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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