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Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, *Pseudotsuga menziesii*

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Abstract

Numerous terpenoid compounds are present in copious amounts in the oleoresin produced by conifers, especially following exposure to insect or fungal pests. CDNA clones for many terpene synthases responsible for the biosynthesis of these defense compounds have been recovered from several conifer species. Here, the use of three terpene synthase sequences as heterologous probes for the discovery of related terpene synthase genes in Douglas-fir, *Pseudotsuga menziesii* (Mirbel) Franco (Pinaceae), is reported. Four full-length terpene synthase cDNAs were recovered from a methyl jasmonate-induced Douglas-fir bark and shoot cDNA library. These clones encode two multi-product monoterpene synthases [a (−)-α-pinene/(−)-camphene synthase and a terpinolene synthase] and two single-product sesquiterpene synthases [an (*E*)-β-farnesene synthase and a (*E*)-γ-bisabolene synthase].

Keywords: *Pseudotsuga menziesii*; Pinaceae; Conifer defense; Oleoresin; Terpenoid synthases

1. Introduction

Conifers maintain a diverse array of both preformed and induced terpenoids (Fig. 1) which are thought to be important for defense against insect herbivores and fungal pathogens. These compounds are hypothesized to act either directly or indirectly against various pests and pathogens (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Bohlmann et al., 2000; Trapp and Croteau, 2001; Alfaro et al., 2002; Huber and Bohlmann, 2004; Huber et al., 2004). While the mixture of terpenoids in conifer resin is complex, quantities of individual terpenoids vary in response to mechanical wounding that simulates insect attack (Steele et al., 1998b; Tomlin et al., 2000; Byun McKay et al., 2003), to actual insect attack (Byun McKay et al., 2003; Miller et al., 2005), or to elicitors, particularly methyl jasmonate (MeJA) (Martin et al., 2002, 2003; Fäldt et al., 2003; Miller et al., 2005). Quantities of individual terpenoids in the resin mixture may also vary across tissues within a tree (Martin et al., 2002, 2003; Huber et al., 2005; Miller et al., 2005). The complexity of the resin mixture changes in the titer of specific terpenoids in the resin are, to a great extent, dependent on the expression of terpene synthase (*TPS*) genes and on the activity and product profile of the associated terpene synthase enzymes (Stofer-Vogel et al., 1996; Bohlmann et al., 1997, 1998a,b, 1999, 2000; Steele et al., 1998a,b; Byun McKay et al., 2003; Fäldt et al., 2003; Phillips et al., 2003; Martin et al., 2004; Miller et al., 2005).

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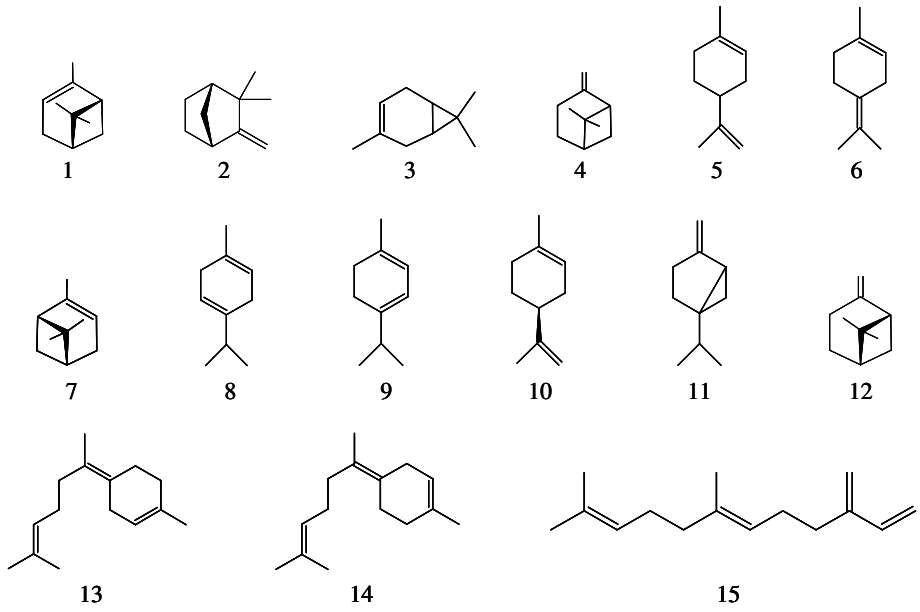

Fig. 1. Structures of (−)-α-pinene (1), (−)-camphene (2), 3-carene (3), β-pinene (4), limonene (5), terpinolene (6), (+)-α-pinene (7), γ-terpinene (8), α-terpinene (9), (−)-limonene (10), sabinene (11), (−)-β-pinene (12), (*Z*)-γ-bisabolene (13), (*E*)-γ-bisabolene (14), and (*E*)-β-farnesene (15).

The ten-carbon monoterpenes, fifteen-carbon sesquiterpenes, and twenty-carbon diterpenes all derive from three basic substrates that are acted on by TPS: ten-carbon geranyl diphosphate (GPP), fifteen-carbon farnesyl diphosphate (FPP) and twenty-carbon geranyl geranyl diphosphate (GGPP), respectively. The diphosphate precursors are formed from prior condensations of two, three, or four units of the five-carbon isopentenyl diphosphate and its isomer, dimethylallyl diphosphate (Koyama and Ogura, 1999). Each TPS may catalyze the formation of one or many terpenoids from a specific, diphosphate precursor. The diverse array of *TPS* genes in conifer genomes (Bohlmann et al., 1998b; Bohlmann and Croteau, 1999; Martin et al., 2004), the differential expression of those genes in different tissues and depending on exogenous stimuli (Steele et al., 1998b; Fäldt et al., 2003; Miller et al., 2005), and the simple or complex product profiles of different TPS all contribute to the ability of conifers to vary the quality and quantity of their resin output to defend against different insect and fungal pests. The multigenic complexity and variability of terpenoid defense may have contributed to the evolutionary success of conifers (Huber et al., 2004). In this paper we describe the cloning and characterization of four TPS from Douglas-fir, two multi-product monoterpene synthases and two single-product sesquiterpene synthases.

2. Results

Following cDNA library screening and successful 5′-RACE, we obtained cDNA sequences for four *TPS* genes from Douglas fir. BLASTP comparisons of the deduced amino acid sequences against the GenBank database suggested that two (*PmeTPS1*, 1910 bp encoding an ORF of 619 amino acids; *PmeTPS2*, 1887 bp encoding an ORF of 625 amino acids) were mono-TPS of the conifer *tps-d1* subfamily (Martin et al., 2004), while the other two (PmeTPS3 2520 bp encoding an ORF of 815 amino acids; PmeTPS4, 2528 bp encoding an ORF of 826 amino acids) were sesqui-TPS of the conifer *tps-d3* subfamily (Martin et al., 2004). Pme TPS1 shares 75% amino acid identity with *Abies grandis* (−)-pinene synthase (GenBank accession number U87909; Bohlmann et al., 1997), 73% identity with *Picea sitchensis* (−)-pinene synthase (GenBank accession number AY237645; Byun McKay et al., 2003), and 74% identity with *A. grandis* (−)-camphene synthase (GenBank accession number U87910; Bohlmann et al., 1999). *Pme*TPS2 shares 74% amino acid sequence identity with *Picea abies* (+)-3-carene synthase (GenBank accession number AF461460; Fäldt et al., 2003), 66% identity with *P. sitchensis* (−)-pinene synthase, and 65% identity with *A. grandis* (−)-pinene synthase. *Pme*TPS3 shares 80% amino acid identity with *A. grandis* (*E*)-α-bisabolene synthase (GenBank accession number AF006195; Bohlmann et al., 1998a) and 79% identity with *P. abies* (*E*)-α-bisabolene synthase (GenBank accession number AY473619; Martin et al., 2004). *Pme* TPS4 shares 76% amino acid identity with *A. grandis* (*E*)-α-bisabolene synthase and 75% identity with *P. abies* (*E*)-α-bisabolene synthase. The four new Douglas-fir cDNA sequences have been deposited in GenBank (GenBank accession nos. AY906866, AY906867, AY906868, and AY906869).

A comparison of the deduced amino acid sequence of *PmeTPS1* and *PmeTPS2* with mono-TPS from other

conifers is presented in Fig. 2. Both the highly conserved RRX_8W motif (residues 51–61 in *Pme*TPS1 and 62–72 in *Pme*TPS2) and the DDXXD active site motif (residues 373–377 in *Pme*TPS1 and 376–380 in *Pme*TPS2) are present. Likewise, a comparison of *PmeTPS3* and *PmeTPS4* with known conifer sesqui-TPS (Fig. 3) also reveals the RRX_8W (residues 17–27 in *Pme*TPS3 and 34–44 in *Pme*TPS4) and the DDXXD motifs (residues 561–565 in *Pme*TPS3 and 571–575 in *Pme*TPS4). Both *Pme*TPS3 and *Pme*TPS4 contain the approximately 200 amino acid motif common to many diterpenes synthases and a few known conifer sesquiterpene synthases (Bohlmann et al., 1998b; Martin et al., 2004). The motif is evident from residues 74–290 in *Pme*TPS3 and from residues 87–301 in *Pme*TPS4.

When incubated with geranyl diphosphate (GPP), *Pme*TPS1 expressed from *Escherichia coli* produced two main products and three minor products (Fig. 4). Mass spectral analyses and retention time matching with authentic standards revealed that the main two products were (−)-α-pinene (1) and (−)-camphene (2) and that the three minor products were 3-carene (3), β-pinene (4), and limonene (5) (Fig. 1). Similar incubation of *Pme*TPS2 with GPP (Fig. 5) produced a main peak of terpinolene (6) and eight minor peaks of (+)-α-pinene (7), γ-terpinene (8), α-terpinene (9), (−)-limonene (10), sabinene (11), (−)-β-pinene (12), and 3-carene (3). In both cases, expressed protein incubated in the absence of their substrates, or with FPP or GGPP did not produce detectable products.

When incubated with farnesyl diphosphate (FPP), *Pme*TPS3 and *Pme*TPS4 expressed from *E. coli* each produced one main product. The best MS library match for the product of *Pme*TPS3 was (*Z*)-γ-bisabolene (13) (Fig. 6). However, retention time matching with an authentic standard and with other likely candidates that were available did not provide a good fit. The Kovat's Index value (Adams, 2001) for the single product of *Pme*TPS3 was calculated to be 1530.70, very closely matching the published value (1531) for (*E*)-γ-bisabolene (14). Hence we suggest the product to be (*E*)-γ-bisabolene. Retention time of authentic standard and MS library matching indicated that the single product of *Pme*TPS4 was (*E*)-β-farnesene (15) (Fig. 7). Again, in both cases, expressed protein incubated in the absence of their substrates, or with GPP or GGPP, did not produce detectable products.

3. Discussion

The cloning and identification of four TPS cDNAs from Douglas-fir provides a first approach towards understanding the molecular genetics and biochemistry of terpenoid resin defenses in this important conifer species. Changes in resin chemistry (Huber et al., 2005) and in anatomical structures related to resin transport and deposition (Hudgins et al., 2003, 2004; Huber et al., 2005) are known to occur in Douglas-fir in response to application of chemical elicitors and to insect herbivory. Resin content of α-pinene (1, 7) and camphene (2) increase significantly in stems and roots of Douglas-fir following root application of MeJA (Huber et al., 2005). For camphene, amounts range from 5.9 $\mu g\, g^{-1}$ dry weight (stems, no MeJA) to 52.2 $\mu g\, g^{-1}$ dry weight (roots, following MeJA application). For α-pinene, amounts range from 404.6 $\mu g\, g^{-1}$ dry weight (stems, no MeJA) to 2794.4 $\mu g\, g^{-1}$ dry weight (roots, following MeJA application). These observed significant changes in the titers of α-pinene and camphene imply an increased expression and/or activity of *Pme*TPS1 following MeJA induction in roots and stems. Camphene (16.5–25.0 $\mu g\, g^{-1}$ dry weight) and α-pinene (425.7–636.6 $\mu g\, g^{-1}$ dry weight) were also observed to be constitutively present in foliage, though no significant change in response to root-treatment with MeJA was observed in that tissue for either compound.

Terpinolene (6) was also observed to be constitutively present in all three tissues, roots, stems, and needles, in amounts ranging from 8.8–165.4 $\mu g\, g^{-1}$ dry weight. Neither (*E*)-γ-bisabolene (14) nor (*E*)-β-farnesene (15) were observed by Huber et al. (2005) in Douglas-fir tissue before or following application of MeJA to roots. The biosynthesis of both may be induced by other stimuli.

Seasonal changes in Douglas-fir resin constituents and the effects of those changes have been particularly well-researched in the context of interactions with a defoliating pest, the western spruce budworm, *Choristoneura occidentalis*. Western spruce budworm prefers to feed on the early buds of Douglas-fir and the phenology of budburst has been attributed to the resistance or susceptibility of the trees to defoliation events by western spruce budworm (Chen et al., 2001, 2002). This is due to the fact that specific compounds, mainly monoterpenoids, accumulate in bud tissue during development. The major products of *Pme*TPS1, α-pinene (1, 7) and camphene (2), and the major product of *Pme*TPS2, terpinolene (6), are variable in needle tissue during the late spring and early summer (Wagner et al., 1989), with all three terpenoids, but particularly the two products of *Pme*TPS1, seeming to play a significant role in Douglas-fir resistance to western spruce budworm (Chen et al., 2002).

Others (Zou and Cates, 1995; Gambliel and Cates, 1995) have also noticed increases in α-pinene (1, 7) and camphene (2) during budbreak and decreases thereafter. Zou and Cates (1995) hypothesized that the variable monoterpenoids would have an effect on western spruce budworm larvae. Further work (Zou and Cates, 1997) revealed that terpinolene, camphene, or mixtures of sesquiterpenoids each affected larval growth. These data imply a potential role of all four of the terpene

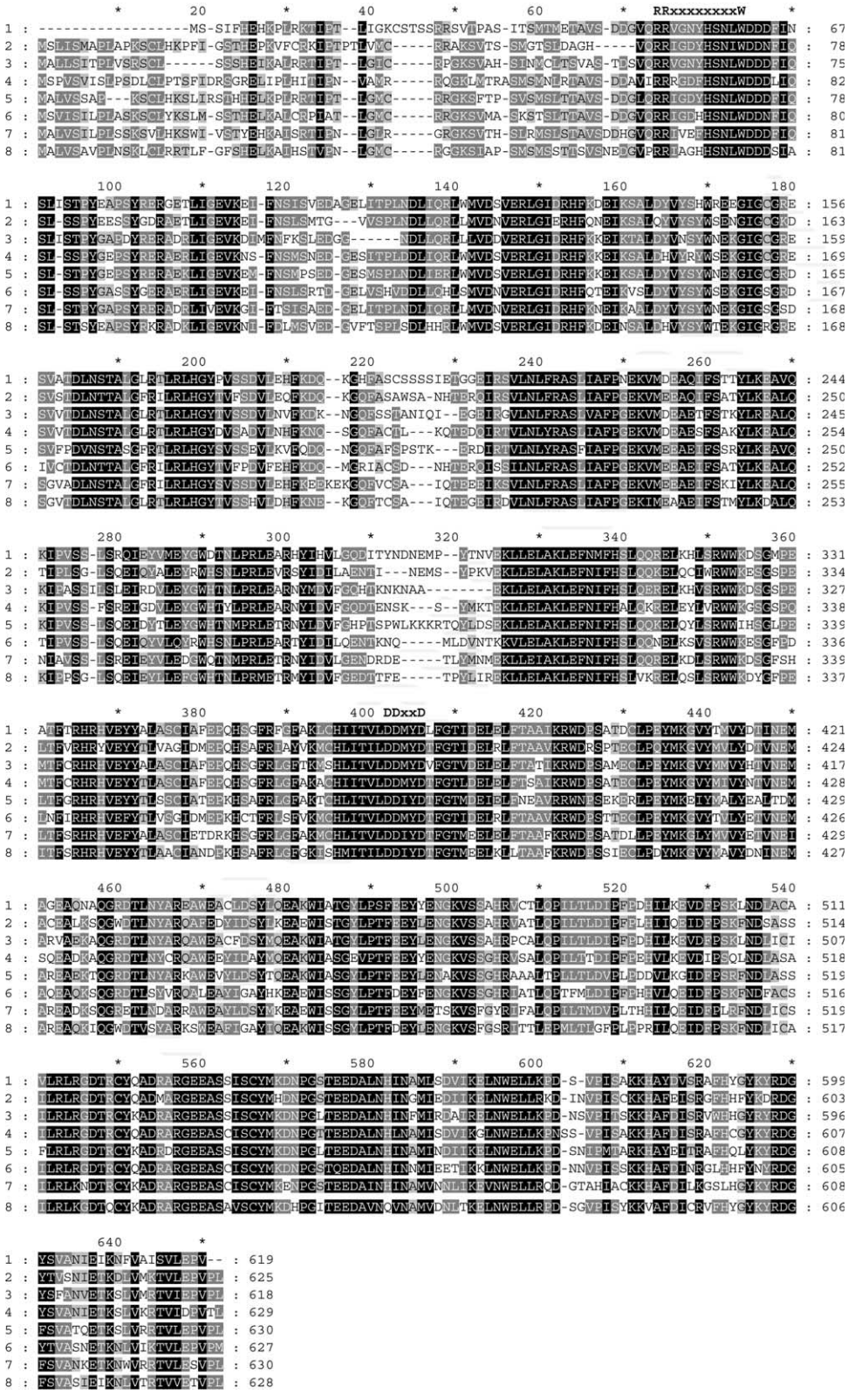

Fig. 2. Alignment of deduced amino acid sequences of two *Pseudotsuga menziesii* monoterpene synthases and six other conifer monoterpene synthases. *Pme*TPS1 (1), *Pme*TPS2 (2), *Abies grandis* (−)-camphene synthase (3, GenBank accession AAB70707), *Pinus taeda* (−)-α-pinene synthase (4, AAO61225), *A. grandis* β-phellandrene synthase (5, AAF61453), *Picea abies* (+)-3-carene synthase (6, AAO73863), *A. grandis* terpinolene synthase (7, AAF61454), and *P. taeda* (+)-α-pinene synthase (8, AAO61228). The locations of the RRX_8W and the DDXXD motifs are shown. Black shaded residues are highly conserved (100% similarity), while dark gray (80%–99% similarity) and light gray (60%–79% similarity) are less conserved. The alignment was completed with Dialign and visualized with GenDoc.

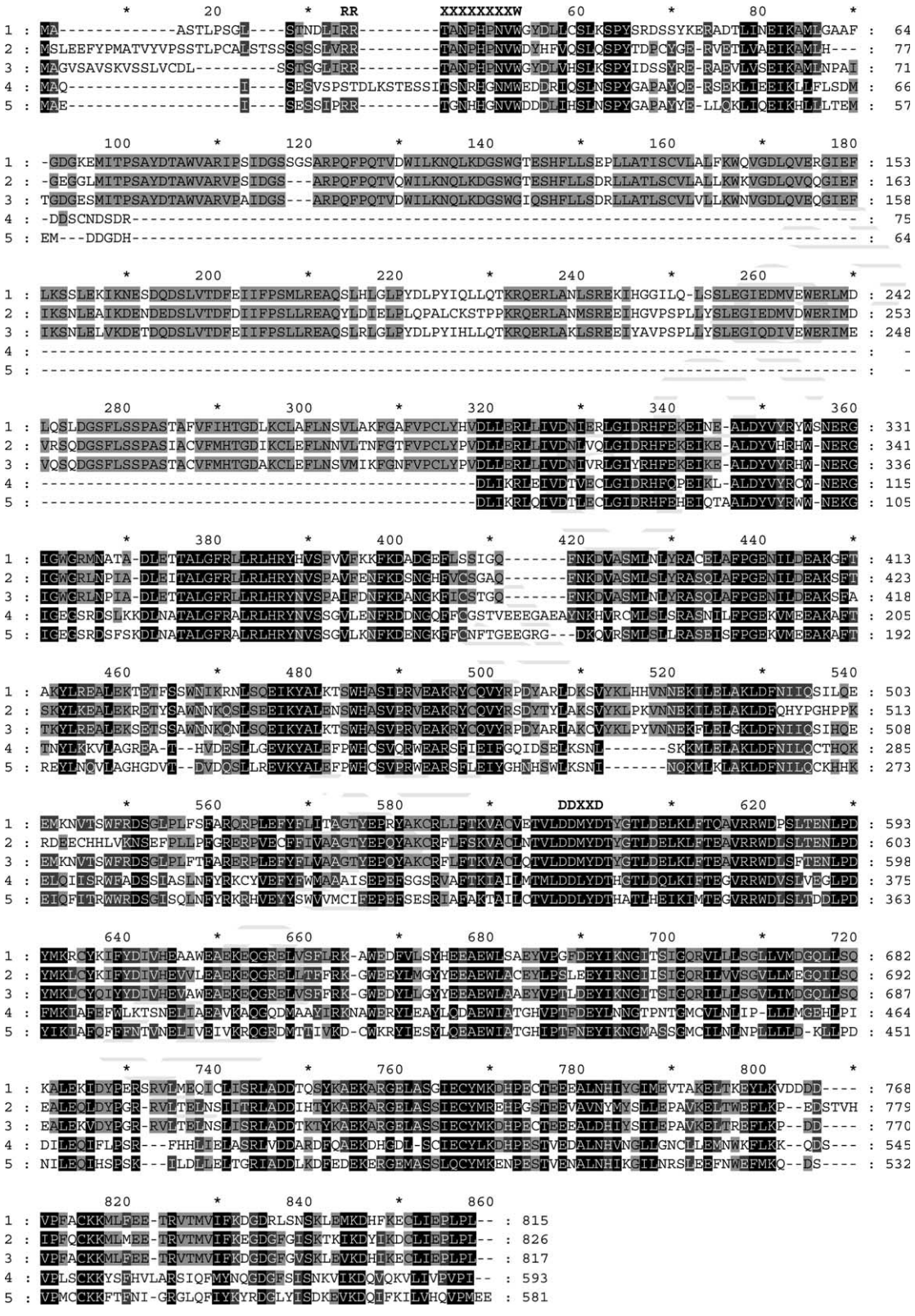

Fig. 3. Alignment of deduced amino acid sequences of two *Pseudotsuga menziesii* sesquiterpene synthases and three other conifer sesquiterpene synthases. *Pme*TPS3 (1), *Pme*TPS4 (2), *Abies grandis* (*E*)-α-bisabolene synthase (3, GenBank accession AAC24192), *A. grandis* γ-humulene synthase (4, AAC05728), and *A. grandis* δ-selinene synthase (5, AAC05727). The locations of the RRX_8W and the DDXXD motifs are shown. The approximately 200 amino acid motif, seen in some terpene synthases, is evident in 1, 2, and 3 from residues 101-318. Black shaded residues are highly conserved (100% similarity), while dark gray (80%–99% similarity) and light gray (60%–79% similarity) are less conserved. The alignment was completed with Dialign and visualized with GenDoc.

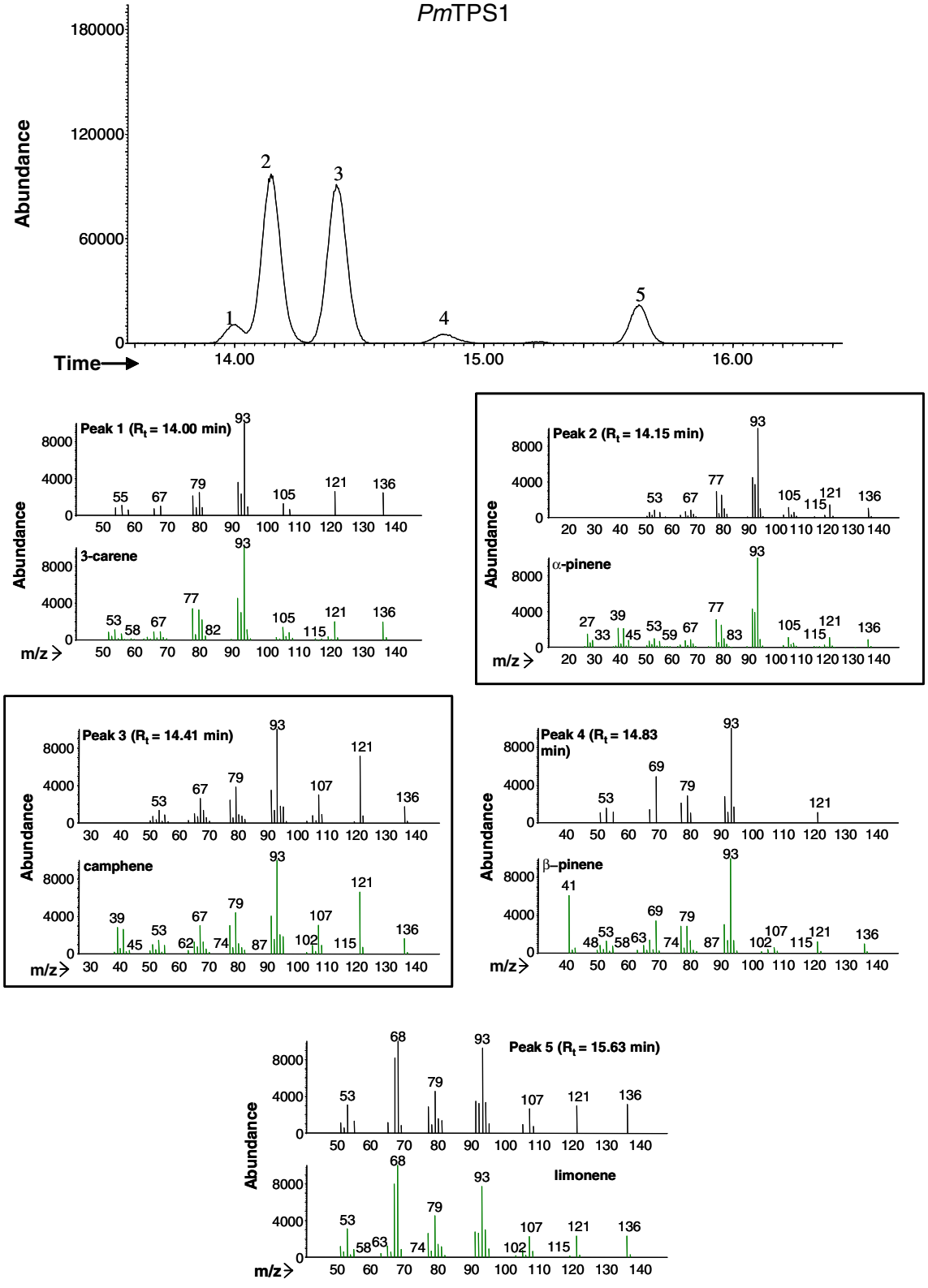

Fig. 4. GC-MS analysis of monoterpene products of the recombinant protein product of *Pme*TPS1 with geranyl diphosphate as the substrate. Mass spectra of products are shown in the top of each panel with the best library match below. Numbers in each panel correspond to numbers above peaks in the chromatogram. The two main products of recombinant *Pme*TPS1 are (−)-α-pinene and (−)-camphene. Three other monoterpenes are also produced at detectable levels.

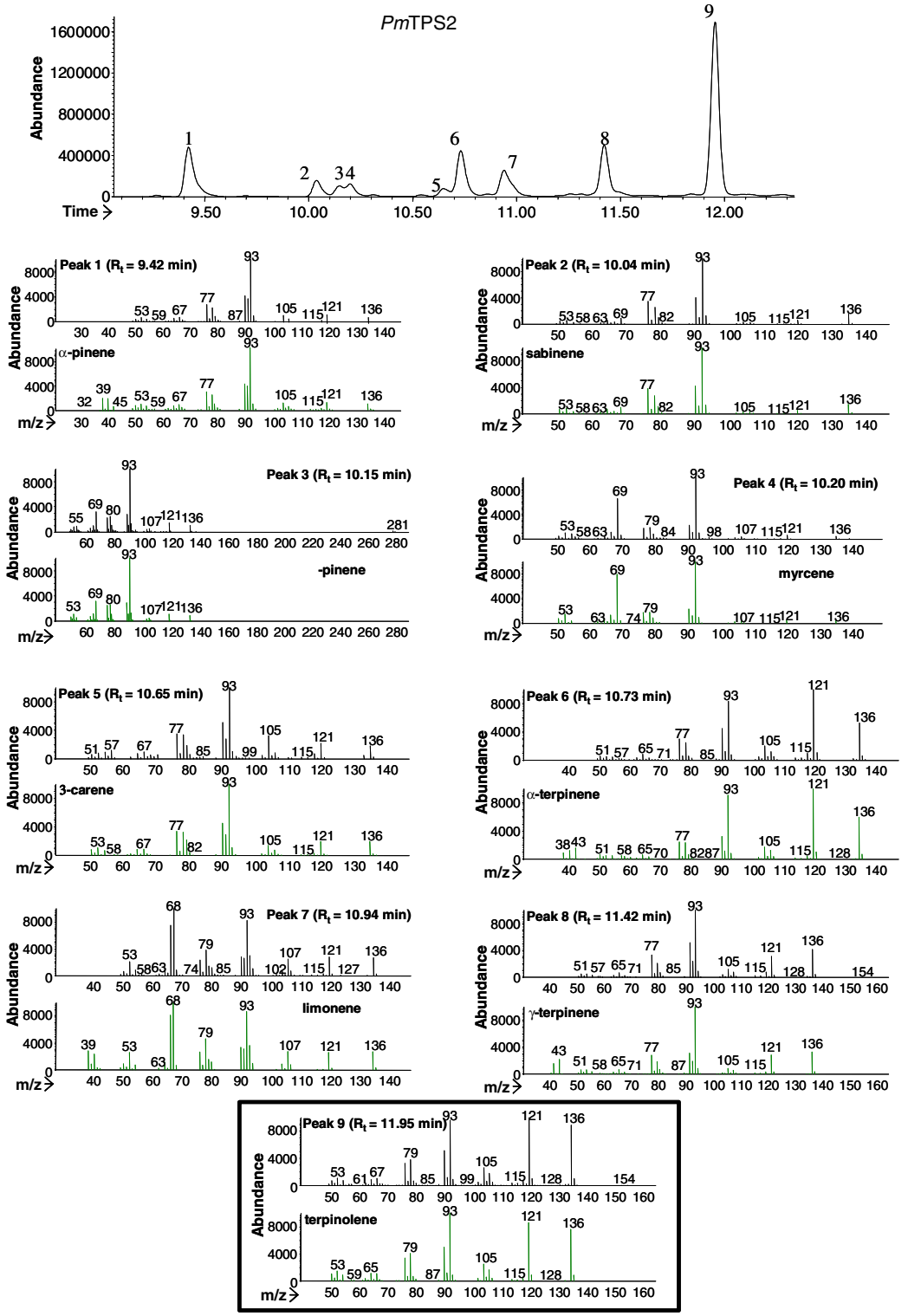

Fig. 5. GC-MS analysis of monoterpene products of the recombinant protein product of *Pme*TPS2 with geranyl diphosphate as the substrate. Mass spectra of products are shown in the top of each panel with the best library match below. Numbers in each panel correspond to numbers above peaks in the chromatogram. The main product of recombinant PmeTPS1 is terpinolene. Eight other monoterpenes are also produced at detectable levels.

synthases that we have characterized in resistance of Douglas-fir to western spruce budworm herbivory. It should be noted that increased CO_2 levels significantly reduce the amount of both α-pinene (1, 7) and camphene (2) in Douglas-fir resin (Snow et al., 2003), implying possible sensitivity of gene expression or protein function of *Pme*TPS1 under such conditions. *Pme*TPS1 may thus be a very important target of research into the effects of

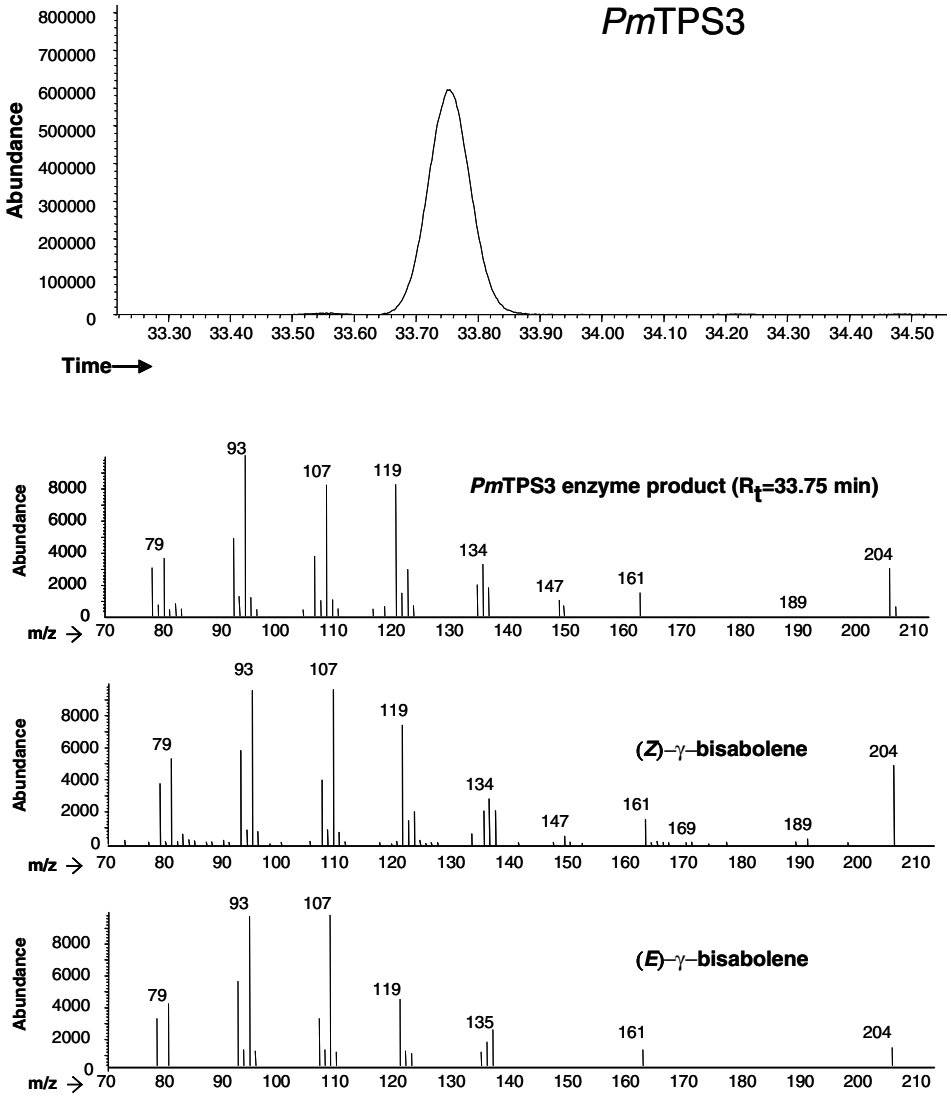

Fig. 6. GC-MS analysis of the sesquiterpene product of the recombinant protein product of *PmeTPS3* with farnesyl diphosphate as the substrate. The mass spectrum of the product is shown in the top of the panel with the best library match below. While the best library match for the single product of PmeTPS3 is (*Z*)-γ-bisabolene, the best match with Kovat's Index (Adams, 2001) indicates that the product actually is (*E*)-γ-bisabolene.

atmospheric CO_2 accumulation as it relates to plant/insect interactions.

Analyses of gene expression of these four synthases, and others yet to be discovered, in various tissues of Douglas-fir in response to seasonal stimuli, atmospheric conditions, insect feeding, fungal infection, and chemical elicitors will provide new data for tree breeders and pest and resource managers to aid in the development and utilization of pest-resistant cultivars.

4. Experimental

4.1. Reagents and strains

EasyTides™ radionucleotides (3000 Ci/mmol) were purchased from Perkin–Elmer (Boston, Massachusetts). All other biochemicals and reagents were purchased from Sigma Chemical Company or Fisher Scientific, unless otherwise noted. *E. coli* strains used were TOP10, XL1-Blue, SOLR, and BL21CodonPlus.

4.2. Plant material

Douglas-fir sibling-group trees, propagated from seeds collected at Cowichan Lake (British Columbia, Canada), were used. Fully regenerated, 1-year-old rooted saplings were grown in 2 l pots in a 3:1 (v:v) ratio of peat:vermiculite, with dolomite (2.4 g/l) and micronutrients (475 mg/l, Micromax). Trees were fertilized to a final concentration of 200 ppm N (3.6 g/l) with Osmocoat 20-8-20 fertilizer (Scotts) and were placed in a greenhouse with ambient conditions equal to the outside temperature for at least 6 months. Trees were used in the

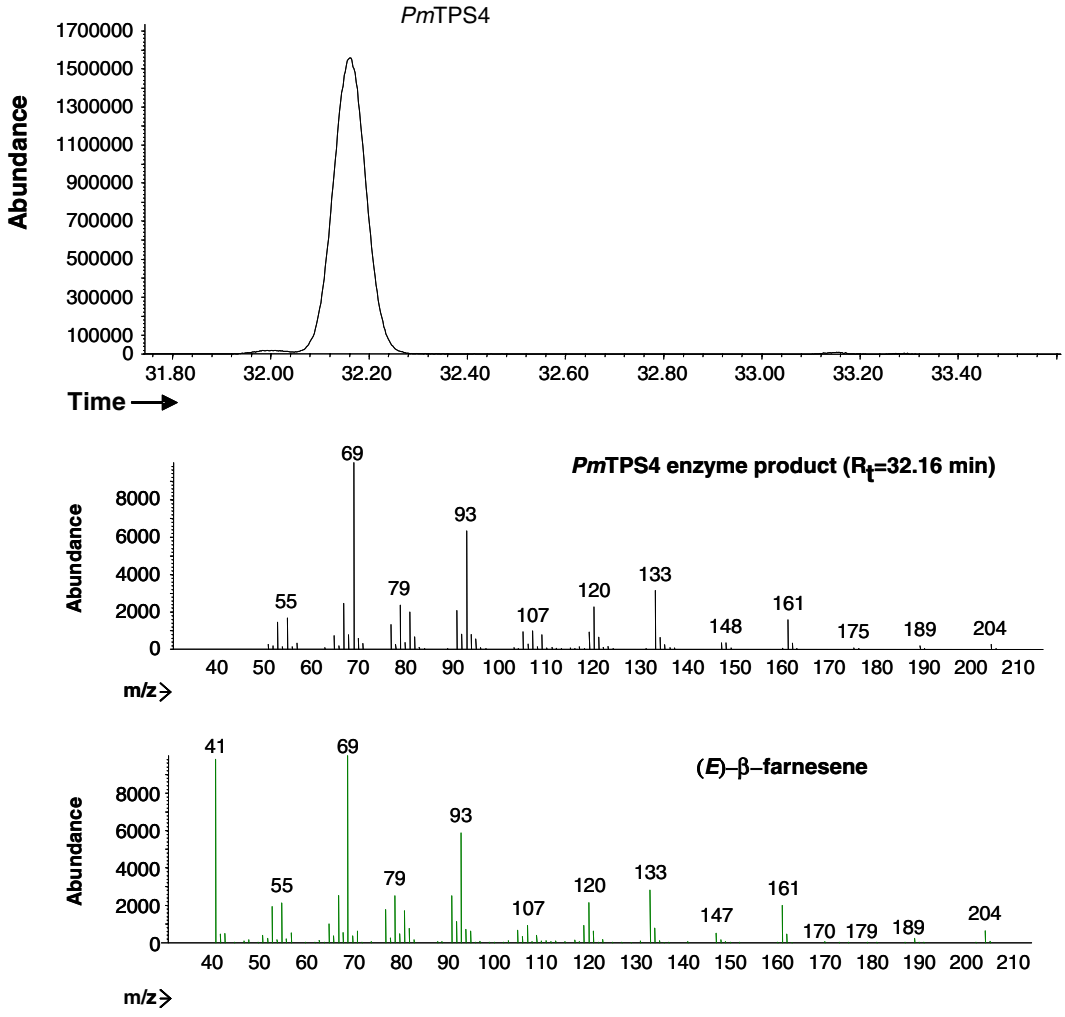

Fig. 7. GC-MS analysis of the sesquiterpene product of the recombinant protein product of *PmeTPS4* with farnesyl diphosphate as the substrate. The mass spectrum of the product is shown in the top of the panel with the best library match below. The single product of PmeTPS4 is (*E*)-β-farnesene.

experiments about six weeks after breaking dormancy, in early April.

4.3. MeJA treatment and harvest of tissues

Trees were treated by spraying the stem and foliage with 100 ml of 0.1% MeJA (95% [w/w] pure, Sigma) aqueous solution plus 0.1% Tween-20. Tissues were harvested ~2 h after MeJA treatment. Fresh green shoots with needles 8-cm long were collected and 6 cm of bark was longitudinally cut with a razor and peeled from the stem. Collected tissue was immediately flash frozen in liquid nitrogen and then stored at −80 °C until use.

4.4. RNA isolation

Water was treated with diethypyrocarbonate (DEPC) and all glassware and metal tools were baked at 180 °C for at least 4 h. Total RNA from MeJA-induced Douglas-fir bark and shoot tissue was isolated by a protocol modified from Lewinsohn et al. (1994). Shoots or bark were ground in liquid nitrogen to a fine powder with a mortar and pestle. About 10 g of ground tissue was added to 30 ml chilled extraction buffer (200 mM Tris–HCl pH 7.5, 300 mM LiCl, 10 mM Na–EDTA; adjust to pH 8.5, then add 1% w/v PVP, 5 mM thiourea, 1 mM aurintricarboxylic acid, and 10 mM dithiothreitol) in a 50 ml conical centrifuge tube containing 300 mg polyvinylpyrrolidone-360 and 600 mg polyvinylpolypyrrolidone, vortexed, and then centrifuged at 5000*g* (Beckman GPR, California) for 20 min at 4 °C. The supernatant was filtered through a sterile Kimwipe into a clean 50 ml centrifuge tube. To the filtered supernatant was added 1 ml of 3.3 M sodium acetate (pH 6.1) and ~10% v/v cold absolute ethanol. The solution was frozen solid at −20 °C, thawed, and then centrifuged at 5000*g* for 20 min at 4 °C. The supernatant was retained and was mixed with 1 volume isopropanol, held

at −20 °C overnight, and centrifuged at 5000*g* for 20 min at 4 °C. The pellet was resuspended in 400 μl DEPC water and transferred to a clean microfuge tube before being centrifuged at 14,000*g* for 15 min at 4 °C. The RNA-containing supernatant was transferred to a fresh microfuge tube. Spectrophotometric analysis and quantitation of the RNA sample was completed with a UV/Vis spectrophotometer (Biochrom Ultrospec 3000 Pro, Cambridge, England) analyzer. mRNA was purified from total RNA with the Ambion (Texas, USA) MicroPoly(A) Pure kit.

4.5. cDNA library construction

A Uni-ZAP XR cDNA library was prepared with purified mRNA following the protocol included with the Zap-cDNA Synthesis Kit (Stratagene, California). Before subcloning, however, cDNAs were size partitioned on an open Sepharose CL-2B gel filtration column. The resulting fractions were extracted with phenol-chloroform to remove contaminating proteins and the cDNA was ethanol-precipitated. The cDNA was run on an 1% agarose gel (BioRad, California) in 1X TAE (Gel-XL Plus system, Labnet International Inc., New Jersey) to separate the amplified fragments. Size-selected (>1.8 kb) cDNAs were cut from the gel, and the agarose was digested with agarase (New England Biolabs, Massachusetts). The large cDNAs were then ligated into the Uni-ZAP XR vector. The resultant cDNA-containing phagemids were then packaged in a highefficiency bacteriophage system (Gigapack III Gold; Stratagene) and plated on the *E. coli* cell line XL1-Blue MRF' for library amplification.

4.6. Library screening

The full length sequences for (*E*)-α-bisabolene synthase from Norway spruce (*Picea abies*) (Martin et al., 2004) was used to probe for sesqui-TPS. A myrcene synthase-like fragment from Norway spruce (Martin et al., 2004) that was obtained by PCR amplification (Table 1) was used to probe for mono-TPS. Each probe was amplified to 100 ng via PCR [(total volume 50 μl containing 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 200 μM of each deoxyribonucleotide triphosphate (dNTP), 0.1 μM primers, and 2.5 units of *Taq*DNA Polymerase (New England Biolabs, Massachusetts), using a PTC-100 thermocycler (MJ Research Inc., Massachusetts) and the following temperature program: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 2.5 min; then 72 °C for 10 min. The resulting PCR products were purified by electrophoresis through 1% agarose gel (BioRad, California) in 1X TAE (100 V, Gel-XL Plus system, Labnet International Inc., New Jersey) and gel extracted using the Qiagen (Hilden, Germany) Qiaquick gel extraction kit. The purified amplicons were randomly labeled with [α-^{32}P]dATP using the Amersham (Buckinghamshire, England) Rediprime II Random Prime DNA Labeling Kit.

Library screening was performed by replica filter hybridization of 10^5 recombinant phage cDNA plaques as previously described (Bohlmann et al., 1997, 1999). The two different radiolabelled probes were pooled and used as a single probe solution. Hybridization was performed for 24 h at 50 °C in hybridization buffer I. Filters were washed twice in hybridization buffer I for 10 min at 50 °C, and once more with hybridization buffer II for 10 min at 65 °C, before being exposed for 24 h to Kodak BioMax-MS film at −80 °C. Of the 76 hits from the first round, 20 clones yielding positive signals were purified through a second round of hybridization. Purified Uni-ZAP XR clones were excised in vivo as Bluescript II SK-phagemids using ExAssist helper phage and transformed into *E. coli* SOLR according to manufacturer's instructions for the ZAP cDNA Synthesis kit (Stratagene, California, USA). The size of each cDNA insert was determined by PCR using T3 and T7 promoter primers, and any amplified inserts over 1.1 kb in size were partially sequenced from both ends.

4.7. 5′ Rapid amplification of cDNA ends

To acquire the 5′-terminus corresponding to each truncated cDNA clone, RNA ligase mediated 5′-rapid amplification of cDNA ends (RLM-RACE) was carried out using the FirstChoice RLM-RACE system (Ambion, Texas) by the manufacturer's protocol. Nested gene-specific RACE primers specific for each clone (Table 1) were used in combination with the primers included in the kit. The primary outside amplification was performed with SuperTaq Plus polymerase (Ambion, Texas), while the inner nested amplification was performed using PfuTurbo DNA polymerase (NEB, Massachusetts) to generate blunt-ended amplified fragments. The resulting full-length cDNA amplicons were cloned into vector pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, California), and then were fully sequenced from both ends.

4.8. Sequence analysis

Using primers designed (Table 1) from our RACE sequencing results, inserts of all five recombinant Bluescript plasmids containing full-length versions of the recovered cDNAs were sequenced on both strands via primer walking. All sequencing work was performed by the NAPS Unit facility at the University of British Columbia, using the BigDye (version 3.1) Terminator Cycle sequencing method from Applied Biosystems.

Table 1
Primers used to generate hybridization probes, in rapid amplification of cDNA Ends, and to generate expression constructs of PmeTPS genes

Name	Research purpose	Sequence (5′–3′)
MYRCF	Myrcene probe forward	GTGGGAGAGATAGTGTTGTTGCTG
MYRCR	Myrcene probe reverse	TGTTACAAGGAGTGAAAGATATTGAACTC
M17Out	PmeTPS1 5′ outer RACE	GTCCTGCCCAAGTACGTGGA
M17In	PmeTPS1 5′ inner RACE	CTGTCGTGAAAGACTGGAGA
M34Out	PmeTPS2 5′ outer RACE	CCAGAAGTTTCTCGACCTTTGGGT
M34In	PmeTPS2 5′ inner RACE	CTCGGCAAATTCGAGTGCCAAC
SS1Out	PmeTPS3 5′ outer RACE	CACGTTGGCCGATGGAAGTGATCC
SS1In	PmeTPS3 5′ inner RACE	TTCACGCCCCTGTTCCTTTTCTGC
SS2Out	PmeTPS4 5′ outer RACE	CGACGCCAATTCTCCACGAGCCTT
SS2In	PmeTPS4 5′ inner RACE	CTTGCGAAAGGATTTGGCCCTCCA
M17F	PmeTPS1 full-length forward	CACCATGGAGTTCTATATTTCATGAG
M17R	PmeTPS1 full-length reverse	TTACACAGGTTCAAGGACGC
M34F1	PmeTPS2 full-length forward	CACCATGTCTCTTATTTCTATGGCGCC
M34F2	PmeTPS2 full-length forward	CACCATGAGACGCATTGGCGATTATCAT
M34R	PmeTPS2 full-length reverse	TTAGAGAGGCACAGGTTCAAGG
SS1F1	PmeTPS3 full-length forward	CACCATGGCAGCTTCTACTCTTCCTT
SS1F2	PmeTPS3 full-length forward	CACCATGCGAAGGACGGCCAATCCTCA
SS1R	PmeTPS3 full-length reverse	TTAGAGAGGAAGTGGTTCAATA
SS2F1	PmeTPS4 full-length forward	CACCATGAGCTTGGAAGAATTTTATCCA
SS2F2	PmeTPS4 full-length forward	CACCATGGCTACCGTTTATGTACCTTCT
SS2F3	PmeTPS4 full-length forward	CACCATGAGAAGGACGGCCAATCCTC
SS2R	PmeTPS4 full-length reverse	TTACAGAGGCAGTGGTTCAATTAG

4.9. Heterologous expression and characterization of terpene synthases

Primers were designed to amplify the full-length coding region of each putative terpene synthase and to insert directionally into the pET-100D expression vector (Invitrogen) as directed by manufacturer (Table 1). PCR reactions were performed in volumes of 50 μl containing 5 ul Roche *Pwo* DNA polymerase 10× buffer, 200 μM each dNTP, 0.6 μM each primer, 2.5 units of recombinant *Pwo* DNA Polymerase (Roche Diagnostics, Germany), and 100 ng of plasmid DNA with the following program: 95 °C, 5 min; 10 cycles of 95 °C for 15 s, 55 °C for 45 s; 72 °C for 2 min; 20 cycles of 95 °C for 15 s, 55 °C for 45 s; 72°C for 3.5 min; then 72 °C for 8 min. The PCR products were purified by agarose gel electrophoresis and used as templates for secondary PCR amplifications with the identical conditions in total volumes of 100 μl each.

Amplified fragments were individually subcloned into pET-100D vectors included in the pET Directional TOPO Expression Kit (Invitrogen, California), following the kit's instructions. These plasmids were then transformed into *E. coli* TOP10 Chemically Competent cells (Invitrogen, California) for sequence characterization and into *E. coli* BL21CodonPlus (Invitrogen, California) for functional characterization.

For expression, bacterial cultures containing each of the full-length cDNAs in BL21CodonPlus were grown to $OD_{600} = 0.6$ at 37 °C in 5 ml of LB medium supplemented with 100 μg of ampicillin/ml. Cultures were then induced by addition of 200 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and grown overnight at 20 °C. Cells were harvested by centrifugation (2000*g*, 15 min, 4 °C) and resuspended in either 1 ml of monoterpene or sesquiterpene assay buffer (Martin et al., 2004). Cells were disrupted by sonication using a Branson Sonifier 250 (Branson Ultrasonic Corporation, Danbury Conn., USA) at constant power (5 W) for 10 s. Homogenates were cleared by centrifugation (15,000*g*, 10 min). Either 2.5 mM geranyl diphosphate (GPP), 2.5 mM farnesyl diphosphate (FPP), or 2.5 mM geranyl geranyl diphosphate was added to separate aliquots of each supernatant. Each assay was overlaid with 1 ml of pentane, and the assay mixtures were incubated in glass tubes in a shaking water bath at 30 °C for 1 h. The aqueous fraction was extracted with pentane by briefly vortexing, and separation of the aqueous and organic fractions was achieved by centrifugation at 2500*g* for 2 min. The pentane overlay was dried with anhydrous $MgSO_4$. Each enzyme assay was extracted with two additional portions of pentane and volume was pooled.

4.10. Product identification

The pooled eluates were concentrated to a volume of ~25 μl with nitrogen gas. The samples were analyzed by GC/MS with an Agilent Hewlett-Packard 6890 GC connected to an Agilent 5973 Network Mass Selective Detector. For putative monoterpene synthase assays, 3ul of the concentrated organic phase was injected at 220 °C on a DB-WAX capillary column (30 m × 0.25 mm with 0.25 mm phase coating; Agilent, California).

Separation was achieved under a He flow rate of 2 ml min^{-1}, inlet temperature of 250 °C, with a temperature program of 3 min at 40 °C, a ramp to 70 °C at 3 °C min^{-1} (1 min hold), and a second ramp from 70 to 240 °C at 6 °C min^{-1} (30 min hold).

For putative sesquiterpene synthase assays, 3 ul of the concentrated organic phase was injected at 220 °C on an HP-5 capillary column (30 m × 0.25 mm with 0.25 mm phase coating; Agilent, California). Separation was achieved under a He flow rate of 2 ml min^{-1}, inlet temperature of 250 °C, with a temperature program of 3 min at 40 °C, a ramp to 180 °C at 5 °C min^{-1}, and a second ramp from 180 to 300 °C at 20 °C min^{-1} (1 min hold).

Chiral analyses for the two main products of *Pme*TPS1 were done on a Cyclo-Sil B column (30 m × 0.25 mm with 0.25 mm phase coating; Agilent, California). Separation was achieved under a He flow rate of 2 ml min^{-1}, inlet temperature of 250 °C, and a temperature program of 60 °C held for 6 min, then a gradual increase of 3 °C min^{-1} up to 160 °C, followed by an increase of 20 °C min^{-1} until 240 °C, which was held for 5 min.

The separated peaks for both the mono-TPS and sesqui-TPS assays were identified by comparing the generated 70-eV mass spectra against an electronic mass spectrometry standards database (Wiley Library BN29C74105 revision C.00.00, John Wiley & Sons, New Jersey) and with retention time matching with authentic standards when available. All mass spectrometry analyses were performed with Hewlett-Packard Chemstation software (ID G1701DA, version D.00.00.38). In one case (*Pme*TPS3) where the MS library match did not correspond to an authentic standard or to other similar sesquiterpenoids that we had available, the Kovat's index of the product peak was calculated (as described by manufacturer) and compared to published results (Adams, 2001). The Kovat's Index analysis (Retention index mixture for GC, Sigma Aldrich, Canada) was done on a DB-1 column under a He flow rate of 2 ml min^{-1}, inlet temperature of 250 °C, with a starting temperature of 40 °C, no hold, and a ramp to 315 °C at 5 °C min^{-1} and a final hold of 5 min.

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