinene synthase [*Ocimum basilicum*]; AmNS nerolidol synthase [*Antirrhinum majus*]; LaBERS α-bergamotene synthase [*Lavandula angustifolia*]; AgHS γ-humulene synthase [*Abies grandis*]; AgSS δ-selinene synthase [*Abies grandis*]. (B) Amino acid sequences neighboring the Y402 residue of *A. annua* β-farnesene synthase are compared among the clustered STSs (β-farnesene, α-bisabolol, amorpha-4,11-diene synthases, see the bracket in A). Accession numbers of HaTPS12_K7 and HaTPS12_K11 are KU674381 and KU674382, respectively.

or form abnormal products (Degenhardt et al., 2009). TPSs often occur as multigene-copy family, which encode functionally identical or very similar enzymes (Bohlmann et al., 1998a,b). The EST databases of model plants like maize, rice, tomato, or *Arabidopsis* show such gene families caused by gene duplications and multiple mutations (Tholl, 2006). The bisabolene synthases from *Arabidopsis* AtTPS12 (used as reference gene in the present study) and AtTPS13 have a sequence identity of 91%, thus providing a good example for such an evolutionary process (Ro et al., 2006b; Tholl, 2006).

The cDNA which lead to the identification of HaTPS12 was gained from RNA of LGT cells. Expression studies in samples from whole bracts, epidermal strips of leaf petioles or stems on which LGT are located, irregularly showed positive results too, whereas no expression of HaTPS12 was found in cotyledons and inner parenchymatic tissues of the plant (data not shown). This indicates a low level of HaTPS12 expression only in specific tissues. Moreover, the expression in LGT was only observed in outdoor grown plants approaching the flowering stage, while it was not detected when plants had been cultivated in the climate chamber under low intensity light. This coincided with earlier microscopic studies (Aschenbrenner et al., 2013; Aschenbrenner, 2014), where the accumulation of metabolites was visible only in LGT from field grown plants at late stages of development. A similar phenomenon of tissue and development dependent enzyme expression was previously described from a germacrene A synthase in sunflower LGT (Göpfert et al., 2010). For other TPSs as well, a limited or induced expression at certain developmental stages and influenced by exogenous factors is known (Ro et al., 2006b). The transcript level of bisabolene synthases, and thus the enzyme concentration is usually low. The bisabolene synthases of *Arabidopsis* (Ro et al., 2006b), for example, have been described as root-specific and wound-induced enzymes. Their expression was increased by mechanical stress. For the α-bisabolene synthase of *Abies grandis*, a wound induction of expression was shown (Bohlmann et al., 1998b). This *de novo* biosynthesis of bisabolenes upon external stress indicates a very specialized function of these natural products. While mono- and diterpenes are often formed during all development stages of the plant and thus serve as a preformed defense in the early phase

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of the attack, the bisabolenes may play a role in the inducible strategy to cope with external stress. The function of these bisabolene-type sesquiterpenes in sunflower is still unclear, although some allelopathic effects were reported from laboratory experiments (Macias et al., 1994, 1999; Munoz-Suano et al., 2009).

4. Experimental section

4.1. Plant material and RNA preparation

Helianthus annuus L. cv. Giganteus plants were grown under greenhouse conditions (16 h light period with additional illumination (330 μmol s^{-1} m^{-2}) followed by 8 h darkness). Alternatively plants were grown in the field (Botanical Garden, University of Hohenheim, Stuttgart, Germany). *H. annuus* linear glandular trichomes (LGT) were manually harvested from the central leaf vein under a stereo microscope using a fine pair of tweezers. For total RNA extraction, trichomes were isolates from fresh plant material and transferred to 200 μl ice-chilled RNA extraction buffer (Aurum total RNA isolation Kit, Biorad, Munich, Germany) in 2 ml vials. A mixer mill (MM20, Retsch, Haan, Germany) was used for cell disruption (16 Hz, 1 min) using 2 ceramic beads (2.8 mm diameter, Precellys, Peqlab, Erlangen, Germany) per sample. After cell disruption, additional 500 μl of lysis buffer was added. All subsequent steps were carried out as described in the manufacturer's manual. RNA quantity and integrity was verified by the Bioanalyzer 2100 using a RNA 6000 Pico Chip (Agilent, Böblingen, Germany). For routine PCR, cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kits (Fermentas, St. Leon-Rot, Germany) with VN_{dT18} primer.

4.2. Identification of HaTPS12_1

Screening of candidate genes in a cDNA pool of LGT was based on conserved amino acid sequences common for all known STSs (Back and Chappell, 1995) and bisabolene synthases in particular. A database research (GenBank, NCBI) was carried out to find sequence similarities especially with a previously identified (*Z*)-γ-bisabolene synthases *AtTPS12* from *A. thaliana* (NCBI Reference Sequence: NM_117401.2; Ro et al., 2006b) in the EST (expressed sequence tags) database of *Helianthus*.

Target sequences with the highest similarities (BLAST, basic local alignment search tool; NCBI) to the reference gene, were used for primer construction specific for *H. annuus*. Based on this bioinformatic approach, a homology-based PCR screening (Phusion DNA Polymerase, Finnzymes, Espoo, Finland) in the cDNA pool of selectively isolated sunflower LGT with the forward primer 5′-AACCTCGAAGGCACTTCACT-3′ and the reverse primer 5′-CCTTC GACTGTTCTATCCCG-3′ was conducted and identified a middle part of a putative bisabolene synthase. By means of RACE-experiments (SMARTer™ RACE cDNA Amplification Kit; Clontech Laboratories Inc., Palo Alto, USA) the full open reading frame was identified. Through control amplification and sequencing of the whole ORF the high similarity to other STSs was confirmed. Sequence comparison of different clones (StrataClone PCR cloning Kit, Stratagene Inc., La Jolla, USA) showed the existence of two potential isoforms of the putative bisabolene synthase, named HaTPS12_K7 and HaTPS12_K11.

4.3. Plasmid construction for expression in S. cerevisiae

For functional screening the full length candidate genes HaTPS12_K7 and K11 were amplified from LGT cDNA with the forward primer 5′-ACGTGGATCCATGTCTATTTCTTCATCTCC-3′ and the reverse Primer 5′-ACGTGCTAGCTTAAACACTTATGGGAGTAACA-3′ using a proofreading Phusion DNA polymerase (Finnzymes, Espoo, Finnland).The amplified PCR products were cloned with a StrataClone Blunt PCR Cloning Kit (Stratagene Inc., La Jolla, USA) and subsequently digested with BamHI and NheI. The digested fragments were ligated to the multiple cloning site 2 (MCS2) of the pESC-Leu2d plasmid Ro et al., 2006a, 2008).

Thioredoxin-fused STSs have been shown to have improved activity (due to higher solubility) in yeast system (Göpfert et al., 2009). A modified *pESC-Leu2d* vector with *pGal1* regulated *thioredoxin* (*Trx*) for the expression of N-terminal Trx-fused proteins from genes cloned into MCS2 was provided by Dae-Kyun Ro (University of Calgary, Canada). To generate the thioredoxin fusion construct for HaTPS12_K7 and K11, the pESC-Leu2d::12_K7/K11 plasmids served as templates and were amplified with the forward primer 5′-ACGTGGGCCCATGTCTATTTCTTCAT-3′ and the reverse primer including the restriction site for NheI described above. After the digestion with ApaI and NheI, the fragments were ligated into the pESC-Leu2d-Trx plasmid.

For all *in vivo* expression experiments, EPY300 *S. cerevisiae* cells (Nguyen et al., 2012) were used. These cells were engineered for a highlevel of FDP production. EPY300 were transformed with purified plasmids following the protocol from Gietz and Woods (2002).

4.4. Protein expression in vivo in S. cerevisiae

For protein expression, transgenic *S. cerevisiae* were inoculated in 2 ml synthetic complete (SC) medium (without the amino acids Met, His, Leu and with 2% glucose supplement) and grown overnight at 30 °C (200 rpm). The overnight culture was diluted 100-fold in SC medium, lacking His and Leu, and supplemented 0.2% glucose, 1.8% galactose and 1 mM Methionine. The yeast culture was overlaid with dodecane (10% of the culture volume) and grown at 30 °C at 200 rpm for 3–4 days in an incubator (Excella E24, New Brunswick Sci. Co. Inc., Edison, USA). After incubation, the cultures were transferred to 50 ml falcon tubes and centrifuged at 10,000 g (5 min). Dodecane was carefully removed, diluted 1:10 with ethyl acetate and used directly for GC-FID and GC–MS analyses to trap any volatile terpenoids released during growth.

To compare the produced quantities of the sesquiterpene product of the two isoforms HaTPS12_K7 and K11, the yeast expression experiment was carries out for 5 clones of each candidate gene. The dodecane overlays were analyzed with GC-FID and product amounts were determined and statistically analyzed (ANOVA).

4.5. GC–MS and GC-FID

A 6890 N gas chromatography coupled with either a 5975B mass spectrometer (GC–MS) or a 6850 flame ionization detector (GC-FID) (Agilent Technology GmbH, Böblingen, Germany) was used to analyze the organic extracts produced by recombinant enzyme in *S. cerevisiae*.

Sample volumes of 1–2 μl were injected at an inlet temperature of 250 °C and analyzed on a HP-5MS column (30 m × 250 μm inner diameter × 0.25 μm film thickness, Agilent Technology GmbH, Böblingen, Germany). Helium (constant flow rate of 1 ml min^{-1}) was used as carrier gas. GC oven temperature program was 80 °C for 1 min, followed by a linear increase of 10 °C min^{-1} to 250 °C and then of 30 °C min^{-1} to 300 °C. Spectra were interpreted with NIST5/Wiley7 Mass Spectra Library (Wiley & Sons, Mississauga, Canada), MASSFINDER4, and by comparison with published literature. Retention indices (RIs) were calculated by use of an alkane standard (C8–C20; Fluka, Bucks, Switzerland) and compared with the values in the literature and the MASSFINDER4 database.

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4.6. Purification and NMR of (Z)-γ-bisabolene

To verify the identity and elucidate the stereo chemistry of enzyme products, nuclear magnetic resonance (NMR) analysis were carried out and required additional amounts of the compounds. For this experiment, tranformants of the plasmid construct pESC-Leu2dTrx::HaTPS12_K7 were used. To fix and accumulate the high volatile compounds during the yeast expression experiment, Amberlite XAD-4 resin (The Dow Chemical Company, Midland, USA) was added to the culture. The culture volume was up-scaled to 500 ml. Further steps were carried out as described above. After cultivation the resin was separated from the culture medium, rinsed with water and extracted with pentane. The pentane extract volume was reduced to 500 µl under nitrogen flow. For NMR studies the compounds had to be dried and redesolved in deuterated solvent. Since complete evaporation of the pentane solvent caused coevaporation of the target compounds, the residual pentane extract was applied to a silica plate and the extracted compounds were separated by TLC in ethylacetate/n-hexane (1:3 v/v) The target fraction for bisabolene was identified according to the Rf value described by Anastasis et al. (1984). For NMR measurements the target fraction was extracted with deuterated chloroform from the silica gel and analyzed with a Unity Inova spektrometer (Varian GmbH, Darmstadt) operating at 500 MHz (^{1}H) and an PFGID probe. For 2D-experiments standard Varian pulse sequences were used.

4.7. Phylogenetic analysis

A phylogenetic tree was reconstructed by MEGA5.2 software using a neighbor-joining algorithm in 1000 replicates. Bootstrap values were calculated in percentage. Sequences were retrieved from the Genbank as follows. AaBFS, AAX39387; MrBFS, AIG92847; MrABS, AIG92846; AaADS, AAF98444; AaABS, AFV40969; GhCDS, P93665; GaCDS, Q39761; CsAFS, NP_001267674; CsCS, NP_001292628; CsVS, AAQ04608; CjFS, Q94JS8; ObGDS, Q5SBP6; CmCDS, NP_001284382; CmAFS, NP_001284384, CaEAS, O65323; AaGAS, ABE03980; AtCS, AAO85539; AtTPS12, NP_193064; AtTPS12, NP_193066; AtBAS, AAX59990; ObCDS, Q5SBP5; AmNS, ABR24417; LaBERS, Q2XSC4; AgHS, O64405; AgSS, O64404.

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