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Cloning and characterization of two different types of geranyl diphosphate synthases from Norway spruce (*Picea abies*)

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Abstract

Geranyl diphosphate (GPP), the universal precursor of monoterpenes, is formed from isopentenyl diphosphate and dimethylallyl diphosphate by the action of geranyl diphosphate synthase, one of the key branchpoint enzymes of terpene biosynthesis. Three types of GPP synthase can be distinguished in plants based on sequence similarity and subunit architecture, but until now individual species have been reported to contain only one of these types. Here we show that the conifer, Norway spruce (*Picea abies*), contains two different types of GPP synthase belonging to two separate groups of homodimeric proteins. One enzyme, designated *Pa*IDS2 (*P. abies* isoprenyl diphosphate synthase 2), has high sequence similarity to other gymnosperm GPP synthases. It produces solely GPP in *in vitro* assays after expression in *Escherichia coli* and likely participates in monoterpene biosynthesis accompanying induced oleoresin formation, based on dramatic increases in transcript level after methyl jasmonate application. The other enzyme, designated *Pa*IDS3, has highest similarity to the previously reported *Arabidopsis thaliana* GPP synthase and several other angiosperm sequences, and is not associated with induced oleoresin formation in Norway spruce. *In vitro* assay of this protein and one encoded by a similar gene sequence from *Quercus robur* gave substantial amounts of the larger prenyl diphosphates, FPP and GGPP, in addition to GPP. Hence these proteins may not be involved in monoterpene formation and could conceivably form products in addition to GPP *in planta*.

Keywords: *Picea abies*; Pinaceae; *Quercus robur*; Fagaceae; Geranyl diphosphate synthase; Isoprenyl diphosphate synthase; Prenyltransferase; Monoterpenes; Gymnosperm; Conifer defense; Methyl jasmonate

1. Introduction

Monoterpenes, the C_{10} representatives of the terpene family, possess multiple functions in plants. They have long been known to play a defensive role in vegetative tissues by repelling insects (Gershenzon and Croteau, 1991), and deterring fungal growth (Hammer et al., 2003). Conifer oleoresin is a good example of a monoterpene-based plant defense which also contains sesquiterpenes and diterpenes (Franceschi et al., 2005; Keeling and Bohlmann, 2006). In addition, monoterpenes are also important in the reproductive biology of certain plants giving flowers and fruit scents that attract pollinating insects and dispersal agents (Pichersky and Gershenzon, 2002; Tholl et al., 2004; Aharoni et al., 2005). More recently, monoterpenes have been demonstrated to have other roles, such as attraction of herbivore enemies (Kessler and Baldwin, 2001; Schnee et al., 2006) and amelioration of oxidative stress (Loreto et al., 2004). Given that at least trace levels of monoterpenes are detectable in nearly all plant species investigated, these compounds may have even more as yet unknown roles. The formation of monoterpenes in plants can sometimes be induced by applying jasmonic acid and other octadecanoids, plant hormones which play an important role in responses to many biotic and abiotic factors in angiosperms

Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IDS, isoprenyl diphosphate synthase; GPPS, geranyl diphosphate synthase; MJ, methyl jasmonate.

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as well as gymnosperms (Martin et al., 2002; Wasternack and Hause, 2002; Hudgins et al., 2003; Hudgins et al., 2004; Zeneli et al., 2006).

Studies on the functions of monoterpenes would be facilitated by greater knowledge of monoterpene biosynthesis since this could provides information on when and where production occurs in the plant and give tools for directly manipulating monoterpene formation. All monoterpenes are derived from the same precursor, the C_{10} intermediate, geranyl diphosphate (GPP), which is synthesized by the condensation of two C_5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). This condensation is catalyzed by geranyl diphosphate synthase (GPPS), an isoprenyl diphosphate synthase (IDS, EC 2.5.1.1). As a group, IDSs catalyze the basic branchpoint reactions of terpenoid biosynthesis also forming farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}), the precursors of sesqui- and diterpenes, respectively. FPP and GGPP are biosynthesized by farnesyl diphosphate synthase (FPPS, EC 2.5.1.10) and geranylgeranyl diphosphate synthase (GGPPS, EC 2.5.1.30), respectively (Chen et al., 1994; Gershenzon and Kreis, 1998; Wang and Ohnuma, 1999; Buchanan et al., 2000). However, FPP and GGPP biosynthesis have been much better investigated at the enzymatic and molecular level than GPP biosynthesis.

Despite the ubiquity of monoterpenes, GPPS genes have been studied in only a handful of species (Croteau and Purkett, 1989; Burke et al., 1999, 2004; Narita et al., 1999; Bouvier et al., 2000; Sitthithaworn et al., 2001; Burke and Croteau, 2002a; Tholl et al., 2004), and there is only one report to date from gymnosperms (Burke and Croteau, 2002a). Based on sequence comparisons, three different classes of plant GPPS can be distinguished, one of which contains heterodimeric proteins, and two of which contain homodimeric proteins. The first heterodimeric GPPS, described from *Mentha* × *piperita* (Burke et al., 1999), was only functional as a heterodimer and the deduced amino acid sequences of the two subunits have only a low similarity to each other. The large subunit has moderate similarity to the sequences of plant GGPP synthases (GGPPS) (~55%) and even showed GGPPS activity when its cDNA was expressed heterologously in *Escherichia coli*. In contrast, the small subunit has much lower similarity to plant GGPPS or GPPS sequences (20–30%) and lacks IDS activity when expressed heterologously Similar heterodimeric GPPS have also been cloned from *Antirrhinum majus* and *Clarkia breweri* (Tholl et al., 2004). Consistent with *M.* × *piperita*, the large subunit of these enzymes has a sequence very similar to that of other plant GGPPS, whereas the small subunit is not.

Of the homodimeric GPPSs, one class is found only in conifers. It includes three GPPSs from *Abies grandis* (grand fir) which are 70–80% identical to conifer GGPPS sequences (Burke and Croteau, 2002a,b; Hefner et al., 1998; Schmidt and Gershenzon, 2007). The other class of homodimeric GPPSs is represented by only one functionally characterized sequence, an *Arabidopsis thaliana* protein which possesses only ~20% amino acid identity to the other group of homodimeric GPPSs as well as with angiosperm or gymnosperm GGPPSs and the large subunit of heterodimeric GPPSs. (Bouvier et al., 2000). Despite its low identity with other IDS sequences, the *A. thaliana* GPPS possesses motifs conserved within this family such as the two aspartate-rich regions. Although this protein is the only homodimeric angiosperm GPPS described to date, several similar but uncharacterized sequences are present in the NCBI-database, e.g., from *Citrus sinensis*, *C. unshiu* and *Quercus robur* (http://www.ncbi.nlm.nih.gov). Additional study of these and other GPPS-like sequences should shed further light on what types of GPPS are most prevalent in plants and how they regulate monoterpene formation.

Our knowledge of GPPS from gymnosperms is especially limited. Hence in this study, we investigated the properties of GPPS from Norway spruce (*Picea abies*), a conifer that produces monoterpene- and diterpene-rich oleoresin as a constitutive and inducible defense (Franceschi et al., 2005). The induced oleoresin response of this species to bark beetles and their associated fungal pathogens can be mimicked by treating trees with methyl jasmonate (MJ) (Martin et al., 2002, 2003; Byun-McKay et al., 2006; Erbilgin et al., 2006; Zeneli et al., 2006). This treatment stimulates the formation of traumatic resin ducts in the developing sapwood, which is associated with an accumulation of monoterpenes and diterpenes (Martin et al., 2002; Franceschi et al., 2005; Zeneli et al., 2006).

Here we report the isolation and heterologous expression of two Norway spruce GPPS. The genes were isolated by homology-based methods and heterologously expressed in *E. coli*. The corresponding proteins each represent a separate group of homodimeric GPPS, and appear to possess separate and distinct roles in the terpenoid metabolism of Norway spruce. In addition, we describe the expression of a GPPS from *Q. robur*, which is very similar to one of the Norway spruce genes.

2. Results

2.1. Isolation and characterization of cDNA clones encoding geranyl diphosphate synthases from Norway spruce

Two distinct GPPS-like cDNA fragments were obtained from Norway spruce by RT-PCR using primers designed to conserved regions of known plant GPPS with RNA isolated from methyl jasmonate (MJ)-treated saplings as a template. Screening of cDNA libraries constructed from wood or bark of MJ-treated spruce saplings yielded clones for each fragment. After extension by 5'RACE-PCR, the complete open reading frames of these genes, designated *PaIDS2* and *PaIDS3* were obtained. These were predicted to encode proteins of 386 and 427 amino acids, respectively, with masses of 42.2 and 47.7 kDa. Since the TargetP

1.1 software (http://www.cbs.dtu.dk/services/TargetP) Emanuelsson et al., 2000) predicted the presence of a signal peptide in the deduced proteins of both genes, these signal sequences were not considered in making sequence comparisons. Thus the deduced mature protein sequences of *PaIDS2* and *PaIDS3* possess only ~20% amino acid identity. The sequence of *PaIDS2* was 71–80% identical to the amino acid sequences of *GPPS1*, *GPPS2* and *GPPS3* from *Abies grandis* (Burke and Croteau, 2002a), and to conifer GGPPS sequences from *A. grandis* (Burke and Croteau, 2002b) and *P. abies* (Schmidt and Gershenzon, 2007). In contrast, *PaIDS3* had high amino acid identity only with the *A. thaliana GPPS* (Bouvier et al., 2000).

To learn more about *PaIDS3*, we selected another database sequence of high similarity for comparison. The gene from *Q. robur* (pedunculate oak), designated *QrIDS1*, was amplified from plasmid DNA. The deduced amino acid sequence consists of 416 residues with a mass of 46.2 kDa. All three sequences studied, *PaIDS2*, *PaIDS3* and *QrIDS1*, contained the two highly conserved aspartate-rich motifs present in all members of the IDS family which are important in substrate binding (Kellogg and Poulter, 1997). The second motif, DDxxD (residues 312–316, Fig. 1), was uniformly conserved in both Norway spruce and pedunculate oak GPPS sequences, but the first aspartate-rich motif, DDxxDxxxxRRG, was somewhat variable. *PaIDS3* and *QrIDS1* both contained this sequence with two additional aspartate residues in the 6th and 8th positions. However, *PaIDS2* had two additional other residues before the 3rd conserved aspartate, giving an DDxxxxDxxxxRRG sequence, and had additional aspartate residues in the 9th and 10th positions (Fig. 1).

2.2. Phylogenetic analysis of Norway spruce GPP synthases

A phylogenetic analysis of the deduced amino acid sequences of *PaIDS*2, *PaIDS*3, *QrIDS*1, and several other angiosperm and gymnosperm GPPSs and GGPPSs was performed (Fig. 2). As expected from previous studies, all of the plant GPPS sequences isolated to date were separated

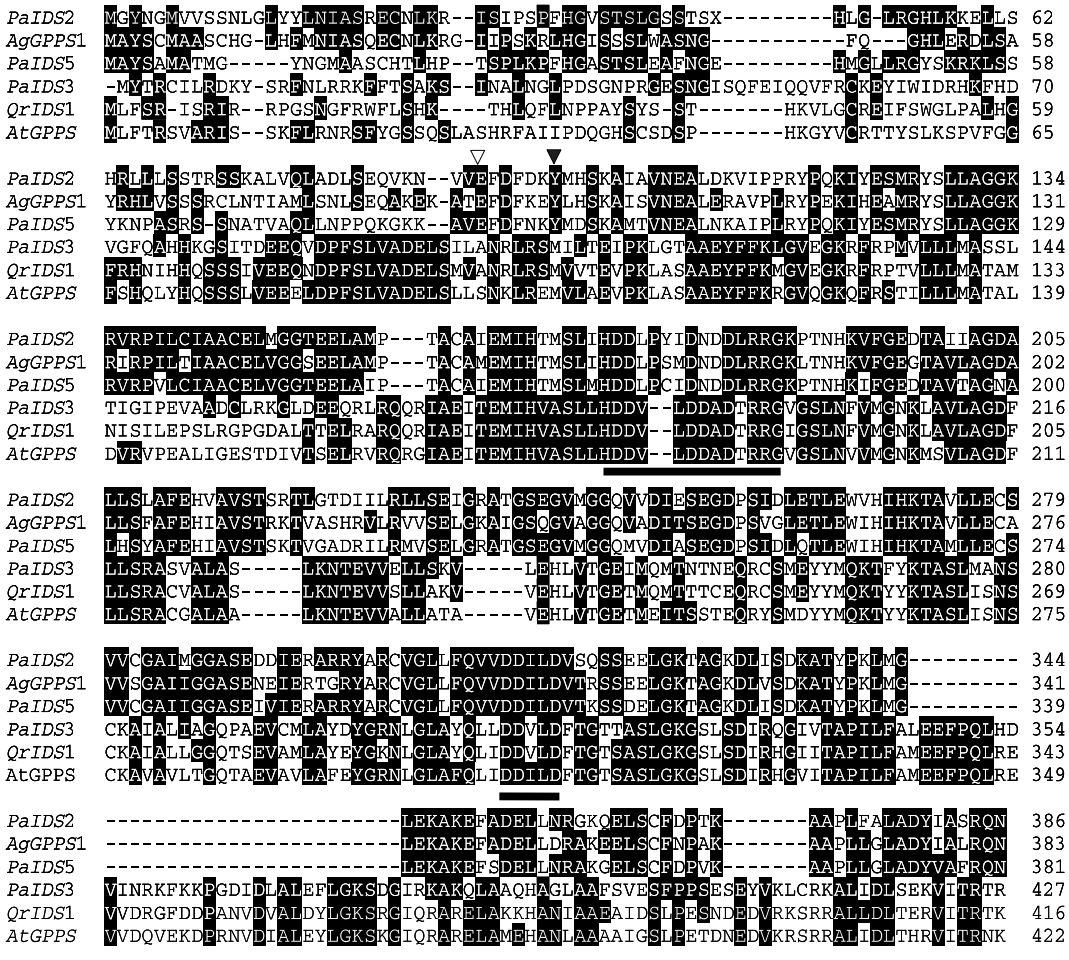

Fig. 1. Alignment of deduced amino acid sequences of *Picea abies IDS*2 (*PaIDS*2), *P. abies IDS*3 (*PaIDS*3), and *Quercus robur IDS*1 (*QrIDS*1, CAC20852) with plant isoprenyl diphosphate sequences of high identity, *Abies grandis GPPS*1 (*AgGPPS*1, AAN01133), *Picea abies IDS*5 (*PaIDS*5), and *Arabidopsis thaliana GPPS* (*At*GPPS, At2g34639). Amino acids identical in at least 3 of the 6 sequences are boxed in black. The artificial translation initiation sites for *PaIDS2* is indicated by an open triangle, the alternative translation initiation site for *PaIDS*3 and *QrIDS*1 are indicated by a filled triangle. The aspartate rich motifs, conserved among isoprenyl diphosphate synthases are underlined.

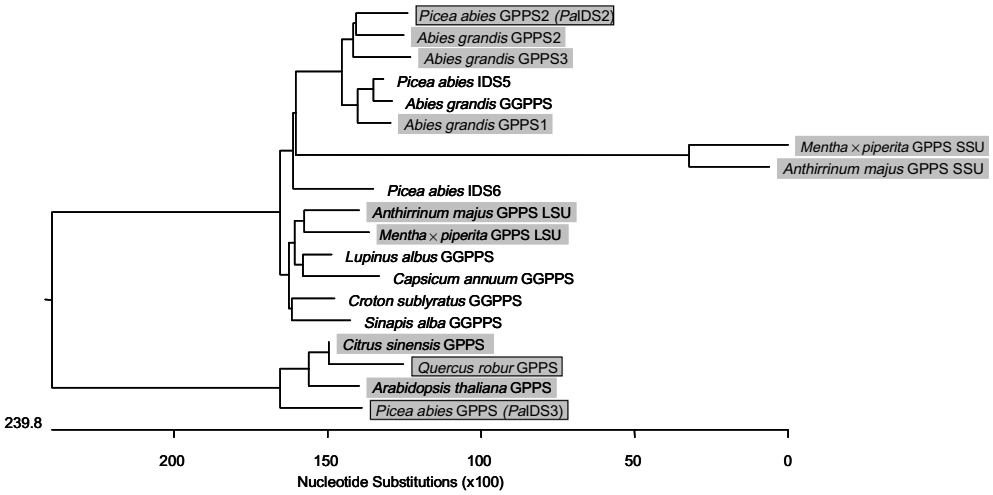

Fig. 2. Phylogenetic relationships of the deduced amino acid sequences of gymnosperm and angiosperm GPPS in comparison with related GGPPS sequences. The program DNA Lasergene MegAlign was used to create a neighbor-joining tree. For the heterodimeric GPPS, "LSU" indicates the large subunit and "SSU" indicates the small subunit. GPPS sequences are shaded in color, while the sequences described in this paper (*Pa*IDS2, *Pa*IDS3) are boxed.

into three main groups. *PaIDS2* clustered with the three *A. grandis* GPPS sequences, *PaIDS*3 and *QrIDS*1 clustered with the *A. thaliana* and *C. sinensis* GPPS sequences, and a third group consisted primarily of angiosperm GPPSs and GGPPSs. The large subunits of the heterodimeric *M.* × *piperita* and *A. majus* GPPS were found in the third group, but the corresponding small subunits of these enzymes formed a separate cluster. When plant GGPPS sequences were added to the analysis, these clustered with *PaIDS2* and the *A. grandis* GPPS in the first group giving a cluster consisting solely of conifer sequences. However, the *PaIDS*3 sequence clustered only with angiosperm GPPS sequences.

To look for other gymnosperm sequences like *PaIDS3* and to confirm the existence of two divergent GPPS gene types in other gymnosperms, an on-line loblolly pine (*Pinus taeda*) EST library (http://compbio.dfci.harvard.edu/tgi) was searched and two nearly complete GPPS-like sequences were found, one (TC66872) with 85% identity to *PaIDS*2, and the other (TC80501) with 96% identity to *PaIDS*3 (data not shown).

2.3. Functional expression of GPP synthases

To study the enzymatic activities of the encoded proteins of *PaIDS*2, *PaIDS*3, and *QrIDS*1, we expressed each full-length cDNA minus its putative signal peptide in *E. coli*. All of the expressed proteins showed GPPS activity. There was no activity in untruncated versions of *PaIDS*2, *PaIDS*3, and *QrIDS*1. However, *Pa*IDS2 catalyzed the biosynthesis of GPP as its sole product, while *Pa*IDS3 and *Qr*IDS1 catalyzed the biosynthesis of larger prenyl diphosphate products as well (Fig. 3). The product profile of *Pa*IDS3 consisted of 42% GPP, 33% FPP, and 25% GGPP (data not shown), while that of *Qr*IDS1 was 55% GPP and 45% FPP, with no GGPP. In all cases, extracts of bacterial strains carrying an empty vector showed no enzyme activity. No change of product specificity was observed in control assays without 250 mM imidazole in the assay buffer (data not shown).

2.4. Expression of Norway spruce GPP synthases in methyl jasmonate-treated spruce saplings

To examine the metabolic roles of *Pa*IDS2 and *Pa*IDS3 in Norway spruce in more detail, qRT-PCR analysis was performed using RNA from MJ-treated *P. abies* saplings. The two genes were expressed in both the bark and wood of stems, but only the expression of *PaIDS*2 was induced by MJ treatment. The temporal pattern of *PaIDS*2 induction was different in the bark vs. the wood. The transcript level of *Pa*IDS2 in bark steadily increased over the time course of the experiment reaching a 120-fold higher expression by day 10, as compared to pre-treatment levels. In contrast, in wood, transcript level reached its maximum at day 1, with a 12-fold higher expression level compared to pre-treatment levels (see Fig. 4).

3. Discussion

Three different types of GPP synthase can be distinguished in plants based on sequence relatedness and subunit architecture. Isolation, sequence analysis and heterologous expression of isoprenyl diphosphate synthases from Norway spruce demonstrated two GPP synthases to be present in this species, each a representative of a different homodimeric type. This is the first time that two different GPP synthase types have been reported from a single species. One enzyme, *Pa*IDS2, possessed a sequence similar to those of previously isolated gymnosperm GPP synthases (Burke and Croteau, 2002a), pro-

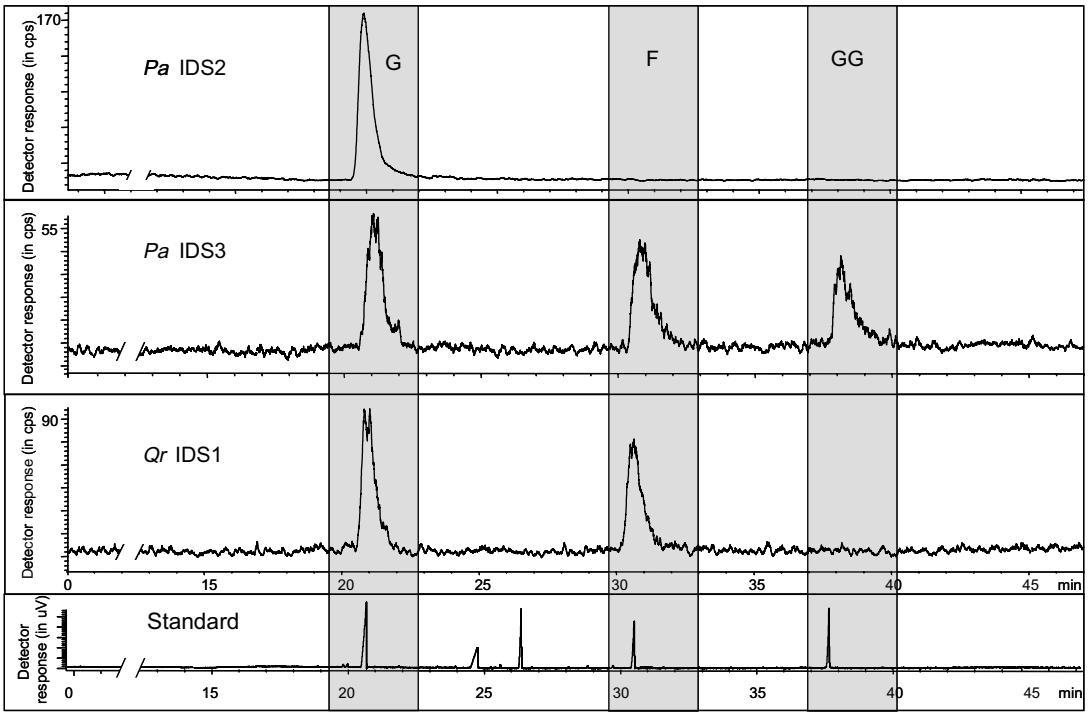

Fig. 3. Catalytic activity of recombinant *Pa*IDS2, *Pa*IDS3 and *Qr*IDS1 after heterologous expression in *Escherichia coli* and assay with DMAPP and [1-^{14}C] IPP. Reaction products were hydrolyzed and the resulting alcohols were analyzed by radio-gas chromatography. The three upper panels show the output of the radioactivity detector. Products were identified by co-injection of authentic unlabeled standards measured with a thermal conductivity detector (bottom panel). Purified crude bacterial extracts expressing empty vector controls did not show any measurable radioactivity (data not shown). G, geraniol; F, farnesol; GG, geranylgeraniol.

duced GPP as its sole *in vitro* product, and showed its gene transcript levels to be strongly induced when saplings were sprayed with methyl jasmonate (MJ). MJ treatment is known to mimic the response of Norway spruce to bark beetle attack by triggering the formation of traumatic resin ducts (TRD) and stimulating production of a defensive oleoresin which contains large quantities of monoterpenes (Martin et al., 2002; Zeneli et al., 2006). The increase of *PaIDS2* transcript after MJ treatment suggests that the GPP produced by the encoded enzyme is an important substrate for monoterpene biosynthesis in oleoresin formation. GPPS enzyme activity was found to increase after MJ application in an earlier study (Martin et al., 2002). Differences in the time-course of expression pattern in wood and bark are presumably be caused by their different involvement in TRD development. Even though TRD are located in the wood, and not in the bark, they begin developing as cambium initials. Since the bark samples used in the present study also contain cambium tissue, the increased *PaIDS2* expression seen in both bark and wood samples is not surprising. *PaIDS2* gene show at least slight activation in the wood which may reflect the general activation of terpenoid metabolism in TRD formation.

A second enzyme, *Pa*IDS3, had a sequence similar to a GPP synthase previously reported from *A. thaliana* (Bouvier et al., 2000) and other angiosperm sequences in the data base, but produced substantial amounts of FPP and GGPP as well as GPP *in vitro*. In addition, its transcript abundance was not correlated with induced monoterpene production after MJ treatment. To gain more information on this enzyme, one of the similar angiosperm sequences, that from *Q. robur*, was also heterologously expressed as part of this study and found to produce high amounts of FPP in addition to GPP, but no GGPP. The *in vitro* product profiles of *Pa*IDS3 and *Qr*IDS1 call into question their assignment as GPP synthases, which is based largely on sequence similarity to the characterized *A. thaliana* GPP synthase (Bouvier et al., 2000). It is also worth noting that neither *A. thaliana* nor *Q. robur* accumulate large amounts of monoterpenes in their tissues, and *PaIDS3* expression is not well associated with monoterpene formation in Norway spruce. Thus it is possible that *Pa*IDS3, *Qr*IDS1 and the *A. thaliana* enzyme are not involved in monoterpene formation *in planta*, but produce GPP for other purposes. They may even catalyze the formation of products other than or in addition to GPP.

The product specificity of IDS enzymes *in planta* could differ from that exhibited in *in vitro* assays. For example, it has been shown that IDSs expressed *in vitro* can catalyze the condensation of an additional C_5 unit to their main products producing a side product with typical abundance in the range of 5–10% of the main product (e.g., Burke and Croteau, 2002a). However, the "side" products of *Pa*IDS3 and *Qr*IDS1 are present in much more substantial amounts. Another possible cause of altered product specificity *in vitro* is the truncation of the predicted signal

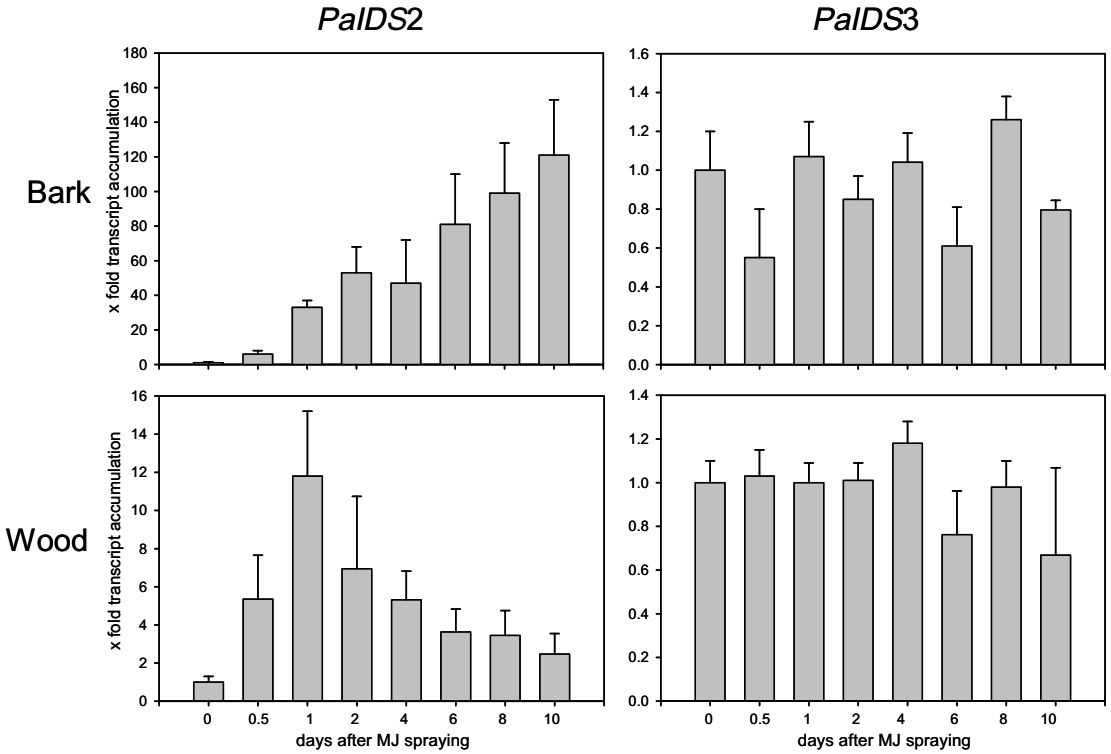

Fig. 4. Relative abundance of mRNA transcripts *PaIDS*2 and *PaIDS*3 genes in methyl jasmonate-treated Norway spruce saplings. Transcript abundance of each gene was measured by quantitative RT-PCR using SYBR Green for detection and ubiquitin for normalization. The measured time points were 0.5, 1, 2, 4, 6, 8 and 10 days after the onset of treatment, using bark and cambium (top panel) and wood (bottom panel). The time zero measurement is the untreated control; its abundance was set to 1.0. Each value is the average of two independent biological replicates, each of which is represented by three technical replicates.

peptide in construction of the expression vector, which is usually thought to be necessary for *in vitro* activity. To test this possibility, we expressed cDNAs of *Pa*IDS3 and *Qr*IDS1 truncated at different positions in the signal peptide, but observed no difference in product spectrum (data not shown).

Additional insight into the function of *Pa*IDS3 and *Qr*IDS1 might be obtained from knowledge of their subcellular location. Analysis of their signal peptides via the TargetP program (http://www.cbs.dtu.dk/services/TargetP, Emanuelsson et al., 2000), predicts that *Pa*IDS2 is localized in the plastids, but that *Pa*IDS3 and *Qr*IDS1 have a high probability of being localized in mitochondria. Analysis of published sequences of *A. grandis GPPS1* and *GPPS2* as well as the *A. thaliana* GPPS and the *C. sinensis* sequence (as yet uncharacterized, Fig. 2) extends these conclusions. Like *Pa*IDS2, the *A. grandis* GPPSs are predicted to be localized to the plastids, while the *A. thaliana* and *C. sinensis* enzymes, like *Pa*IDS3, are predicted to be found in the mitochondria. While these theoretical predictions must still be verified by experimental methods, they suggest that the two enzyme clusters, exemplified by *Pa*IDS2 on the one hand and *Pa*IDS3 on the other, play different roles in the plant cell. Our discovery of sequences representing these two enzyme types in *P. taeda* (Section 2.2) suggests that the same two enzyme types exist in other gymnosperms as well.

Interestingly, we found no evidence for Norway spruce GPPS sequences similar to the heterodimeric enzymes described from *M.* × *piperita* (Burke et al., 1999), *Clarkia breweri* (Tholl et al., 2004) and *A. majus* (Tholl et al., 2004), despite the use of appropriate primers in numerous PCR reactions with spruce cDNA. Based on the phylogenetic relationships depicted in Fig. 2 and the demonstrated GGPPS activity of the individual large subunits of the heterodimeric protein (Tholl et al., 2004), the heterodimeric GPPSs seem to have been derived from GGPPS sequences. However, both clusters of IDS represented by *Pa*IDS2 and *Pa*IDS3 seem to represent evolutionary lines separate from that leading to the heterodimeric enzymes. Additional study of plant IDS involving gene cloning, *in vitro* expression and *in planta* investigations are necessary before a full functional and evolutionary picture of GPP synthases can be obtained.

4. Experimental

4.1. Chemicals

All chemicals and solvents were of analytical grade and were obtained from Merck (Germany), Serva (Germany) or Sigma (Germany). The substrate [1-^{14}C] IPP (55 Ci mol^{-1}) was purchased from Biotrend (Germany) while

unlabeled DMAPP and IPP were obtained from Echelon Res. Lab. Inc. (Salt Lake City, UT, USA).

4.2. Plant material

Four-year-old Norway spruce *P. abies* monoclonal saplings (clone 3369 Schongau) were purchased from Samenklenge und Pflanzgarten Laufen (Germany). Saplings were grown in standard soil under a 21 °C day/16 °C night temperature cycle, controlled light conditions (16 h day at 150–250 μE, mixture of cool white fluorescent and incandescent light) and a humidity of 70% in a climate chamber (Vötsch, Germany). Saplings used for experiments were kept for 3 weeks under these growing conditions. To induce saplings, they were sprayed with 100 μM methyl jasmonate (MJ) in 0.5% (v/v) Tween 20 as detergent. At various time intervals, bark (including the cambium layer)), and wood were peeled from the uppermost internode (representing the previous year's growth) and frozen in liquid nitrogen.

4.3. Amplification and cloning of GPPS-like fragments

Degenerate primers were designed based on conserved regions of GPPS sequences from *M.* ×*piperita*, and *P. taeda* (EST NXSI_058_F04_F, http://www.ncbi.nlm.nih.gov), *C. sinensis* and *A. thaliana* (Table 1). Total RNA was isolated from MJ-treated spruce bark harvested at various time points after treatment (6, 12, and 24 h; 2, 4, 6, 10, and 20 days) based on the method of Wang et al. (2000). Reverse transcription of the pooled RNA was carried out with oligo-(dT) primers using Superscript™ II (Invitrogen) according to the manufacturer's instructions. Subsequently, PCR was performed with 35 cycles of 94 °C for 1 min, annealing for 1 min, and extension at 72 °C for 1 min in a Robocycler (Stratagene). To identify the optimal annealing temperature, a temperature gradient with 2 °C intervals ranging from 42 °C to 64 °C was used. The resulting PCR products were used as templates in nested PCR to amplify the GPPS-like cDNA fragments. A 165 bp fragment and a 544 bp fragment were subcloned into pCR™ 4-TOPO™ (Invitrogen) according to the manufacturer's instructions.

4.4. Isolation of full length spruce and oak GPPS cDNA clones

Poly $(A)^+$ RNA was isolated from total spruce RNA (extracted as described above) using Dynabeads™ (Dynal). cDNA was synthesized using the SMART™ cDNA-Library-Construction Kit (Clontech). *In vitro* packaging with Gigapack™ III Gold (Stratagene) yielded a library of 8×10^7 plaque-forming units, which was subsequently screened for sequence homology to *GPPS* using the ^{32}P-labeled 165 bp and 544 bp PCR gene-specific fragments as probes. Single plaques were isolated from positive signals and λDNA was converted into pDNA in *E. coli* BM25.8. The sequence obtained from screening with the 165 bp fragments was designated *PaIDS2* and that obtained from screening with the 544 bp fragment was designated *PaIDS3*.

For *PaIDS*2 the selected pDNA clone of 820 bp was elongated with nested 5'-RACE PCR using the SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions (primers appear in Table 1) to obtain a full length open reading frame of 1158 bp. The complete open reading frame of *PaIDS3* was 1281 bp. The oak (*Q. robur*) *IDS* sequence was isolated from a plasmid generously provided by Wolfgang Zimmer (Institute for Meteorology and Climate Research, Garmisch-Partenkirchen, Germany) and found to contain 1248 bp. All sequence analyses were carried out using an ABI 3100 automatic sequencer (Applied Biosystems).

Table 1
Primers used for screening, heterologous expression in *Escherichia coli* and expression in *Picea abies*

Gene	Purpose	Forward primer	Reverse primer
PaIDS2	Screen	GC(G/C)GCITGCGAGCTCGTCGGCGG	CC(G/C)I(C/T)(C/T)AICA(G/T)ICCCTCCG(A/G)(C/T)CCG
PaIDS2	Screen-n	GCCATGG(A/C)IGCCGCGGCGGC(G/C)	GCCAII(A/T)(A/G)CTCGAAC(G/C)C(G/T)A(A/G)IG(A/G)G
PaIDS3	Screen	GGTTCC(A/T)AAGCTTGCCTC(A/T)GCTGC	GCGTA(A/G)(C/T)TGAGG(A/G)AACTCTTCCATGGC
PaIDS3	Screen-n	GCTGATGGC(A/G)ACAGCTCTG(A/G)ATGT(A/G)CG	CCCTTTCC(A/G)AGAGAGGC(A/T)GATGTGCC
PaIDS2	RACE		CAGCCTCAAAATTATATCAGTCCCC
PaIDS2	RACE-n		CCCATAAGCTCGCACGC
PaIDS2	Expr	ATGTTTGATTTCGATAAGTACATGCATTCC	GTTTTGACTTGAAGCAATGTAATCTGCC
PaIDS3	Expr	ATGTCTGCAAAATCTATCAATGCTCTG	CCTTGTTCTGGTGATCACTTTTTC
QrIDS1	Expr	ATGCACAAAACCCACCTTCAATTC	CTTGGTCCTTGTAATGACTCTTTC
PaIDS2	RT-PCR	GTTGTTGTCACATAGACTTCTGC	CTGTTCAGACAGATCAGCTAG
PaIDS3	RT-PCR	GATGATTCTTACCGAGATTCC	CTTTTGCCCTCCACTCC
Ubi	RT-PCR	GTTGATTTTTGCTGGCAAGC	CACCTCTCAGACGAAGTAC

4.5. Heterologous expression of GPPS sequences in E. coli

The coding sequences of cDNA clones *PaIDS*2, *PaIDS3* and *QrIDS1* were amplified with the primer combinations shown in Table 1. The cleavage site of the signal peptide was predicted with the chloroP program (http://www.cbs.dtu.dk) and by comparison with the truncation sites of heterologously-expressed *A. thaliana* and *A. grandis* GPPSs (Bouvier et al., 2000; Burke and Croteau, 2002a). Amplification of the truncated cDNAs was carried out with the Expand High Fidelity PCR System (La Roche) and the resulting cDNA fragments were cloned into the expression vector pCR™-T7 CT TOPO™ (Invitrogen) which adds a tag containing six histidine residues to the C-terminus. Positive clones were first transferred into *E. coli* strain TOP10F' (Invitrogen) and then into strain BL 21(DE3)pLysS (Invitrogen). Bacterial cultures expressing *PaIDS*2 were grown to an OD of 0.6 and transformants were induced with 2 mM IPTG according to the manufacturer's instructions, except that they were grown exclusively at 18 °C. *PaIDS*3 and *QrIDS*1 were expressed using the Overnight Express™ Autoinduction System 1 (Novagen). Bacterial pellets were resuspended in an assay buffer containing 20 mM Mopso, pH 7.0, 10% (v/v) glycerol and 10 mM $MgCl_2$ and sonicated. The spruce IDS2 and IDS3 proteins, and the oak IDS1 protein were purified individually over Ni-NTA agarose columns (Qiagen) according to the manufacturer's instructions. The recombinant proteins were eluted with 250 mM imidazole in the assay buffer. After adding 2 mM DTT to the assay buffer containing 250 mM imidazole, proteins were checked for purity by SDS–PAGE and used to determine enzyme activity.

4.6. Assay of recombinant PaIDS2, PaIDS3 and QrIDS1 proteins

Assays were carried out in a final volume of 500 µl containing 20 mM Mopso (pH 7.0), 10 mM of $MgCl_2$, 10% (v/v) glycerol, 2 mM DTT, 40 µM [1-^{14}C] IPP (2 MBq/µmol) and 40 µM DMAPP. Assays were carried out in triplicates for at least three biologically independent replicates. The reaction was initiated by addition of recombinant protein, and the assay mixture was overlaid with 1 ml pentane and incubated overnight at 30 °C. To stop the assay and hydrolyze all diphosphate esters, a 1 ml solution of 2 U of calf intestine alkaline phosphatase (Sigma) and 2 U of potato apyrase (Sigma) in 0.2 M Tris–HCl, pH 9.5, was added to each assay and incubated at 30 °C overnight. After enzymatic hydrolysis, the resulting isoprenyl alcohols were extracted into 2 ml of diethyl ether, and after a standard terpene mixture was added, the organic extracts were evaporated under N_2 and used for radio-GC measurements. Radio-GC analysis was performed on a Hewlett–Packard HP6890 gas chromatograph (injector at 220 °C, TCD at 250 °C) in combination with a Raga radioactivity detector (Raytest, Straubenhardt, Germany) using a DB5-MS capillary column (J&W scientific) (30 m × 0.25 mm with 0.25 µm phase coating). Separation of the injected concentrated organic phase (1 µl) was achieved under a H_2 flow rate of 2 ml/min with a temperature program of 3 min at 70 °C, followed by a gradient from 70 to 240 °C at 6 °C/min with a 3 min hold at 240 °C. Products, measured by radio-GC, were identified by comparing retention times with those of co-injected authentic non-radioactive terpene standards, measured via a thermal conductivity detector. Protein concentrations in enzyme assays were measured according to Bradford (1976) using the BioRad reagent with bovine serum albumin (BSA) as standard. The protein concentrations used in each assay were between 50 and 100 µg/ml.

4.7. Quantitative PCR of PaIDS2 and PaIDS3 in Norway spruce

Total RNA from different time points and tissues used for RT-PCR reaction was isolated according to Wang et al. (2000) except that an additional DNA digestion step was included (RNase Free DNase Set, Qiagen). Using identical amounts of total RNA, template cDNA for the subsequent PCR reactions was generated using Superscript™ III (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed with Brilliant® SYBR Green QPCR Master Mix (Stratagene) and ubiquitin was used to normalize transcripts of interest. The primers used are given in Table 1. Reactions were carried out with a M×3000P Real Time PCR Thermocycler (Stratagene) using a program with a maximum of 45 cycles of 95 °C for 30 s, an annealing temperature of 55 °C (for *PaIDS2*) or 52 °C (for *PaIDS*3) for 30 s, and 72 °C for 30 s, followed by a melting curve analysis of transcripts). The relative amount of transcript was normalized to ubiquitin using the M×3000P software with the amount at the onset of treatment used as calibrator. Every measurement was repeated with two independent biological duplicates, each of which was represented by at least three technical replicates.

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