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Diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*): Functional characterization of abietadiene/levopimaradiene synthase (*PtTPS-LAS*) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienal oxidase (PtAO, CYP720B1)

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We wish to dedicate this paper to Rodney Croteau on the occasion of his 60th birthday in recognition of a great mentor and his many outstanding contributions to the field of terpenoid biochemistry.

Abstract

Diterpene resin acids are prominent defense compounds against insect pests and pathogens in conifers. Biochemical and molecular analyses in grand fir (*Abies grandis*), Norway spruce (*Picea abies*), and loblolly pine (*Pinus taeda*) have identified two classes of genes and enzymes that generate much of the structural diversity of terpenoid defense compounds: The terpenoid synthases (TPS) and cytochrome P450 monooxgenases (P450). Using a single substrate, geranylgeranyl diphosphate, families of single-product and multi-product diterpene synthases generate an array of cyclic diterpene olefins. These diterpenes are converted to diterpene resin acids by activity of one or more P450 enzymes. A few conifer diterpene synthases have previously been cloned and characterized in grand fir and in Norway spruce. We have also previously shown that the loblolly pine P450 abietadienol/abietadienal oxidase (PtAO) catalyzes multiple oxidations of several diterpene alcohols and aldehydes. Conifer diterpene synthases are thought to function in plastids while P450s can also be localized to plastids or to the endoplasmic reticulum (ER). Here, we show that a loblolly pine cDNA (*PtTPS-LAS*) encodes a typical multi-product conifer diterpene synthase that forms levopimaradiene, abietadiene, palustradiene, and neoabietadiene similar to the grand fir abietadiene synthase and Norway spruce levopimaradiene/abietadiene synthase. Subcellular targeting of PtTPS-LAS and PtAO to plastids and ER, respectively, was shown with green fluorescent fusion protein expression in tobacco cells. These data suggest that enzymes for conifer diterpene resin acid biosynthesis are localized to at least two different subcellular compartments, plastids and ER, requiring efficient transport of intermediates and secretion of diterpene resin acids into the extracelluar space.

Keywords: Terpene synthase; Cytochrome P450 monooxygenase; Abietic acid; Conifer defense; Oleoresin

1. Introduction

Many conifer species of the pine family (*Pinaceae*) synthesize copious amounts of oleoresin, composed mainly of monoterpenes and diterpene resin acids in addition to smaller amounts of sesquiterpenes (Langenheim, 2003). Together the mono-, sesqui- and diterpenes display a diverse array of hundreds of different chemical compounds for potential defense against the many possible pests or pathogens that may threaten a long-lived conifer tree during its lifetime. Oleoresin terpenoids are sequestered and stored in specialized anatomical structures, namely resin cells, resin blisters or resin ducts, in the stems, roots, nee-

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dles and cones of conifer trees, where they provide an effective defense barrier against most herbivores or pathogens. Research on the enzymes and genes for the biosynthesis of conifer oleoresin terpenoids has been pioneered by Rodney Croteau and coworkers. This fascinating subject of natural product biochemistry has been extensively reviewed in recent years (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001; Bohlmann et al., 2004; Huber et al., 2004; Martin and Bohlmann, 2005).

In brief, much of the chemical diversity of terpenoids present in conifer oleoresin is generated by families of terpene synthases (TPS) and cytochrome P450 monooxyegneases (P450). The conifer TPS utilize the three prenyl diphosphate substrates, geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), yielding the many different carbon skeletons of cyclic and acyclic mono-, sesqui-, and diterpenes, respectively (Bohlmann et al., 1998; Martin et al., 2004). Most of the monoterpenes that form much of the turpentine fraction of conifer oleoresin are the olefinic products of monoterpene synthase enzyme activities encoded in a monophyletic conifer TPS-d1 gene family (Bohlmann et al., 1997; Bohlmann et al., 1999; Fäldt et al., 2003; Phillips et al., 2003; Martin et al., 2004). Formation of diterpene resin acids has been studied at the molecular genetic levels in grand fir (*Abies grandis*), Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). These studies have identified both single-product and multi-product diterpene synthases that generate an array of cyclic diterpene olefins (Stofer Vogel et al., 1996; Peters et al., 2000; Martin et al., 2004). Diterpene olefins generated by TPS-d3 family members of conifer diterpene synthases (Martin et al., 2004) are commonly oxidized by P450s to form the diterpene resin acids of conifer oleoresin (Funk and Croetau, 1994; Ro et al., 2005). Recently, we cloned and characterized the first P450 involved in diterpene resin acid biosynthesis. The multifunctional and multi-substrate loblolly pine P450 abietadienol/abietadienal oxidase (PtAO) catalyzes a matrix of at least eight different diterpene conversions involving oxidations of both alcohols and aldehydes in the formation of various diterpene resin acids found in this conifer species (Ro et al., 2005).

The formation of terpenoids in plant cells involves several subcellular compartments. For example, a recent immuno-cytochemical analysis of enzymes in the biosynthesis of monoterpenes showed that four subcellular compartments (cytosol, ER, plastids, and mitochondria) are necessary to produce *p*-menthane in mint (Turner and Croteau, 2004). Based on the presence of putative N-terminal plastid targeting sequences, conifer diterpene synthases are predicted to function in plastids of diterpene resin acid producing cells (Bohlmann et al., 1998; Martin et al., 2004). Although most plant P450s are associated with the ER, several plant P450s are also known to be localized to plastids (Froehlich et al., 2001; Helliwell et al., 2001; Watson et al., 2001). In a first attempt to demonstrate subcellular compartmentation of conifer diterpene resin acid formation, we functionally characterized a recently cloned diterpene synthase cDNA from loblolly pine (*PtTPS-LAS*; Ro et al., 2005) and tested subcellular targeting of two enzymes of loblolly pine diterpene resin acid formation, PtTPS-LAS and PtAO. In this paper, we show that *PtTPS-LAS* encodes a typical multi-product conifer diterpene synthase that forms levopimaradiene, abietadiene, palustradiene, and neoabietadiene. Subcellular targeting of PtTPS-LAS and PtAO to plastids and ER, respectively, was shown by confocal in vivo microscopy with green fluorescent fusion proteins in transiently transformed tobacco leaf cells.

2. Results and discussion

2.1. Functional characterization of diterpene synthase cDNA *PtTPS-LAS*

We have previously isolated a cDNA (*PtTPS-LAS*) for a methyl jasmonate inducible diterpene synthase from loblolly pine (Ro et al., 2005). The longest predicted open reading frame (ORF) of *PtTPS-LAS* encodes for a protein of 850 amino acids with a calculated pI of 5.52 and molecular mass of 97.5 kDa (Fig. 1). The predicted PtTPS-LAS protein is 84% and 88% identical with isopimara-7,15-diene synthase (PaTPS-Iso) and levopimaradiene/abietadiene synthase (PaTPS-LAS) from Norway spruce, respectively (Martin et al., 2004). It also shows 84% identity to abietadiene synthase from grand fir (AgTPS-LAS; Stofer Vogel et al., 1996) and 62% identity to levopimaradiene synthase from the more distantly related species *Ginkgo biloba* (GbTPS-Lev; Schepmann et al., 2001). For functional characterization of PtTPS-LAS, we expressed a truncated version of the protein lacking the N-terminal 57-amino acids in *Escherichia coli* and tested the recombinant protein in enzyme assays with GPP, FPP or GGPP as described in Martin et al. (2004). Gas-chromatography coupled with mass spectrometry (GC–MS) analysis of the product profiles of in vitro enzyme assays showed that the recombinant PtTPS-LAS enzyme was active with GGPP, but did not catalyze a terpene synthase reaction with GPP or FPP. The PtTPS-LAS enzyme efficiently converts GGPP to four different diterpenoid products, levopimaradiene (1) (Fig. 2, peak 1), abietadiene (2) (peak 2), neoabietadiene (3) (peak 3), and palustradiene (4) (peak 4). Each of these products was identified by comparison of their electron impact (EI) fragmentation patterns and retention index with those of the respective authentic standards (Fig. 2 and Table 1). When enzyme assays were conducted at pH 7.2, levopimaradiene (45.5% ± 1.9; average ± SD, $n = 4$) was the major diterpenoid product followed by abietadiene (27.6% ± 0.8), neoabietadiene (22.4% ± 1.1) and palustradiene (4.5% ± 0.5). The PtTPS-LAS product profile is nearly identical to that of Norway spruce PaTPS-LAS when tested under the same conditions [levopimaradiene (47.1% ± 1.9; average ± SD, $n = 4$), abietadiene (27.1% ± 1.2), neoabietadiene (22.5% ± 0.7), and palustradiene (3.2% ± 0.3)] but is

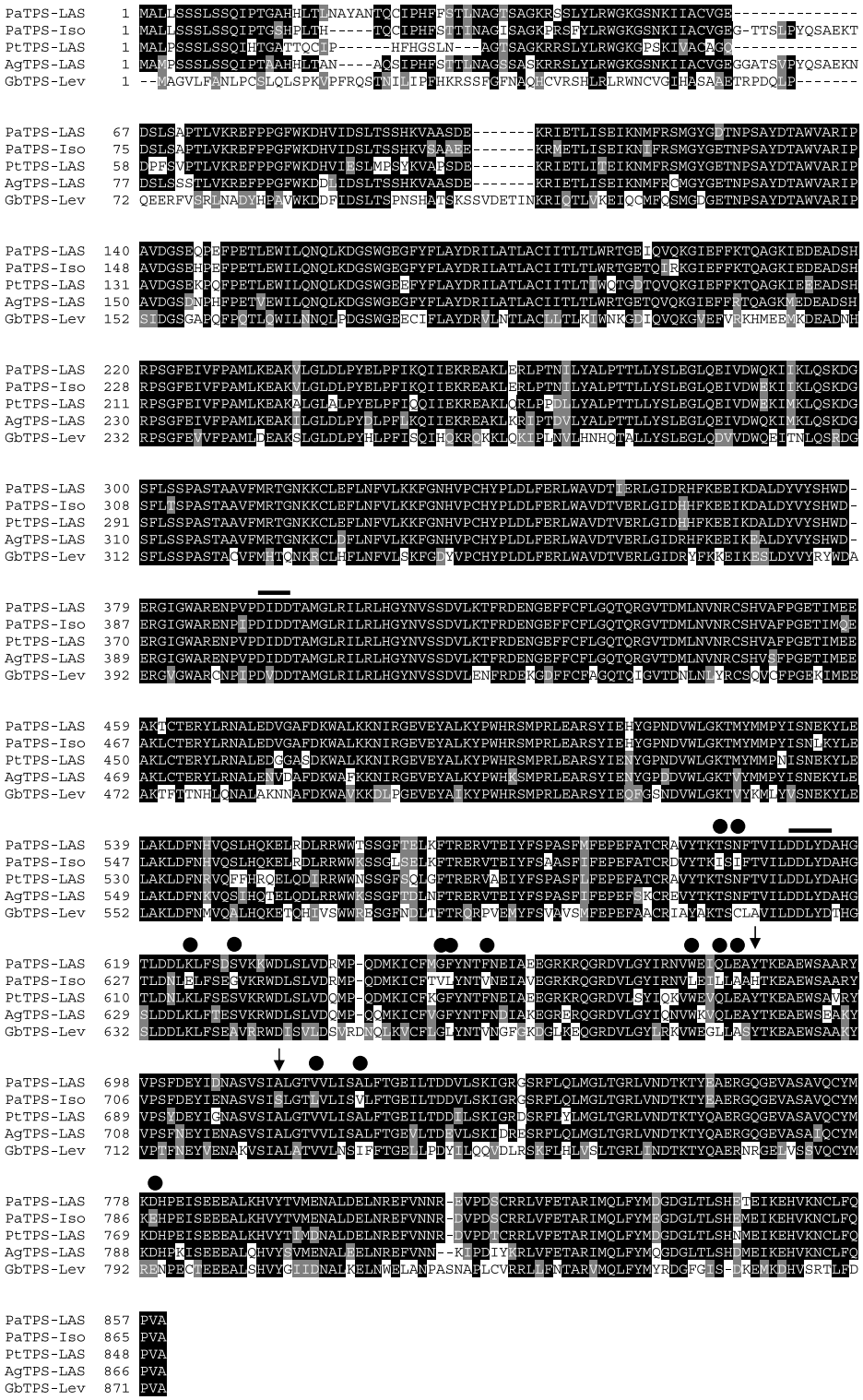

Fig. 1. Alignment of deduced amino acid sequences of diterpene synthases from Norway spruce (PaTPS-LAS and PaTPS-Iso), loblolly pine (PtTPS-LAS), grand fir (AgTPS-LAS), and *Ginkgo biloba* (GbTPS-Lev). Aspartate-rich motifs (DxDD and DDxxD) that are conserved in the two active sites (Peters et al., 2001) of these conifer diterpene synthase are shown with black lines above the alignment. Two amino acids predicted to be important in determining product profiles of LAS enzymes are shown with arrows (Martin et al., 2004). Indicated with black circles are additional residues in the C-terminal domain, that are conserved in the three LAS type enzymes from loblolly pine, grand fir, and Norway spruce, but are different in the PaTPS-Iso enzyme.

substantially different from the single product profile of Norway spruce PaTPS-Iso. Two amino acid positions (Y677 and A705 in PtTPS-LAS) that were previously predicted to contribute to differences in product profiles of the Norway spruce multi-product PaTPS-LAS and single-product PaTPS-Iso (Martin et al., 2004) are conserved in

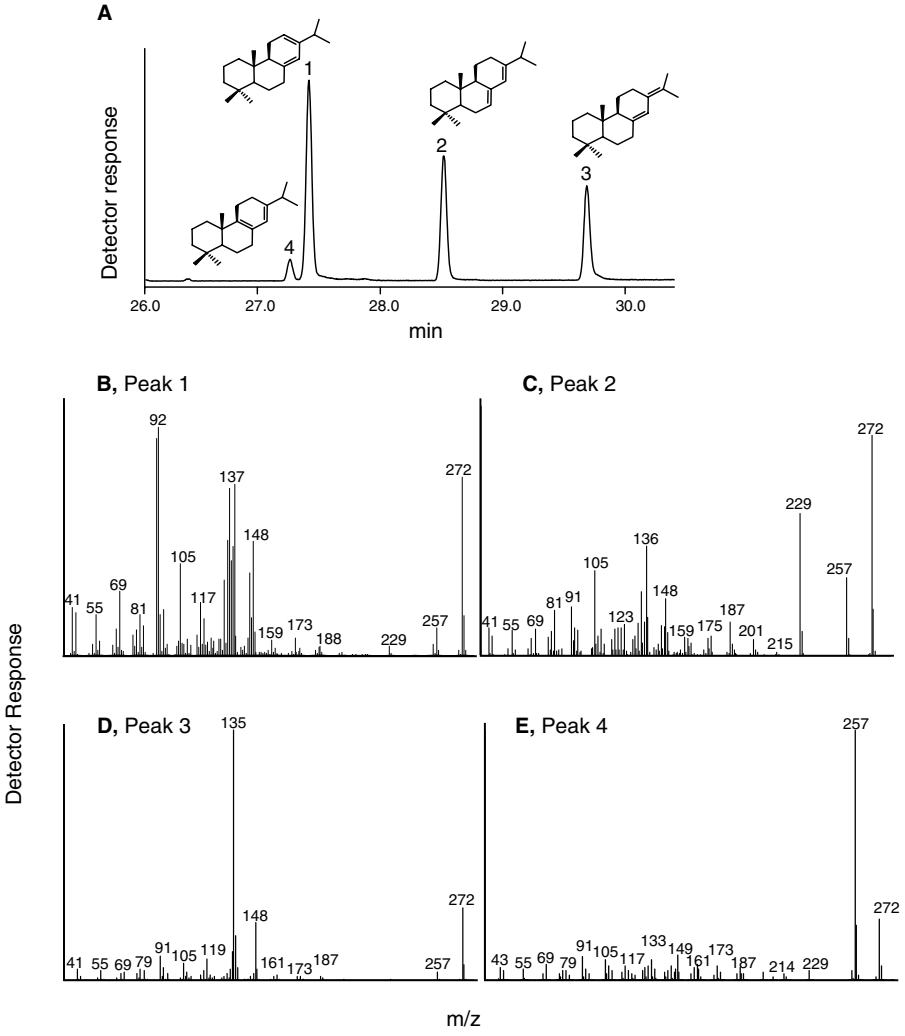

Fig. 2. GC–MS analyses of diterpene products formed by PtTPS-LAS recombinant protein. Total ion chromatogram of the four diterpene products **1**–**4** from in vitro enzyme assays (A), and mass spectra for each product in order of decreasing abundance: (B) levopimaradiene, peak 1; (C) abietadiene, peak 2; (D) neoabietadiene, peak 3; (E) palustradiene, peak 4. Additional data supporting identification of each product by comparison with authentic standards are shown in Table 1.

Table 1
Identification of diterpene products formed by recombinant PtTPS-LAS

Peak/Standard	Retention index	Relative ion abundance of parental and characteristic ions							
		272	148	137	136	134	133	92	91
Peak 1	2057	78	50	75	48	73	50	100	95
Levopimaradiene standard	2056	75	51	75	47	69	50	100	99
		272	257	229	148	136	133	105	91
Peak 2	2119	100	35	64	26	50	29	39	22
Abietadiene standard	2119	100	35	62	26	52	30	42	23
		272	148	136	135	134	119	105	91
Peak 3	2187	29	23	18	100	12	9	7	10
Neoabietadiene standard	2187	31	26	18	100	14	12	8	12
		272	258	257	161	149	133	105	91
Peak 4	2048	25	22	100	6	10	8	8	9
Palustradiene standard	2048	23	21	100	6	9	7	8	8

Peak numbers are according to Fig. 2. Each product peak is matched with its authentic standard.

all three multi-product LAS-type enzymes from loblolly pine, Norway spruce, and grand fir as well in the levopimaradiene synthase from *G. biloba* (Fig. 1, marked with arrows).

2.2. Subcellular targeting of PtTPS-LAS and PtAO

Biosynthesis of diterpene resin acids presumably occurs in specialized epithelial cells that line the surface of resin

ducts or resin blisters. The formation of diterpene resin acids involves the enzyme activities of diterpene synthases, one or more P450 enzymes, and possibly an aldehyde dehydrogenase (Funk and Croetau, 1994; Ro et al., 2005). To determine subcellular targeting of loblolly pine diterpene synthase PtTPS-LAS and diterpene P450 oxidase PtAO, we used confocal microscopy of GFP-fusion proteins in transiently transformed tobacco cells. For PtTPS-LAS the first 80 amino acids and for PtAO the first 48 amino acids were fused to the N-terminus of green fluorescent protein (GFP) and expressed in tobacco under control of Cauliflower Mosaic Virus 35S promoter. As a control, GFP alone was expressed under control of the same promoter. All constructs were transformed and transiently expressed in tobacco leaves using *Agrobacterium* infiltration. In these transient expression assays, only some of the leaf cells were transformed while neighboring non-transformed cells served as a further negative control.

Using the GFP-control construct, green fluorescence was detectable by confocal in vivo microscopy in the cytoplasmic space between plastids and in the vacuole, but GFP was not found in plastids of mesophyll cells (Fig. 3A–C). In contrast, green fluorescence derived from GFP fusions with PtTPS-LAS co-localized with red auto-fluorescence of chloroplasts (Fig. 3D–F) providing evidence that the N-terminal 80 amino acids of PtTPS-LAS are sufficient for targeting protein to plastids in vivo. Using the same GFP-fusion protein expression analysis with PtAO we found green fluorescence associated with reticulate structures of mesophyll cells (Fig. 3G–I). This pattern of PtAO-GFP association with a reticulate subcellular structure was even more distinct when expression was analyzed in epidermal cells (Fig. 3K). Control GFP expression only showed diffuse green fluorescence in epidermal cells (Fig. 3J) supporting the finding that the N-terminal 48 amino acids of PtAO target protein to the ER system.

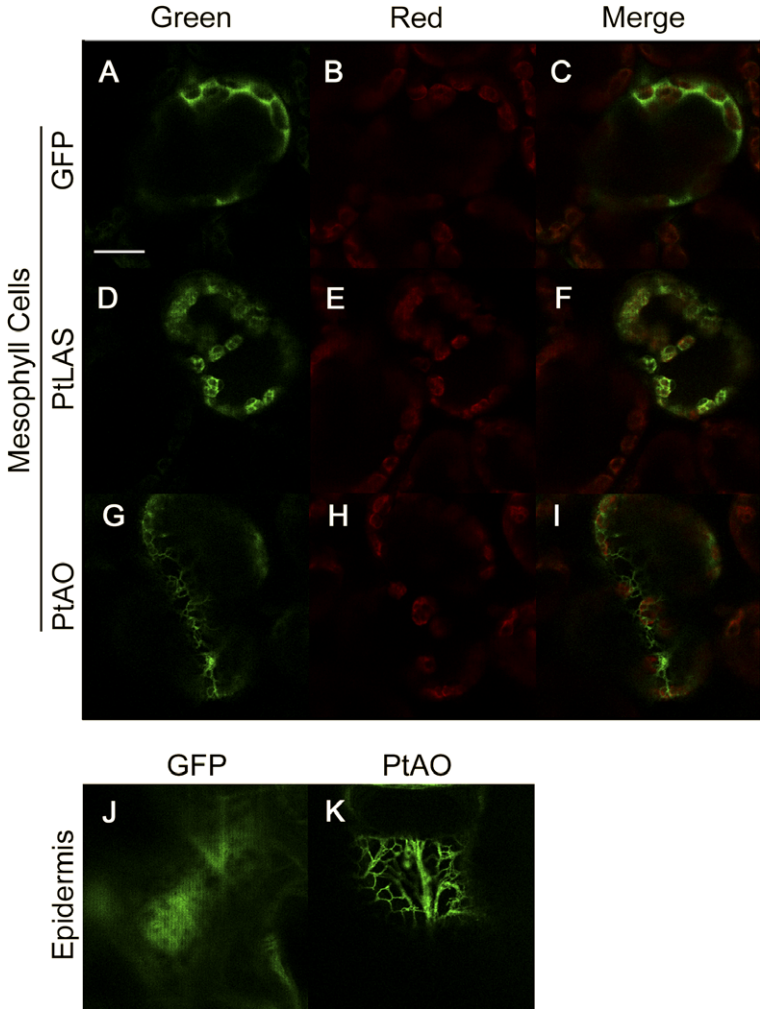

Fig. 3. Subcellular targeting of PtTPS-LAS and PtAO GFP fusion proteins. Mesophyll cells (A–I) and epidermis cells (J and K) of tobacco leaves transiently expressing GFP, PtTPS-LAS(l-80)::GFP, or PtAO(1-48)::GFP were analyzed by confocal in vivo microscopy. Green fluorescent signals are shown in A, D, and G. Red auto-fluorescence from chloroplasts is shown in B, E, and H. Merged images obtained for green fluorescence and red chloroplast auto-fluorescence are shown in C, F, and I. The proteins monitored are indicated on the left side of each row of images A–C, D–F, and G–I, and above images J and K.

Taken together, in silico predictions of plastid target peptides for conifer diterpene synthases (Martin et al., 2004), in silico predictions of membrane targeting domains of PtAO (Ro et al., 2005), microsomal association of PtAO in recombinant yeast cells (Ro et al., 2005), and the present data of GFP targeting detected by in vivo confocal microscopy, all support a model according to which diterpene synthase protein PtTPS-LAS and diterpene P450 protein PtAO are targeted to two different subcelluar compartments, plastids and ER, respectively. As most diterpene olefins do not accumulate in conifers but are instead rapidly oxidized by P450 activity to diterpene resin acids, an efficient transport system must exist to move the hydrophobic diterpene olefins produced by the diterpene synthases to the cytosol/ER. Such transport systems, potentially ABC transporters or lipid transfer proteins, as well as transporters for secretion of diterpene resin acids into the extracellular space of resin ducts or resin blisters are important targets for gene and protein discovery in conifers. While recent work provided considerable knowledge about enzymes involved in the biosynthesis of monoterpenes, sesquiterpenes and diterpene resin acids of conifer oleoresin defense, much future work is needed to elucidate the cellular and subcellular transport and secretion machinery of this fascinating secondary metabolite system.

3. Materials and methods

3.1. Expression of PtTPS-LAS in pET100 vector

A truncated version of cDNA clone *PtTPS-LAS* (Accession No. AY779541) lacking nucleotides encoding for amino acid 1–57 was amplified using *Pfu* polymerase (Stratagene, La Jolla, CA), forward primer 5′-CACCGATCCGTTTTCAGTTCCTACACTG-3′, and a reverse primer, 5′-CTAAGCAACCGGCTGGAAGAGGC-3′. The amplification product was ligated into expression vector pET100/D-TOPO (Invitrogen, Carlsbad, CA). The expression construct vector was transformed into *E. coli* Codon plus BL21 RIL cells (Stratagene, La Jolla, CA). One milliliter overnight culture of transformed cells was added to 250 mL of Luria-Bertani medium and cultured at 37 °C for 6 h. The culture was cooled to room temperature and induced by addition of 200 μM isopropylthio-β-galactoside and further incubation at 22 °C for 16 h. Cells were pelleted, resuspended in 5 mL of 10 mM sodium phosphate buffer (pH 7.5), and treated with 1 mg mL^{-1} lysozyme on ice for 30 min, followed by sonication for 10 s five times. Cell fragments were pelleted for 15 min at 14,000*g*. Supernatant (3 mL) was filtered through Sephadex G-50 gel filtration (BD Bioscience, Palo Alto, CA) and further concentrated on Amicon Ultra Centrifuge Filter (Millipore, Billerica, MA) to a final volume of 500 μL, adjusted to 20% glycerol, and stored at −80 °C.

3.2. Diterpene synthase enzyme assay

A total of 0.5–1 mg protein was used for each enzyme assay in 1 mL assay buffer [50 mM HEPES, pH 7.2, 100 mM KCl, 7.5 mM $MgCl_2$, 20 μM $MnCl_2$, 5% (v/v) glycerol, 5 mM fresh DTT]. Assays were incubated with 50 μM GGPP (Sigma–Aldrich, St. Louis, MO) at 30 °C for 1 h. Diterpene products were extracted twice with pentane (1 mL). Pentane extracts were dried over sodium sulfate and concentrated under N_2 gas to a final volume of 100 μL, of which 2 μL was injected on an Agilent 6890 GC-MSD system with an HP-5 capillary column (Agilent Technologies, Palo Alto, CA; 0.25 mm × 0.25 μm × 30 m) using the following temperature program: 100 °C initial temperature and 5 °C min^{-1} increment to 300 °C. The injection port was set to 220 °C and a column flow was 1 mL He min^{-1}. Diterpenes were identified by comparison with authentic standards.

3.3. Construction of GFP fusion proteins

As starting material we used a construct in which GFP was fused to the C-terminus of poplar cinnamate-4-hydroxylase (C4H) (Ro et al., 2001). The C4H portion from C4H::GFP in pSL1180 was removed by digestion with ApaI and XbaI. A 240-bp fragment from the 5′-end of the ORF of *PtTPS-LAS* was PCR amplified using *Pfu* polymerase, forward primer 5′-TACAGCGGGCCCATCATGGCCTTGCCTTCCTCTTCATTGT-3′, and reverse primer 5′-CAGCTCTAGAGATAACATGATCCTTCCAGAAACCT-3′. The 144-bp fragment from the 5′-end of the ORF of *PtAO* was amplified using forward primer 5′-TACAGCGGGCCCATCATGGCAGACCAAATATCATTACTGCT-3′ and reverse primer 5′-CAGCTCTAGAACCAGGTGGTAGATGGAGCTCCTGA-3′. The amplified fragments of *PtTPS-LAS* and *PtAO* were digested with ApaI and XbaI, gel purified, and ligated into ApaI- and XbaI-digested GFP::pSL1180 vector resulting in in-frame PtAS (80 amino acids)-GFP and PtAO (48 amino acids)-GFP fusions. The PCR-amplified regions were sequenced to verify PCR-fidelity. Fusion constructs in pSL1180 were digested by *Sal*I and *Spe*I for transfer into *Xho*I- and *Xba*I-digested binary vector pRT101-Bin19 which contained CMV 35S promoter, a multiple cloning site, and terminator sequence (Ro et al., 2001). The PtTPS-LAS and PtAO GFP fusion constructs in binary vectors were independently transformed into *Agrobacterium tumefaciens* GV3101 strain by electroporation. A GFP control construct in the same vector background (Ro et al., 2001) was used in the same fashion.

3.4. Plant growth and transient gene expression by leaf infiltration

Tobacco seeds were germinated in 0.5 × Murashige and Skoog medium and transferred to soil. Seedlings were grown for 2 months. *Agrobacterium* strains harboring the

fusion constructs or GFP control constructs were infiltrated on the abaxial side of tobacco leaves (Yang et al., 2000). Two to three days after infiltration, GFP fluorescence or chloroplast auto-fluorescence was measured by a series of 0.2 μm optical sections in epidermal and mesophyll cells using multiphoton confocal microscopy (BioRad Radiance 2000 on a Nikon Eclipse TE300). A line of 488 nm krypton laser with HQ515/30 emission filter was used to collect GFP signals, and a line of 568 nm krypton laser with HQ600/50 filter was used to collect chlorophyll auto-fluorescence. ImageJ 1.32J (National Institute of Health) was used to process a series of confocal images.

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