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Cloning and characterization of isoprenyl diphosphate synthases with farnesyl diphosphate and geranylgeranyl diphosphate synthase activity from Norway spruce (*Picea abies*) and their relation to induced oleoresin formation

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Abstract

The conifer *Picea abies* (Norway spruce) employs terpenoid-based oleoresins as part of its constitutive and induced defense responses to herbivores and pathogens. The isoprenyl diphosphate synthases are branch-point enzymes of terpenoid biosynthesis leading to the various terpene classes. We isolated three genes encoding isoprenyl diphosphate synthases from *P. abies* cDNA libraries prepared from the bark and wood of methyl jasmonate-treated saplings and screened via a homology-based PCR approach using degenerate primers. Enzyme assays of the purified recombinant proteins expressed in *Escherichia coli* demonstrated that one gene (*PaIDS* 4) encodes a farnesyl diphosphate synthase and the other two (*PaIDS* 5 and *PaIDS* 6) encode geranylgeranyl diphosphate synthases. The sequences have moderate similarity to those of farnesyl diphosphate and geranylgeranyl diphosphate synthases already known from plants, and the kinetic properties of the enzymes are not unlike those of other isoprenyl diphosphate synthases. Of the three genes, only *PaIDS* 5 displayed a significant increase in transcript level in response to methyl jasmonate spraying, suggesting its involvement in induced oleoresin biosynthesis.

Keywords: *Picea abies*; Pinaceae; Gymnosperm; Conifer defense; Prenyltransferase; Methyl jasmonate; Enzyme kinetics, Phylogenetic analysis

1. Introduction

Conifers of the family Pinaceae frequently come under attack from herbivorous insects, such as the tree-killing bark beetle, *Ips typographus*, and its fungal associate, *Ceratocystis polonica* (Phillips and Croteau, 1999; Trapp and Croteau, 2001; Baier et al., 2002; Franceschi et al., 2005). However, the long life spans of many conifers and their evolutionary persistence indicate the presence of effective defenses. The best known example of conifer defense is oleoresin, a pungent, viscous mixture of terpenoids found in specialized ducts, blisters and cells in stems and foliage. Oleoresin is both a constitutive and an inducible defense. For instance, some species of the Pinaceae, especially in the subfamily Piceoideae and to some degree the Abietoideae, form new (traumatic) resin ducts (TRDs) in response to attack by stem-boring insects and their associated fungi. These oleoresin-filled structures are found mainly in the sapwood and bark of coniferous trees, and are believed to help resist attack by augmenting the constitutive resin flow, thus providing a strong barrier against most herbivores and pathogens (Nagy et al., 2000; Martin et al., 2002; Hudgins et al., 2004; Franceschi et al., 2005; Byun-McKay et al., 2006; Keeling and Bohlmann, 2006).

Abbreviations: TRD, traumatic resin duct; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IDS, isoprenyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; MJ, methyl jasmonate.

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With more than 30,000 structural variants; terpenes are the largest class of plant secondary metabolites. Oleoresin consists mainly of monoterpenes (C_{10}) and diterpene resin acids (C_{20}) as well as smaller amounts of sesquiterpenes (C_{15}) (Langenheim, 2003). The biosynthesis of all terpenoids is initiated by the synthesis of isopentenyl diphosphate (IPP) via the mevalonic acid pathway or the methylerythritol phosphate pathway (Gershenzon and Kreis, 1999) (Fig. 1). IPP and its isomer, dimethylallyl diphosphate (DMAPP), are the five-carbon building blocks that undergo sequential condensation reactions to form geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), and geranylgeranyl diphosphate (GGPP, C_{20}), the precursors of monoterpenes, sesquiterpenes and diterpenes, respectively. The enzymes catalyzing these sequential condensations are a group of prenyltransferases referred to collectively as isoprenyl diphosphate synthases. GPP, FPP, and GGPP are each believed to be formed by a specific isoprenyl diphosphate synthase (IDS) named for its product: geranyl diphosphate synthase (GPPS), FPP synthase (FPPS), and GGPP synthase (GGPPS) (Gershenzon and Kreis, 1999) (Fig. 2). In subsequent steps, these linear diphosphates serve as substrates for terpene synthases, which form a complex variety of mono-, sesqui-, and diterpene carbon skeletons (Martin et al., 2004; Keeling and Bohlmann, 2006). An important feature of terpenoid biosynthesis is the way this process is partitioned among different cell compartments. The formation of GPP and GGPP and their subsequent conversion to mono- and diterpenes occur in the plastids, whereas the biosynthesis of FPP and its conversion to sesquiterpenes are localized in the cytosol (Fig. 2).

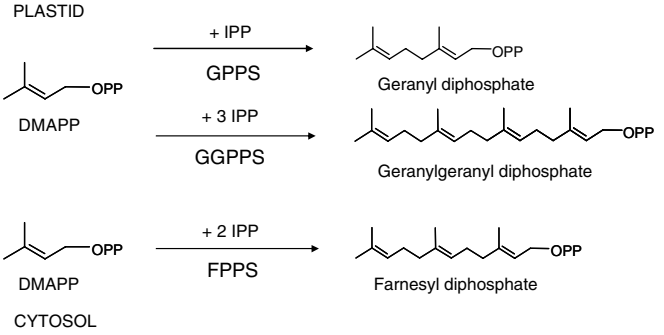

Fig. 2. Scheme for IDS catalysis in terpenoid biosynthesis. Each enzyme catalyzes 1, 2 or 3 sequential condensations to give a single product without significant release of intermediates. GPPS and GGPPS occur in the plastid, whereas FPPS is localized in the cytosol.

We are currently attempting to characterize the molecular and biochemical basis of conifer defenses using Norway spruce (*Picea abies*) L. Karst as a model. Norway spruce stems are constitutively defended by oleoresin, but also possess a suite of induced defenses. The induced defense response of this species to bark beetle attack can be mimicked by spraying trees with methyl jasmonate (MJ) (Martin et al., 2002; Byun-McKay et al., 2006; Erbilgin et al., 2006; Zeneli et al., 2006). This treatment triggers the formation of

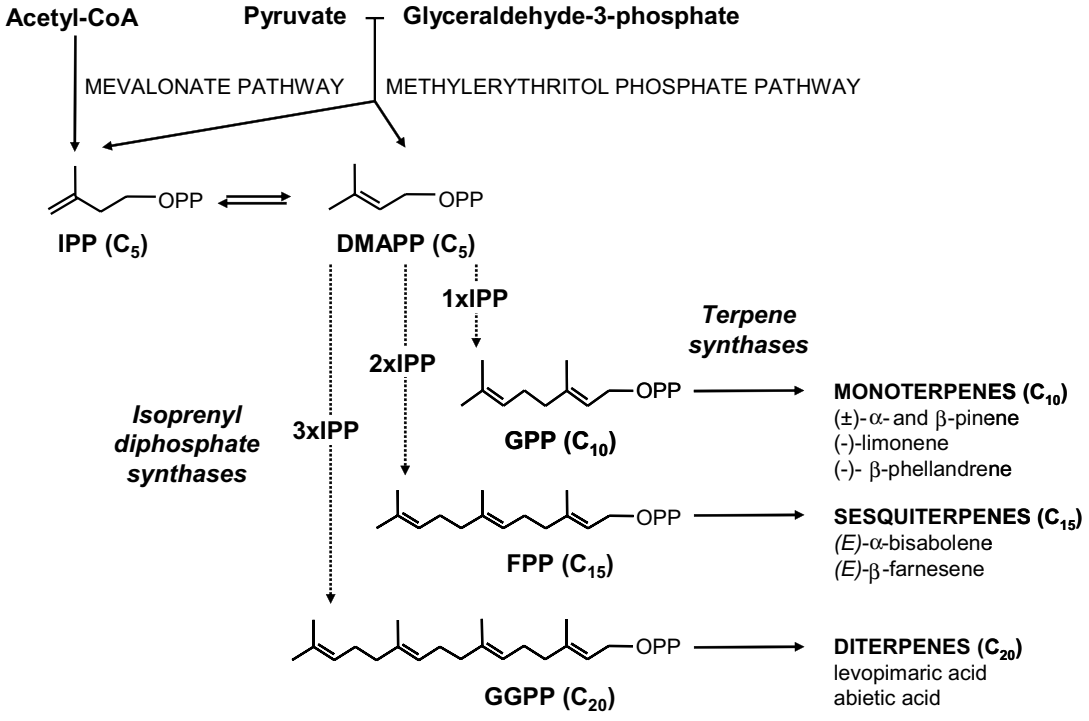

Fig. 1. Outline of terpenoid oleoresin biosynthesis in conifers showing the formation of IPP and DMAPP, the condensations to make larger prenyl diphosphates and the subsequent cyclizations to form the major products in *P. abies*. The isoprenyl diphosphate synthases catalyze the branch-point reactions leading to the different terpene classes.

TRDs in the developing sapwood and is associated with an accumulation of mono- and diterpenes (Franceschi et al., 2002; Martin et al., 2002; Zeneli et al., 2006). Numerous genes and enzymes involved in conifer defense have been characterized recently, but attention has focused on later steps, including terpene synthases (Fäldt et al., 2003; Hietala et al., 2004; Martin et al., 2004; Huber et al., 2005; Byun-McKay et al., 2006; Ro and Bohlmann, 2006) and cytochrome *P* 450 mono-oxygenases (Hamberger and Bohlmann, 2006). Very little information is available about the isoprenyl diphosphate synthases (IDSs) of the Pinaceae (Hefner et al., 1998; Tholl et al., 2001; Burke and Croteau, 2002; Schmidt et al., 2005) despite the fact that these enzymes catalyze the branch-point reactions leading to GPP, FPP and GGPP, and so may channel flux among the different terpene classes present in oleoresin.

IDSs have been extensively studied at the molecular level in angiosperms, which generally produce much lower amounts of terpenes than conifers. FPPS and GGPPS activity have been investigated in *Arabidopsis thaliana*, rubber tree (*Hevea brasiliensis*), banana (*Musa acuminata*), escobilla (*Scoparia dulcis*) and maize (*Zea mays*) (Kojima et al., 2000; Okada et al., 2000; Masferrer et al., 2002; Takaya et al., 2003; Cervantes-Cervantes et al., 2006). The pool size of GPP, FPP, and GGPP has been suggested to limit terpenoid biosynthesis in these plants (Aharoni et al., 2003; Han et al., 2006), thus implicating IDSs as primary regulators of terpenoid formation.

The IDS activities of gymnosperms are only poorly known. An FPPS from *Abies grandis* has been purified (Tholl et al., 2001), and GGPPSs genes were characterized from *A. grandis* and Canadian yew (*Taxus canadensis*) (Hefner et al., 1998; Burke and Croteau, 2002). However, *T. canadensis* contains no oleoresin and *A. grandis* produces resin only on induction. Our model species, *P. abies*, possesses both constitutive and inducible resin duct systems in its wood, and so may require much more complex controls over terpene biosynthesis. Here we report the cloning and expression of three *IDS* genes from *P. abies*. The expressed clones possessed FPPS and GGPPS activity are useful for understanding the molecular regulation of oleoresin biosynthesis in conifers.

2. Results

2.1. Isolation and sequence comparisons of cDNA clones encoding Norway spruce isoprenyl diphosphate synthases (IDSs)

To isolate Norway spruce *IDS* genes, cDNA fragments were obtained by PCR using primers designed to conserved regions of known plant farnesyl diphosphate synthases (FPPS) and geranylgeranyl diphosphate synthases (GGPPS) with Norway spruce RNA as a template. These fragments were then employed to screen cDNA libraries constructed with mRNA isolated from Norway spruce treated with methyl jasmonate (MJ) to induce terpene oleoresin biosynthesis. Positive clones were isolated and subcloned into pTriplEx2 yielding three *P. abies* (*Pa*) *IDS* cDNA clones, designated *PaIDS* 4, *PaIDS* 5 and *PaIDS* 6.

Of the three clones, *PaIDS* 4 had the highest similarity to other plant FPPS sequences based on comparisons at the amino acid level. Seven different *PaIDS* 4 sequences were actually found that differed from each other only in the 3′-untranslated region, consisting of 330–699 nucleotides between the stop codon and the poly $(A)^+$ tail. The protein encoded by *PaIDS* 4 consisted of 347 amino acids and had a calculated mass of 39.9 kDa. The highest homology of the deduced amino acid sequence (Fig. 3, Table 1) was to an FPPS of the maidenhair tree (*Ginkgo biloba*) with 86% identity and an FPPS from banana (*M. acuminata*) with 77% identity. The two active site, aspartate-rich DDxxD motifs, found in other plant FPPSs are both conserved in the *P. abies* gene. Neither the *PaIDS* 4 nor any other isolated plant FPPS contains a putative transit peptide at the 5′-end of the cDNA (Fig. 3). When the *PaIDS* 4 sequence was subjected to a phylogenetic analysis with all known plant *IDSs*, it clustered with other plant *FPPS* genes apart from the plant *GPPS* and *GGPPS* genes. Because of limited sequence information, gymnosperm and angiosperm *FPPSs* cannot be differentiated (Fig. 4).

Seven Norway spruce *IDS* clones had high similarity to other plant GGPPSs sequences based on comparisons at the amino acid level. These fell into two groups that shared only 60% identity in the deduced amino acid sequences of their coding regions. Within each group, differences among the amino acid sequences were only 1-3%, with more substantial differences in the 110–325 nucleotides 3′-untranslated region. Two representative cDNA clones of each class, designated *PaIDS* 5 and *PaIDS* 6, with lengths of 1146 bp and 1155 bp in the coding region, respectively, were selected for further study. The proteins encoded by *PaIDS* 5 and *PaIDS* 6 contain 382 and 385 amino acids, respectively, and have calculated masses of 42.0 and 41.7 kDa (Fig. 3). The highest similarity of the deduced amino acid sequence of *PaIDS* 5 was to the sequences of gymnosperm GGPPSs (90% identity) and to the *A. grandis* GPPS (82% identity). In contrast, the deduced amino acid sequence of *PaIDS* 6 showed the highest identity (72%) to the angiosperm *Adonis palaestina* GGPPS and the large subunit of snapdragon (*Antirrhinum majus*) GPPS (64% identity) (Table 1). Unlike *PaIDS* 4, both *PaIDS* 5 and *PaIDS* 6 contain a putative transit peptide for plastid targeting and possess the same conserved aspartate-rich DDxxxxDxDD and DDxxD motifs as other plant GGPPSs (Fig. 3). When sequences of GPPS and GGPPS are analyzed phylogenetically, there is no distinction between the two, but an almost complete separation between angiosperm and gymnosperm sequences appears. A cluster of gymnosperm GPPS and GGPPS genes is evident in which *PaIDS* 5 is present, while a second cluster contains angiosperm GGPPS genes along with *PaIDS* 6 (Fig. 4).

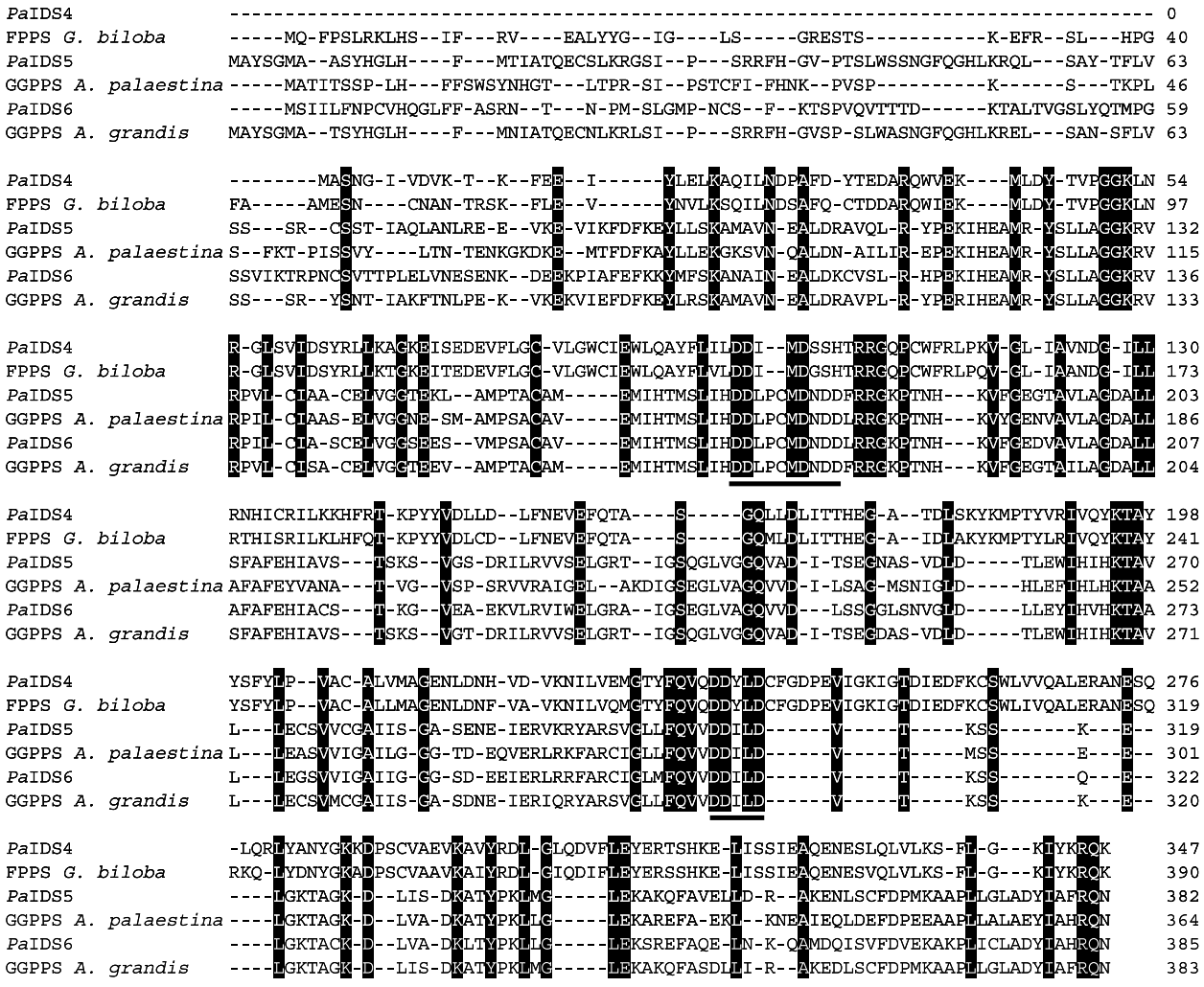

Fig. 3. Alignment of *PaIDS* 4, *PaIDS* 5 and *PaIDS* 6 with the plant *IDS* sequences of highest deduced amino acid sequence identity. The listed sequences are FPPS from *Ginkgo biloba* (AY389818), GGPPS from *Adonis palaestina* (AY661707) and GGPPS from *Abies grandis* (AF425235). Amino acid residues with pair-wise identity between *Pa*IDS 4 and GGPPS *G. biloba*, *PaIDS* 5 and GGPPS *A. palaestina* and *Pa*IDS 6 and GGPPS *A. grandis* are indicated by black boxes. Dashes indicate sequence gaps introduced to optimize the alignment. The conserved, aspartate-rich motifs are indicated by the black underline.

Table 1
Sequence similarity among *PaIDS* 4, *PaIDS* 5 and *PaIDS* 6, and other *IDS* genes, *Ginkgo biloba* FPPS, *Adonis palaestina* GGPPS and *Abies grandis* GGPPS, in percent

FPPS *G. biloba*	86.5				
*Pa*IDS 5	32.3	30.5			
GGPPS *A. palaestina*	29.7	28.5	60.5		
*Pa*IDS 6	30.3	28.2	62.3	71.4	
GGPPS *A. grandis*	31.7	30.0	91.1	62.6	61.3
	*Pa*IDS 4	FPPS *G. biloba*	*Pa*IDS 5	GGPPS *A. palaestina*	*Pa*IDS 6

The analysis was performed using ClustalX and DNAstar. The accession numbers of the non-*P. abies* genes are given in the legend of Fig. 4.

2.2. Heterologous expression of Norway spruce isoprenyl diphosphate synthase genes

To confirm that *PaIDS* 4, *PaIDS* 5, and *PaIDS* 6 are functional isoprenyl diphosphate synthase, we expressed full length cDNA in *E. coli* in a vector adding a C-terminal His tag. For *PaIDS* 5 and *PaIDS* 6, the signal sequences at the 5′-end of the coding region for directing plastidial import were first truncated. Analysis of the crude bacterial extracts of the expressed proteins with SDS–PAGE revealed recombinant protein bands with an apparent molecular mass of approximately 40 kDa for *Pa*IDS 4, and 37 kDa for both the truncated *Pa*IDS 5 and *Pa*IDS 6. The proteins were subsequently purified with Ni-NTA

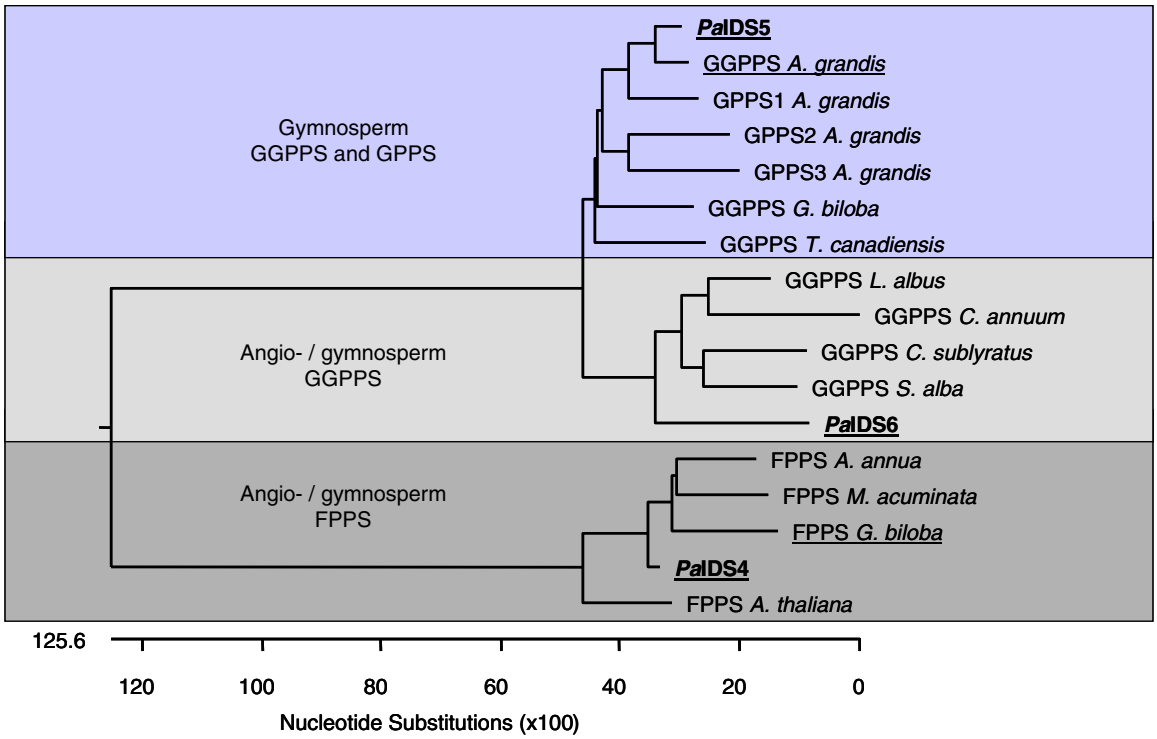

Fig. 4. Phylogenetic tree of the deduced amino acid sequences of *PaIDS* 4, *PaIDS* 5 and *PaIDS* 6 and other gymnosperm and angiosperm GPPS, GGPPS and FPPS sequences. Analysis was performed using Clustal X and DNAstar. The accession number of the sequences are as follows: GGPPS *Abies grandis* AF425235, GPPS1 *A. grandis* AF513111, GPPS2 *A. grandis* AF513112, GPPS3 *A. grandis* AF513113, GGPPS *Ginkgo biloba* AY371321, GGPPS *Taxus canadensis* AF081514, GGPPS *Lupinus albus* U15778, GGPPS *Capsicum annuum* X80267, GGPPS *Croton sublyratus* AB034249, GGPPS *S. alba* X98795, FPPS *Artemisia annua* AF112881, FPPS *M. acuminata* AAL82595, FPPS *G. biloba* AAR27053, FPPS *Arabidopsis thaliana* Q43315. Not all known plant FPPS and GGPPS sequences were included in the tree.

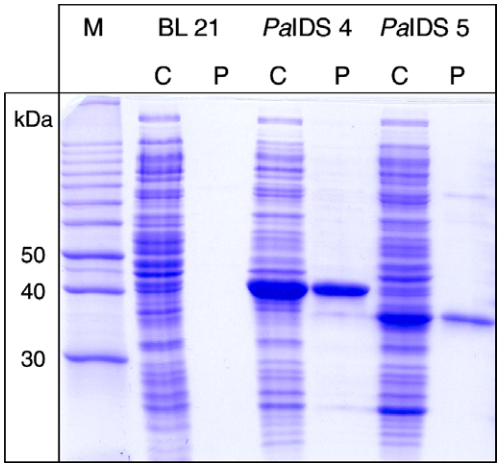

Fig. 5. SDS–PAGE analysis of recombinant IDS proteins expressed in *E. coli* and visualized by Coomassie staining. *M*, molecular mass markers; *BL21*, bacterial extract expressing empty vector; *C*, crude bacterial extracts; *P*, purified recombinant proteins after Ni-NTA agarose chromatography. The expression and purification of *Pa*IDS 5 was similar to that of *Pa*IDS 6.

agarose columns (Fig. 5). The purified *Pa*IDS 4 protein exhibited FPPS enzyme activity, while both *Pa*IDS 5 and *Pa*IDS 6 showed GGPPS enzyme activity when incubated with [1-^{14}C] IPP and DMAPP as substrates. *Pa*IDS 4 was also able to catalyze the synthesis of a small amount of GPP, while no side products were observed in the *Pa*IDS 5 and *Pa*IDS 6 assays (Fig. 6). Neither the empty vector controls nor heat-treated assays showed any significant enzyme activity. No change of enzyme activity was observed in control assays without 250 mM imidazole in the assay buffer (data not shown). Thus the expected product specificities, FPP for *Pa*IDS 4 and GGPP for *Pa*IDS 5 and *Pa*IDS 6, were confirmed.

2.3. Kinetic properties of Norway spruce isoprenyl diphosphate synthases

To compare the biochemical properties of the *P. abies* enzymes to other plant IDSs, the kinetic constants of the recombinant *P. abies* FPPS (*Pa*IDS 4) and GGPPS (*Pa*IDS 6) were measured under linear conditions with respect to time and protein concentration (Table 2). The data are similar to those measured for other conifer IDSs, such as native *A. grandis* FPPS (Tholl et al., 2001) and recombinant *T. canadensis* GGPPS (Burke and Croteau, 2002). *Pa*IDS 4 had an apparent K_m value of 71 μM for DMAPP and 230 μM for IPP, while *Pa*IDS 6 had a K_m value of 181 μM for DMAPP and 72 μM for IPP. Maximum velocity V_{Max} of 0.95 ng/mg for *Pa*IDS 4 and 0.18 ng/mg for *Pa*IDS 6 was calculated. As for *A. grandis* FPPS (Tholl et al., 2001) and *T. canadensis* GGPPS (Burke and

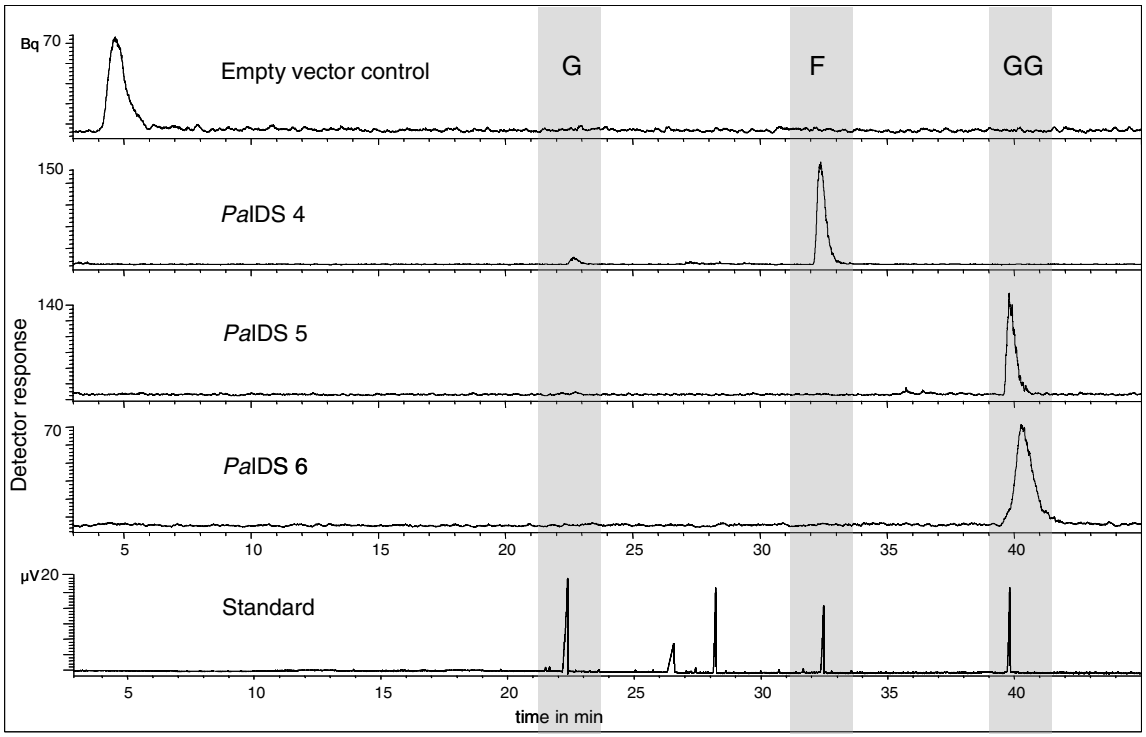

Fig. 6. Catalytic activities of recombinant *Pa*IDS 4, *Pa*IDS 5 and *Pa*IDS 6 proteins heterologously expressed in *E. coli* after assay with [1-^{14}C] IPP and DMAPP. Reaction products were enzymatically hydrolyzed and the resulting alcohols were analyzed by radio-gas chromatography. The four upper panels show the output of the radioactivity detector. The main product of the *Pa*IDS 4 reaction was farnesol (F) with minor amounts of geraniol (G), and the products of the *Pa*IDS 5 and the *Pa*IDS 6 reactions were geranylgeraniol (GG). The empty vector control showed no activity. Compounds were identified by coinjection of standards as depicted in the thermal conductivity detector trace (bottom panel).

Table 2
Kinetic constants for the recombinant enzymes *Pa*IDS 4 and *Pa*IDS 6, calculated by Lineweaver–Burk plotting

	Apparent saturation IPP (μM)	Apparent saturation DMAPP (μM)	K_m DMAPP (μM)	K_m IPP (μM)	V_{Max} (nkat/mg)
*Pa*IDS 4	800	200	71±5.2	230±8.4	0.95±0.35
*Pa*IDS 6	250	800	181±7.2	72±4.2	0.18±0.02

Each value is the average of three independent biological replicates.

Croteau, 2002), the *P. abies* enzymes were both dependent on MgCl2. *Pa*IDS 4 activity was optimal with 5 mM $MgCl_2$ and *Pa*IDS 5 activity with 10 mM $MgCl_2$.

2.4. Expression of Norway spruce isoprenyl diphosphate synthase genes after oleoresin induction

To determine the potential role of *PaIDS* 4, *PaIDS* 5, and *PaIDS* 6 in the induced oleoresin formation of *P. abies*, transcript levels were measured over a 10 day time course after saplings had been sprayed with MJ. In bark (and attached cambium), the only transcript that increased in abundance in response to MJ treatment was from *PaIDS* 5. Transcripts of this *GGPPS* gene increased continuously and reached a maximum at day 8 representing a 120-fold increase in transcript. In contrast, *PaIDS* 4 and *PaIDS* 6 transcript levels remained relatively constant after spraying. In wood, each of the *PaIDS* genes showed some transcript increase upon MJ spraying, but the magnitudes of these increases were much less than that of *PaIDS* 5 in the bark. The maximum induction of *PaIDS* 4 transcript occurred 1 day after MJ treatment whereas induction of *PaIDS* 5 was at its maximum at 2 days and the induction of *PaIDS* 6 occurred much more slowly, over a period of 8 days (Fig. 7). No significant transcript accumulation for the investigated genes was found in control saplings (data not shown).

3. Discussion

The isoprenyl diphosphate synthases (IDS) catalyze the branch-point reactions leading to the different classes of terpenes. To learn more about how conifers control production of the major constituents of their defensive oleoresin, we cloned three genes encoding IDSs from Norway spruce (*P. abies*) using a homology-based, nested PCR approach. One of the genes, *PaIDS* 4, encodes a farnesyl

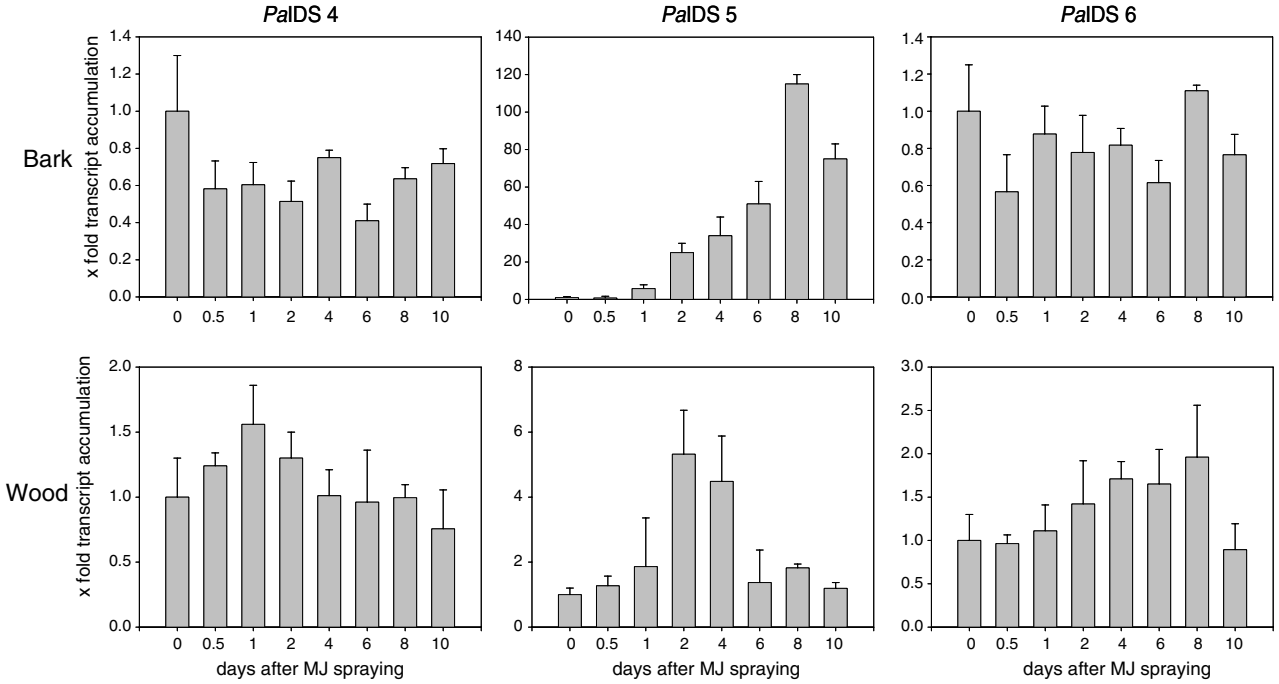

Fig. 7. Relative abundance of mRNA transcripts of *PaIDS* 4, *PaIDS* 5 and *PaIDS* 6 genes in bark and wood of MJ-treated Norway spruce saplings. Transcript abundance of each *IDS* gene was measured by quantitative RT-PCR using SYBR-Green for detection and normalized against ubiquitin. The transcript abundance at day 0 was set to 1.0. Each value is the average of two independent biological replicates, each of which is represented by three technical replicates.

diphosphate synthase (FPPS). This is the first FPPS gene to be isolated from a conifer, although there are many examples known from angiosperms (Cunillera et al., 1996, 1997; Hemmerlin et al., 2003; Cervantes-Cervantes et al., 2006; Han et al., 2006). The other two genes, *PaIDS* 5 and *PaIDS* 6, both encode geranylgeranyl diphosphate synthases (GGPPS). Multiple *GGPPS* genes have previously been described from *H. brasiliensis* and *A. thaliana* (Okada et al., 1999; Takaya et al., 2003). All three *P. abies* genes contain two aspartate-rich motifs that are thought to be responsible for substrate binding and are found in all IDS described to date (Chen et al., 1994).

A phylogenetic analysis of a range of known plant IDS genes, excluding heterodimeric forms, shows that FPPS sequences cluster separately from GPPS and GGPPS sequences (Fig. 4). In this gene tree, the *P. abies* FPPS and GGPPS genes cluster with others of their respective types. However, there are two groups of GPPS/GGPPS sequences, each of which contains one of the *P. abies* GGPPS genes. The upper cluster (Fig. 4) contains other gymnosperm GPPS and GGPPS plus *PaIDS* 5, while the lower contains angiosperm GGPPS plus *PaIDS* 6. These relationships suggest that *PaIDS* 5 may have a specific function only in gymnosperm, like the active formation of oleoresin and other terpene secondary metabolites, while *PaIDS* 6 has a more general function in all plants, perhaps in making GGPP for the synthesis of gibberellins, carotenoids or the phytol side chain of chlorophyll.

In keeping with the compartmentalization of terpenoid metabolism in the plant cell, the encoded IDS proteins are localized to different sites. FPPSs are located principally in the cytosol. However, for the *A. thaliana FPPS1* gene, it has been shown that the use of an alternative translation start site yields a longer pre-protein which is targeted to the mitochondria (Cunillera et al., 1997a). Nevertheless, the *P. abies* IDS 4 enzyme is likely targeted to the cytosol since no plastidial transit peptide could be detected by the available algorithms (see Section 4).

Unlike FPPSs, most GGPPSs are localized to the plastids although some are found in mitochondria or in the endoplasmic reticulum (Cunillera et al., 1997b; Okada et al., 2000; Bick and Lange, 2003). The *PaIDS* 5 and *PaIDS* 6 cDNA sequences appear to have plastidial targeting signals. To successfully express them in *E. coli*, it was necessary to truncate the first 79 amino acids from the open reading frame of each clone.

The catalytic properties of the recombinant *P. abies* FPPS and GGPPS measured *in vitro* are similar to those that have been reported for other plant enzymes of these types (e.g., Cunillera et al., 1996; Hefner et al., 1998; Tholl et al., 2001; Burke and Croteau, 2002; Hemmerlin et al., 2003). Product specificity of both recombinant proteins is usually quite high. As in our study small amounts of the intermediate product GPP was occasionally observed for FPPS.

The kinetic constants of the recombinant *P. abies* enzymes are also of the same magnitude as previously

reported in the literature, in keeping with the high level of amino acid identity with these groups.

FPP and GGPP are central intermediates in plant terpenoid metabolism which each have a diversity of metabolic fates. For example, FPP is a precursor of sterols, ubiquinone and dolichols, as well as sesquiterpenes. GGPP is a precursor of phytol, gibberellins and carotenoids, as well as diterpenes. To determine the possible involvement of the isolated *P. abies* FPPS and GGPPS genes in forming the sesquiterpenes and diterpenes of defensive oleoresin, we followed changes in their transcript levels after spraying of saplings with MJ. Treatment with MJ mimics the response of *P. abies* to bark beetle attack by inducing traumatic resin ducts (TRDs), which leads to increased oleoresin biosynthesis and accumulation (Martin et al., 2002; Byun-McKay et al., 2006; Erbilgin et al., 2006; Zeneli et al., 2006). The transcript levels of one of the GGPPS genes (*PaIDS* 5) were increased by MJ, but there was much less effect on the other GGPPS gene (*PaIDS* 6) and the FPPS gene (*PaIDS* 4).

The increase of *PaIDS* 5 transcripts in bark (and attached cambium) in response to MJ was over three orders of magnitude (Fig. 7), a clear suggestion that this gene has an important role in the induced formation of oleoresin diterpenes. This conclusion is in agreement with the phylogenetic analysis (Fig. 4). Here *PaIDS* 5 was shown to be most closely related to other gymnosperm sequences, and so may be assumed to share a role in typical gymnosperm functions, like diterpene oleoresin production. The large increase in *PaIDS* 5 transcript level observed corresponds with the large increases in GGPP synthase activity and diterpene accumulation seen after sapling treatment with MJ (Martin et al., 2002). Even though TRDs are located in the wood, strictly speaking, 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 *PaIDS* 5 expression seen in both bark and wood samples is not surprising. All three IDS genes show at least slight activation in the wood which may reflect the general activation of terpenoid metabolism in TRD formation, including formation not only of oleoresin components, but also of common cell components, such as sterols. Given the large variety of terpenoids produced in *P. abies*, there are clearly additional *IDS* genes in this species. The cloning and characterization of these genes is ongoing.

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, USA).

4.2. Plant material

Four-year-old Norway spruce (*P. abies*) monoclonal saplings were used that had been purchased from the Samenklenge und Pflanzgarten Laufen (Germany) and are members of clone 3369-Schongau. Saplings were grown in standard soil under a 21 °C day/16 °C night temperature cycle, controlled light conditions (16 h per 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 grown for 3 weeks under these conditions, and induced by spraying them with 100 μM methyl jasmonate (MJ) in 0.5% (v/v) Tween 20 as detergent. Control trees were sprayed only with Tween 20. After various time intervals, bark (including the cambium layer) and wood were peeled off from the uppermost internode (representing the previous year's growth) and frozen in liquid nitrogen in preparation for RNA isolation. For each time point and treatment at least two saplings were used, respectively.

4.3. Amplification and cloning of IDS sequences

Using FPPS sequences from *A. thaliana*, *Z. mays*, *H. brasiliensis*, and *Lupinus albus*, and GGPPS sequences from *T. canadensis*, *Helianthus annus*, *C. sublyratus*, *Mentha x piperita*, and *L. albus*, degenerate primers were designed to different conserved regions. For the amplification of Norway spruce FPPS cDNA fragments, the upstream primer was (5′-GG(C/T)TGGTG(C/T)ATTGAATGGCT(C/T)CA(A/G)GC-3′) and the downstream primer was (5′-CTA(C/T)TT(C/T)TGCCTCTT(A/G)TAIAT(C/T)TTII(G/C)-3′). For the amplification of GGPPS fragments, the primer combination (5′-CIATG(A/C)G(G/T)TA(C/T)TCI CTICTIGCIGG-3′) and (5′-CTTATCIG(C/T)(A/C)AICA AATC(C/T)TT(C/T)CC-3′) was used. Reverse transcription was carried out with pooled total RNA from MJ-treated spruce bark harvested at various time points after treatment (6, 12 and 24 h; 2, 4, 6, 10 and 20 days) and 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 then 72 °C for 1 min in a thermocycler (Stratagene). To identify the optimal annealing temperature, a temperature gradient was used with 2 °C intervals ranging from 42 °C to 64 °C. The resulting PCR products were used as templates in nested PCR with (5′-GTTGGI(A/C)TG(A/G)TTGCII(C/T)IAA(C/T)GA(C/T)-GG-3′) and (5′-CC(A/T)ATCTTICC(A/C)A(G/T)I(A/G)I(C/T)TCIGGA(G/T)CA(G/C)C-3′) as up- und downstream primers, respectively, to amplify FPPS fragments and (5′-GA(A/G)ATGATICA(C/T)ACIATGTC-3′) and (5′-CATC(A/C)ACIAC(C/T)TGAAACA(A/G)(A/C)A(A/G)(C/T)CC-3′) to amplify GGPPS fragments. A 655-bp FPPS fragment and a 430-bp GGPPS fragment were generated at 44 °C and subcloned into pCR™ 4-TOPO™ (Invitrogen) according to the manufacturer's instructions.

4.4. Isolation of full length IDS cDNA clones

Total RNA was isolated according to a method developed by Wang (Wang et al., 2000) from *P. abies* bark harvested at various time points (as above) after being sprayed with 100 μM MJ. From total pooled RNA, poly $(A)^+$ RNA was isolated 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 with the ^{32}P-labeled 655 bp *FPPS* and 430 bp *GGPPS* fragments previously amplified by PCR. Single plaques were isolated from positive colonies and λDNA was converted into pDNA by subcloning into pTriplEx2 vector and transformation in *E. coli* BM25.8. Sequence analysis was carried out using an ABI 3100 automatic sequencer (Applied Biosystems). One sequence named *PaIDS* 4 was obtained from screening with the *FPPS* fragment, and two others named *PaIDS* 5 and *PaIDS* 6 from screening with the *GGPPS* fragment.

4.5. Expression of IDS sequences in E. coli

The entire coding sequence of cDNA clone *PaIDS* 4 and truncated sequences of clones *PaIDS* 5 and *PaIDS* 6 lacking the predicted signal peptide for plastidial transport (based on analysis at http://www.cbs.dtu.dk/services) were amplified with primers that included the start and stop codon for translation. The primer combination (5′-ATGGCTTCAAACGGCATCGTCGACG-3′) and (5′-CTTCTGCCGCTTGTATATCTTCCC-3′) was used to express *PaIDS* 4. For amplifying *PaIDS* 5 and *PaIDS* 6, the primer pairs (5′-ATGGAAGAAGTAAAGGAGGT C-3′) and (5′-GTTTTGTCTGAATGCAATGTAATCTG C-3′), and (5′-ATGAATGAATCAGAAAACAAAGAT C-3′) and (5′-GTTCTGTCTGTGGGCAATGTAATC-3′) were used, respectively. The amplification 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 BL21 (DE3)pLysS (Invitrogen). Bacterial cultures were grown to an OD of 0.6 and transformants were induced with 2 mM IPTG as described by the manufacturer's instructions, except that they were grown exclusively at 18 °C. Bacterial pellets were resuspended in a buffer containing 20 mM Mopso, pH 7.0, 10% (v/v) glycerol and 10 mM $MgCl_2$, and sonified by using a Sonopuls HD 2070 (Bandelin, Berlin, Germany) for 4 min, cycle 2, power 60%. *Pa*IDS 4, *Pa*IDS 5 and *Pa*IDS 6 proteins were individually purified 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 of 2 mM DTT to the assay buffer containing 250 mM imidazole, proteins were checked for purity by SDS–PAGE and their enzyme activity measured.

4.6. Assay of recombinant PaIDS 4, PaIDS 5 and PaIDS 6

Small-scale IDS assays were carried out in a final volume of 50 μl containing 20 mM Mopso (pH 7.0), 5 mM $MgCl_2$ (for *Pa*IDS 4) or 10 mM $MgCl_2$ (for *Pa*IDS 5), 10% (v/v) glycerol, 2 mM DTT, and [1-^{14}C] IPP (2 MBq/μmol) and DMAPP at saturating concentrations (800 μM IPP and 200 μM DMAPP for *Pa*IDS 4, and 800 μM IPP and 250 μM DMAPP for both *Pa*IDS 5 and *Pa*IDS 6). The reaction was initiated by addition of recombinant protein, and the assay mixture was overlaid with 1 ml hexane and incubated for 20 min (*Pa*IDS 4) or 40 min (*Pa*IDS 5 and *Pa*IDS 6) at 30 °C. Assays were stopped by addition of 10 μl of 3 N HCl and incubated for an additional 20 min at 30 °C to solvolyze the acid-labile allylic diphosphates formed during the assay to their corresponding alcohols. Solvolysis products were extracted into the hexane phase by vigorous mixing for 15 s. After centrifugation, 300 μl of the hexane phase were used for total radioactivity determination by liquid scintillation counting. Protein concentration was measured according to Bradford (Bradford, 1976) using the BioRad reagent with bovine serum albumin (BSA) as standard. The *Pa*IDS 4 reaction was linear for up to 60 min and the *Pa*IDS 5 reaction for up to 30 min at protein concentrations of 40 μg/ml. To calculate kinetic parameters, the Sigma plot-Enzyme kinetics Module (SPSS, Chicago, IL, USA) was used. All assays were carried out in triplicate.

For the identification of reaction products of for *Pa*IDS 4, *Pa*IDS 5 and *Pa*IDS 6, larger scale assays were carried out in a final volume of 500 μl. The assay mixture was as described above but concentrations of 40 μM [1-^{14}C] IPP, 40 μM DMAPP and a 1 ml layer of pentane were used. Assays were incubated overnight at 30 °C. To stop the assay and hydrolyze all diphosphate esters, a 1 ml solution of 2 units of calf intestine alkaline phosphatase (Sigma) and 2 units potato apyrase (Sigma) in 0.2 M Tris–HCl, pH 9.5, was added to each assay and followed by overnight incubation at 30 °C. After enzymatic hydrolysis, the resulting isoprenyl alcohols were extracted into 2 ml of diethyl ether and, after addition of a standard terpene mixture; the organic extracts were evaporated under N_2 and used for radio-GC measurements. Radio-GC analysis was performed on a Hewlett Packard HP 6890 gas chromatograph (injector at 220 °C, TCD at 250 °C) in combination with a Raga radioactivity detector (Raytest, Straubenhardt, Germany) using a DB 5-MS capillary column (J&W scientific) (30 m × 0.25 mM with a 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 comparison of retention times with those of co-injected

authentic non-radioactive terpene standards, monitored via a thermal conductivity detector.

4.7. Measurement of PaIDS 4, PaIDS 5, and PaIDS 6 transcripts by quantitative PCR

Total RNA from each time points and tissues was isolated according to the method of Wang, (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 subsequent PCR reactions was generated using Superscript™ III (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed with Brillant® SYBR Green QPCR Master Mix (Stratagene) and a ubiquitin fragment of *P. abies* to normalize transcripts of interest. The primers used were as follows: ubiquitin forward (5′-GTTGATTTTTGCTGGCAAGC-3′) and reverse (5′-CACCTCTCAGACGAAGTAC-3′), *PaIDS* 4 forward (5′-GTCTGTAATAGACAGCTACAGG-3′) and reverse (5′-CCAGCCAAGCACACATCC-3′), *PaIDS* 5 forward (5′-CATTTCTGGTATCATCATCTAG-3′) and reverse (5′-GTCCTCCTTTACTTCTTCACG-3′), and *PaIDS* 6 forward (5′-GTTGGTTCTCTTTATCAGAC-3′) and reverse (5′-CTAATGGTGTCGTTACACTG-3′). At least 8 amplicons from each primer pair were cloned and sequenced to confirm primer specificity. Transcript abundance was quantified with a Mx3000P Real Time PCR Thermocycler (Stratagene) using a program with a maximum of 45 cycles of 95 °C for 30 s, 55 °C (or 52 °C for *Pa*IDS 4 and *Pa*IDS 6) for 30 s and 72 °C for 30 s, followed by a melting curve analysis of transcripts. The transcript abundance of each *PaIDS* gene was normalized to ubiquitin, and the relative amount of transcript was calculated using the software of the thermocycler. The relative amount of transcript at the onset of treatment was used as calibrator. Each measurement was repeated with two independent biological replicates, each of which was represented by at least three technical replicates.

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