CONIFER TERPENE SYNTHASES: FUNCTIONS IN INDUCED PLANT DEFENSE, PHYLOGENETIC ANALYSES, & MOLECULAR MODELING

by

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ABSTRACT

Terpenes and terpene biosynthesis play major roles in conifer secondary metabolism. Oleoresin, a combination of monoterpenes, sesquiterpenes and diterpenes, is produced by conifers in copious quantities and stored in specialized anatomical structures. These chemicals serve as toxic protectants and mechanical barriers against invading herbivores and pathogens. Norway spruce, *Picea abies*, is capable of constitutive and inducible terpene-based defenses. Following insect attack or biological elicitation, Norway spruce is induced to form traumatic resin ducts. The studies presented here describe the complex changes induced after methyl jasmonate (MeJA) treatment in this species. Terpene defenses are characterized in a tissue specific manner on anatomical, chemical, biochemical, and molecular levels. The formation of traumatic resin ducts in the developing xylem is initiated after MeJA treatment. Similarly, the accumulation of monoterpenes in both bark and wood and the accumulation of diterpenes in wood begin rapidly after MeJA treatment. Terpene accumulation is preceded temporally by the increase in GGPP synthase, monoterpene synthase (mono-TPS) and diterpene synthase (di-TPS) activities in wood. *Mono-TPS* and *di-TPS* transcripts increase in abundance in stem tissue and mono-TPS transcripts increase in foliage following MeJA treatment. MeJA treatment also induces the release of large amounts of volatile oxygenated monoterpenes and sesquiterpenes, compounds that are not emitted from untreated control plants. This emission of volatile terpenes coincides with a two-fold accumulation on terpenes in foliage and a five-fold increase in TPS activities. Ten Norway spruce *TPS* genes, involved in these constitutive and inducible defenses, were cloned, functionally expressed and characterized. This suite of TPS contains members of the mono-TPS, sesqui-TPS and di-TPS biochemical classes including several of previously not characterized functions. Furthermore, these TPS have provided a new basis to re-examine the phylogeny of the gymnosperm TPS-d subfamily and the TPS family overall. A model of gymnosperm TPS evolution is discussed as are some striking parallels between gymnosperm and angiosperm TPS evolution.

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ABBREVIATIONS

di-TPS	Diterpene synthase
DMADP	Dimethylallyl diphosphate
FDP	Farnesyl diphosphate
FID	Flame ionization detector
GC	Gas chromatography
GDP	Geranyl diphosphate
GGDP	Geranylgeranyl diphosphate
HEPES	[*N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)]
I	Identity
IDP	Isopentenyl diphosphate
JA	Jamonate(s)
MeJA	Methyl Jasmonate
MEP	2-C-methyl erythritol-4-phosphate
mono-TPS	Monoterpene synthase
MS	Mass spectroscopy
PCR	Polymerase Chain Reaction
PT	Prenyltransferases
Radio	Radioisotope Detector
S	Similarity
sesqui-TPS	Sesquiterpene Synthase
TCD	Termal Conductivity Detector
TD	Traumatic Resin Ducts
TPS	Terpene Synthase

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1. INTRODUCTION

The field of plant secondary metabolism fosters enthusiastic interest and continued research into how these metabolites can function in defense and/or attraction (Croteau et al., 2000) as well as how the evolution of these biochemical pathways occurs (Pichersky and Gang, 2000; Facchini et al., 2004). For this discussion, the terms secondary metabolites and natural products will be used interchangeably and they will be used to designate compounds believed to function in defense and/or attraction. Secondary metabolites enable plants to respond to a variety of pathogen or insect challenges and are critical to the health and survival of an individual species. The terpenoids represent the largest group of secondary metabolites with over 30,000 members (Croteau et al., 2000). Natural products and the utilization of these compounds in defense are essential to gymnosperms where long sessile life spans necessitate means of preservation. Three classes of terpenes, monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20), are especially prominent among the conifer defense compounds as they constitute conifer oleoresin (Figure 2.1, p. 16). These terpenoids can be acyclic, cyclic or multicyclic. Additionally, some of these compounds may be oxygeneated to terpene alcohols. Class specific terpene synthases (TPS) are responsible for the formation of the many simple (single ring structure or acyclic) and intricate (two or more ring structures) terpene skeletons known. TPS can also be responsible for producing terpene alcohols. The cloning and functional characterization of these enzymes provides valuable information concerning the biochemistry of defense and establishes the necessary tools for gene expression and structure-function analyses. Additionally, the cloning of conifer *TPS* enables the phlyogenetic re-construction of the TPS family thereby shedding light on the evolution of all TPS.

This introduction will bring together the major concepts and supporting literature that helped ideate my research on terpene defenses and TPS phylogeny in Norway spruce (*Picea abies* L. Karst). Each chapter presented here represents either a published work [Chapters 2 (Martin et al., 2002), 3 (Fäldt et al., 2003b), and 4 (Martin et al., 2003b)] or a work currently under in press [Chapter 5 (Martin et al., 2004)]. The introductions contained in each chapter are specific for the topics covered therein. Some of this research has been published for almost two years and has spawned further studies by others into other aspects of conifer defense. These studies will be thoroughly covered in the discussion (p. 132).

TERPENE BIOSYNTHESIS

While the secondary metabolic terpenes figure prominently in plant defense, there are numerous examples of primary metabolic terpenes such as gibberllin hormones and phytol (C20), steroids (C30), and carotenoids (C40). Terpene biosynthesis begins with the formation of the five carbon (C5) building blocks, isopentenyl diphosphate (IDP) and its isomer, dimethylallyl diphosphate (DMADP) [see Figure 2.2 p. 17 and (Cane, 1999)]. Two pathways exist for the formation of these precursors (Eisenreich et al., 1998). The mevalonate pathway is found in the cytosol / endoplasmic reticulum and the 2-C-methyl erythritol-4-phosphate (MEP) pathway, which proceeds via 1-deoxyxylulose-5-phosphate, occurs in plastids. While the sy

Upon the formation of IDP and DMADP, prenyltransferases (PT) perform 1'-4 condensation reaction coupling IDP with an allylic chain. Class specific PT form the C10, C15 and C20 prenyl diphosphate precursors respectively (see Figure 2.2 p. 17). The initial reaction, coupling these two C5 compounds, IDP and DMAPP, synthesizes geranyl diphosphate (GDP). This is the product of geranyl diphosphate synthase and is the ubiquitous C10 precursor of the monoterpenes. Farnesyl diphosphate synthase synthesizes a product five carbons longer by a subsequent addition of IDP to GDP resulting in farnesyl diphosphate (FDP), precursor of all sesquiterpenes. The C20, geranylgeranyl diphosphate (GGDP), is formed by a further addition of IDP to FDP and is produced by geranylgeranyl diphosphate synthase. With the exception of a small subunit recently characterized as part of a GDP synthase heterodimer (Burke and Croteau, 2002), PT share many common features including sequence similarities, a divalent metal ion requirement for catalysis, and the similar catalytic motif DDXXD (Alonso and Croteau, 1993; Croteau and Cane, 1985). While the synthesis of GDP and GGDP is localized to the plastid and the synthesis of FDP is localized to the cytosol / endoplasmic reticulum, crosstalk between these two pathways has been reported (Bick and Lange, 2003). These studies indicate that export of IDP, GDP and to a lesser degree FDP and DMADP can occur from plastids and may represent a mechanism by which to share pathway intermediates and control flux through these pathways.

The PT are critical components of terpene biosynthesis but little is known about PT activities involved in induced defense responses. It is possible that a level of regulation resides in the biosynthesis of TPS substrates by the class-specific PT. Furthermore, it was considered possible that differential control over PT may occur in individual tissues. Therefore, objectives of this work were 1) to determine how the activity of each of the three classes of PT changed during induced defense responses and 2) to determine how these

activities differed between bark and wood tissues of Norway spruce. The work presented here (Chapter 2) explores the role of these enzymes in induced terpene biosynthesis and reveals tissue-specific PT activities.

The prenyl diphosphate precursors are used by TPS as the first committed step in the production of monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) (see Figure 2.2 p. 17). All members of the TPS family utilize an electrophilic reaction mechanism, assisted by divalent metal ion cofactors (Cane, 1999; Wise and Croteau, 1999; Davis and Croteau, 2000). As catalysis proceeds, the prenyl diphosphate substrates are ionized or protonated to produce reactive carbocation intermediates. Depending on whether the TPS is a single or multiple product enzyme, these carbocations can be rearranged and eventually quenched in one or several different ways to create the wide variety of cyclic and acyclic terpene skeletons known (Lesburg et al., 1997; Starks et al., 1997; Cane, 1999; Wise and Croteau, 1999; Davis and Croteau, 2000; Whittington et al., 2002). While secondary transformations of terpenes can occur, the formation of terpene skeletal structures is almost entirely controlled by TPS. Furthermore, TPS tend to exert tight control over the stereochemistry of products formed and as a result, one enantiomer usually dominates any TPS profile.

A collection of conifer *TPS* has previously been cloned from *Abies grandis* (Bohlmann et al., 1998a; Bohlmann et al., 1997; Bohlmann et al., 1999; Steele et al., 1998a; Stofer Vogel et al., 1996) and *Pinus taeda* (Phillips et al., 2003), and the enantiomeric specificity of these enzymes has been investigated. Furthermore, the distribution of monoterpene enantiomers and the location of enantiomer-specific mono-TPS has been well characterized in *P. taeda* (Phillips et al., 1999). However, little is known about how changes in the accumulation of specific enantiomers occurs in induced defense responses. Additionally, the number of conifer TPS functions known prior to this study does not adequately represent the variety of known terpene skeletons present in oleoresin. The objectives of this work were 1) to identify tissue specific changes in accumulation of specific terpene enantiomers, 2) clone and characterize TPS displaying new functions, and 3) determine the enantiomeric distribution of terpenes formed by Norway spruce TPS. Chapters 2 and 4 detail the complexity of induced conifer oleoresin accumulation and the induced release of volatiles in Norway spruce, including the chirality of these terpenes. TPS involved in this response also showed enantiomeric specificity (Chapter 4). Ten TPS displaying new functions were identified and nearly all were found to exert stereochemical control over the products formed (Chapters 3 and 5).

CONIFER TERPENOID DEFENSES

Chemical Ecology

Oleoresin biosynthesis and accumulation is a critical component of conifer ecology. As some of the most ancient living organisms, conifers have developed significant induced and/or constitutive terpene defenses enabling them to combat herbivores and pathogens (Seybold et al., 2000; Trapp and Croteau, 2001a). Terpenes are known to play important ecological roles not only in the defense of the tree, but also in bark beetle chemical ecology. Beetles use volatile terpenes as a means of locating host tissues (Klepzig et al., 1996; Wallin and Raffa, 2000) and preferentially choose tissues with lower levels of monoterpenes (Klepzig et al., 1996). Additionally, the complexity and variation in terpene constituents between conifer species also affects beetle landing and host acceptance (Wallin and Raffa, 2000). Species-specific oleoresin of a distinct chemical profile enables bark beetles to preferentially choose one or a few species of host trees and emphasizes the co-evolution of herbivore and host (Mitton and Sturgeon, 1982). While some species of bark beetles may be able to produce their own monoterpene pheromone precursors (Martin et al., 2003a), bark beetles commonly rely also on host-derived monoterpenes as precursors to their own sex and aggregation pheromones (Seybold et al., 2000). Monoterpene pheromones are used in a chiral-specific manner by bark beetles such that only one enantiomer is able to serve as a sex or aggregation pheromone while the other enantiomer often acts as an anti-aggregation pheromone precursor. Since both enantiomeric host-derived monoterpene precursors are accepted and chemically modified by the beetle (and/or symbionts) (Byers, 1995), the exact monoterpene enantiomeric mixture of conifer resin is significant to both the beetle and to the trees' ability to survive an attack (Borden, 1984). Given the importance of conifer forests, the role of terpenes in conifer defense as well as regulation of terpenoid biosynthesis has been the subject of considerable research (Bohlmann et al., 2000a; Bohlmann and Croteau, 1999; Bohlmann et al., 1999; Phillips et al., 1999; Steele et al., 1998a; Steele et al., 1998b; Litvak and Monson, 1998; Steele et al., 1995; Lewinsohn et al., 1991a).

The resin or pitch, of conifer oleoresin is a complex mixture composed of approximately equal molar concentrations of monoterpenes and diterpenes accompanied by a smaller fraction of sesquiterpenes. Recent reviews have underscored the complexity of terpene-based conifer defenses (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Seybold et al., 2000; Trapp and Croteau, 2001a). These chemicals function to protect the tree through toxicity to invaders, by providing mechanical barriers, by interrupting insect physiology and by signaling predators and parasites to the attacking herbivore. As toxicants,

many terpenes, mono-, sesqui- and diterpenes, have demonstrated anti-biotic or repelling activities against a variety of insects and insect-mediated fungal symbionts (Seybold et al., 2000). Some terpenes are also able to interrupt the normal development and maturation of insects by mimicking herbivore hormones. Examples of chemicals with this activity are the sesquiterpenes, juvabione, which was originally isolated from *Abies balsamea* (Slama and Williams, 1965), farnesol, and farnesal (Schmialek, 1963). The precursor to juvabione is *E*-α-bisabolene whose synthesis is wound-inducible in *Abies grandis* (Bohlmann et al., 1998a). The mechanical component of terpene defenses enables the tree to physically seal wounds and insect invaders are often pushed out of the tree in this process (Croteau and Johnson, 1985). When an opening is created, volatile monoterpene constituents act as solvents to facilitate the movement of the diterpene resin acids to the wound. Subsequently, the monoterpenes evaporate and the crystallization of the resin acids forms a protective barrier sealing the point of injury.

Recently, attention has been drawn to the action of conifer terpenes in tritrophic or indirect defense interactions. Volatile terpenes may function in indirect or tritrophic defense interactions by signaling predators or parasites of an attacking herbivore. Indirect defense interactions involving Norway spruce (*P. abies*), the European spruce bark beetle (*Dendroctonus micans*), and the predatory beetle (*Rhizophagus grandis*) are mediated through volatile oxygenated monoterpenes (Gregoire et al., 1992; Gregoire et al., 1991) where these cues also stimulate ovipositioning behavior from the predator (Gregoire et al., 1991). An investigation of predator responses to *Ips spp.* pheromones revealed that *Pinus*-derived monoterpenes act synergistically on the attractiveness of beetle pheromones to the predators, *Thanasimus dubius*, *Platysoma cylindrica*, and *Corticeus parallelus* (Erbilgin and Raffa, 2001; Raffa and Klepzig, 1989). Furthermore, the specific chirality of these compounds is important in guiding the predators to hosts. Volatile terpenes from *Pinus sylvestris* where pine sawfly eggs were deposited were also found to attract the egg parasitoid, *Chrysonotomyia ruforum* (Hilker et al., 2002). Evidently, the biological activities of terpenes in either direct defense or attraction of predators and/or parasites can be unique to a given molecule/enantiomer or to a group of terpenes.

Conifer chemical ecology includes many other herbivores, predators and pathogens aside from the bark beetles, and in these diverse interactions, volatile emissions as well as accumulated terpenes play important roles in defense. In spruce species, the feeding and brooding damage from white pine weevil, *Pissodes strobi*, is the cause of reduced fitness and widespread sapling mortality (Wallace and Sullivan, 1985; Alfaro et al., 2002). Defoliating insects such as budworms (*Choristoneura spp.*), sawflies (*Diprion pini*) and tiger moth larvae

(*Halisdota ingens*) are also major sources of damage for many conifers (Zhang and Alfaro, 2002; Alfaro et al., 2001; Litvak and Monson, 1998). In an effort to understand foliar responses to defoliating herbivores, the activities of mono-TPS and the accumulation of monoterpenes were studied (Litvak and Monson, 1998). Mono-TPS activities increased in response to herbivory and simulated herbivory in the foliage of several conifer species, but this was not related to terpene accumulation. This study provided an initial glimpse into the involvement of mono-TPS in foliar defense responses and provided a rationale to further investigate the role of foliar volatile emissions and TPS in conifer defense. Both monoterpenes and sesquiterpenes are common volatile constituents, but little is known about conifer sesquiterpene volatiles. Furthermore, the regulation and biochemistry of TPS activity within foliage and the temporal control over volatile release has not been examined for any conifer species. Objectives of this research included 1) determining if foliar terpene accumulation differed between control and induced Norway spruce plants, 2) determining which volatile terpenes were released from Norway spruce foliage and whether volatile emissions could be induced, 3) analyzing quantitatively and qualitatively differences in induced volatile emissions vs noninduced volatile emissions, and 4) defining the *TPS* gene expression profiles within foliage. In this thesis, I present chemical and biochemical characteristics of induced volatile emissions from Norway spruce (Chapter 4). Induced *TPS* gene expression analyses are presented in chapter 3. These volatile studies were the first of this kind in a conifer species and represent an exciting beginning for future work in understanding the role of induced volatile emissions in conifers.

The Structural Anatomy of Resin Accumulation

Conifers have developed unique specialized structures to accumulate terpenes. A classical taxonomy of the Coniferales is based on Bannan's classification of these structures (Bannan, 1936). Terpene sequestration structures can be as simple and short-lived as the resin blisters found in true firs (*Abies*), cedars (*Cedrus*), hemlock (*Tsuga*) and golden larches (*Pseudolarix*), or they can be a complex, long-lasting, highly organized system of interconnected resin-filled canals such as those found in spruces (*Picea*), larch (*Larix*), pine (*Pinus*), and Douglas-fir (*Pseudotsuga*). The complexity and the longevity of these structures are defining characteristics for members of this order (Langenheim, 2003). Species producing high constitutive levels of terpenes have a well-developed system of resin canals, while those with very little constitutive resin lack these pre-existing ducts, but retain the ability to

induce terpenoid defenses when challenged (Lewinsohn et al., 1991b; Phillips and Croteau, 1999).

Traumatic Resin Ducts and Traumatic Resinosis

Spruces represent an interesting example displaying both complex constitutive resin canals along with the intense inducible response of traumatic resin duct development. Spruce cortical tissue has numerous axial resin canals surrounded by epithelial cells capable of producing terpenes for many years (Trapp and Croteau, 2001a). However, under normal circumstances, there is little duct development within the xylem. The formation of traumatic resin canals in spruces occurs in a tangential series within the newest xylem annual ring and can extend over great distances longitudinally. Recent work on Norway spruce has cytologically described the induced formation of traumatic resin ducts (Nagy et al., 2000). The developmental onset and the anatomy of these ducts indicate that xylem mother cells are the primary source of duct formation. Consequently, the production of traumatic resin ducts is due to a change in cambial activity that initiates resin duct epithelial cells in lieu of tracheids. Nagy et al. (2000) also found lumenal continuity between the traumatic resin ducts and the radial duct rays and direct contact between the epithelial cells and the ray parenchyma cells. Together these features enable enhanced resin flow along with transport of components to the outer bark.

In *Picea spp.* and *Cedrus*, traumatic resin canals can be induced by a wide variety of both external and internal stimuli. These include, but are not limited to, insect attack (Alfaro, 1995) pathogens (Christiansen et al., 1999; Krekling et al., 2004; Berryman, 1988) mechanical wounding (Christiansen et al., 1999), plant growth hormones (Fahn et al., 1979), preparations made from fungal cell walls (Lieutier and Berryman, 1988), and environmental stress (Wimmer and Grabner, 1997). However, under normal growth conditions these ducts rarely appear, or if they do, they are few in numbers and scattered as opposed to the nearly complete concentric circles of ducts observed under conditions of pathogen or insect response.

Studies with *P. abies* and *P. glauca* have also linked intensity of the induced duct formation with those of resistance to insect and fungus (Tomlin et al., 1998; Nagy et al., 2000). Resistant trees display a more rapid induction of traumatic resin duct formation than do susceptible clones. These studies also documented that resistant spruce clones were less damaged than susceptible clones when inoculated with fungus. The correlation between resistance and intense traumatic resin canal formation in *P. glauca* has also been well

documented by Alfaro et al. (1996). They recognized this phenomenon in trees attacked by the white pine weevil, *P. strobi*, where resistant trees had enhanced survival rates when attacked.

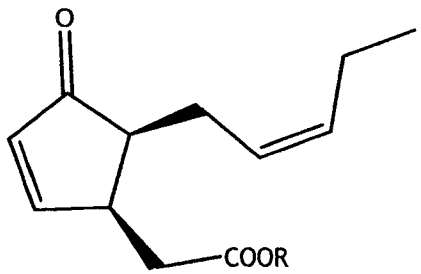
Traumatic resinosis in *P. abies*, *P. glauca*, and *Pinus strobus*, is correlated with the decreased survival of *Pissodes strobi* larvae (Dixon and Hourseweart, 1982; Overhulser and Gara, 1981; Sullivan, 1960). In *P. glauca*, *P. stichensis*, and *P. abies*, the rather lengthy time (about 2 - 3- weeks) for traumatic resin duct development corresponds well with *P. strobi* larval development and indeed, the ducts, by spilling their terpenoid contents into the larval cavity, were able to effectively increase brood mortality (Alfaro, 1995). It has also been noted that some trees will produce successive rings of traumatic ducts up to three years after a bark beetle attack (Franceschi et al., 2000) or when a tree undergoes a second attack in a given year, the development of a second ring of traumatic resin ducts is often greatly enhanced (Alfaro, personal communication). These results indicate a degree of biological memory of an insect attack, an idea that has been supported by Krokene et al. (1999) who found that an initial fungal inoculation was able to effectively protect trees from a normally lethal fungal application later in the season. Although the mechanism by which this takes place is still unknown, these observations again indicate a crucial role for traumatic resin duct development and the accompanying induced resinosis in conifer defense.

There has been considerable research on induced terpene biosynthesis within the stem tissue of *A. grandis*. Monoterpene and diterpene synthase activities and gene expression were shown to be wound-inducible, although, sesquiterpene synthases appear to be less inducible (Funk and Croteau, 1994; Bohlmann et al., 1997; Bohlmann et al., 1998a; Lewinsohn et al., 1991a; Steele et al., 1998a; Steele et al., 1998b; Gijzen et al., 1991, 1992). In all cases, the increases in TPS activities and *TPS* gene expression were reflected in the quality and quantity of oleoresin accumulation. Additionally, several TPS enzymes and their corresponding products were found to be upregulated or accumulating at increased concentrations only in the wounded tissues (Lewinsohn et al., 1991a). Work in *P. taeda* has described the distribution of α-pinene enantiomers within foliage, xylem, phloem and root tissues and has investigated the properties of mono-TPS responsible for some of these products (Phillips et al., 1999). (–)-α-pinene and (+)-α-pinene synthases were cloned and characterized from this species along with two other TPS (Phillips et al., 2003). While several studies have concentrated on spruce terpene chemistry (Ekman, 1979; Brooks et al., 1987; Lorbeer and Zelman, 1988; Hanover, 1974), data on induced qualitative changes and the role of induced enzyme and gene activities in Norway spruce had not been described.

The complexity and the inducible biosynthesis of conifer oleoresin make this defense useful against a wide range of pests. Since terpene defenses function by mechanical and chemical means, the amount of terpenes produced is as important as the quality of the given blend. Furthermore the ability to mount an induced defense can be paramount to the survival of individual trees. It is clear that conifer terpenoid defenses are multifaceted as they can be released as volatiles, sequestered in constitutive resin canals or may be added to newly developing TD. Characterization of inducible conifer defenses as well as the genes and enzymes involved in terpene biosynthesis is critical to the understanding of these defenses and the processes governing them. Main objectives of this work included 1) the characterization of induced terpene defenses in wood, bark and foliage, 2) the correlation of these induced defenses to the development of TD and any increases in TPS activity, and 3) the cloning and characterization of TPS involved in the induced resinosis of *P. abies*. I present information concerning inducible terpene defense responses, the induction of dramatic quantitative and qualitative changes in terpene accumulation and volatile release, the formation of TD and induced *TPS* gene expression (Chapters 2, 3, and 4). A suite of ten TPS involved in this response were cloned and functionally characterized (Chapters 3 and 5) and represent one of the largest collections of *TPS* cDNAs isolated and characterized from a single species.

METHYL JASMONATE AS A BIOLOGICAL ELICITOR

Plants control and coordinate metabolic processes and development through the use of hormones and other regulatory factors. Chemical signals/hormones are critical to the orchestration of secondary metabolic plant defenses as they enable plants to respond in different manners to pathogen or herbivore challenges. Tailoring of the defense responses to an individual threat is accomplished by utilizing different signal molecules or a combination of signal molecules such as jasmonate or other octadecanoids and oxylipids, ethylene and salicylate to induce defense-related genes (Kessler and Baldwin, 2002).



R = H jasmonic acid

R = CH_3 methyl jasmonate

Figure 1.1: Defense-related signal compounds of the octadecanoid pathway

The octadecanoid pathway is one of the best characterized signaling pathways in angiosperms. Jasmonates (JA) have a demonstrated involvement in

many developmental processes including the maturation of anthers/pollen (Xie et al., 1998), tendril curling (Weiler et al., 1993), and seed germination (Wilen et al., 1991). Addtionally, JA is known to induce a wide variety of plant defense responses including secondary metabolite synthesis including the release of volatiles and synthesis of proteinase inhibitor proteins (Baldwin, 1999; Creelman and Mullet, 1997). Additionally, methyl jasmonate (MeJA) is believed to play a role in interplant communications (Karban et al., 2000) and has been used experimentally to induce plant defense responses in a number of angiosperm species including tomato, tobacco and *Arabidopsis* (Farmer and Ryan, 1990; Creelman and Mullet, 1997; Baldwin, 1999). JA and MeJA are also known to induce the emission of volatiles in numerous angiosperm species (Hopke et al., 1994; Dicke et al., 1999; Gols et al., 1999; Koch et al., 1999; Halitschke et al., 2000; Kessler and Baldwin, 2001; Rodriguez-Saona et al., 2001; Schmelz et al., 2001; Arimura et al., 2004). JA elicitation functions by activating transcription factors which then bind to promoter regions to induce transcription of genes (Memelink et al., 2001). In this manner, plants are able to upregulate the activity of key enzymes involved in the synthesis of plant natural products that function as herbivory deterrents or signal herbivore predators and parasites through the release of volatiles.

Much less is known about jasmonate-induced secondary metabolism in gymnosperms. In the gymnosperm *Taxus*, enzymes involved in the synthesis of the diterpene taxol are induced by MeJA in suspension cultures (Hefner et al., 1998; Yukimune et al., 1996; Ketchum et al., 1999). Studies in white spruce seedlings and cell cultures have shown that MeJA treatment induces the expression of a 14-3-3 protein and chalcone synthase (Richard et al., 2000; Lapointe et al., 2001). Activities of JA have been studied in both symbiotic and antagonistic interactions between spruce seedlings and fungi. Apparently, MeJA application assists ectomycorrhizal colonization of Norway spruce roots (Regvar et al., 1997). MeJA application to Norway spruce seedlings also enhances their survival rate when they were challenged by *Pythium ultimun* (Kozlowski et al., 1999).

While work in *A. grandis* has resulted in a better understanding of induced defense responses (Gijzen et al., 1991; Lewinsohn et al., 1991b; Lewinsohn et al., 1991a; Lewinsohn et al., 1993b; Bohlmann et al., 1998a; Steele et al., 1998a; Steele et al., 1998b), these studies have largely relied on wounding to stimulate induced defenses. Similarily, previous studies looking at induced terpene defenses and TD development in spruce have also utilized wounding and/or insect attack to elicit a response (Nagy et al., 2000; Litvak and Monson, 1998; Tomlin et al., 2000; Tomlin et al., 1998; Krokene et al., 1999). In my research, MeJA was used to study induced terpene responses on a whole plant basis in Norway spruce. MeJA provided a means by which to induce plant defense responses without mechanically injuring

the organism. This is especially vital to the quantification of induced terpene defenses where any injury not only limits the biological capacity of the tissue to respond, but also, would enhance the loss of terpenes through the woundsite. I used MeJA to address the following questions 1) does MeJA treatment induce terpene-based defenses in spruce, 2) does this induction occur in a dose-specific manner, 3) does MeJA induce the development of TD and what is the time frame of development, 4) does MeJA induce volatile emissions, and 5) does MeJA induce *TPS* gene expression. The application of MeJA was utilized to study induced terpene accumulation in wood, bark, and foliage and to investigate the TPS activities induced in these tissues as well (Chapters 2 and 4). MeJA was also shown to induce volatile emissions and these emissions were characterized for the first time in a conifer (Chapter 4). Furthermore, MeJA treatment was used to study induced *TPS* transcript levels in stem and foliage tissue (Chapter 3), and MeJA treatment was used to develop induced RACE templates for the cloning of *TPS* cDNAs (Chapter 5).

MOLECULAR BIOLOGY AND PHYLOGENY OF THE *TPS* FAMILY

The known plant TPS are part of a large family of enzymes containing members from angiosperms and gymnosperms. Based on sequence similarities, catalytic mechanisms, and exon/intron patterns, all plant TPS are believed to have arisen from a common ancestor (Bohlmann et al., 1998b; Trapp and Croteau, 2001b). This ancestral TPS may have resembled the known conifer di-TPS and/or may have been involved in gibberellic acid metabolism as known TPS with these functions all share an ancestral ~ 200 amino acid motif (Figure 5.2, p. 94) (Bohlmann et al., 1998b; Trapp and Croteau, 2001b). The evolution of TPS occurred separately in angiosperms and gymnosperms and appears to have involved the discrete loss of exons and introns which gave rise to the gene structures of known *TPS* (Bohlmann et al., 1998b; Trapp and Croteau, 2001b). Based on amino acid similarity, the *TPS* gene family has been divided into seven subfamilies designated TPS-a through TPS-g (Figure 5.13, p.113) (Bohlmann et al., 1998b; Dudareva et al., 2003). TPS subfamilies are designated by distinct branching patterns visualized by phylogenetic analyses and each subfamily usually consists of TPS from the same biochemical class. Members of the TPS-a group include angiosperm sesqui-TPS and one known di-TPS with a similar catalytic mechanism to that of sesqui-TPS. The TPS-b group is composed of mono-TPS and one hemi-TPS, isoprene synthase (Miller et al., 2001), all of angiosperm origin. The TPS-g family was recently identified and contains members of mono-TPS from *Antirrhinum majus* (Dudareva et al., 2003) and *Arabidopsis thaliana* flowers (Chen et al., 2003) that form acyclic products. The TPS-f branch contains the monotype

linalool synthase from *Clarkia brewerii* that is likely an inoperative di-TPS that gained a mono-TPS function. This mono-TPS is the only characterized mono-TPS to contain the ancestral ~ 200 amino acid motif. Also coding for this motif, angiosperm TPS producing gibberellic acid pathway precursors are grouped into TPS-c (copalyl diphosphate synthases) and TPS-e (ent-kaurene synthases). All TPS of conifer origin, regardless of the biochemical class and whether or not the ancestral ~ 200 amino acid motif is present, are grouped into the TPS-d subfamily (Figure 5.12, p.109).

TPS of secondary metabolism are believed to have evolved from TPS involved in primary metabolism. Through gene duplication events followed by functional diversification, TPS evolved to fill new niches and gained functions to create the wealth of known terpene products (Bohlmann et al., 1998b; Trapp and Croteau, 2001b). Within the TPS family, there are highly related enzymes that make different products, but there are also highly divergent enzymes which make the same products (Bohlmann et al., 1998b; Bohlmann et al., 2000a). Furthermore, several TPS appear similar to one biochemical class of enzymes on an amino acid level, while their revealed function places them within an entirely different biochemical class. Casbene synthase, a di-TPS from *Ricinus communis* (Mau and West, 1994), is one such example. On a primary sequence level, this enzyme resembles known sesqui-TPS. For these reasons, accurate *a priori* predictions of enzyme function cannot be made. Each enzyme must be functionally characterized to ascertain its biochemical role.

While there are many representatives of angiosperm TPS known, there are relatively few known gymnosperm TPS. Furthermore, the TPS-d subfamily is almost exclusively made up of TPS from *A. grandis* and a phylogenetic analysis of this group has not been completed since the cloning of four *P. taeda TPS* (Phillips et al., 2003), a mono-*TPS* from *Picea sitchensis* (Byun McKay et al., 2003), and a di-*TPS* from *Ginkgo biloba* (Schepmann et al., 2001). For these reasons, the phylogeny of the gymnosperm TPS-d subfamily needs to be re-addressed. Moreover, it is not known whether the inclusion of additional conifer TPS will divide the TPS-d family based on sequence structure and functional assessment, or if this subfamily will stay as a cohesive unit undelineated into biochemical classes. A major objective of my work was to clone and functionally characterize a number of TPS from Norway spruce. By including these genes in a phylogenetic analysis of the gymnosperm TPS-d subfamily, it was anticipated that evolutionary relationships within this subfamily would come to light. The cloning and characterization of ten new *TPS* genes from Norway spruce (Chapters 3 and 5) supplied the necessary sequence information to re-examine the phylogeny of the TPS-d subfamily and the TPS family on the whole (Chapter 5). The model of *TPS* gene evolution has been reconstructed to reflect these findings. Additionally, the cloning of this suite of *TPS* genes has enabled me

to use similar conifer TPS in comparative modeling experiments (Chapter 5). The goal of this latter work is to further understand which amino acids may be present in the active sites of similar TPS and to guide future studies in site-directed mutagenesis where the role of these modeled amino acids will be investigated.

OBJECTIVES

While mentioned previously in this introduction, this list will serve as a comprehensive itemization of this project's goals. The first part of this work will concentrate on MeJA induced changes in Norway spruce occurring on anatomical, chemical, biochemical and molecular biological levels. Foremost among the objectives in this first part will be to determine whether MeJA has the ability to induce terpene defense responses in *P. abies* in a similar manner in which they are induced during insect attack. This will include a detailed analysis of any anatomical changes that accompany MeJA treatment. It will include an analysis of mono-, sesqui-, and diterpene accumulation in wood, bark and foliage for 35 days following MeJA treatment. TPS and PT activities will also be examined in these tissues. Induced volatile release will also be characterized. The last piece of this examination will be to establish whether TPS gene expression is induced by MeJA treatment. Each of these experiments will be performed in replicate and each will be contrasted and compared to appropriate controls.

In the subsequent sections of this thesis, the focus will be on the cloning and functional characterization of TPS involved in the production of *P. abies* oleoresin and induced volatiles. TPS of each of the three biochemical classes, mono-TPS, sesqui-TPS and di-TPS, will be targeted to achieve broard coverage of TPS present in this species. The newly cloned and characterized TPS will then be included in phylogenetic analyses of the tps-d subfamily as well as the entire TPS family that included both gymnosperms and angiosperms. It is hoped that these analyses will resolve some lingering questions regarding the evolution of all TPS. Lastly, several orthologous TPS from *P. abies*, *P. sitchensis* and *A. grandis* will be comparatively modeled in an effort to gain a further understanding of differences in these enzyme's active sites.

2. METHYL JASMONATE INDUCES TRAUMATIC RESIN DUCTS, TERPENOID RESIN BIOSYNTHESIS AND TERPENOID ACCUMULATION IN DEVELOPING XYLEM OF NORWAY SPRUCE (*Picea abies*) STEMS[1,2]

ABSTRACT

Picea abies (L.) Karst (Norway spruce) produces an oleoresin characterized by a diverse array of terpenoids, monoterpenoids, sesquiterpenoids and diterpene resin acids, that can protect conifers against potential herbivores and pathogens. Oleoresin accumulates constitutively in resin ducts in the cortex and phloem (bark) of Norway spruce stems. *De novo* formation of traumatic resin ducts (TDs) is observed in the developing secondary xylem (wood) after insect attack, fungal elicitation and mechanical wounding. Here I characterize the methyl jasmonate (MeJA)-induced formation of TDs in Norway spruce by microscopy, chemical analyses of resin composition, and assays of terpenoid biosynthetic enzymes. The response involves tissue-specific differentiation of TDs, terpenoid accumulation, and induction of enzyme activities of both prenyltransferases and terpene synthases in the developing spruce xylem, a tissue that lacks constitutive axial resin ducts. The induction of a complex defense response in Norway spruce by MeJA application provides new avenues to evaluate the role of resin defenses in protection of conifers against destructive pests such as weevils, bark beetles and insect-associated tree pathogens.

[1] This work has been published in Martin et al. (2002)

[2] The investigation of MeJA induced traumatic resinosis (Martin et al., 2002) presented in chapter two was exclusively done by the author, save for the one experiment involving prenyl transferases (Figure 2.8) that was completed by the co-author Dr. Dorthea Tholl. Drs. Jörg Bohlmann and Jonathan Gershenzon served as supervisors for this work. The writing of the manuscript was completed by Drs. Jörg Bohlmann and Jonathan Gershenzon and the author.

INTRODUCTION

Conifers produce extensive terpenoid-based resins that have long been studied for their industrial importance and role in defense against herbivores and pathogens (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001a). Composed of approximately equimolar amounts of monoterpenes (10 carbon atoms) and diterpenes (20 carbon atoms), conifer resin also contains a smaller proportion of sesquiterpenes (15 carbon atoms) (Figure 2.1). The monoterpenes and sesquiterpenes constitute the volatile turpentine fraction of conifer oleoresin, while the diterpene resin acids form the rosin. Conifers have specialized anatomical structures for accumulation of resin terpenes, which can be as simple as the resin blisters found in species of true fir (*Abies*), or more complex such as the resin-filled canals of spruce (*Picea*) and pine (*Pinus*) that are interconnected in a three-dimensional reticulate system (Bannan, 1936; Fahn et al., 1979).

The study of plant terpenoid biosynthesis has made rapid progress in recent years (McGarvey and Croteau, 1995; Chappell, 1995; Gershenzon and Kreis, 1999; Bohlmann et al., 2000a) (Figure 2.2). Two pathways exist for the formation of the five-carbon biosynthetic building block, isopentenyl diphosphate (IDP). The mevalonate pathway is found in the cytosol / endoplasmic reticulum and the 2-C-methyl erythritol-4-phosphate (MEP) pathway, which proceeds via 1-deoxyxylulose-5-phosphate, occurs in plastids (Eisenreich et al., 1998; Lichtenthaler, 1999). Condensation of IDP and its isomer, dimethyallyl diphosphate (DMADP), catalyzed by class-specific prenyltransferases (PTs) supplies the three central intermediates of the isoprenoid pathway, geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP) (Alonso and Croteau, 1993). The basic terpene skeletons are then formed from GDP, FDP or GGDP by catalysis of terpene synthases (TPS) resulting in monoterpenes (10 carbon atoms), sesquiterpenes (15 carbon atoms) and diterpenes (20 carbon atoms), respectively (Bohlmann et al., 1998b; Davis and Croteau, 2000). These enzymes function through the divalent metal ion-assisted generation of carbocation intermediates from the prenyl diphosphate precursor and give rise to the hundreds of cyclic and acyclic parent skeletons typical of plant terpenoids. Many TPS yield only one or a few closely related products, while some TPS form complex product mixtures (Bohlmann et al., 1998b; Steele et al., 1998a).

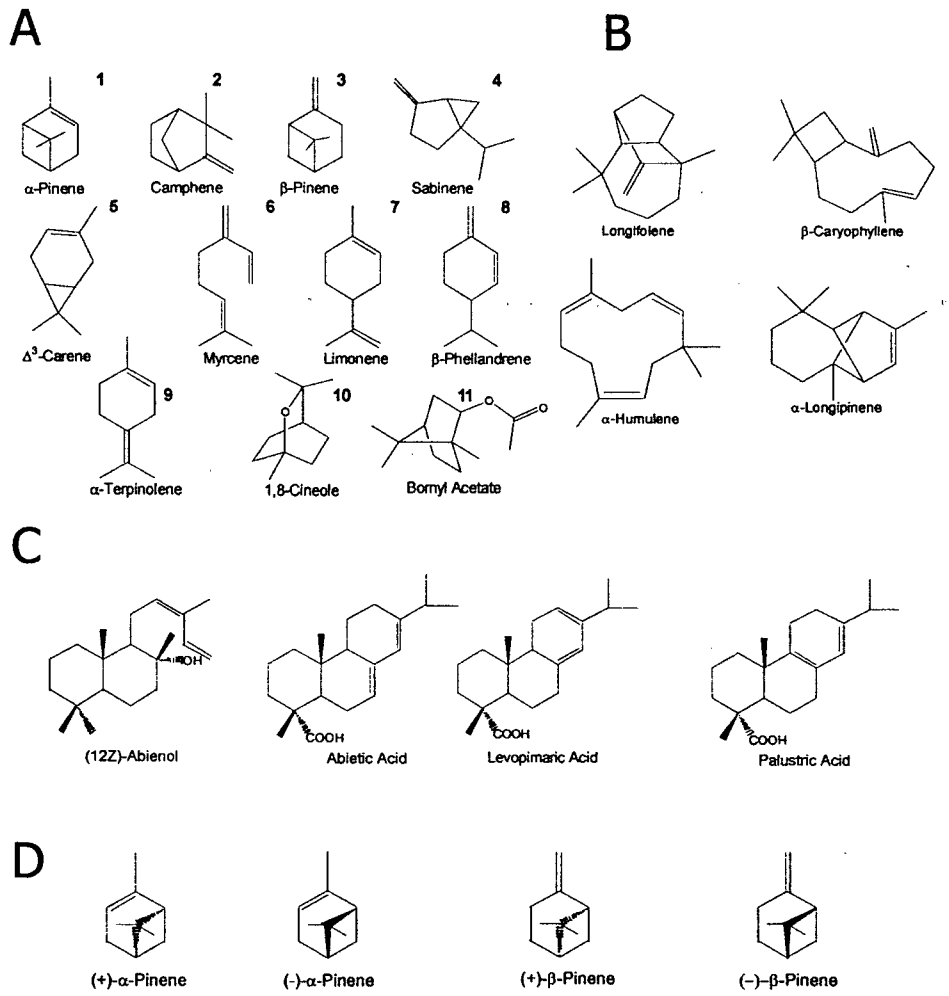


Figure 2.1: Representative structures of terpenoids of Norway spruce.

A and D, monoterpenes (10 carbon atoms). B, sesquiterpenes (15 carbon atoms). C, diterpene resin acids (20 carbon atoms). Monoterpenes are numbered corresponding to peak numbers in Figure 9.

Most of the research on the biosynthesis of conifer terpenoids to date has focused on the TPS enzymes and their genetic regulation in *Abies grandis*, grand fir, (Lewinsohn et al., 1991b; Stofer Vogel et al., 1996; Steele et al., 1998a; Steele et al., 1998b; Bohlmann et al., 1998b; Bohlmann et al., 1998a; Bohlmann and Croteau, 1999; Bohlmann et al., 1999; Bohlmann et al., 1997). These studies revealed a wound-induced resin response in stem tissues based on up-regulation of the *TPS* genes and enzymes. However, the possible role of prenyltransferases for regulation of induced resin formation has not been as thoroughly studied (Tholl et al., 2001). In addition, very little is known about the biochemical processes of induced terpenoid formation in other conifer species.

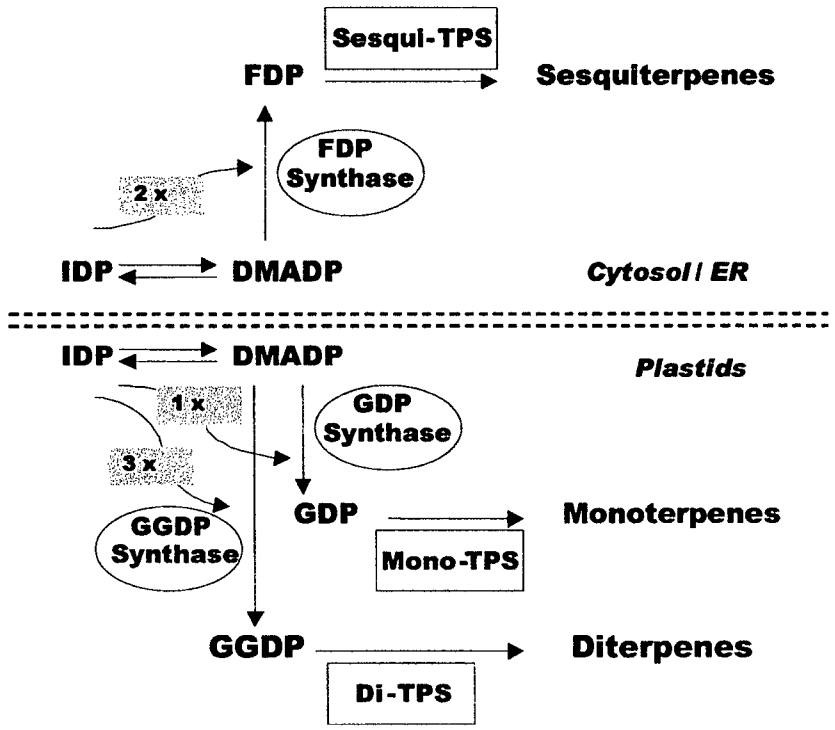


Figure 2.2: Scheme of the pathways of terpenoid biosynthesis in conifers.

The five-carbon precursors, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), are formed via two pathways, the mevalonate pathway in the cytosol / endoplasmic reticulum and the 2-C-methylerythritol-4-phosphate pathway (via 1-deoxyxylulose-5-phosphate) in plastids. Prenyltransferases, catalyze (1'-4) head-to-tail condensations of DMADP with one, two or three molecules of IDP to form geranyl diphosphate (GDP; GDP synthase), farnesyl diphophate (FDP; FDP synthase) and geranylgeranyl diphosphate (GGDP; GGDP synthase), respectively. Terpene synthases (cyclases) (TPS) of three classes, monoterpene synthases (mono-TPS), sesquiterpene synthases (sesqui-TPS), and diterpene synthases (di-TPS), convert the three prenyl diphosphate intermediates into the hundreds of cyclic and acyclic terpenoids characteristic of conifers. For intermediates in these pathways, OPP denotes a diphosphate moiety.

The genus *Picea* includes some of the economically most important species of forest trees. In *Picea*, stem resin accumulates constitutively in axial resin canals in the cortex and in axial traumatic resin ducts (TDs), which appear within the developing xylem after mechanical wounding, insect feeding or fungal elicitation (Figure 2.3). Recent microscopic work with Norway spruce (*Picea abies*) has described details of the TD formation (Nagy et al., 2000). The production of TDs is due to a change in the developmental program of cambial activity whereby some of the xylem mother cells initiate epithelial cells (which eventually come to

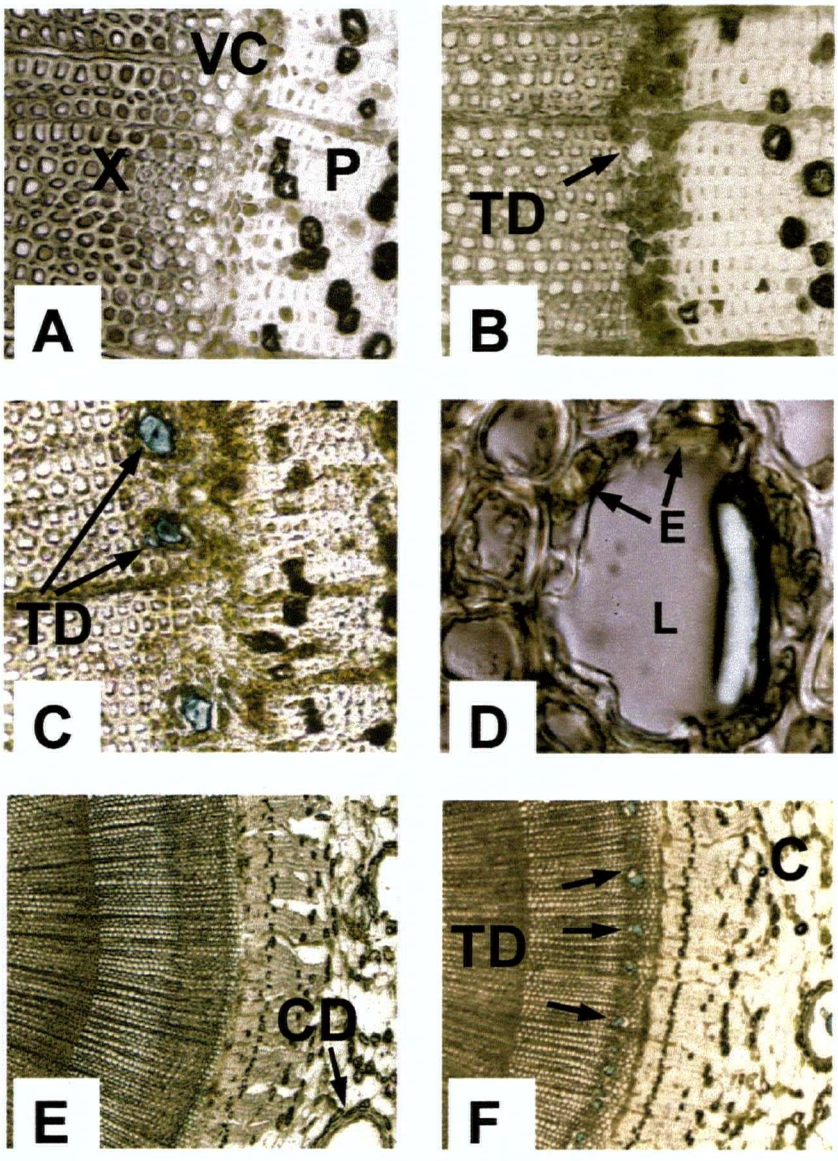


Figure 2.3: Light microscopy of induced traumatic resin duct differentiation in cross sections of Norway spruce stems.

A, E: Untreated control trees show the absence of resin ducts in the constitutive xylem (X) and phloem (P), but large constitutive resin ducts (CD) are present in the cortex. B: Nine days after 10 mM MeJA treatment, early stages of induced development of traumatic resin ducts (TD) can be observed in the xylem next to the vascular cambium. C: 25 Days after MeJA treatment, accumulation of resin in the lumen of the fully differentiated TD is visualized by staining with copper acetate. D: Two months after treatment, lumen (L) of TD with resin droplet (blue) and remainder of epithelial resin duct cells (E). F: Two months after treatment showing a ring of fully formed traumatic ducts in the newly developed xylem.

surround the TD lumen) in lieu of tracheids. However, it is not known whether duct formation is associated with *de novo* biosynthesis of resin terpenoids in the xylem, or if preformed resin stored under pressure in constitutive resin canals in the bark tissue is mobilized to these new sites for resin accumulation. *De novo* formation could result in an increase in total resin quantity and a change in resin composition.

Previous studies of the anatomy and resin chemistry of TDs in spruce involved mechanical wounding or fungal inoculation of trees as a means to induce TD formation (Nagy

et al., 2000; Tomlin et al., 1998; Tomlin et al., 2000; Franceschi et al., 2000; Alfaro, 1995). In this study, I developed a non-invasive procedure for TD induction in Norway spruce based on topical application of methyl jasmonate (MeJA). This treatment enabled a detailed chemical and biochemical analysis of the traumatic resin response.

RESULTS

Induced Formation of Traumatic Resin Ducts

Formation of traumatic resin ducts in spruce is elicited by stem boring insects and microbial pathogens as a defense response that can also be induced by mechanical wounding or by wounding and fungal inoculation of trees (Nagy et al., 2000; Tomlin et al., 1998; Tomlin et al., 2000; Franceschi et al., 2000; Alfaro, 1995). Because wounding of trees can cause massive bleeding and volatilization of oleoresin and disruption of the tissues that are possibly involved in de novo resin formation, it was important to develop a non-invasive method for TD induction to enable a detailed chemical and biochemical analysis of the traumatic resin response. To determine if methyl jasmonate (MeJA) induces the formation of traumatic resin ducts, two-year old Norway spruce saplings were sprayed with MeJA in aqueous solution and stem samples were examined by light microscopy over a period of two months following treatment. Copper acetate staining was used to visualize the terpenes accumulating in the constitutive and traumatic resin ducts (Figure 2.3).

In unsprayed saplings, axial resin ducts were largely restricted to the bark (all tissues outside of the cambium, i.e. phloem, cortex and periderm). However, after MeJA treatment, striking morphological changes became apparent as early as 6 - 9 days after application. As seen in Figure 2.3B, some new xylem cells immediately adjacent to the cambium had denser cytoplasm and thinner walls than surrounding xylem cells and appeared to constitute the epithelial cells of nascent TDs which surround the terpene-rich lumen. Within 15 days after treatment, lumens of the developing ducts were clearly discernible in a ring within the youngest portion of the xylem. Starting at this time, the lumen began filling with resin, presumed to be secreted from the epithelial cells, which were still visible at day 25 (Figure 2.3C). Two months after induction, the lumen had enlarged further and the epithelial cells had disappeared or diminished in size considerably (Figure 2.3D and 2.3F). During the same period, resin ducts in the bark were not visibly affected.

A range of concentrations of MeJA (1 mM - 100 mM) applied as a surface spray was shown to induce TD formation in spruce stems. Response at 10 mM was greater than that at 1 mM, but at 100 mM MeJA xylem development was minimal following TD initiation and several of the treated saplings shed needles and suffered severely reduced growth. Thus, 10 mM was routinely employed in further experiments. However, the MeJA concentration that is effective in the responding tissues is probably much lower than that of the applied surface spray, because this elicitor is unlikely to penetrate the thick cortex of spruce stems. This anatomical feature precludes any direct comparison of effective MeJA concentration to that

previously used in studies of herbaceous angiosperms or plant cell suspension cultures. A full TD response was also induced by much lower concentrations of MeJA (100 µM to 500 µM MeJA) when 0.1 % (v/v) Tween 20 was added to the spray solution (data not shown).

Table 2.1: Monoterpene composition of constitutive and induced* resin in wood and bark of Norway spruce.

Monoterpene	Control Bark	MeJA Bark	Control Wood	MeJA Wood
	µg g^{-1} dry weight			
Santene	19.2	19.0	nd	nd
Tricyclene	14.9	21.4	6.0	5.8
α-Pinene	1609.3	2744.8	212.6	522.6
Camphene	68.0	127.7	15.9	22.0
β-Pinene	4586.3	6388.1	225.6	836.8
Sabinene	22.5	34.0	1.7	7.0
3-Carene	109.8	123.2	nd	4.3
α-Phellandrene	11.8	15.3	nd	nd
Myrcene	240.8	337.8	15.7	56.1
Limonene	100.5	463.1	10.0	35.6
β-Phellandrene	623.5	845.9	38.3	142.6
1,8-Cineole	nd	31.1	3.4	1.8
α-Terpinolene	31.5	41.0	1.5	6.4
α-Fenchone	31.6	44.9	3.1	2.1
Bornyl acetate	37.1	62.3	0.8	6.6
Other	37.5	54.4	1.4	1.5
Total	7544.3	11354.0	536.0	1651.2

*** Trees were induced with 10 mM MeJA**

Induced Accumulation of Monoterpenes and Diterpenes

The initiation of traumatic resin duct formation in the developing xylem by MeJA suggested that increased accumulation of resin terpenoids would also be observed. To test this possibility, the effect of MeJA on resin terpenoid composition was evaluated in both wood and in bark. Saplings were treated with 1 mM, 10 mM, or 100 mM MeJA as above and the saplings were harvested for resin analysis two months later when TDs were fully developed.

The three MeJA treatments significantly increased the total accumulation of monoterpenes (Table 2.1) and diterpenes (Table 2.3) within the wood tissue of sapling stems (Figure 2.4), but sesquiterpene (Table 2.2) concentrations were unchanged. Monoterpenes showed a five-fold increase at 1 mM MeJA, a 12-fold increase at 10 mM and a 7-fold increase

at 100 mM compared to control saplings. Diterpene accumulations in the wood reflected the same trend with an 11-fold increase at 1 mM, a 38-fold increase at 10 mM and a 20-fold increase at 100 mM. The lower accumulation levels in the saplings treated with 100 mM MeJA as compared to 10 mM MeJA may reflect the negative effect this high concentration had on growth and development as described above. In bark tissue, there was only a minor relative increase in monoterpene accumulation (Figure 2.4C, Table 2.1), and no consistent change in diterpene levels (Figure 2.4D, Table 2.3). It should be mentioned that clonal trees of Norway spruce with low constitutive amounts of monoterpenes and diterpenes in the xylem (clone 1015-903, Figure 2.4) revealed a stronger relative induction than clonal trees with higher constitutive amounts of monoterpenes and diterpenes (clone 3166-728). However, the absolute amounts of induced monoterpenes and diterpenes were similar in these clones.

Table 2.2: Sesquiterpene composition of constitutive and induced* resin in wood and bark of Norway spruce.

Sesquiterpene	Control Bark	MeJA Bark	Control Wood	MeJA Wood
		$\mu g\ g^{-1}$ dry weight		
Longipinene	14.8	25.3	0.9	0.4
Longifolene	38.4	52.5	2.5	1.2
α-Cedrene	14.4	17.5	1.2	0.0
β-Caryophyllene	134.1	189.5	1.3	5.5
Z-β-Farnesene	23.2	23.6	0.7	1.5
α-Humulene	25.9	41.5	0.5	1.5
Z,E-α-Farnesene	12.6	17.7	0.3	0.8
E-β-Farnesene	5.7	8.0	nd	nd
Z-α-Bisabolene	3.9	3.7	2.2	3.4
E-α-Bisabolene	4.9	5.8	1.3	0.9
Other	20.0	25.5	4.5	7.0
Total	297.9	410.6	15.4	22.2

*** Trees were induced with 10 mM MeJA**

Time Courses of Induced Monoterpenoid and Diterpenoid Accumulation

The temporal pattern of monoterpenoid, sesquiterpenoid and diterpenoid accumulation in wood and bark tissue was analyzed over a five-week period following treatment with 10 mM MeJA (Figure 2.5). There was a significant increase in the accumulation of both monoterpenoids and diterpenoids in the wood compared to untreated controls starting 10 to 15 days after treatment (Figure 2.5). Monoterpene concentrations peaked (1.7

mg g^{-1} tissue dry wt) at 18 days post-treatment (Figure 2.5A) and then fell slightly over the remainder of the 35 day time course, although they remained greater than those seen in the controls. A similar pattern was seen for wood diterpenes, with the first significant increase over controls becoming evident at day 15 (Figure 2.5B). The maximum diterpene concentration was observed at day 25 (4 mg g^{-1} tissue dry weight) slightly later than the peak for monoterpene accumulation. Thus, unlike other JA-induced plant defense responses, the increase in resin terpenoids after MeJA treatment persists over an extended period, consistent with the persistence of the TDs.

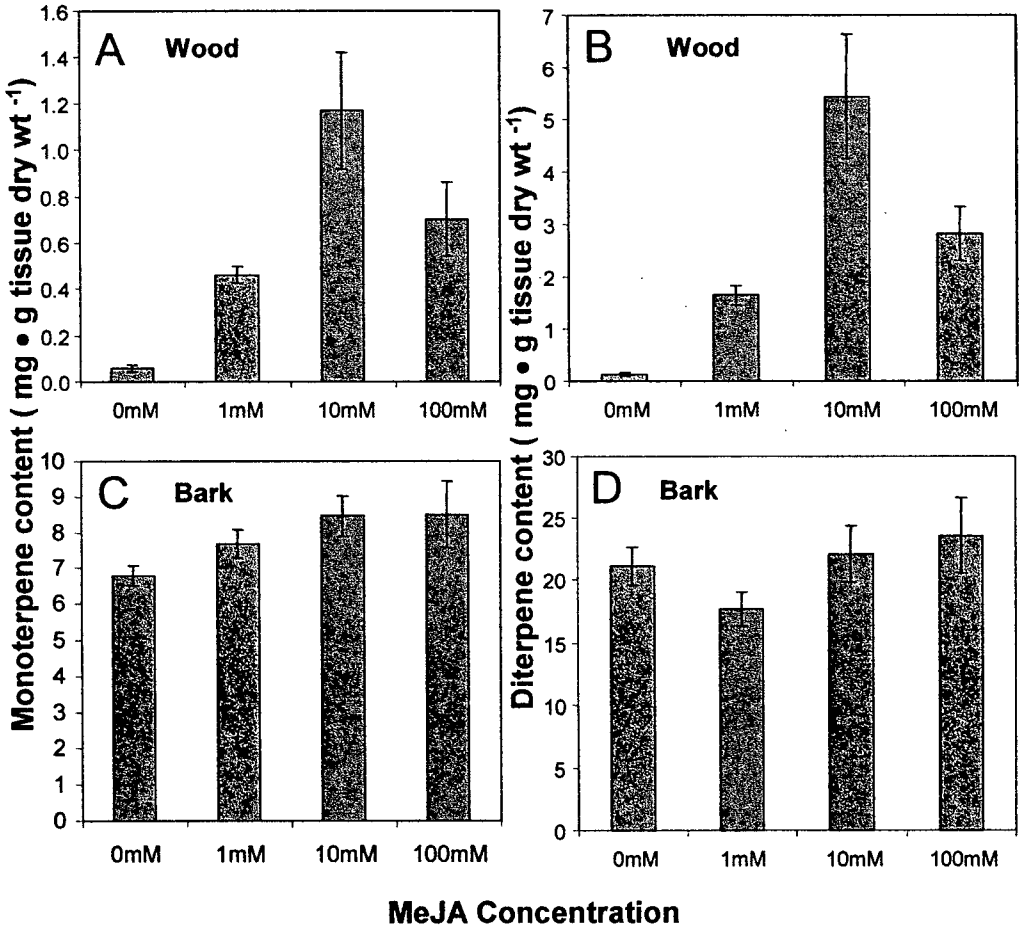


Figure 2.4: Tissue specific and dose-dependent changes in monoterpene and diterpene accumulation in wood and bark after treatment of trees with MeJA.

A: Monoterpenoids, wood. B: Monoterpenoids, bark. C: Diterpenoids, wood. D: Diterpenoids, bark. Each concentration was tested on four trees. Analysis was performed in duplicate and results are presented as the mean with standard error. Wood denotes the entire xylem tissue of a stem section. Bark denotes all tissues outside the vascular cambium, including phloem, cortex and periderm. Lower concentrations of MeJA of 100 µM to 500 µM induced chemical changes of wood

resin terpenoids similar to those induced with 10 mM MeJA when Tween 20 was added at 0.1 % (v/v) to the surface spray solution.

By contrast, the bark tissue, already rich in resin from constitutive ducts, exhibited much smaller relative changes in terpene concentrations over the 35 day time course after MeJA treatment. Monoterpenes reached a maximum concentration (12 mg g^{-1} tissue dry wt) at day 15 after MeJA treatment (Figure 2.5C), and decreased to approximately the same levels as the controls by day 35. Significant increases were already seen by day six, indicating that the bark responds more rapidly to MeJA treatment than the wood, possibly because the response in the bark does not require de novo differentiation of resin-producing cells as with TDs in the xylem. For bark diterpenes, MeJA treatment had no significant effects over the period of this time course (Figure 2.5D).

Table 2.3: Diterpene composition of constitutive and induced* resin in wood and bark of Norway spruce.

Diterpene	Control Bark	MeJA Bark	Control Wood	MeJA Wood
		μg g^{-1} dry weight		
Manoyl oxide	460.1	1004.0	nd	nd
Abienol	4674.3	4528.4	301.3	365.1
Pimaric acid	373.5	374.5	66.3	160.8
Sandaracopimaric acid	377.7	506.2	83.7	171.7
Dehydroabietinal	145.7	145.1	nd	nd
Isopimaric acid	1712.7	1918.2	195.4	208.2
Palustic acid	447.7	400.3	83.1	43.6
Levopimaric acid	3623.7	5046.9	443.0	2309.4
Dehydroabietic acid	703.3	970.0	72.7	47.5
Abietic acid	2837.2	2713.5	119.8	241.8
Neoabietic acid	1881.1	2127.0	74.0	181.9
Other	993.2	1042.0	66.3	160.8
Total	18230.2	20776.1	1505.6	3890.8

*** Trees were induced with 10 mM MeJA**

For the third class of resin terpenoids, the sesquiterpenoids, MeJA treatment had no effect on their accumulation in wood tissue and resulted in a weak response in the bark (less than two-fold) over the monitored time course with maximum increase in accumulation between day 10 and day 25 (Figure 2.6). The time course of induction of sequiterpenoid

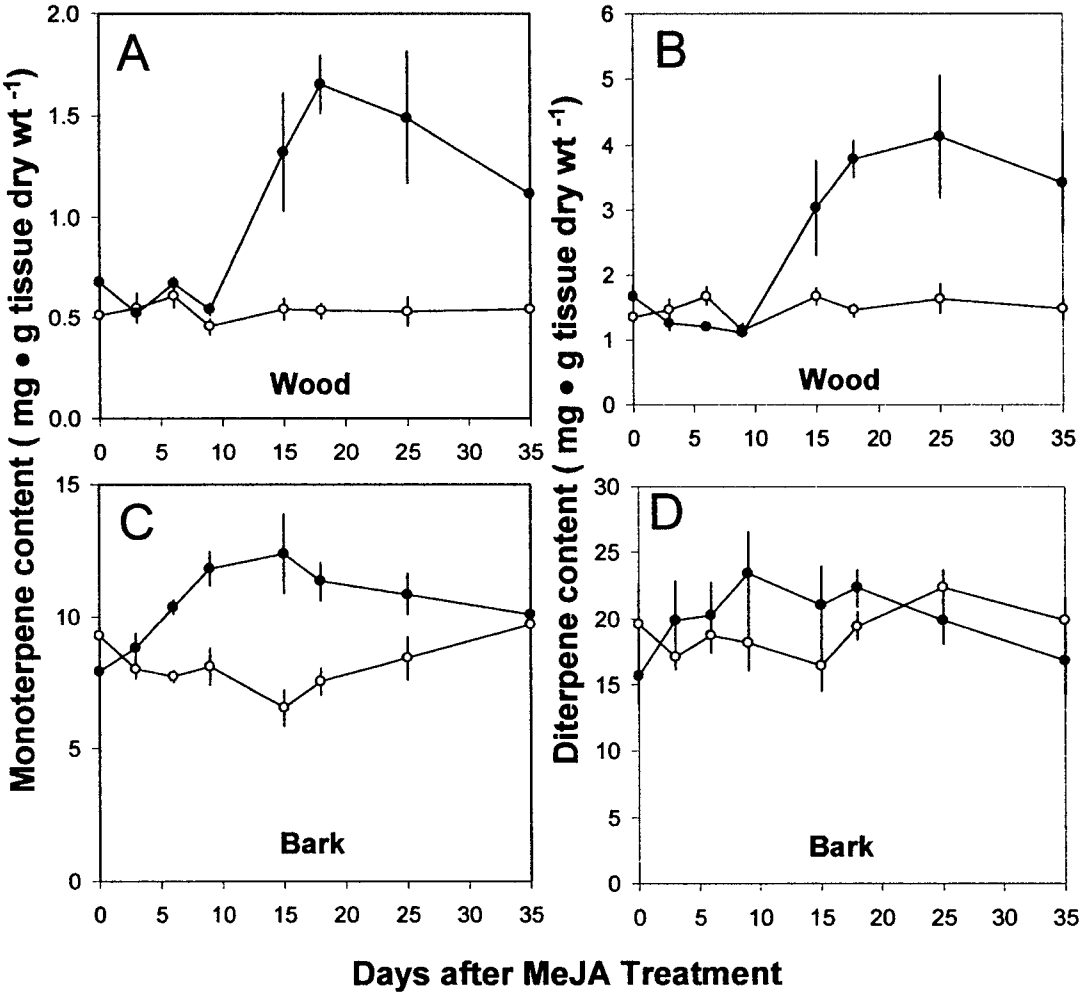


Figure 2.5: Time course of total monoterpenoid and diterpenoid content in wood and bark after treatment with MeJA.

A: Monoterpenoids, wood. B: Monoterpenoids, bark. C: Diterpenoids, wood. D: Diterpenoids, bark. Data are presented as the means with standard error of duplicate or triplicate assays of extracts from treated (•) and control (○) trees.

accumulation in the bark lacks a clear peak and appears more transient than that seen for the monoterpenoid and diterpenoid resin components, which likely explains why sesquiterpenoid induction was not observed when resin accumulation was monitored at the end of the two month experiment with the 1 mM, 10 mM and 100 mM MeJA-treated saplings.

Composition of Constitutive and Induced Resin Terpenoids

Analyses of resin from MeJA-treated and control saplings 18 days after treatment revealed a number of differences in composition. The seven most abundant monoterpenoids in both bark and wood tissues were, in order of decreasing abundance in induced tissues, β-pinene, α-pinene, β-phellandrene, limonene, myrcene, 3-carene, and camphene (Table 2.1, Figure 2.1). In the bark, most of these abundant compounds increased 1.4 to 2-fold upon MeJA treatment with a higher increase (4-fold) for limonene and a lower increase (1.13-fold) for 3-carene accumulation in induced bark tissue.

In the wood, the site of TD formation, changes in individual monoterpene concentrations were more pronounced. While α-pinene and camphene increased by only 2.5 and 1.5-fold, respectively, the concentrations of most of the remaining major monoterpenes (β-pinene, myrcene, limonene, β-phellandrene), increased by 3.7 to 4.3-fold. 3-Carene was not detectable before treatment. The two major monoterpene resin constituents in the wood, α-pinene and β-pinene, were differentially affected by MeJA. These two compounds are found in the wood in nearly equal amounts in control saplings, but the proportion of β-pinene to α-pinene increases upon MeJA treatment reaching a ratio of 2:1 at day 15. In the bark, this proportion is 3:1 regardless of treatment. Several of the oxygenated monoterpenoids and minor hydrocarbons also exhibited altered concentrations upon MeJA application including 1,8-cineole (not present in the control), α-fenchone (1.4-fold increase), and bornyl acetate (1.7-fold increase). In the wood, increases of bornyl acetate (8-fold) and α-terpinolene (4.3-fold) were found. Chiral analysis of wood extracts showed an increase in the relative amounts of (–)-α-pinene and (–)-

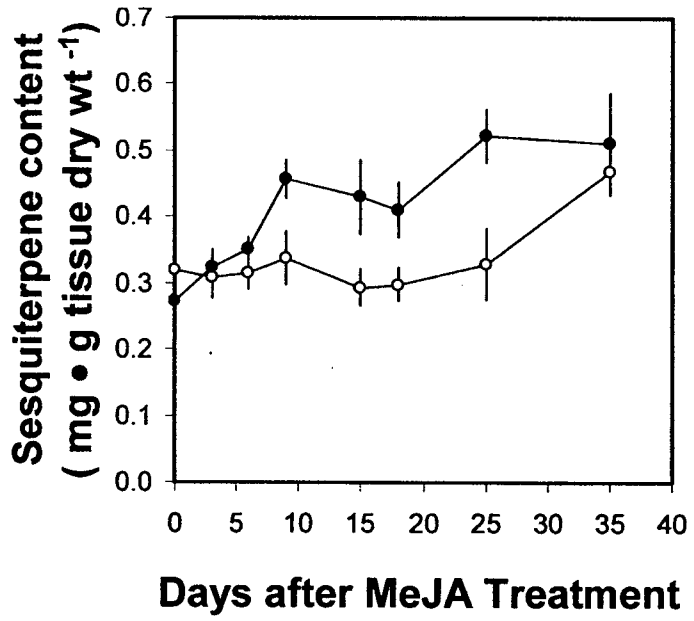


Figure 2.6: Time course of total sequiterpenoid content in bark after treatment with MeJA.

Data are presented as the means with standard error of duplicate or triplicate assays of extracts from treated (•) and control (○) trees.

ß-pinene in relation to their respective (+)-enantiomers (data not shown), indicating the existence of at least two different pinene synthases in the stem tissue of Norway spruce, as in *A. grandis* (Bohlmann et al., 1997; Bohlmann et al., 1999) and in *P. taeda* (Phillips et al., 1999). In both bark and wood, most of the individual sesquiterpenes increased by 1.4-fold following MeJA treatment (Table 2.2). However, wood extracts showed a 4.2-fold increase in ß-caryophyllene and a 2 to 3-fold increase in *Z*-ß-farnesene, α-humulene, and *E*-ß-farnesene (Figure 2.1).

While diterpene concentrations did not differ between control and MeJA treated bark extracts, there was an overall 2.6-fold induction in the wood (Table 2.3). Concentrations of levopimaric acid (Figure 2.1) showed the highest increase (5.2-fold) while neoabietic acid, pimaric acid and three unidentified peaks exhibited approximately 2.5-fold increases in treated over control saplings. Noted also in this analysis was the constant concentration of abienol, the major diterpene alcohol found in this tissue in induced and control trees. Another interesting observation is the decrease in dehydroabietic acid (1.5-fold) seen in treated versus control trees.

Effect of Induction on Prenyltransferase Activities

Accumulation of resin terpenoids in developing xylem after MeJA treatment may reflect *de novo* synthesis or mobilization of resin from sites of constitutive accumulation in the bark. To test for induced *de novo* biosynthesis, protein extracts from bark and wood of saplings were assayed in vitro for activities of prenyl transferases and terpene synthases, two principal steps in terpene biosynthesis (Figure 2.2).

Three different types of prenyltransferases were measured, GDP synthase, FDP synthase and GGDP synthase, which provide the precursors for all monoterpene, sesquiterpene and diterpene formation, respectively. All three activities were detectable in bark extracts but were unaffected by treatment with 10 mM MeJA over the time course of 35 days (Figure 2.7). In the xylem, the activities of GDP synthase and FDP synthase also did not differ between MeJA treated and control trees (Figures 2.7A and 2.7B). However, GGDP synthase activity, which was barely detectable in constitutive wood extracts, was strongly induced by treatment with MeJA starting three days post treatment and reaching a maximum specific activity (10 pmol µg protein^{-1} hour^{-1}) at day 15 (Figure 2.7C). At days 18 and 25, the activity declined, but remained at higher levels relative to the control. The induction of GGDP synthase in the xylem reflects the tissue specific response to MeJA previously observed at the microscopic structural level and by terpenoid analysis.

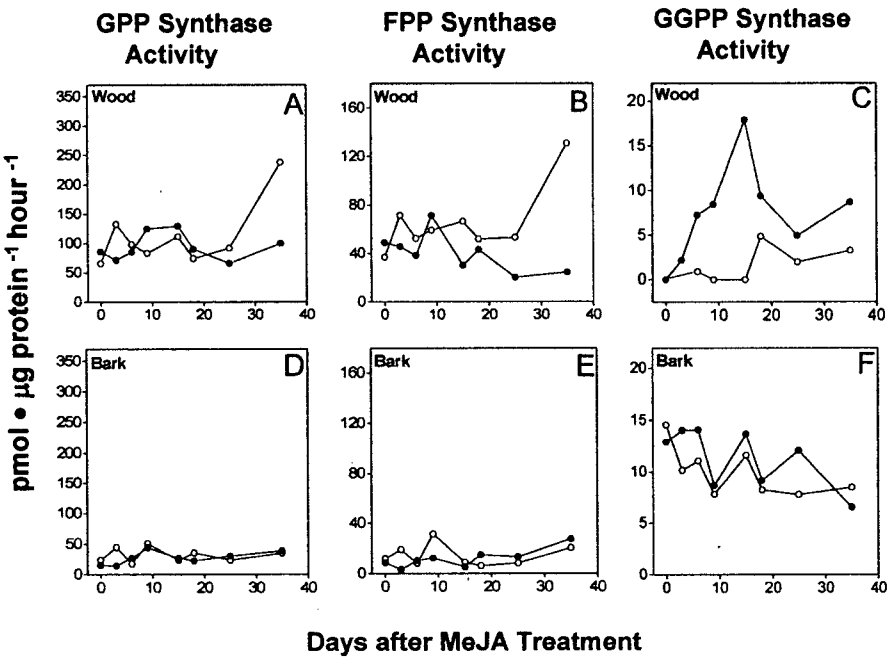


Figure 2.7: Time course of prenyltransferase activity in wood and bark after treatment with MeJA.

A: GDP synthase activity, wood. B: FPP synthase activity, wood. C: GGDP synthase activity, wood. D: GDP synthase activity, bark. E: FPP synthase activity, bark. F: GGDP synthase activity, bark. Values are the means of duplicate or triplicate assays of extracts from treated (•) and control (○) trees. A rapid increase in enzyme activity was found only for GGDP synthase in induced wood samples. The increase in specific activities of GDP synthase and FPP synthase at day 35 reflects a decrease of total protein in these samples. Ranges of duplicate assays were normally 1-25 % of the mean but were 45-60 % of the mean in control day 35 in A and B and in MeJA days 25 and 35 in B.

Effect of Induction on Terpene Synthase Activities

The activity of two classes of terpene synthases, monoterpene synthases and diterpene synthases, were measured. In unsprayed saplings, these activities were at best barely detectable in wood extracts, but revealed strong activity in the bark (Figure 2.8). Treatment with MeJA did not affect the activity of bark monoterpene synthases over a time course of 35 days. In contrast, monoterpene synthase activity rose rapidly in wood with increased specific activity detectable three days after treatment with 10 mM MeJA. The peak at nine days (28 pmol µg protein^{-1} hour^{-1}) (Figure 2.8A) corresponded to a 28-fold increase in activity over the control, and was five-fold higher than the specific monoterpene synthase activities in bark, the site of constitutive resin formation and resin accumulation. By days 18 and 25, the activity in induced wood had dropped but remained approximately 12 times that of the control until day 35 when similar levels of activity were found in treated and control trees.

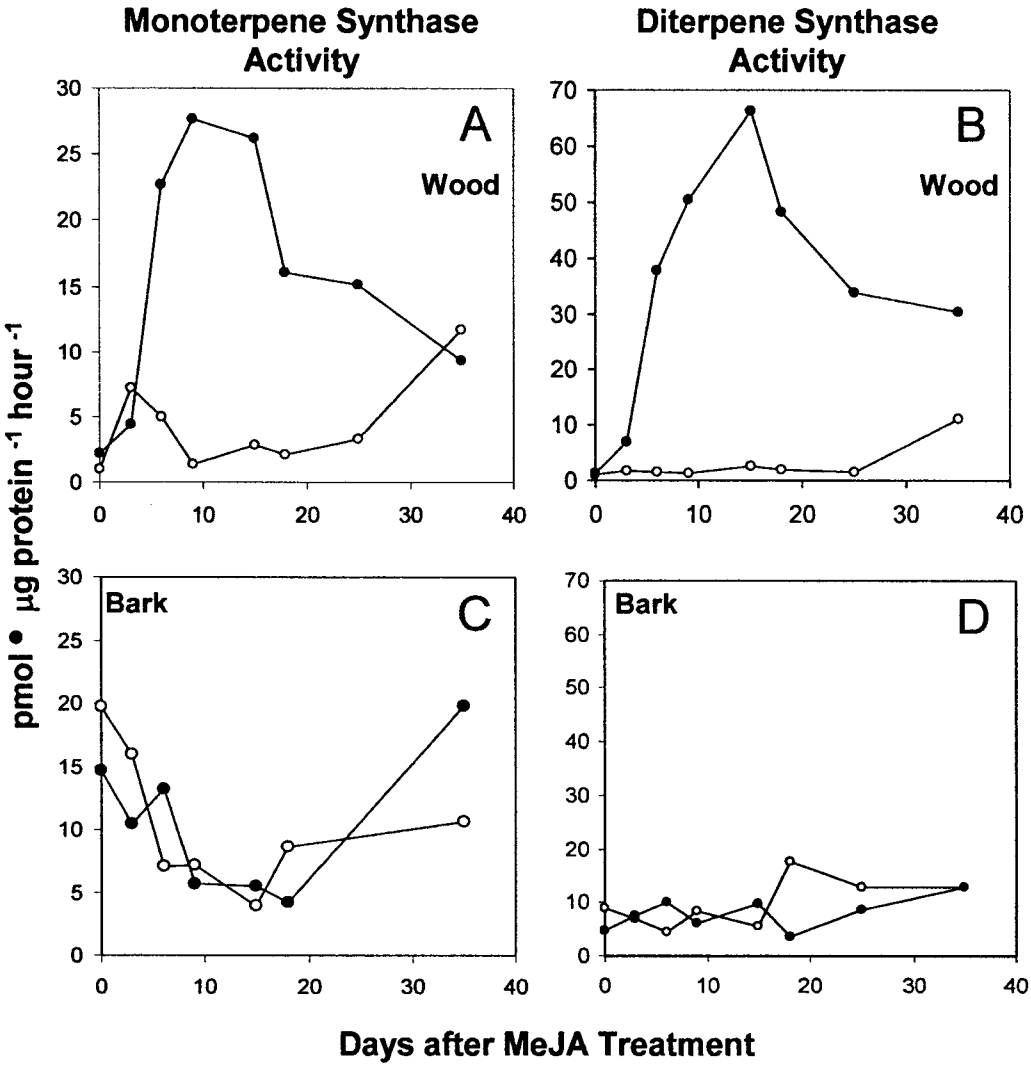


Figure 2.8: Time course of monoterpenoid synthase activity and diterpenoid synthase activity in wood and bark after treatment with MeJA.

A: Monoterpenoid synthase activity, wood. B: Monoterpenoid synthase activity, bark. C: Diterpenoid synthase activity, wood. D: Diterpenoid synthase activity, bark. Data are the means of duplicate or triplicate assays of extracts from treated (•) and control (○) trees.

This transient increase of enzyme activity following MeJA application was also observed for diterpene synthases in wood which exhibited the same rapid induction detectable three days post treatment (Figure 2.8B). Induced diterpene synthase activity reached a maximum at day 15 with a 22-fold increase (66 pmol µg protein^{-1} hour^{-1}) in activity over the control. This activity exceeds the specific activity of diterpene synthases from bark tissue at the same time point by more than 10-fold. Following the maximum at 15 days, diterpene synthase activity remained at an elevated level over the entire 35 day time course.

This transient induction of enzyme activities in terpene-accumulating wood tissue coupled with the appearance of TDs is highly suggestive of the occurrence of MeJA-induced *de novo* synthesis.

Products of the Induced Terpene Synthase Activities

Product analysis of the monoterpene synthase assays of induced wood tissue by Radio-GC (Figure 2.9) revealed the presence of the six major monoterpene components that are all found in xylem resin: α-pinene (peak 1), camphene (peak 2), β-pinene (peak 3), myrcene (peak 5) limonene (peak 6), and β-phellandrene (peak 7). The individual components were found in approximately the same ratios as they occur in the xylem resin (Table 1). Additionally, an increase of β-pinene relative to α-pinene paralleled that observed in the resin extracts from induced xylem.

Products of the diterpene synthase assays analyzed by Radio-GC and GC-MS revealed the presence of a single peak (Figure 2.10), identified as abietadiene by comparing the mass spectrum with that of an authentic standard. The result is consistent with the activity of the monospecific abietadiene-producing *A. grandis* diterpene synthase described previously (LaFever et al., 1994). Peters et al. (2000) recently found that under modified conditions the cloned *A. grandis* abietadiene synthase actually produces abietadiene, levopimaradiene, and

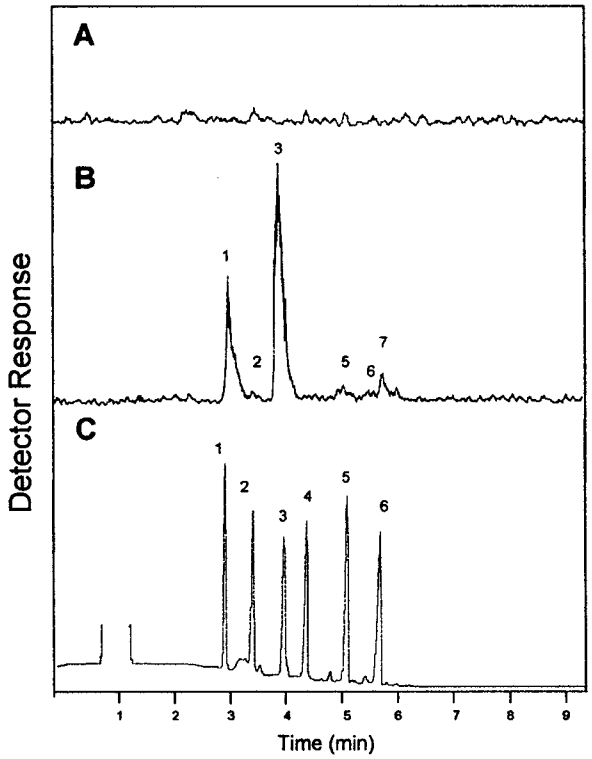


Figure 2.9: Analysis of products formed in vitro by constitutive and induced monoterpenoid synthase activity from wood tissue.

A: Radio-GC traces for monoterpenoid synthase assay products from wood of control saplings. B: Radio-GC trace for monoterpenoid synthase assay products from wood of saplings treated with 10 mM MeJA. C: TCD trace for monoterpene standards. Standard for β-phellandrene not shown. Peak 1, α-pinene; 2, camphene; 3, β-pinene; 4, 3-carene; 5, myrcene; 6, limonene; 7, β-phellandrene.

neoabietadiene in nearly equal ratios as well as three other minor products. The Norway spruce enzyme might also produce additional products under similar conditions, which will be tested in future work once the corresponding spruce diterpene synthase gene has been cloned.

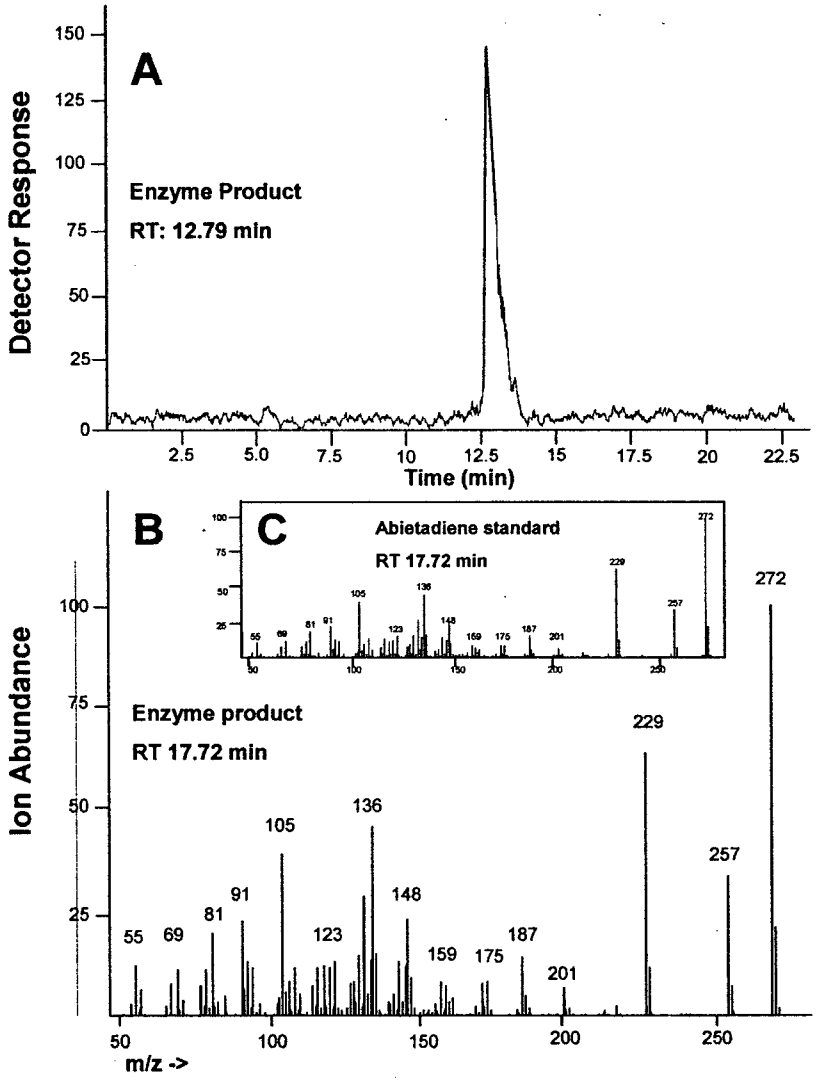


Figure 2.10: Analysis of products formed in vitro by induced diterpenoid synthase activity from wood tissue.

A: Radio-GC trace for diterpene synthase assay products. B: GC-MS fragmentation pattern for major diterpene synthase assay product. C: GC-MS fragmentation pattern for authentic abietadiene standard.

DISCUSSION

Conifers include some of the longest living of all organisms, which, during their extended lifetimes, must survive countless challenges by a diverse array of herbivore and pathogen species (Seybold et al., 2000). Constitutive and inducible resin terpenoids are characteristic defense compounds of many conifer genera of the pine family (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001a). Species of *Picea*, like other conifers, produce copious amounts of resin terpenoids in the bark as a constitutive outer defense barrier, but do not accumulate significant amounts of resin in the constitutive wood and developing xylem in contrast to some other conifers. However, induced resin terpenoids accumulate in newly initiated axial resin ducts (TDs) in the developing xylem of *Picea* (spruce) species (Nagy et al., 2000). Induced terpenoid defense responses in *Picea* are activated by stem boring insects, such as coniferophagous bark beetles (Scolytidae) and the white pine weevil (*Pissodes strobi*), which are among the most destructive insect pests of conifer forests worldwide, as well as by insect-associated fungi, such as the blue stain fungus, *Ceratocystis polonica* (Alfaro, 1995; Franceschi et al., 2000; Tomlin et al., 1998). *De novo* formation of resin ducts in the developing xylem has been described as a possible resistance mechanism against insects and pathogens in white spruce (*P. glauca*), Sitka spruce (*P. sitchensis*) and Norway spruce (*P. abies*) (Alfaro, 1995; Franceschi et al., 2000; Tomlin et al., 1998).

In an effort to characterize the induced changes in terpene chemistry as well as changes in terpenoid biosynthetic activities during the development of traumatic resinosis in Norway spruce, I first tested the effect of MeJA to establish a non-destructive method of inducing the resin defense response. While the effect of exogenous MeJA and the role of endogenous octadecanoids have been well characterized in herbaceous angiosperm species (Farmer and Ryan, 1990; Creelman and Mullet, 1997; Baldwin, 1999), the effect of jasmonates has been demonstrated in only a few conifers, mainly in cell cultures. MeJA induces the transformation of *E*-α-bisabolene into todomatuic acid, a precursor of juvabione type insect juvenile hormone analogues, in suspension culture of *A. grandis* (Bohlmann et al., 1998a). In cell cultures of *Taxus*, MeJA induces production of paclitaxel (taxol) and other taxoids with the concomitant increase in GGDP synthase and acetyl-CoA:taxadienol-O-acetyl transferase enzyme activities, both of which are involved in taxol biosynthesis (Ketchum et al., 1999; Hefner et al., 1998; Yukimune et al., 1996). Spruce cell cultures treated with MeJA show transcript accumulations of chalcone synthase and a 14-3-3 protein (Lapointe et al., 2001). In Douglas fir, MeJA was found to induce the expression of two low-molecular weight heat shock proteins in dormant seeds (Kaukinen et al., 1996). Other work with jasmonates in conifers has

focused on jasmonate-induced effects on interactions with fungi. Jasmonates have been implicated in both symbiotic and antagonistic relationships of seedlings with fungi. Apparently, MeJA application assists ectomycorrhizal colonization of *P. abies* roots (Regvar et al., 1997). MeJA application to *P. abies* seedlings also enhances their survival rate when they were challenged by *Pythium ultimun* (Kozlowski et al., 1999).

Compared to the numerous effects of jasmonates reported for angiosperms and the effects previously described for conifers, the induction of terpenoid defenses in Norway spruce is probably among the most complex responses. Similar to a stem-boring insect attack, treatment with MeJA completely alters the developmental program of xylem mother cells within the vascular cambium, switching differentiation from tracheid formation to formation of traumatic resin duct cells, two cell types with enormous contrasts in structure and function (Esau, 1977). In addition, induced *de novo* differentiation of resin ducts in the xylem is also associated with resin terpenoid accumulation and *de novo* resin biosynthesis. Interestingly, this effect is strictly tissue-specific because existing resin canals in the phloem and cortex are not affected in the young trees of this study. Unlike older trees, young Norway spruce trees do not display significant radial resin duct formation in the phloem. Because the phloem of older trees is the primary side of attack by bark beetles, the possibility that radial ducts in the phloem of older trees could also be activated by MeJA will be tested in future work.

Differential effects of MeJA in Norway spruce stem tissues are further observed at the level of terpenoid biosynthesis and terpenoid accumulation. Induced accumulation of resin terpenoids is most apparent in the wood and barely detectable in the bark, where high constitutive resin levels already exist. Among the resin terpenoids, levels of the two major classes of resin components, monoterpenes and diterpenes, are strongly increased while the sesquiterpenes, the least abundant class of resin terpenoids, are only weakly effected in the induced xylem. The formation of traumatic resin occurs on a much longer time scale than that of most other induced defenses. However, it has been demonstrated that the site of TD development not only corresponds to the area in which bark beetle or weevil larvae could potentially develop, but that the time of TD development is synchronized with the time during which these insects would be emerging from their eggs thereby flooding the brood with toxic and immobilizing resin constituents (Alfaro, 1995).

In measuring terpenoid biosynthetic enzymes, I observed a dramatic response in both monoterpenoid and diterpenoid pathway activities in wood tissue, whereas sesquiterpene biosynthesis was only weakly and transiently affected in the bark. Of the three prenyltransferases of resin terpenoid biosynthesis (Figure 2.2) only GGDP synthase was induced, but not GDP synthase and FDP synthase. The lack of FDP synthase activation reflects

the weak effect of MeJA on sesquiterpene accumulation associated with TD development. Similarly, sesquiterpene synthases activities in these tissues are below detection limits (data not shown). However, despite a strong induction of monoterpene accumulation and monoterpene synthase induction in developing xylem, GDP synthase is not induced, suggesting that GDP synthase activity is not limiting for induced monoterpene formation and accumulation. Similar results were obtained from *A. grandis* saplings which showed no increase in GDP synthase activity over a 20 day period after mechanical wounding of the stem (Tholl et al., 2001), even though monoterpene synthase enzyme activity and gene expression increased strongly during this same period (Steele et al., 1998b; Bohlmann et al., 1997). In contrast, GGDP synthase is active at a very low level in the constitutive xylem and induced accumulation of diterpene resin acids is preceded by coordinately increased activities of both GGDP synthase and diterpene synthases in xylem of MeJA treated spruce trees. These results suggest that unlike monoterpene accumulation, the induction of diterpenes is controlled not only by enzyme activity at the level of terpene synthases but also at the level of the prenyltransferase that yields the 20-carbon precursor. The differential enzymatic regulation of the three classes of resin terpenoids, monoterpenoids, sesquiterpenoids and diterpenoids, has important implications for attempts to alter resin composition in spruce through genetic engineering. For instance, increased monoterpenoid formation may not require altering expression of GDP synthase, while altering GGDP synthase together with diterpene synthase may be necessary to alter diterpene formation.

This is the first study that demonstrates the constitutive and induced activity of terpenoid synthases at a tissue-specific level in a gymnosperm system. Enzymatic and molecular studies of terpenoids at the tissue-specific level have previously been reported only in some angiosperm systems, the glandular trichomes of *Mentha* (McConkey et al., 2000), the trichomes of *Nicotiana* (Guo and Wagner, 1995), or the scent-producing floral tissues of *Clarkia* (Dudareva et al., 1996). The results described here justify future analysis of MeJA-induced TD differentiation and regulation of terpenoid biosynthesis in spruce at the cellular and subcelluar level to elucidate the control mechanisms responsible for such localized expression.

This study did not intend to investigate the role of jasmonates as endogenous signal molecules in conifers. Treatment of trees with MeJA was tested and developed solely as a procedure to study in detail the chemical, biochemical and anatomical processes of traumatic resin defense in spruce. Nevertheless, our results could imply that octadecanoids are involved in insect-induced defense in conifers, a possibility which will be tested in future work. Since this is the first demonstration of traumatic resin duct formation by a signal molecule, the

effect of other signal molecules to elucidate the endogenous signal cascade active in xylem specific resinosis and TD development in response to insects, pathogens or wounding need to be evaluated. The non-invasive and dose-dependent activation of defenses in spruce by MeJA treatment provides new opportunities to evaluate the significance of induced responses in conifers in bioassays with insects and pathogens, as recently demonstrated for agricultural crops (Thaler, 1999). Considering restrictions on use of pesticides for insect and pathogen control in forest systems, pretreatment of trees with elicitors can be explored as a strategy for tree protection. Furthermore, non-destructive induction of traumatic resinosis in spruce provides a superb system for discovery of genes associated with resin canal differentiation in the developing xylem and genes of induced terpenoid formation and accumulation, a striking characteristic of conifers.

MATERIALS AND METHODS

Plant Materials

Norway spruce (*Picea abies* Karst) trees of clonal lines 3166-728 and 1015-903 were propagated from lateral branches of current and previous year growth at the Niedersächsische Forstliche Versuchsanstalt, Escherode, Germany. Fully regenerated, two-year old, rooted saplings were grown in 2 L pots in a 2:3 ratio of peat to universal planting mix for at least six months prior to experiments. Trees were fertilized with Osmocoat® and maintained in growth chambers under illumination with high-pressuresodium vapor lamps. The photoperiod and ambient temperature cycled from 1 h at 220 μmol/m^{-2} s^{-1} (20°C), 4 h at 440 μmol m^{-2} s^{-1} (20°C), 3 h at 660 μmol m^{-2} s^{-1} [22°C (2 h), 24°C (1h)], 7 h at 440 μmol m^{-2} s^{-1} [24°C (1 h), 22°C (2 h), 20°C (4 h)], and 1 h at 220 μmol m^{-2} s^{-1} (18°C). This was followed by 8 h of darkness (18°C). The relative humidity was maintained at 50% throughout the entire cycle. Saplings were introduced to these growth conditions four weeks prior to use in experiments to ensure a complete break of dormancy.

Substrates, Standards and Reagents

Chemical reagents were from Sigma-Aldrich or Roth. Terpene standards were from Sigma-Aldrich, Roth, Bedoukian and Helix Biotech and were of the highest purity available. All solvents were GC grade. The substrates, [1-^{14}C] IDP (54 Ci mol^{-1}), [1-^{3}H]-geranyl diphosphate (GDP) (20 Ci mol^{-1}) and all-trans-[1-^{3}H]-geranylgeranyl diphosphate (GGDP) (58 Ci mol^{-1}) were from Biotrend (Köln, Germany). [1-^{3}H]-Farnesyl diphosphate (FDP) (125 Ci mol^{-1}) was the gift of Rodney Croteau, Washington State University, Pullman. Unlabeled DMADP, GDP and FDP were from Echelon Research Laboratories Inc. (Salt Lake City, USA). Diazomethane was made fresh from Diazald® (Aldrich) by a standard procedure (Aldrich technical information bulletin number 180).

Methyl Jasmonate Treatment and Harvest of Tissues

To test dose-dependent effects of MeJA, saplings (clone 1015-903) were sprayed with 1 mM, 10 mM, or 100 mM solutions of MeJA (95% pure, Sigma-Aldrich, Steinheim, Germany) dissolved in distilled water. Time course experiments were done with saplings (clone# 3166-728) sprayed with 10 mM MeJA dissolved in distilled water. Saplings were placed in a ventilated fume hood and each tree was sprayed with 150 mL of MeJA solution over a period of 30 minutes to obtain a complete and even coating. Saplings were kept in fume hoods for 1

h after treatment to allow evaporation of excess MeJA solution prior to transferring to growth chambers. Control saplings (4 for dose-dependent experiment and 4 for each time point for the time course) were sprayed with water. Control and MeJA-treated saplings were kept in separate growth chambers. To determine dose-dependent effects of MeJA, trees were harvested after 42 days by cutting the stem above the ground and just below the upper internode and freezing the entire section in liquid nitrogen. Needles were removed and the tissue was stored at -80°C. The lowest 3 cm of the stem section was discarded. The next 3 - 4 cm of stem tissue was prepared for resin extractions. This section was cut into two pieces of equal length. The bark of these stem sections was sliced longitudinally with a razor blade, and while still frozen, was peeled away from the wood. Bark and wood were extracted separately for resin analysis. An additional 2 cm of the stem was used for light microscopy analysis of traumatic resin duct development. Saplings for each of the time points were harvested in the same manner for microscopy and resin analysis. An extra 7 cm of the harvested stem was used for enzyme preparations. For enzyme extracts, bark and wood tissues were separated as indicated and the similar tissues of four saplings for each time point were combined into one protein extract.

Light Microscopy

Samples were prepared for cryosectioning by soaking small sections (2 to 3 cm long) of the stems in a solution of 4 % formaldehyde and 100 mM K_2HPO_4 (pH 7.5) for 4 h. The samples were then washed with dH_2O and submerged in saturated (aq.) copper acetate overnight. Prior to sectioning, the samples were removed from the copper acetate solution and frozen at -20°C. Cyrosectioning of the samples took place at -20°C and each section was sliced into 18 µm cross-sections. A polyvinyl alcohol-based glue was used to attach the coverslip immediately after sectioning. After visualizing by light microscopy, (Axiophot, Zeiss GmbH, Jena, Germany), digital images were taken of the samples.

Extraction of Resin Terpenes

Extraction of terpene constituents was modified from Lewinsohn et al. (1993). All steps of this procedure were carried out in 2 mL vials (glass with a teflon-coated screw cap, Hewlett-Packard). Bark and wood tissue samples of approximately 1 cm to 1.5 cm length were submerged separately into 1.5 mL tert-butyl methyl ether (MTBE) in a 2 mL vial containing 150 µg mL^{-1} isobutylbenzene and 200 µg mL^{-1} dichlorodehydroabietic acid as internal

standards. The tissue samples were extracted over 14 h with constant shaking at room temperature. To purify extracted terpenes from other small organic acids, the etheral extract (approximately 1.5 mL) was transferred to a fresh vial and washed with 0.3 mL of 0.1 M $(NH_4)_2CO_3$ (pH 8.0). Diterpene acids were then methylated by adding 0.4 mL of the washed etheral extract to 0.16 mL methanol and 0.15 mL diazomethane solution in a separate vial, which was then capped and left at room temperature for 30 min to allow the methylation reaction to go to completion. Then, the solvent was evaporated under nitrogen leaving the residual diterpene fraction. The monoterpenes, sesquiterpenes, and diterpenes were then recombined by dissolving the methylated diterpene residue in 0.6 mL of the washed etheral extract. The extract was prepared for capillary gas chromatography (GC) or GC-mass spectroscopy (MS) analysis by filtering through a Pasteur pipette column filled with 0.3 g silica gel (Sigma 60 Å) overlaid with 0.2 g anhydrous $MgSO_4$. The column was further eluted with 1 mL of diethyl ether to release bound oxygenated terpenes and both eluants were collected in a fresh vial. Finally, the sample was evaporated to an approximate volume of 100 µL that was stored at -20°C. The dry weights of each extracted tissue were determined after drying at 70°C 20 hours in order to calculate terpene constituent concentrations on a mg g^{-1} dry weight basis. Standard errors were calculated from 8 independent extracts per treatment.

Analysis of Monoterpenes, Sesquiterpenes and Diterpenes

For monoterpene and sesquiterpene analysis, a Hewlett-Packard 6890 GC was equipped with a flame ionization detector (FID) fitted with a DB-WAX column (0.25 mm x 0.25 µm x 30 m J&W Scientific). The flow rate was 2 mL H_2 min^{-1} and the FID was operated at 300°C. One µl of extract was introduced into the injection port at 220°C and was split in either a 10:1 ratio for the bark extracts or a 5:1 ratio for the wood extracts. The GC was programmed with an initial oven temperature of 40°C (3 min hold), temperature increased at a rate of 3°C min^{-1} until 80°C, followed by 5°C min^{-1} until 180°C and then 15°C min^{-1} up to 240°C (5 min hold). GC-MS analysis was accomplished with a Hewlett-Packard 6890 GC-MSD system (70eV), using a DB-WAX column as described above. Split injections (1 µl etheral extract) were made at a ratio of 5:1 (bark extracts) or 3:1 (wood extracts) with an injector temperature of 220°C. The instrument was programmed from initial temperature of 40°C (3 min hold) and increased at a rate of 1.5°C min^{-1} until 45°C, then increasing at 3°C min^{-1} up to 80°C, 5°C min^{-1} until 180°C, followed by an additional ramp of 10°C min^{-1} up to 240°C (5 min hold). Helium was used at a constant flow of 1 mL min^{-1}. Chiral analysis of monoterpene constituents utilized the same GC-FID equipped with a Cyclodex-B (0.25 mm x 0.25 µm x 30

m, J & W Scientific). The same etheral samples were injected in a split ratio of 20:1 (220°C injection port). The oven was programmed initially at 40°C increasing at 1°C min^{-1} until 45°C, then at 5°C min^{-1} until 65°C, followed by 20°C min^{-1} until 230°C (2 min hold). All other conditions were identical to those used in analysis with the DB-Wax column mentioned above.

Analysis of diterpene constituents was performed on the same GC-FID and GC-MS instruments fitted with an HP-5 column (0.25 mm x 0.25 μm x 30 m, Hewlett Packard). Injections were 1 μl of the etheral extracts. For GC-FID analysis, the split ratios were 40:1 or 20:1 for bark and wood extracts, respectively. The injection port was operated at 250°C and the FID was maintained at 300°C. The oven was programmed from an initial temperature of 120°C to 150°C at a rate of 1°C min^{-1} followed by 5°C min^{-1} until 280°C (5min hold). GC-MS split ratios were 20:1 (bark extracts) or 10:1 (wood extracts) with an injector temperature of 220°C. The instrument was programmed from an initial temperature of 120°C and increased at a rate of 1°C min^{-1} until 150°C followed by 5°C min^{-1} up to 280°C (6-min hold). Helium was at a constant flow of 1mL min^{-1}.

GC-FID generated peaks were integrated using Hewlett-Packard Chemstation software. Terpene concentrations were calculated by comparing the integrated peak area to that of the internal standard. Isobutylbenzene was used as the internal standard for both monoterpenes and sesquiterpenes. Methylated dichlorodehydroabietic acid was employed as an internal standard to calculate diterpene concentrations. Identification of terpenes was based on comparison of retention times and mass spectra with authentic standards or with mass spectra in the Wiley or NIST libraries.

Protein Extraction

Tissue samples (bark or wood) from each of the four saplings per time point were combined into one protein preparation and extracted as previously described (Lewinsohn et al., 1991b). Using an analytical grinding mill (A10, IKA WORKS, Cincinnati, OH), the tissue was ground to a fine powder in liquid nitrogen and combined with extraction buffer (50 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) pH 6.8, 5 mM ascorbic acid, 5 mM sodium bisulfite, 5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (w/v) polyvinylpyrrolidone (10,000 MW), 4 % (w/v) polyvinylpolypyrrolidone, 4 % (w/v) Amberlite ® XAD4, 0.1 % (v/v) Tween 20) in a ratio of 1 : 10 (g tissue : ml buffer). The preparations were allowed to shake at 4°C for 30 min and were then centrifuged at 10,000 *g* for 30 min. The supernatant was then filtered through two layers of Whatman #1 filter paper, divided into 4

mL aliquots, frozen in liquid nitrogen and kept at - 80 °C. Extracts were thawed only once prior to enzyme assay. Total protein concentration of each protein extract was determined using the BioRad Coomassie reagent and protocol (BioRad).

Prenyltransferase Enzyme Assays

Wood and bark protein extracts were desalted into a buffer containing 20 mM MOPSO, pH 7.0, 10 mM $MgCl_2$, 10% (v/v) glycerol and 2 mM. Assays were carried out in duplicate or triplicate in a final volume of 500 µl containing 40 µM [1-^{14}C]-IDP (54 Ci mol^{-1}) and 40 µM DMADP. To reduce competing IDP isomerase activity, 5 mM iodoacetamide was added. After the reaction was initiated by addition of the enzyme preparation, the assay mixture was immediately overlaid with 1 ml pentane and incubated for 1 h at 30°C. To stop the assay and hydrolyze all diphosphate esters (both unreacted substrate as well as products), a 500 µL solution of calf intestine alkaline phosphatase (Sigma, 4 units) and potato apyrase (Sigma, 4 units) in 0.2 M tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 9.5 was added to each assay and incubated at 30°C for 8-12 h. After enzymatic hydrolysis, the resulting prenyl alcohols were extracted into 2 ml diethyl ether and, after addition of a mixture of terpene standards, the organic extract was prepared for radio-GC as described previously (Burke et al. 1999). 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 radio detector (Raytest, Giessen). The concentrated organic phase (one to two µL) was injected on a DB-wax capillary column (J&W scientific) (30 m x 0.25 mm with 0.25 µm phase coating). Separation was achieved under a He flow rate of 2 ml min^{-1} with a temperature program of 3 min at 40°C, a ramp to 70°C at 3°C min^{-1} (one min hold) and a second ramp from 70 to 240°C at 6°C min^{-1} (30 min hold). Both mass and radioactivity traces were monitored simultaneously. Products were identified by comparison of retention times with those of co-injected authentic standards.

Preliminary enzyme assays had shown that ^{3}H-labeled GDP and FDP were not incorporated into longer C-15 or C-20 prenyl diphosphate products, when GDP or FDP concentrations of 2-5 µM were used in the presence of 40 µM IDP and 40 µM DMADP. Thus, individual prenyltransferase activities could be clearly distinguished.

Terpene Synthase Enzyme Assays

Terpene synthase activities were determined by published procedures (Lewinsohn et al., 1991b; Lafever et al., 1994; Bohlmann et al., 1997) with minor modifications. Before

assaying enzyme activity, the frozen protein extracts were placed at 37°C until just thawed. The protein extracts were desalted in BioRad Econo Pac 10DG sizing columns pre-equilibrated with appropriate assay buffers: monoterpene synthase buffer [25 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (pH 7.5), 5 mM dithiothreitol, 10 % (v/v) glycerol, 1 mM $MnCl_2$, and 100 mM KCl], sesquiterpene synthase buffer [25mM HEPES (pH 7.3), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 10% (v/v) glycerol] or diterpene synthase buffer [30 mM HEPES (pH 7.2), 7.5 mM $MgCl_2$, 20 µM $MnCl_2$, 5 % (v/v) glycerol and 5 mM dithiothreitol]. Enzyme activity was assessed with 1 mL of the desalted extracts with the addition of 10 µM GDP (with 1 µCi ^{3}H-GDP) for monoterpene synthase activities, or 10 µM GGDP (0.5 µCi ^{3}H-GGDP) as substrate for diterpene synthase assays. All enzyme assays were done in duplicate, overlaid with 1 mL of pentane to collect released volatiles, and incubated at 30°C for 1.5 h. To stop all enzyme activity, the extracts were immediately frozen. After thawing, the aqueous assay fraction was rapidly extracted with the pentane fraction by vortexing, and separation of the aqueous and organic fractions was achieved by centrifugation at 2,500 *g* for 2 min. The one mL pentane overlay was removed and filtered through a Pasteur pipette filled with 0.4 g silica gel (Sigma 60 Å) overlaid with 0.6 g $MgSO_4$ to remove non-specific substrate hydrolysis products and to dry the pentane extract. Each enzyme assay was extracted with an additional two portions of pentane, vortexed and centrifuged as before. These sequential extractions were also passed over the same column and pooled with the initial column eluent. Subsequently, the column was washed with pentane (2 x 1 mL) and the total volume was determined. The extracts were analyzed by liquid scintillation counting, 0.1 mL in 0.3 mL of Lipoluma (JT Baker) and GC.

The conditions for all enzyme assays, including pH optimum, incubation time, substrate concentration, and temperature optimum, were optimized for this system such that maximum activity was achieved in a linear range of product generation. In addition, the possibility that enzyme activities in induced tissues might have been inhibited by additional resin or phenolic substances was ruled out by experiments in which extracts from different stages of the time course were mixed together. In all cases, the resulting enzyme activity was additive, implying that additional compounds found in induced tissues had no effect on enzyme activity.

Analysis of Terpene Synthase Assay Products

The extracts of duplicate assays were combined and evaporated on ice to 50-100 µL. From this, 2 µL were analyzed by radio-GC coupled with a thermal conductivity detector

(TCD). The radio detector enables the detection of radioactive substances and the TCD provides a non-destructive method to determine retention times of coinjected, unlabeled standards. For the monoterpene synthase assays, the Hewlett-Packard 6890 GC was fitted with a DB-WAX column (described above). The column flow rate was 2 mL H_2 min^{-1}, and the TCD was kept at 250°C with an additional 7 mL H_2 min^{-1} (make-up gas). Parameters for the RAGA radio-detector (Radiomatic, Giessen, Germany) were as follows: the platinum catalyst was operated at 740°C, the total H_2 flow rate including the make-up gas was 20 mL min^{-1}, and methane quench gas flowed through a 2 mL-counting tube at 5 mL min^{-1}. The samples were introduced to the injection port at 220°C and were split in either a 5:1 ratio for the bark assay extractions or a 3:1 ratio for the wood assay extractions. The oven was programmed with an initial temperature of 40 °C (3 min hold), and increased at a rate of 1.5°C min^{-1} until 70°C followed by an increase of 15°C min^{-1} until 240°C (3 min hold). Monoterpenes were identified by comparing retentions times with those of known standards.

Extracts of diterpene synthase assays were analyzed by GC-MS employing a DB-WAX column (described above). Extracts from the bark and wood assays were split 10:1 or 5:1, respectively. The injection port was operated at 250°C and the column flow and the radio-detector were maintained as for the monoterpene analysis. The oven was programmed from an initial temperature of 180°C to 240°C at a rate of 4°C min^{-1}, which was followed by a 6-min hold. The extracts were further analyzed by GC-MS. For this analysis, the same DB-WAX column was used. A splitless injector was kept at 220°C and a flow rate of 1 mL He min^{-1} was maintained. The initial temperature was 50°C and the rate increased at a constant 10°C min^{-1} up to 280°C (3 min hold). All other parameters were as described for the GC-MS diterpene resin analysis. For peak identification, the 70eV mass spectra generated were compared to an authentic abietadiene standard (Rodney Croteau). All analysis was performed with Hewlett-Packard Chemstation software.

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3. TRAUMATIC RESIN DEFENSE IN NORWAY SPRUCE (*Picea abies*): METHYL JASMONATE-INDUCED TERPENE SYNTHASE GENE EXPRESSION, AND CDNA CLONING AND FUNCTIONAL CHARACTERIZATION OF (+)-3-CARENE SYNTHASE[3,4]

ABSTRACT

Picea abies (L.) Karst. (Norway spruce) employs constitutive and induced resin terpenoids as major chemical and physical defense-shields against insects and pathogens. In recent work, I showed that a suite of terpenoids, monoterpenoids, and diterpenoids, was induced in stems of Norway spruce after treatment of trees with methyl jasmonate (MeJA) (Martin et al., 2002). Increase of enzyme activities of terpenoid biosynthesis and accumulation of terpenoids was associated with MeJA-induced *de novo* differentiation of xylem resin ducts. The formation of defense-related traumatic resin ducts was also found in Norway spruce after attack by stem-boring insects or after infestation with fungal pathogens. In the present study, we analyzed the traumatic resin response in Norway spruce further at the molecular genetic level. Treatment of trees with MeJA induced transient transcript accumulation of monoterpenoid synthases and diterpenoid synthases in stem tissues of Norway spruce. In screening for defense-related terpenoid synthase (*TPS*) genes from Norway spruce, a full-length monoterpenoid synthase cDNA, *PaJF67*, was isolated and the recombinant enzyme expressed in *E. coli* and functionally characterized *in vitro*. The cloned *PaJF67* cDNA represents a new monoterpenoid synthase gene and the gene product was identified as 3-carene synthase. The enzyme encoded by *PaJF67* forms stereospecifically (+)-3-carene (78 % of total product) together with minor acyclic and cyclic monoterpenes, including the mechanistically closely related terpinolene (11 % of total product). (+)-3-Carene is a characteristic monoterpene of constitutive and induced oleoresin defense of Norway spruce and other members of the Pinaceae.

[3] This work has been published in Fält et al. (2003b).

[4] The work detailing the cloning of 3-carene synthase (Fäldt et al., 2003b) was completed by the author, and Drs. Jenny Fäldt and Suman Rawat. The author was involved in teaching molecular biology to Dr. Fäldt. Furthermore, the author generated the probe and the cDNA library discussed in this work. The autor was also responsible for the phylogenetic analysis presented in Figure 3.8. Dr. Fäldt cloned, expressed and identified the products of this (+)-3-carene synthase. Dr. Suman Rawat performed the northern experiments and Dr. Jörg Bohlmann acted as supervisor for this project. The writing of the manuscript was completed by Drs. Bohlmann and Fäldt and the author.

INTRODUCTION

Conifer resins are composed of a large suite of structurally diverse terpenoid natural products that have long been recognized for their many functions in the chemical ecology of conifers and coniferophagus insects (Bohlmann and Croteau, 1999; Langenheim, 1994; Seybold et al., 2000; Trapp and Croteau, 2001a), as well as for their industrial value (Schrader and Berger, 2001; Dawson, 1994). The oleoresin of Norway spruce (*Picea abies*) consists mainly of monoterpenoids (10-carbon), sesquiterpenoids (15-carbon), and diterpenoids (20-carbon). Monoterpenoids and diterpenoids are equally abundant and together account for more than 98 % of resin terpenoids in stems of Norway spruce (Martin et al., 2002). The turpentine fraction of Norway spruce resin includes more than 20 different monoterpenoids, several of which exist as pairs of their respective stereoisomers (Borg-Karlson et al., 1993; Persson et al., 1996; Persson et al., 1993) (Figure 3.1).

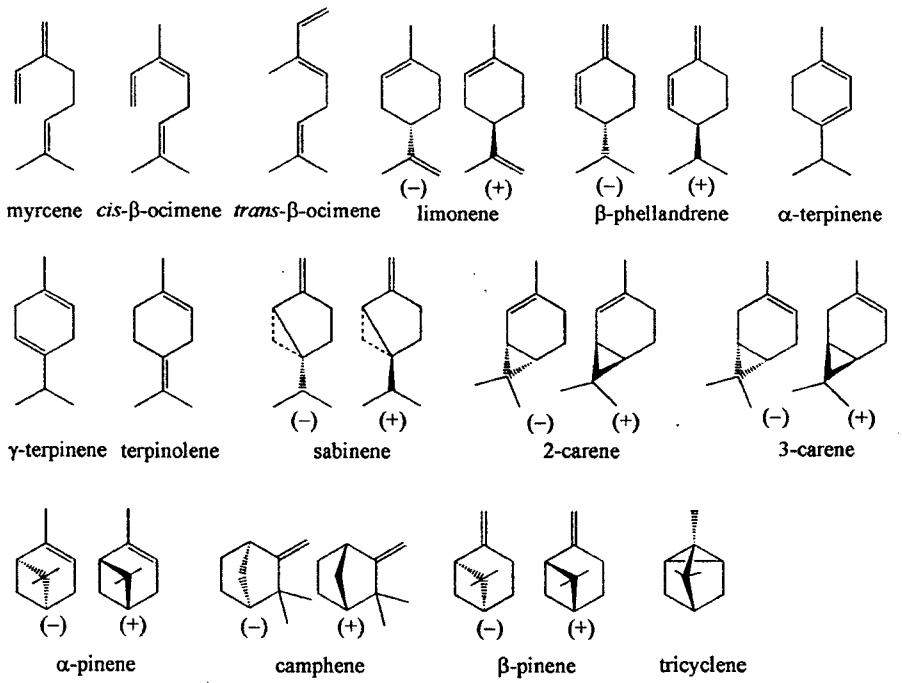

Figure 3.1: Characteristic monoterpenes of the turpentine fraction of Norway spruce oleoresin and their enantiomers.

The 10-carbon monoterpenes occur as acyclic, monocyclic, bicyclic or tricyclic structures. Several monoterpenes, such as α-pinene and β-pinene, can exist as pairs of two enantiomers in the turpentine of conifers. Other monoterpenes are found only in form of one of two possible enantiomers. For instance only the (+)-enantiomer of 3-carene was found in Norway spruce.

Recent chemical, biochemical and histological studies demonstrated that accumulation of monoterpenoids and diterpenoids in sapling stems of Norway spruce is elicited by treatment of trees with methyl jasmonate (MeJA) (Martin et al., 2002). Induced terpenoid accumulation is the result of a xylem-specific increase in enzyme activities of terpenoid biosynthesis, namely prenyl transferases and terpenoid synthases. These chemical and biochemical responses are associated with defense-related *de novo* formation of traumatic resin ducts in the cambium zone and in the differentiating xylem. The MeJA-induced oleoresin response described in Norway spruce is similar and possibly identical to the defense response induced by stem-boring insects and microbial pathogens in several species of spruce, including Norway spruce, Sitka spruce (*Picea sitchensis*) and white spruce (*Picea glauca*) (Alfaro, 1995; Alfaro et al., 2002; Tomlin et al., 1998; Tomlin et al., 2000; Nagy et al., 2000).

Induced terpenoid defense in Norway spruce xylem includes accumulation of 3-carene, a bicyclic monoterpene hydrocarbon with an unusual cyclopropyl function (Figure 3.1). 3-Carene is a common component of many conifer turpentines. However, proportions of 3-carene relative to other resin monoterpenes and absolute amounts of 3-carene can vary drastically between different conifer species and between chemotypes of the same species (Sjödin and Borg-Karlson, 2000; Sjödin et al., 1996; Plomion et al., 1996; Persson et al., 1996; Hiltunen et al., 1973). In some conifer-pest interactions, quantitative variation of 3-carene has been associated with resistance or susceptibility of trees. For example, in lodgepole pine (*Pinus contorta*) resistance to Douglas-fir pitch moth (*Synanthedon novaroensis*) correlates with high amounts of 3-carene (Rocchini et al., 2000). In clones of Scots pine (*Pinus sylvestris*) high levels of 3-carene were found to correlate with low larval survival of the sawfly *Diprion pini* (Passquier-Barre et al., 2001). As the major monoterpene induced in roots of Scots pine upon infection with the ectomycorrhizal fungus, *Boletus variegates*, 3-carene was suggested to be involved in development of the root-mycorrhizae symbiosis and in disease resistance of the mycorrhizal system (Krupa and Fries, 1971). Infection of wound sites of lodgepole pine stems with the fungus *Ceratocystis clavigera* resulted in an increase of 3-carene (Croteau et al., 1987), a pattern that was also found in Scots pine phloem infected by *Leptographium wingfieldii* (Fäldt et al., 2004).

Terpene synthase (TPS) enzyme activities responsible for the formation of 3-carene have been characterized *in vitro* in stems of Douglas fir (*Pseudotsuga menziesii*) and in partially purified stem extracts of lodgepole pine (Savage and Croteau, 1993). These studies established both the regio- and stereochemistry of the formation of (+)-3-carene. 3-Carene is formed from geranyl diphosphate (GDP), the common precursor of all monoterpenes (Wise

and Croteau, 1999), by monoterpene synthase activity via a coupled isomerization-cyclization mechanism that proceeds through linalyl diphosphate (LPP) and the α-terpinyl cation (Figure 3.2) (Wise and Croteau, 1999; Savage and Croteau, 1993). The cyclopropyl function of 3-carene is formed by an *anti*-1,3-elimination of a C-5 proton in the α-terpinyl cation. Induced monoterpene synthase activities that catalyze the cyclization of GDP to 3-carene have been described for grand fir (*Abies grandis*) (Katoh and Croteau, 1998; Gijzen et al., 1991) and for Norway spruce (*P. abies*) (Martin et al., 2002).

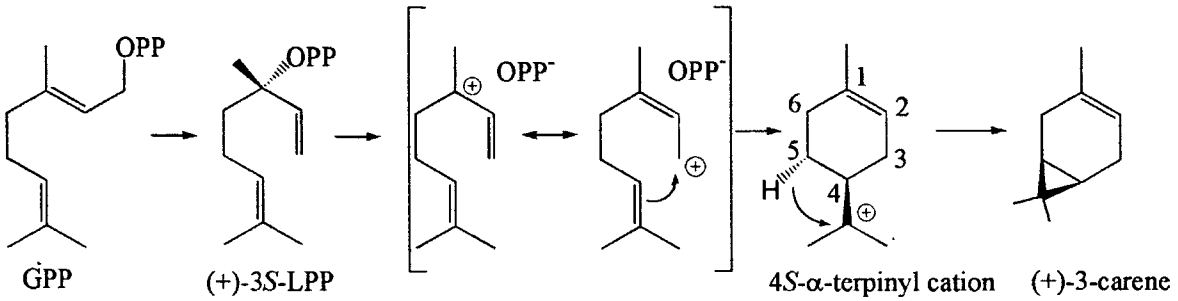

Figure 3.2: Proposed scheme for the formation of 3-carene from geranyl diphosphate (GPP).

3-Carene is formed from GPP via a coupled isomerization-cyclization mechanism that proceeds through linalyl diphosphate (LPP) and an α-terpinyl cation (Savage and Croteau, 1993). The cyclopropyl function of 3-carene is formed by *anti*-1,3-elimination of a C-5 proton in the α-terpinyl cation. OPP denotes the diphosphate moiety.

In a combined molecular genetic and biochemical approach, a group of constitutive and induced monoterpene synthases was cloned as cDNAs from grand fir and the recombinant enzymes were functionally characterized (Bohlmann et al., 1997; Bohlmann et al., 1999). Although the grand fir *TPS* cDNAs account for many of the monoterpenes characteristic of conifer oleoresin, a *TPS* gene or cDNA yielding 3-carene eluded isolation. Genetic analysis of quantitative variation of 3-carene allowed mapping of a quantitative trait locus for this monoterpene in maritime pine (*Pinus pinaster*) (Plomion et al., 1996), but did not yet result in the isolation of a gene that controls formation of 3-carene.

In the present study, we used molecular probes for *TPS* gene expression analysis of the MeJA-induced traumatic resin response and for full-length *TPS* cDNA isolation. Functional characterization of a new monoterpene synthase cDNA revealed for the first time a gene for the formation of (+)-3-carene.

RESULTS AND DISCUSSION

MeJA-induced TPS gene expression in Norway spruce

In recent work, Martin *et al.* (2002) demonstrated that treatment of Norway spruce saplings with MeJA induced a multi-layered resinosis response in stem tissues. The MeJA-induced response involves a series of histological, biochemical and chemical changes in the developing xylem that include (i) *de novo* differentiation of traumatic resin duct cells and resin canals, (ii) induced activities of prenyl transferases (geranylgeranyl diphosphate synthase) and terpene synthases (monoterpene synthases and diterpene synthases), and (iii) strongly increased accumulation of monoterpenoids and diterpenoids in the newly formed resin ducts. This MeJA-induced response of Norway spruce saplings closely resembles the traumatic resin response evoked by stem-boring insects and microbial pathogens observed in several species of spruce, such as Norway spruce, Sitka spruce and white spruce (Alfaro, 1995; Alfaro et al., 2002; Nagy et al., 2000; Tomlin et al., 1998; Tomlin et al., 2000). To further characterize the resinosis response of spruce stem tissues at the level of transcript accumulation, northern hybridizations were performed for transcripts of monoterpene synthases and diterpene synthases. A 481-bp monoterpene synthase cDNA fragment, *PaTPS13* (GenBank accession AF461459) was isolated from Norway spruce and used to evaluate monoterpene synthase gene expression under

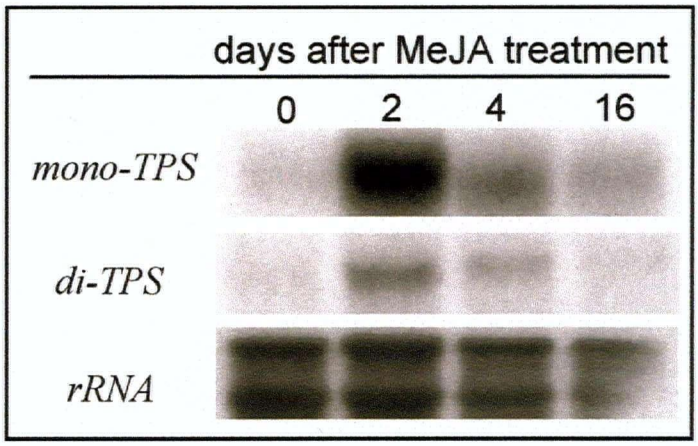

Figure 3.3 MeJA-induced monoterpene synthase and diterpene synthase transcript accumulation in stems of Norway spruce.

Total RNA was extracted from combined bark and xylem of control trees and from trees treated with 0.01 % (v/v) MeJA. Northern blots were hybridized with class-specific monoterpene synthase and diterpene synthase probes, *PaTPS13* (this study) and grand fir abietadiene synthase (Stofer Vogel *et al.*, 1996). Conifer monoterpene synthases and diterpene synthases are less than 30 - 40 % identical at the nucleic acid level. No cross hybridization was found between the two probes under the conditions of our experiments. Equal loading of total RNA in each lane was monitored by ethidium bromide staining of ribosomal RNA (not shown) and hybridization to ribosomal RNA probe.

constitutive and induced conditions. Monoterpene synthases exist in species of conifers as small gene families of members that are from 65 % to 99 % identical at the nucleotide level (Bohlmann et al., 1998b; Bohlmann and Croteau, 1999). Therefore, we used probe *PaTPS13* at hybridization conditions that could reveal possible expression of all members of such a monoterpene synthase family in Norway spruce. Since it is known that grand fir cDNA abietadiene synthase, a conifer diterpene synthase (Stofer Vogel et al., 1996), does not cross-hybridize with monoterpene synthase transcripts (Steele et al., 1998b), this gene was employed as a heterologous probe to measure diterpene synthase transcripts in Norway spruce. Northern analysis revealed strong accumulation of monterpene synthase transcripts in stems of Norway spruce within two days after treatment with MeJA applied at a concentration of 0.01 % (v/v) as a headspace fumigant. Lower levels of monoterpene transcripts were detected four days and 16 days after treatment (Figure 3.3). The time course of rapid monoterpene synthase transcript accumulation clearly precedes MeJA-induced increase in monoterpene synthase enzyme activities (maximum ten to 15 days after treatment) and induced accumulation of monoterpenoids in Norway spruce (maximum between days 15 to 20 after treatment) (Martin et al., 2002). Although weaker signals were found for diterpene synthase transcripts (Figure 3.3), possibly due to the heterologous origin of the hybridization probe, similar time courses are followed for monoterpene synthase and diterpene synthase transcripts in stems in response to MeJA treatment. This finding is also consistent with time courses of induced diterpene synthase enzyme activities and diterpenoid accumulation in MeJA treated stem tissues (Martin et al., 2002). Similar overall induction of transcript accumulation for monoterpene synthases and diterpene synthases was previously found in grand fir stems as

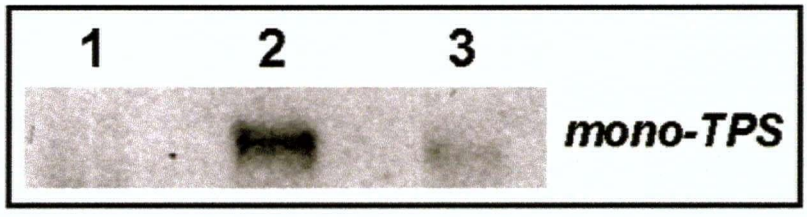

Figure 3.4: Constitutive monoterpene synthase transcript accumulation in foliage and stems of Norway spruce.

RNA was extracted from combined bark and xylem of one year old stems (lane 1), from young shoots and foliage four to six weeks after bud burst (lane 2), or from one-year old foliage (lane 3). Northern blots were hybridized with monoterpene synthase probe *PaTPS13*. Equal loading of total RNA in each lane was monitored by ethidium bromide staining of ribosomal RNA (not shown). Constitutive accumulation of monoterpene synthase transcripts was low in one-year old stems and foliage of Norway spruce, but was higher in young developing needles and shoots within four to six weeks of bud burst.

the result of mechanical wounding (Steele et al., 1998b).

Isolation of full-length monoterpene synthase cDNA PaJF67

Although several monoterpene synthase genes were previously isolated from grand fir (Bohlmann et al., 1997; Bohlmann et al., 1999), additional monoterpene synthase genes of unique biochemical functions must exist for the formation of some typical conifer resin components such as 3-carene. Because Norway spruce possesses high constitutive monoterpene synthase enzyme activity (Fischbach et al., 2000) and high constitutive monoterpene synthase gene expression in young foliage and developing shoots (Figure 3.4), these tissues are an ideal source for cDNA isolation of new monoterpene synthases. Partial cDNA PaTPS13 clone was employed as a probe for screening for monoterpene synthases in Norway spruce. This effort resulted in the isolation of a new full-length cDNA clone, PaJF67 (GenBank accession AF461460), which encodes for a deduced protein of 627 amino acids with a predicted molecular weight of 71,912 Da and predicted pI of 6.17 (Figure 3.5). The deduced amino acid sequence of cDNA clone PaJF67 resembles typical monoterpene synthases (Bohlmann et al., 1998b) and most closely grand fir monoterpene synthases of the gymnosperm *TPS-d* group (Bohlmann et al., 1997; Bohlmann et al., 1999) (Figure 3.5). Multiple sequence alignment of the predicted PaJF67 protein with grand fir monoterpene synthases showed that these sequences are, in many regions, highly conserved (Figure 3.5). PaJF67 protein shares from 63 % identity (78 % similarity) with grand fir (–)-4*S*-limonene synthase to 66 % identity (80 % similarity) with grand fir terpinolene synthase (Bohlmann et al., 1997; Bohlmann et al., 1999), but shares less identity with angiosperm monoterpene synthases such as mint limonene synthase (25 % identity, 44 % similarity) (Colby et al., 1993) or *Arabidopsis thaliana* myrcene/ocimene synthase (22 % identity, 43 % similarity) (Bohlmann et al., 2000b). The PaJF67 protein is also less similar to the known sesquiterpene synthases of grand fir, showing 37 % identity with γ-humulene synthase (59 % similarity) and δ-selinene synthase (58 % similarity) (Steele et al., 1998a) and 26 % identity (44 % similarity) with *E*-α-bisabolene synthase (Bohlmann et al., 1998a). PaJF67 protein is only 26 % identical (39 % similar) with abietadiene synthase, a diterpene synthase from grand fir (Stofer Vogel et al., 1996). These data suggest that *PaJF67* encodes a monoterpene synthase, rather than a sesquiterpene synthase or a diterpene synthase.

The predicted PaJF67 protein contains the twin-arginine/tryptophan motif RRx_8W in position 64 to 74 from the N-terminus (Figure 3.5). This motif is characteristic of all monoterpene synthases (Bohlmann et al., 1998b; Bohlmann et al., 2000a) and was shown to be involved in the initial diphosphate migration reaction step of the coupled isomerization-

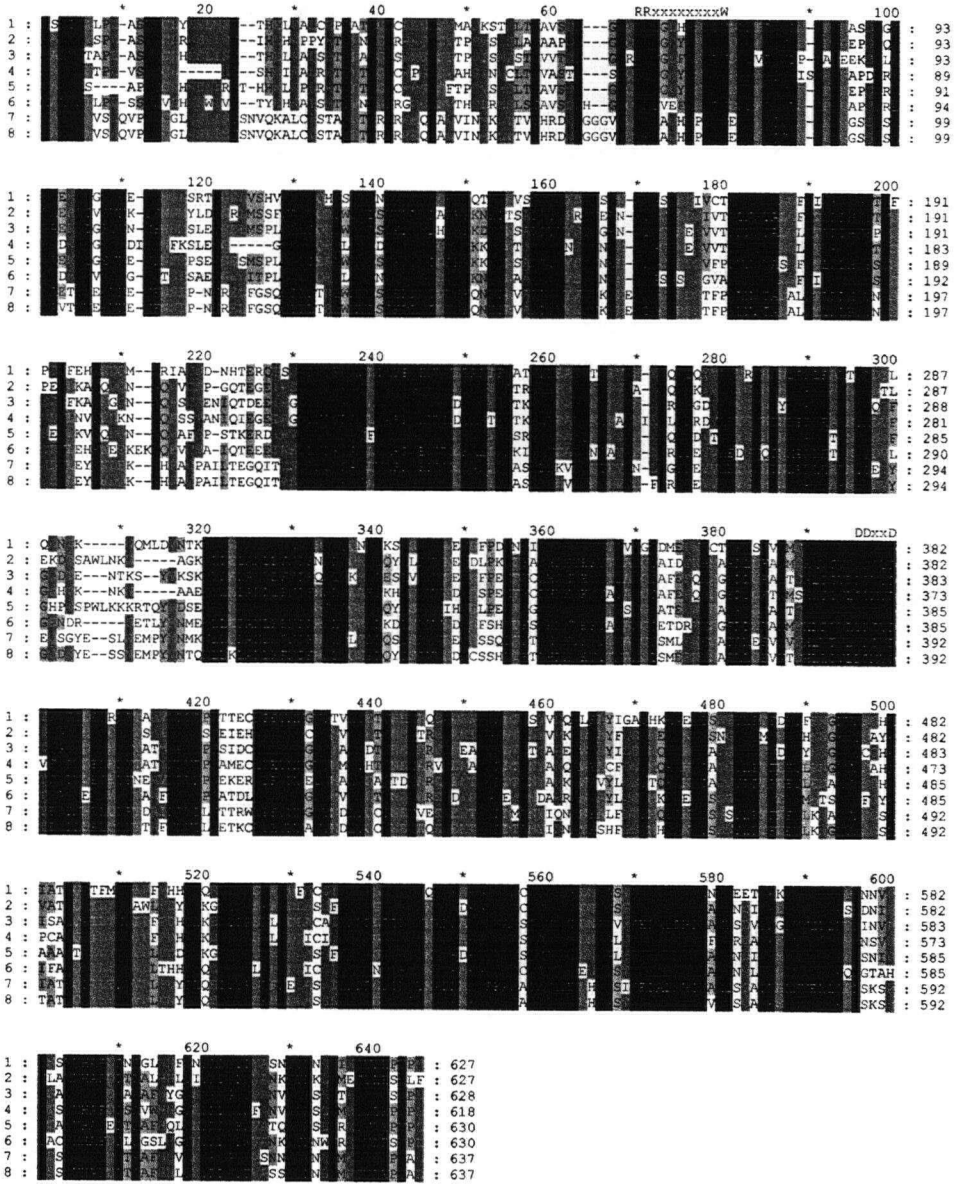

Figure 3.5: Amino acid sequence alignment of Norway spruce PaJF67 predicted protein and grand fir monoterpene synthases.

Sequences are Norway spruce PaJF67 (AF461460, this study) [1], and the grand fir monoterpene synthases myrcene synthase (U87908) [2], (–)-pinene synthase (U87909) [3], (–)-camphene synthase (U87910) [4], ß-phellandrene synthase (AF139205) [5], terpinolene synthase (AF139206) [6], (–)-limonene synthase (AF006193) [7], and (–)-limonene/(–)-α-pinene synthase (AF139207) [8]. Residues shaded in black (100 % similarity), dark grey (above 80 % similarity) or light grey (above 60 % similarity) are conserved in all sequences of the comparison. Conserved motifs RRx_8W and DDxxD of known function in TPS reaction mechanism are indicated. The alignment was created using ClustalX and visualized using GeneDoc software.

cyclization reaction mechanism of monoterpene synthases (Williams et al., 1998). The region upstream of the twin-arginine residues bears typical features of a plastid transit peptide characteristic of nuclear-encoded monoterpene synthase preproteins (Williams et al., 1998; Bohlmann et al., 1998b). A conserved DDxxD motif previously identified to be involved in divalent metal ion assisted substrate binding (Davis and Croteau, 2000) was also located in PaJF67 (Figure 3.5).

Functional expression of PaJF67 in E. coli

Monoterpene synthases are members of a family of *TPS* genes in plants that apparently evolved from a common ancestor by repeated gene duplication, structural diversification and functional specialization (Bohlmann et al., 1998b; Bohlmann et al., 2000a). Members of the *TPS* family cluster into six subfamilies based on sequence similarity. However, specific functions of TPS enzymes cannot be predicted based on sequence relatedness, because similar functions did evolve independently in separate subfamilies, and functions of members of the same subfamily can be quite diverse (Bohlmann et al., 1998b). Many monoterpene synthases also yield multiple products from the same substrate as a consequence of a cationic reaction mechanism that allows for the formation of a suite of similar products from common intermediates (Davis and Croteau, 2000; Wise and Croteau, 1999). It was therefore necessary to express and biochemically characterize the PaJF67 protein to obtain correct functional identification of the gene product and thus to provide accurate annotation of gene function.

cDNA *PaJF67* was subcloned into pET100/D-TOPO vector for T7-RNA polymerase directed expression of the active protein in *E. coli* BL21-CodonPlus(DE3) cells. Expressed recombinant PaJF67 protein was tested in cell-free extracts of transformed *E. coli* for monoterpene synthase, sesquiterpene synthase and diterpene synthase activities using the corresponding prenyl diphosphate substrates geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP). Of these substrates, only GDP was efficiently converted into monoterpene hydrocarbons by the enzyme activity of PaJF67 protein. Extract prepared from *E. coli* BL21(DE3) transformed with pET100/D-TOPO without *PaJF67* insert served as a control for product formation independent of recombinant TPS enzyme. These extracts did not yield detectable amounts of enzymatic terpenoid products.

Product identification of PaJF67 enzyme activity

Analysis of the monoterpene hydrocarbon product fraction of recombinant PaJF67 enzyme by GC and GC/MS revealed 3-carene as the major product (77.7 %) when using GDP as

substrate (Figure 3.6). Additional cyclic and acyclic monoterpene products were identified as terpinolene (11 %), sabinene (5.1 %), myrcene (3 %), γ-terpinene (1 %), α-pinene (0.9 %), β-phellandrene (0.7 %), α-terpinene (0.6 %) and limonene (0.4 %). The co-occurrence of 3-carene and terpinolene as the two most abundant products can be rationalized based on similar reaction mechanisms of their formation involving, respectively, proton elimination at C-5 and C-4 of the α-terpinyl cation intermediate (Figure 3.7) (Savage and Croteau, 1993; Wise and Croteau, 1999). The multiple cyclic products of 3-carene synthase relate to a highly reactive α-terpinyl cation intermediate that can, within the constraints of the enzyme active site, undergo a variety of transformations prior to ultimate quenching of the carbocation (Wise and Croteau, 1999). No oxygenated or phosphorylated monoterpenes were found in detectable amounts in the product fraction of PaJF67 enzyme assays.

The PaJF67 enzyme produces enantiomerically pure (+)-3-carene, with no detectable amounts of the (–)-enantiomer as determined by 2D-GC. The stereochemistry of (+)-3-carene is consistent with the stereochemistry of all other chiral products of this enzyme activity, (–)-sabinene, (–)-α-pinene, (–)-β-phellandrene, and (–)-limonene, because of all of these products relate mechanistically to the 4S-configuration of the intermediate α-terpinyl cation (Figure 7) (Wise and Croteau, 1998). Interestingly, this multi-product enzyme does not catalyze the biosynthesis of constituents such as (–)-β-pinene and (–)-α-thujene, which are structurally and mechanistically similar to (–)-α-pinene and (–)-sabinene, respectively. cDNA cloning, functional expression and biochemical identification of (+)-3-carene synthase from spruce provides new means for future evaluation of possible active site residues responsible for the formation of a monoterpene cyclopropyl function. A comparison of spruce 3-carene synthase and grand fir terpinolene synthase is of particular interest, because these enzymes provide a pair of conifer monoterpene synthases that are closely related in their primary structures (Figure 3.5 and Figure 3.8) and enzyme mechanisms (Figure 3.7).

Previously, a group of monoterpene synthases was cloned from grand fir (Bohlmann et al., 1999). However, these cloned synthases produced 3-carene as neither a major or a minor product and therefore, could not account for the formation of 3-carene and the quantitative variability of 3-carene relative to other monoterpenes in chemotypes of grand fir (Katoh and Croteau, 1998). Our findings in Norway spruce prove the existence of a gene for a highly specialized 3-carene synthase in conifers and provide genetic and biochemical

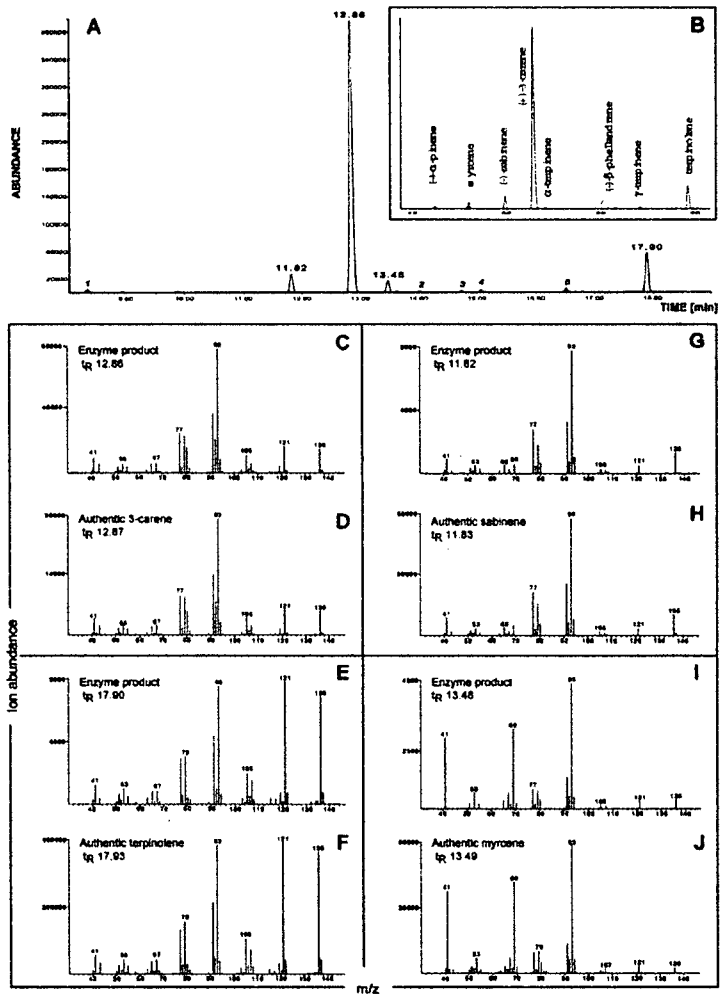

Figure 3.6: Gas chromatography (GC) and mass spectroscopy (MS) of the monoterpene products derived from geranyl diphosphate by PaJF67 (+)-3-carene synthase.

(A) Total ion chromatogram of products of PaJF76 enzyme activity formed from GPP and separated on a DB-WAX column. The most abundant products (more than 1 % of total product) are by order of retention time: sabinene (5 % of total product, tR 11.82 min.), 3-carene (78 % of total product, tR 12.86 min.), myrcene (3 % of total product, tR 13.48 min.), and terpinolene (11 % of total product, tR 17.90 min.). Low abundance products (1 % or less of total product) are: α-pinene (0.9 %, peak 1), α-terpinene (0.6 %, peak 2), limonene (0.4 %, peak 3), β-phellandrene (0.7 %, peak 4), and γ-terpinene (1 %, peak 5). (B) Total ion chromatogram of monoterpene products of PaJF76 enzyme separated on a Cyclodex B column. Enantiomers of chiral components were identified by co-injection with enantiomerically pure standards. (C) Mass spectrum of enzyme product tR 12.86 min. (D) Mass spectrum of authentic 3-carene. (E) Mass spectrum of enzyme product tR 17.90 min. (F) Mass spectrum of authentic terpinolene. (G) Mass spectrum of enzyme product tR 11.82 min. (H) Mass spectrum of authentic sabinene. (I) Mass spectrum of enzyme product tR 13.48. (J) Mass spectrum of authentic myrcene.

mechanisms to account for previously observed quantitative variation of 3-carene relative to other monoterpenes in species and chemotypes of conifers (Sjödin and Borg-Karlson, 2000; Sjödin et al., 1996; Plomion et al., 1996; Persson et al., 1996; Hiltunen et al., 1973).

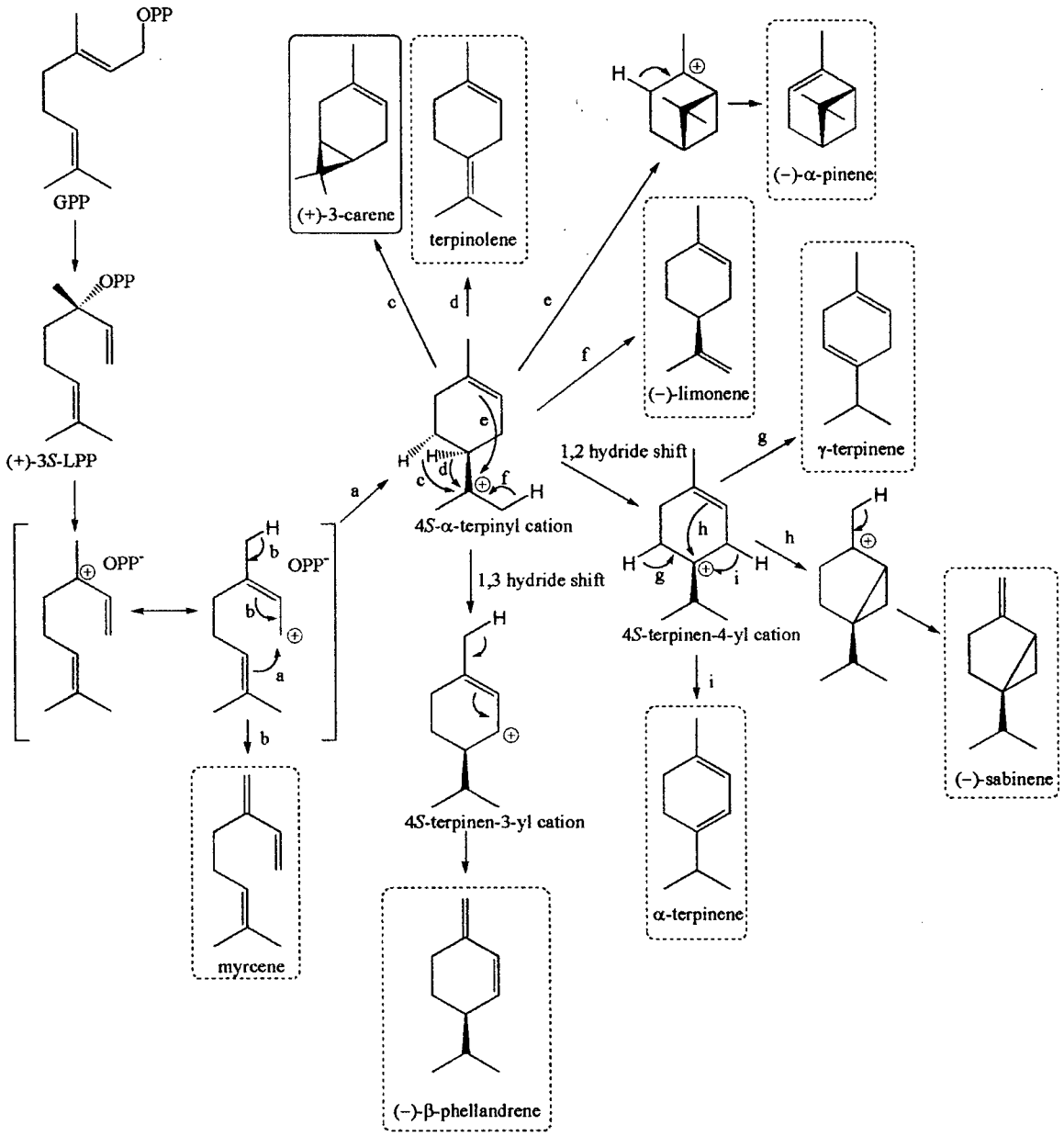

Figure 3.7: Proposed mechanism for monoterpene formation by (+)-3-carene synthase (PaJF67).

The proposed isomerization-cyclization scheme accounts for all of the major and minor regio-chemically different monoterpenes of the multi-product 3-carene synthase. The stereochemistry determined for all products can be rationalized via the intermediates of (+)-3S-linalyl diphosphate (LPP) and 4S-α-terpinyl cation. The two most abundant products, 3-carene (78 %) and terpinolene (11 %) are derived by closely related mechanisms involving, respectively, proton elimination at C-5 and C-4 of the 4S-α-terpinyl cation intermediate (Savage and Croteau, 1993; Wise and Croteau, 1998). OPP denotes the diphosphate moiety.

Phylogenetic relatedness of (+)-3-carene and TPS-d genes

Norway spruce 3-carene synthase is a new member of the gymnosperm *TPS-d* group of the *TPS* family that includes monoterpene synthases, sesquiterpene synthases and diterpene synthases of plant origin (Figure 3.8) (Bohlmann et al., 1998b). 3-Carene synthase is most closely related to other conifer monoterpene synthases and shares a common origin in a putative ancestral gene with the mechanistically similar grand fir terpinolene synthase (Bohlmann et al., 1999). Norway spruce 3-carene synthase is only distantly related to angiosperm monoterpene synthases. It is known that specific functions of plant monoterpene synthases evolved independently in angiosperms and gymnosperms (Bohlmann et al., 1998b). To date, an orthologue 3-carene synthase has not been reported from any other species, including the conifer grand fir. Therefore, it is currently not possible to conclude whether specific biochemical functions of conifer monoterpene synthases, such as 3-carene synthase, evolved prior to speciation of extant members of the Pinaceae. Alternatively, it is possible that functional specialization of conifer monoterpene synthases occurred independently in different genera or species of gymnosperms. To test these models, additional monoterpene synthases of members of the Pinaceae will be cloned and characterized in future work.

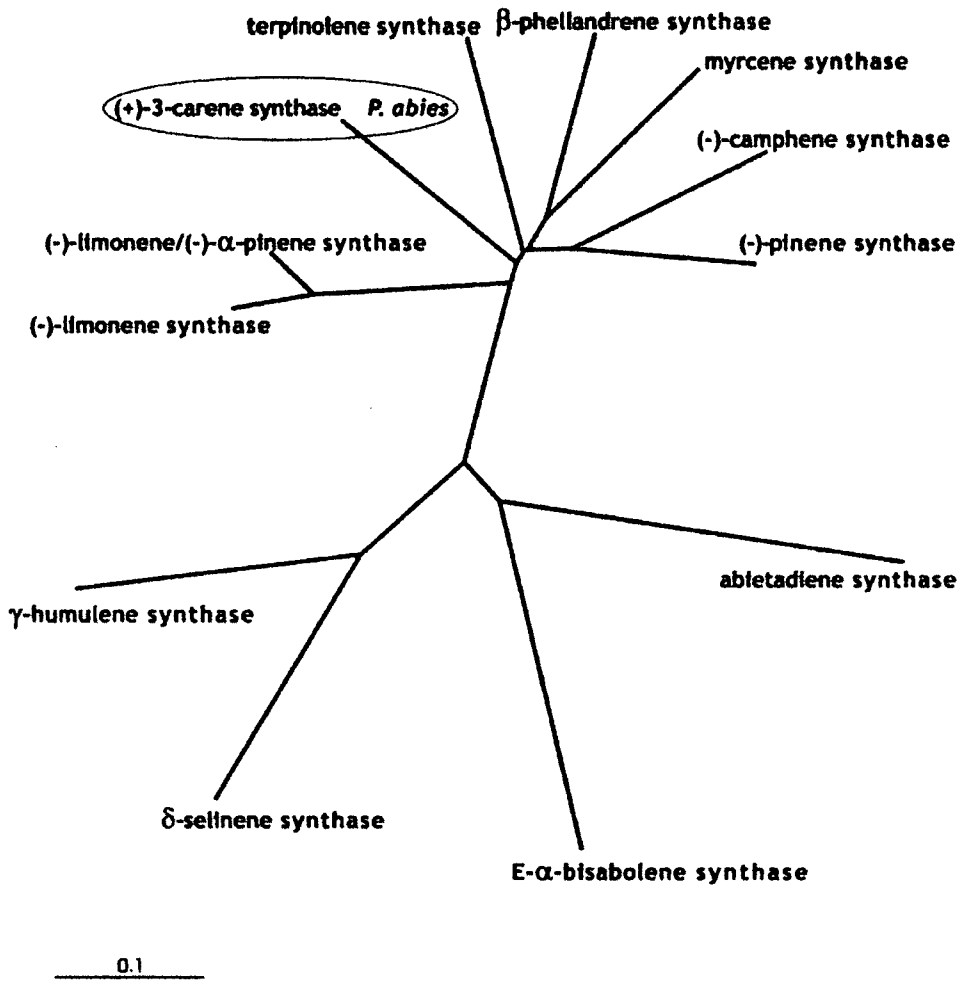

Figure 3.8: Sequence relatedness of Norway spruce (+)-3-carene synthase (PaJF67) and grand fir monoterpene synthases, sesquiterpene synthases and diterpene synthases.

Deduced amino acid sequences were compared using ClustalX and trees were visualized using Treedraw. Norway spruce 3-carene synthase is a member of the gymnosperm TPS-d family (Bohlmann et al., 1998b) and is most closely related to the mechanistically similar terpinolene synthase from grand fir (Bohlmann et al., 1999). Except for (+)-3-carene synthase from Norway spruce, all other sequences of this dendrogram are from grand fir: monoterpene synthases (myrcene synthase (U87908), (–)-pinene synthase (U87909), (–)-camphene synthase (U87910), β-phellandrene synthase (AF139205), terpinolene synthase (AF139206), (–)-limonene synthase (AF006193), (–)-limonene/(–)-α-pinene synthase (AF139207)), sesquiterpene synthases (γ-humulene synthase (U92267), δ-selinene synthase (U92266), E-α-bisabolene synthase (AF006195)), and a diterpene synthase (abietadiene synthase (U50768)).

CONCLUSIONS

Using homologous and heterologous *TPS* probes that are class-specific for conifer monoterpene synthases or diterpene synthases we demonstrated that the multi-layered traumatic resin defense response in Norway spruce involves increased transcript accumulation for two classes of *TPS* genes, monoterpene synthases and diterpene synthases. Screening for Norway spruce monoterpene synthases resulted in the discovery and functional characterization of a new *TPS* cDNA that is highly specialized for the stereospecific formation of (+)-3-carene, a common component of constitutive and induced conifer resins. In earlier studies, 3-carene was associated with defense and resistance of conifers to a suite of insect pests and pathogens (Fäldt et al., 2004; Krupa and Fries, 1971; Passquier-Barre et al., 2001; Rocchini et al., 2000). Isolation of a 3-carene synthase cDNA and efficient methods for conifer transformation (Peña and Seguin, 2001) provide a means for future directed manipulation of 3-carene synthesis and the evaluation of proposed roles of 3-carene in conifer chemical ecology.

The cloned 3-carene synthase forms additional minor products, a characteristic feature of many plant terpene synthases (Davis and Croteau, 2000; Bohlmann et al., 1998b). Terpinolene is the second most abundant product of Norway spruce 3-carene synthase and is mechanistically closely related to the major product, 3-carene (Figure 3.7). Interestingly, the deduced protein sequence of 3-carene synthase from Norway spruce is most similar to grand fir terpinolene synthase (Figure 3.5 *and* Figure 3.8). Future work will address the structure-function relationships of the formation of the bicyclic 3-carene and monocyclic terpinolene and will be guided by structure and sequence comparison of the closely related conifer monoterpene synthases.

MATERIALS AND METHODS

Plant Materials

Norway spruce (*Picea abies* (L.) Karst.) trees of clonal line 244-932 were from the Niedersächsische Forstliche Versuchsanstalt, Escherode, Germany, grown as described previously by Martin *et al.* (2002).

Substrates, Standards and Reagents

[1-^{3}H]Geranyl diphosphate (GDP) (150 Ci/mol), [1-^{3}H]farnesyl diphosphate (FDP) (125 Ci/mol) and [1-^{3}H]geranylgeranyl diphosphate (GGDP) (120 Ci/mol) were gifts of Dr. Rodney Croteau, Washington State University, Pullman WA, USA. Non-labeled GDP (1 mg/mL) was from Echelon Research Laboratories Inc. (Nevada, USA). Terpenoid standards, other chemicals and reagents were purchased from Sigma Chemical Co., Fluka Chemical Co or Aldrich Chemical Co., unless otherwise noted.

Treatment of trees with methyl jasmonate (MeJA)

Two-year old trees were treated with MeJA as described during the period of active growth approximately six weeks after bud burst (Martin *et al.*, 2002). In brief, individual trees were sprayed with 150 mL of a solution of 0.01 % (v/v) methyl jasmonate (95 % pure) dissolved in 0.1 % (v/v) Tween 20 (Fisher Scientific). Control trees were sprayed with 0.1 % (v/v) Tween 20. Trees were harvested two, four and 16 days after spraying by removing all branches and needles from stems and cutting stem sections of second year growth into 10 cm fragments. The bark and developing xylem of each section was sliced longitudinally with razor blades and peeled off the wood. All tissues were immediately frozen in liquid nitrogen and stored at - 80 °C.

cDNA library construction

Young developing shoots and foliage of Norway spruce were harvested within four weeks of bud burst and RNA was extracted according to a procedure of Lewinsohn *et al.* (1994) with the following modifications. Triton X-100 (1 % (v/v)), sodium deoxycholate (1 % (w/v)), and sodium N-lauryl sarcosine (1.5 % (w/v)) were added to the mRNA extraction buffer. Tissues were extracted in a ratio of 1 g per 5 mL extraction buffer. Poly-A mRNA was

purified using Streptavidin MagneSphere® Paramagnetic Particles (Promega, Madison WI, USA). A cDNA library was constructed using the λ-ZAPII system (Stratagene, La Jolla CA, USA) and recombinant phage DNA packaged using the Gigapack Gold system (Stratagene).

Probe isolation

A probe for monoterpene synthase screening and Northern analysis was generated by two rounds of PCR from the Norway spruce cDNA library using two pairs of nested primers derived from alignments of grand fir monoterpene synthases (Bohlmann et al., 1997; Bohlmann et al., 1999) and partial *TPS*-like expressed sequence tags (ESTs) of other conifers identified in the NCBI dbest database (http://www.ncbi.nlm.nih.gov). PCRs were performed each in a total volume of 50 µL containing 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM each of a forward primer and a reverse primer (Operon technologies, Inc., Alameda CA, USA), 1 U of *Taq* polymerase (Gibco BRL, Burlington, Canada) and 5 µL of template. As template in the primary PCR, 5 µL of the Norway spruce cDNA library (5 x 10^8 pfu/mL) was used in combination with the primary forward primer (5'-GCGCTGGATTACGTGTACAGTTATTGG-3') and the primary reverse primer (5'-CCGGTTTCTTTCCACCATCTGGAG-3'). Five µL of the completed primary PCR served as template in a secondary amplification with the secondary forward primer (5'-GTGGGAGAGATAGTGTTGTTGCTG-3') and the secondary reverse primer (5'-CTGTTACAAGGAGTGAAAGATATTGAACTC-3'). The temperature program for primary and secondary PCR was: denaturing at 95 °C for 2 min., 35 cycles: 30 sec. at 95 °C, 30 sec. at 45 °C (for the primary reaction) or at 60 °C (for the secondary PCR reaction), 1 min. at 72 °C. PCR reactions were analyzed by agarose gel electrophoresis. The amplicon of the secondary reaction was ligated into pCR2.1-TOPO vector (Invitrogen, Burlington, Canada), and transformed into *E. coli* TOP10F' cells (Invitrogen). Plasmid DNA was prepared from individual transformants and the inserts were sequenced (BigDye Terminator Cycle Sequencing kit, Perkin Elmer Applied Biosystems, Streetsville, Canada). A 481-bp *TPS*-like insert sequence was identified and was designated as probe *PaTPS13*.

Northern blot analysis

RNA isolation was performed following the procedure of Wang SX et al. (2000). Samples of 10 µg of total RNA were separated under denaturing conditions using formaldehyde in a 1.2 % agarose gel at 4-5 V/cm for 5-6 h according to the protocol described

in Sambrook and Russel (2001). Ribosomal RNA was visualized (ChemiImager™ 5500 with AlphaEaseFC™ software, Alpha Innotech Corporation, San Leandro CA, USA) and the RNA was transferred overnight by capillary action to positively charged nitrocellulose (Hybond-N+, Amersham Pharmacia Biotech,) using standard protocols (Sambrook and Russel, 2001). Gene fragments for hybridization probes were generated by PCR using 1 U *Taq* DNA-polymerase (Gibco BRL), 1 x PCR buffer (Gibco BRL), 4.0 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each primer (Operon technologies, Inc.), and 1 ng plasmid-DNA in a volume of 50 µl. Primers for amplification of monoterpene synthase probe *PaTPS13* were as described above. A diterpene synthase probe was generated from grand fir abietadiene synthase (Stofer Vogel *et al.*, 1996) using primers 5'-GCCATGCCTTCCTCTTCATTG-3' and 5'-AGGCAACTGGTTGGAAGAGGC-3'. The temperature program for PCR was: 36 cycles: 30 sec. at 94 °C, 1 min. at 55 - 65 °C, 1.5 min. at 72 °C. The PCR products were purified using QIAquick system (Qiagen). DNA labeling of purified PCR products was performed following instruction of the Strip-EZ™ DNA Kit (Ambion Inc., Austin, USA) using (α-^{32}P)dATP (3000 Ci/mmol, 10 mCi/ml, Perkin Elmer). Unincorporated label was removed on a gel filtration column (Microspin™ S-300, Amersham Pharmacia Biotech). After one hour of prehybridization at 65 °C in hybridization buffer (0.05 M $Na_4P_2O_7$ x 10 H_2O, 0.115 M NaH_2PO_4, 7 % SDS, 1 mM EDTA, 100 µg/ml salmon sperm DNA) blotted membranes were incubated in hybridization buffer with heat-denatured terpene synthase probes for 16 hours at 65 °C. Membranes were rinsed in 10 mL prewarmed wash buffer (0.05 M $Na_4P_2O_7$ x 10 H_2O, 0.115 M NaH_2PO_4, 1 % SDS, 1 mM EDTA) and washed twice in 40 mL wash buffer at 65 °C and once in 40 mL wash buffer at 68 °C. To detect the hybridization signals the membranes were exposed to autoradiography (Kodak BioMax MS film, Kodak BioMax intensifying screen) for 2-20 h at -80 °C.

Library screening

PaTPS13 probe (50n g) was purified by agarose gel electrophoresis and gel extraction (Qiagen gel extraction kit, Mississauga, Canada), labelled with [α-^{32}P]dCTP (Easytide, Perkin Elmer) using the Readiprime™ II random prime labelling system (Amersham Pharmacia Biotech Inc., Piscataway, USA), and used to screen replica filters of 3 x 10^5 plaques of the Norway spruce cDNA library plated on *E. coli* XL1 Blue MRF'. Hybridization was performed for 18 h at 55 °C in 3 x SSPE and 0.1 % SDS. Filters were washed three times for 10 min. each at 55 °C in 3 x SSPE with 0.1 % SDS and exposed for 17 h to Kodak BioMax-MS film at –80 °C. Of 274 clones yielding positive signals, all of which were purified through a second round of hybridization, 72 pBluescript SK(-) phagemids were excised *in vivo* in *E. coli* XL1 Blue MRF'

and transformed into *E. coli* SOLR. Plasmid DNA was prepared from individual transformants according to standard protocols (Sambrook and Russel, 2001).

DNA sequencing and sequence analysis

Inserts of all recombinant plasmids were completely sequenced on both strands via primer walking using the cycle sequencing dideoxy chain termination reaction with BigDye terminators (Perkin Elmer Applied Biosystems). Sequence analysis was done using programs from the Lasergene DNA-Star Package version 5, ClustalX (http://www.igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.htm) and Genedoc (http://www.psc.edu/biomed/genedoc). Sequence relatedness was analyzed using ClustalX using the Neighbor Joining method and an unrooted tree was visualized using Treedraw (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

cDNA expression in E. coli and enzyme assays

The full-length *PaJF67* cDNA insert of plasmid pBluescript-*PaJF67* was subcloned into the pET100/D-TOPO expression vector (Invitrogen) following the manufacturer's instructions. The *PaJF67* insert was amplified by PCR using forward primer (5'-CACCATGTCTGTTATTTCCATTTTGCCG-3') in combination with reverse primer (5'-CTTACATAGGCACAGGTTCAAGAAC-3'). PCRs were performed in volumes of 100 µL containing 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100, 10 µg BSA, 200 µM of each dNTP, 0.1 µM of each primer, 2.5 U of high fidelity Turbo *Pfu* polymerase (Stratagene) and 2 ng plasmid pBluescript-*PaJF67*. After a denaturing step at 95 °C for 2 min., 30 cycles of amplification were performed employing the following temperature program in a MJ PTC100-thermocycler: 30 sec. at 95 °C, 30 sec. at 59 °C, 2 min. at 72 °C, followed by 10 min. final extension at 72 °C. The amplified segment was cloned into pET100/D-TOPO vector and recombinant plasmids were transformed into *E. coli* TOP10 F' cells according to the protocol for ligation and transformation (Invitrogen). The plasmid pET-*PaJF67* was purified and transformed for expression into *E. coli* BL21-CodonPlus(DE3) (Stratagene). Positive clones were analyzed by PCR using insert and/or vector based primers.

For functional expression, bacterial strain *E. coli* BL21-CodonPlus(DE3)/pET-*PaJF67* was grown to A_{600} = 0.5 at 37 °C in 5 ml of LB medium supplemented with 20 µg/mL ampicillin. Cultures were then induced by addition of isopropyl–1–thio–ß–D-galactopyranoside to a final concentration of 1 mM and grown for another 12 h at 20 °C. Cells

were harvested by centrifugation, resuspended in 1 mL monoterpene synthase buffer (25 mM HEPES, pH 7.2, 100 mM KCl, 10 mM $MnCl_2$, 10 % glycerol, 5mM dithiothreitol) and disrupted by sonication using a Branson Sonifier 250 (Branson Ultrasonic Corporation, Danbury CT, USA) at constant power (5W) for 10 sec. Lysates were cleared by centrifugation and the resulting supernatants containing the soluble enzyme were assayed for monoterpene, sesquiterpene and diterpene synthase activity using the appropriate radio-labelled substrates as described previously (Bohlmann et al., 1997; Bohlmann et al., 1999). In the case of the monoterpene synthase and sesquiterpene synthase assay, the assay mixture (1 mL) was overlaid with 1 mL of pentane to trap volatile products. In all cases, after incubation at 30 °C for 1 h, the reaction mixture was extracted with pentane (3 x 1 mL) and the combined extract was passed through a 400 µL column of equal amounts of anhydrous $MgSO_4$ and silica gel (60 Å) to obtain the terpenoid hydrocarbon fraction free of oxygenated metabolites. To collect possible oxygenated products and to control for the possibility the products were a result of rearrangements (caused by the presence of silica gel), an independent set of assay mixtures were extracted with hexane (3 x 1 mL). The hexane fractions were combined, extracted with water and the hexane fraction was dried with anhydrous $MgSO_4$. Aliquots of each fraction were taken for liquid scintillation counting to determine activity. The counter efficiency was estimated to be 57 % by using [1-^{3}H]toluene as standard (2.24 x 10^6 dpm/mL, ARC, St. Louis, USA). To obtain product for analysis by GC-FID and GC-MS, we used unlabeled GDP at a final concentration of 137 µM. Controls for product formation independent of *PaJF67* cDNA were performed using extracts of *E. coli* BL21-CodonPlus(DE3) transformed with pET100/D-TOPO plasmid without the *PaJF67* insert.

Monoterpene product identification by gas chromatography (GC) and mass spectrometry (MS)

Monoterpenes were identified and quantified by GC-MS analysis on an Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector (70 eV) using a DB-WAX (J&W Scientific, Palo Alto CA, USA) capillary column (0.25 mm i.d. x 30 m with 25-µm film). A Cyclodex B (permethylated β-cyclodextrin in DB-1701, J&W Scientific) capillary column (0.25 mm i.d. x 30 m with 25-µm film) was used for chiral separation of monoterpene products. An injector (6 sec. splitless) was used at 200 °C and a column flow of 1 mL He min^{-1}. Using the DB-WAX column the following temperature program was used: initial temperature of 40 °C (4 min. hold) which was then increased to 150 °C at 4 °C min^{-1} followed by a 20 °C min^{-1} ramp until 230 °C (5 min. hold). For chiral separations (Cyclodex B) of α-pinene,

sabinene, limonene, ß-phellandrene, the following temperature program was used: initial temperature was 55 °C (1 min. hold) which was then increased to 100 °C at 1 °C min^{-1} followed by a 10 °C min^{-1} ramp until 230 °C (10 min. hold). For assignment of the stereochemistry of 3-carene, the enzyme product was co-injected with pure (+)-3-carene (Sigma-Aldrich) or with a (–)-3-carene standard prepared from black pepper (Anna-Karin Borg-Karlson, personal communication). Enantiomers of 3-carene were separated (baseline separation) on a Lipodex E column (dipentylbuturyl-γ-cyclodextrin, 0.25 mm i.d. x 25 m) (Macherey Nagel, Switzerland) using a two-dimensional GC (2D-GC) system coupled to a flame ionization detector (FID) (Borg-Karlson et al., 1993; Sjödin and Borg-Karlson, 2000) with the following temperature program: first GC (DB-WAX): initial temperature of 40 °C (1 min. hold) which was then increased to 180 °C at 6 °C min^{-1} followed by a 15 °C min^{-1} ramp to 230 °C (5 min. hold); second GC (Lipodex E): 30 °C isothermal for 50 min. Compounds were identified by comparing mass spectra using Agilent Technologies software and Wiley126 MS-library, as well as by comparing retention times and mass spectra with those of authentic standards. Stereochemistry of products was assigned based on matching of retention times with enantiomerically pure standards.

ACKNOWLEDGEMENTS

We thank Anna-Karin Borg-Karlson (Royal Institute of Technology, Stockholm, Sweden) for access to the 2D-GC facilities, Michael Phillips for technical advice, and Rodney Croteau (Washington State University, Pullman, USA) for the grand fir abietadiene synthase probe and substrates.

4. INDUCTION OF VOLATILE TERPENE BIOSYNTHESIS AND DIURNAL EMISSION BY METHYL JASMONATE IN FOLIAGE OF NORWAY SPRUCE (*Picea abies*)[5],[6]

ABSTRACT

Terpenoids are characteristic constitutive and inducible defense chemicals of conifers. The biochemical regulation of terpene formation, accumulation and release from conifer needles was studied in Norway spruce [*Picea abies* L. (Karst)] saplings using methyl jasmonate (MeJA) to induce defensive responses without inflicting physical damage to terpene storage structures. MeJA treatment caused a two-fold increase in monoterpene and sesquiterpene accumulation in needles without changes in terpene composition, much less than the 10- and 40-fold increases in monoterpenes and diterpenes, respectively, observed in wood tissue after MeJA treatment [Martin et al. (2002) Plant Physiol. 129: 1003-1018]. At the same time, MeJA triggered a five-fold increase in total terpene emission from foliage, with a shift in composition to a blend dominated by oxygenated monoterpenes (e.g., linalool) and sesquiterpenes (e.g., *E*-ß-farnesene) that also included methyl salicylate. The rate of linalool emission increased more than 100-fold, and that of sesquiterpenes increased more than 30-fold. Emission of these compounds followed a pronounced diurnal rhythm with the maximum amount released during the light period. The major MeJA-induced volatile terpenes appear to be synthesized *de novo* after treatment, rather than being released from stored terpene pools, since they are almost completely absent from needle oleoresin and are the major products of terpene synthase activity measured after MeJA treatment. Based on precedents in other species, the induced emission of terpenes from Norway spruce foliage may have ecological and physiological significance.

[5] This work has been published in Martin et al. (2003b)

[6] This work detailed in this chapter on MeJA induction of volatile terpenes (Martin et al., 2003b) was completed solely by the author. Again, Drs. Jörg Bohlmann and Jonathan Gershenzon served as supervisors for this work. The writing of the manuscript was completed by Drs. Bohlmann and Gershenzon and the author.

INTRODUCTION

Terpenes contribute to the characteristic defense chemistry of conifers in many forms. Monoterpenes (C_{10}) and diterpenes (C_{20}) are major components of conifer oleroresins (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Seybold et al., 2000; Trapp and Croteau, 2001a), while sesquiterpenes (C_{15}), though less abundant in resin, have enormous structural diversity (Steele et al., 1998b) (Figure 4.1). Terpene-containing oleoresin is mobilized to wound and infection sites, and serves to protect conifers against herbivore and pathogen invasion because of its toxicity and deterrency (Phillips and Croteau, 1999). After exposure to the atmosphere, the volatile mono- and sesquiterpenes evaporate, while the diterpene acids polymerize sealing the wound.

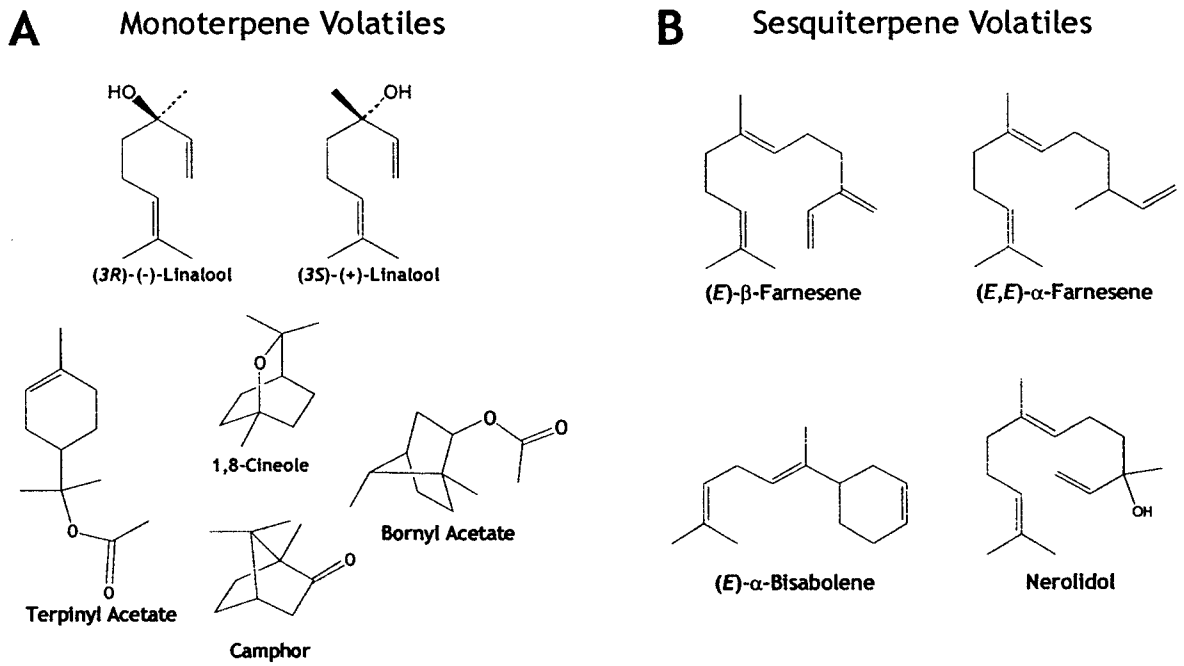

Figure 4.1: Structures of monoterpenes (A) and sesquiterpenes (B) emitted by MeJA-treated Norway spruce saplings.

Oleoresin terpenes are products of constitutive and inducible pathways that have been characterized in some detail at the biochemical and molecular level in grand fir (*Abies grandis*) (Lewinsohn et al., 1991a; Steele et al., 1998b; Bohlmann et al., 1998a; Bohlmann and Croteau, 1999) and in Norway spruce (*Picea abies*) (Martin et al., 2002; Fäldt et al., 2004; Fischbach et al., 2000). Much of this work has focused on the terpene synthases (TPS), which catalyze the formation of the basic terpene carbon skeletons from acyclic phosphorylated intermediates (Bohlmann et al., 1998b). These enzymes convert geranyl diphosphate (GDP,

C_{10}), farnesyl diphosphate (FDP, C_{15}) and geranylgeranyl diphosphate (GGDP, C_{20}) to monoterpenes, sesquiterpenes and diterpenes, respectively.

Research on conifer oleoresin biosynthesis to date has focused primarily on induced resin biosynthesis in the stem tissues. For example, it was shown recently that treatment of Norway spruce with methyl jasmonate (MeJA) induces a complex, traumatic oleoresin response including de novo differentiation of traumatic resin ducts in the developing xylem, increased accumulation of monoterpenes and diterpenes, and induced enzyme activities and gene expression of monoterpene synthases and diterpene synthases (Martin et al., 2002; Fäldt et al., 2004). In this regard, MeJA-induced resinosis in Norway spruce stems closely resembles responses to stem boring insects and pathogens in conifers (Alfaro et al., 2002; Franceschi, 2002; Martin et al., 2002).

In Norway spruce and other conifers, terpenes are accumulated not only in stems, but also in needles (Schönwitz et al., 1987; Schönwitz et al., 1990a; Persson et al., 1996). However, much less is known about the biochemical regulation of terpene formation and accumulation in conifer needles. Simulated herbivory on needles was shown to increase monoterpene synthase activities in the foliage of different conifer species, but had no effect on monoterpene accumulation (Litvak and Monson, 1998; Fischbach et al., 2000). In Norway spruce, monoterpene synthase activities have been characterized in the needles (Litvak and Monson, 1998; Fischbach et al., 2000), and a gene encoding (+)-3-carene synthase was cloned from a cDNA library prepared from young shoots and foliage (Fäldt et al., 2003).

Besides their storage of terpenes, conifer needles also release a profusion of volatile terpenes into the environment, a topic studied by numerous researchers seeking to inventory the volatile organic compounds of the atmosphere (Kesselmeier and Staudt, 1999). Emission is influenced by physical factors, such as light and temperature, as well as biotic stress. Terpenes are also emitted from the foliage of a variety of angiosperm species as well, and much recent research has focused on emission in response to damage by insects or other arthropods. Terpenes serve as airborne signals that deter herbivores, attract predators and parasites of herbivores, or possibly act in plant-to-plant signaling (Dicke and Vet, 1999; Paré and Tumlinson, 1999; Pichersky and Gershenzon, 2002; Kessler and Baldwin, 2002; Turlings et al., 1990) Herbivore-induced volatiles from gymnosperm foliage have also been reported. Egg deposition by the pine sawfly, *Diprion pini*, on *Pinus sylvestris* needles triggers the release of volatiles, most likely monoterpenes and sesquiterpenes, which attract an egg parasitoid (Hilker et al., 2002; Seybold et al., 2000). Knowledge of the regulation of volatile terpene formation is essential in understanding the mechanism of these responses and manipulating them in ecological studies.

The present study examines the biochemical regulation of terpene formation, accumulation and release in the needles of Norway spruce. Based on the success of previous work with stems of this species (Martin et al., 2002), MeJA was chosen as a means to induce terpene-based responses. Unlike mechanical wounding which has been used in several previous studies to trigger defense responses in conifers (Litvak and Monson, 1998; Steele et al., 1998b), MeJA does not inflict physical damage on terpene storage sites and does not destroy the tissues that are potentially involved in terpene biosynthesis, terpene accumulation, or terpene emission. This is of particular importance for attempts to characterize volatile emission in conifers that constitutively accumulate large quantities of low molecular weight terpenes in resin canals or resin cells in needles. After application of MeJA, I monitored changes in the accumulation and emission of terpenes and the activity of terpene synthases.

RESULTS

MeJA Induces a Two-fold Increase in Terpene Accumulation in Needles

Based on recent reports that MeJA induces terpene biosynthesis in stem tissues of Norway spruce (Martin et al., 2002; Franceschi, 2002; Fäldt et al., 2004), I examined whether changes in terpene levels also occurred in needles of this species after MeJA treatment. Needles of young saplings (clone IA) harvested over a 25 day time course after treatment showed a transient, two-fold increase in monoterpene and sesquiterpene accumulation compared to needles from untreated control saplings (Figure 4.2A and 4.2B). Maximum accumulation was observed 15 days after MeJA treatment with declines to control levels by 20 days. There was no significant increase in diterpene concentrations in treated saplings compared to untreated saplings over the time course of this experiment (Figure 4.2C). The terpene composition of the needles was also not significantly affected by treatment (Table 4.1). A total of 27 different monoterpenes, sesquiterpenes, and diterpenes were detected and most of them identified, of which the monoterpenes, bornyl acetate, α-pinene, camphene and limonene, and the diterpenes, manool and dehydroabietate, were the most abundant.

MeJA Induces a Five-fold Increase in Terpene Synthase Activity in Needles

To determine if MeJA-induced terpene accumulation was due to de novo biosynthesis, I measured the activities of terpene synthases in cell-free extracts of needles of MeJA-treated and control saplings (clone IA) over a 25-day time course after treatment. While the accumulation of monoterpenes and sesquiterpenes increased two-fold in needles over this period in response to MeJA (Figure 4.2), the activities of monoterpene synthases and sesqui terpene synthases increased over five-fold, reaching a peak at day 10 or 12 and then declining precipitously after day 15 (Figure 4.3). Diterpene synthase activity was not detectable in needle extracts from either treated or control trees, consistent with the lack of induction of diterpene accumulation in needles. The constitutive levels of diterpenes in mature needles (Figure 4.2) could be due to enzyme activity in young needles and their accumulation and longevity in resin ducts.

The Major Terpene Synthase Products are Linalool and E-β-Farnesene

To investigate the discrepancy between the rates of terpene synthase activity and terpene accumulation in needles after MeJA treatment, products of monoterpene synthase assays and sesquiterpene synthase assays were analyzed by GC-mass spectrometry (GC-MS).

Table 4.1: Composition of monoterpenes, sesquiterpenes, and diterpenes of Norway spruce needles after treatment with 10 mM MeJA.

Clone IA 15 days of trees is shown in these comparisons of needles from untreated controls. Data are the means ± SE of triplicate analyses.

Terpene	Control (µg /g dry weight)	MeJA (µg /g dry weight)
MONOTERPENES		
tricyclene	33.8 ± 1.8	64.4 ± 14.8
α-pinene	165.9 ± 10.8	338.0 ± 62.0
camphene	262.7 ± 17.6	542.1 ± 97.8
ß-pinene	36.7 ± 1.0	84.4 ± 17.8
sabinene	0.0 ± 0.0	5.2 ± 3.4
3-carene	16.8 ± 0.2	22.4 ± 5.0
myrcene	26.2 ± 1.6	52.1 ± 9.0
limonene	54.9 ± 3.2	117.9 ± 20.0
ß-phellandrene	2.6 ± 2.6	5.6 ± 3.8
1,8-cineole	34.5 ± 1.2	77.8 ± 14.0
camphor	5.7 ± 3.0	6.1 ± 4.0
bornyl acetate	313.3 ± 27.0	624.3 ± 101.2
camphenilol	58.8 ± 5.6	110.1 ± 19.0
terpinyl acetate	7.4 ± 3.8	16.4 ± 2.4
α-terpineol	14.4 ± 2.0	24.7 ± 3.2
borneol	54.6 ± 10.0	106.1 ± 18.2
piperitone	6.0 ± 3.0	15.4 ± 2.4
total monoterpenes	1094.5 ± 86.4	2213.2 ± 372.6
SESQUITERPENES		
longifolene	3.7 ± 3.8	0 ± 0.0
ß-caryophyllene	21.3 ± 2.8	40.2 ± 7.2
α-humulene	18.0 ± 2.2	34.3 ± 6.0
total sesquiterpenes	43.1 ± 2.0	74.5 ± 6.6
DITERPENES		
manool	663.9 ± 35.8	820.7 ± 140.6
sandaracopimarate	110.4 ± 3.6	140.8 ± 19.9
dehydroabietal	22.6 ± 1.6	31.9 ± 1.1
dehydroabietate	354.0 ± 13.6	546.3 ± 86.3
unknown 1	302.0 ± 12.4	346.4 ± 67.7
unknown 2	209.1 ± 12.9	325.5 ± 27.0
unknown 3	105.0 ± 11.7	136.4 ± 21.0
total diterpenes	1767.0 ± 57.4	2348.0 ± 324.4

Two clonal lines of different genetic background (IA and II) were used for these experiments thereby allowing us to address variation that might exist between these individual lines. In clone IA, the major product (55.4 %) of the monoterpene synthase assays of MeJA-treated needles was the alcohol linalool (Table 4.2), with an enantiomeric ratio of approximately 1:2 (3R : 3S) (Figure 4.1). The terpene hydrocarbons, α-pinene (33.9 %) and β-pinene (10.7 %) were formed as additional products by monoterpene synthase activity in this clone (Table 4.2). Linalool was also the major monoterpene product (72.9 %) in enzyme assays with extracts of MeJA-treated needles of a second, unrelated Norway spruce clone (clone II) (Figure 4.4). In addition, enzyme assays with clone II also produced a number of minor monoterpene products (Figure 4.4) each constituting 5 to 6 % or less of the total product profile. Extracts of needles from control saplings of each clone (IA and II) showed only slight activity and any products formed were below the limits for identification by radio-GC. No detectable linalool synthase activity was present in extracts from control needles.

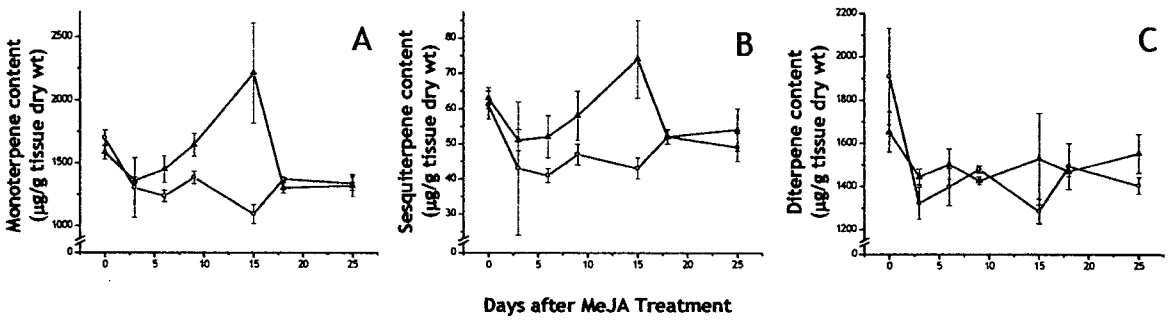

Figure 4.2: Time course of accumulation of monoterpenes (A), sesquiterpenes (B) and diterpenes (C) in needles of Norway spruce saplings (clone IA) after treatment with MeJA.

Data are presented as the means with standard error of triplicate analyses from extracts of treated (▲) and control (○) trees.

The only product of the sesquiterpene synthase assays of MeJA-treated needles of clone IA was *E*-β-farnesene (Table 4.2) as analyzed by radio-GC. Sesquiterpene synthase assays with extracts of MeJA-treated needles from clone II also produced *E*-β-farnesene (17.2 %) as one of four major products. Additional major sesquiterpene synthase enzyme products of clone II were *E,E*-α-farnesene (8.1 %), *E*-α-bisabolene (30.3 %), and nerolidol (33.8 %), together with five minor sesquiterpene hydrocarbons (Figure 4.5). Each of the minor constituents contributed less than 3 % of total sesquiterpene synthase products. Extract of needles from control untreated saplings showed low levels of sesquiterpene synthase enzyme

activity, but product formation was below the limits for identification of individual sesquiterpene synthase products.

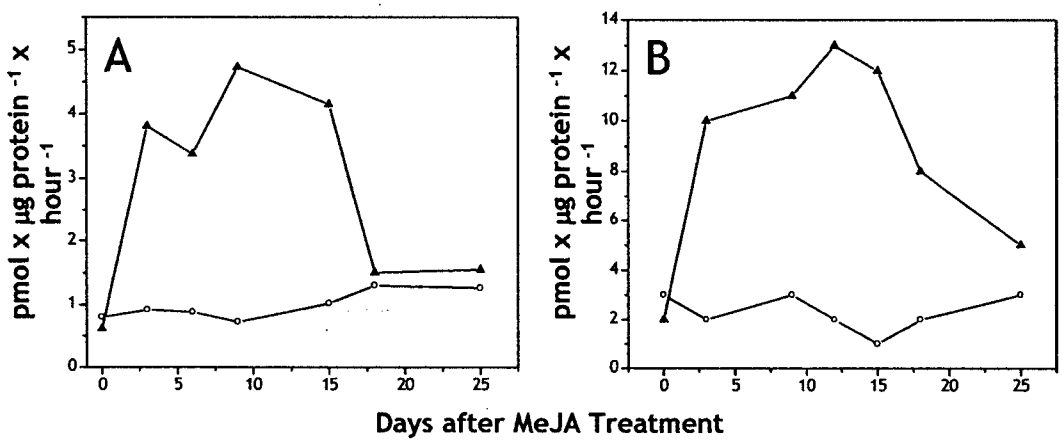

Figure 4.3: Time courses of monoterpene synthase (A) and sesquiterpene synthase (B) activities in the needles of Norway spruce saplings (clone IA) after treatment with MeJA.

Data presented are the averages of duplicate assays from extracts of treated (▲) and control (○) trees. SE are shown for treated tree samples. SE for controls were in the range of 0.0002 pmol x mg protein^{-1} x hour^{-1}.

MeJA Induces Increases in Terpene Emissions and Drastic Changes in the Composition of Terpene Emissions

Because the major products of the induced needle monoterpene synthase and sesquiterpene synthase enzyme activities (Table 4.2) in clone IA were not observed to accumulate in the needles (Table 4.1), I analyzed volatile terpene emissions from Norway spruce saplings after MeJA treatment to access whether significant amounts of these products were released. Because of limited numbers of trees of the identical genetic background, I used a related (similar chemotype) clone IB for volatile analysis. Headspace collections revealed that the rate of terpene emission after MeJA treatment was approximately five-fold that of unsprayed control saplings, 281.4 vs. 54.3 µg per gram needle fresh weight per hour, averaged over a collection period of four days following treatment for two replicates of each treatment with two saplings in each (Figure 4.6).

MeJA treatment also had a dramatic effect on the composition of volatile terpenes emitted by Norway spruce saplings measured at peak emission at around 24 to 30 hours, or approximately one day, after treatment. During this peak emission, composition of volatiles

collected over a period of two hours (26.5 - 28.5 h after treatment) was analyzed (Table 4.3). The rate of sesquiterpene emission was increased approximately 30-fold by MeJA one day after treatment, while that of monoterpenes was increased only 2.2-fold. Hence sesquiterpenes, which made up only 19 % of the terpene emission of untreated saplings, were 77 % of the total terpene at peak emission after MeJA treatment. These compositional changes were largely maintained throughout the time course of up to 7 days. Among all terpenes emitted, the sesquiterpene *E*-ß-farnesene became the most abundant compound after MeJA application (Table 4.3). Its rate of emission increased over 100-fold, accounting for nearly 3/4 of the total sesquiterpenes. Among the monoterpenes, the oxygenated monoterpenes, linalool and 1,8-cineole showed the greatest proportional increases after MeJA

Table 4.2: Products of terpene synthase activities in needles after MeJA treatment.

Assays were performed on pooled extracts harvested from clone IA 3-15 days after treatment and analyzed by radio-GC. Product identification was confirmed by co-chromatography of authentic standards and GC-MS. Assay products with needles from untreated saplings were below the level required for identification of individual components. Products of phosphohydrolase activity seen in controls assays with buffer only are not listed.

Assay Product	% of Total Products
Monoterpene synthase assays	
Linalool	55.4
α-Pinene	33.9
ß-Pinene	10.7
Sesquiterpene synthase assays	
E-ß-Farnesene	100.0

treatment, and came to make up nearly 40 % of the total monoterpenes. The prominence of linalool and *E*-ß-farnesene emission after MeJA treatment in clone IB is consistent with them being the principal products of monoterpene and sesquiterpene synthase assays found in the similar clone IA.

It was surprising to find that the major terpene synthase assay products were volatilized rather than accumulating in the needles. To confirm this unexpected result, I investigated saplings of a second clone (clone II) from a completely different genetic background. After spraying with MeJA, the principal emitted monoterpenes, sampled by SPME, were ß-pinene and linalool (Table 4.4), while the principal sesquiterpenes detected

were *E*-β-farnesene, *E,E*-α-farnesene and *E*-α-bisabolene. The major terpene synthase assay products identified by GC-MS for this clone corresponded closely with the volatiles. Linalool was the dominant product formed in monoterpene synthase assays (Figure 4.4), while *E*-β-farnesene, *E,E*-α-farnesene, *E*-α-bisabolene and *E*-nerolidol were the dominant products formed in sesquiterpene synthase assays (Figure 4.5). Thus, the biosynthetic machinery after

Table 4.3: Composition of monoterpene and sesquiterpenes volatiles emitted from MeJA treated and control trees.

Terpenes were emitted from Norway spruce clone IB and collected over a period of two hours between 26.5 and 28.5 hours after treatment of trees with 10 mM MeJA in comparison to emission from untreated control saplings.

Terpene	Control	MeJA
MONOTERPENES	ng / hour / g needles fresh weight	
α-pinene	106.6	166.7
camphene	28.1	40.7
β-pinene	175.2	283.4
sabinene	14.4	23.3
3-carene	16.0	8.4
myrcene	40.6	51.9
limonene	37.3	30.4
β-phellandrene	31.2	18.1
1,8-cineole	20.4	101.6
γ-terpinene	0.0	17.4
linalool	6.5	313.6
bornyl acetate	0.0	3.4
terpinen-4-ol	0.0	4.1
α-terpinyl acetate	4.4	4.6
α-terpineol	6.0	19.7
borneol	6.9	7.3
other monoterpenes	8.3	26.0
Total monoterpenes	502.0	1120.7
SESQUITERPENES		
Z-α-Bergamotene	0.0	10.5
E-β-Farnesene	26.7	2653.4
Z,E-α-Farnesene	0.0	12.6
E,E-α-Farnesene	29.4	34.2
E-α-Bisabolene	62.9	972.3
Other sesquiterpenes	0.0	10.5
Total sesquiterpenes	118.9	3693.5

Table 4.4: Relative composition of terpene headspace volatiles emitted from saplings of Norway spruce (clone II) at different time points after treatment with 10 mM MeJA.

Compound	% Total Terpenes		
	5.5 h after treatment	8.5 h after treatment	32.5 h after treatment
α-pinene	0	0	0
camphene	0	0	2.1
β-pinene	0	2.8	5.5
β-phellandrene	0	0	0.9
1,8-cineole	0	0	2.0
linalool	0	0	6.4
camphor	13.7	2.1	0.8
α-terpineol	0	0	1.1
bornyl acetate	86.3	5.8	2.1
E-β-farnesene	0	38.1	26.0
Z,E-α-farnesene	0	0	1.4
E,E-α-farnesene	0	34.1	26.4
E-α-bisabolene	0	17.1	23.1
nerolidol	0	0.0	2.1

MeJA induction appears to be devoted principally to the formation of emitted compounds as was shown with two independent Norway spruce clones.

Induced Terpene Emission Follows a Diurnal Rhythm

Over a time course of 7 days after MeJA treatment, the emission of induced volatiles of clone IB followed a day-night cycle with increased release rates during the light period and lower emission during the dark period (Figure 4.7). Maximum levels of monoterpene and sesquiterpene emission were detected during the light period approximately 24 or 48 h after MeJA treatment (Figure 4.7). Over the rest of the time course, the, amplitude of the daytime maximum slowly declined. The more strongly-induced classes of terpenes, the oxygenated monoterpenes and sesquiterpenes, followed a much more pronounced diurnal rhythm than the monoterpene olefins. When rhythmic emission profiles of individual oxygenated monoterpenes and sesquiterpenes were monitored, I found that few compounds such as terpinen-4-ol peaked very early within the first 12 h after treatment and then diminished rapidly during subsequent light periods, while other compounds such as linalool, 1,8-cineole, and *E*-β-farnesene were elevated during the light periods over the entire time course. In

addition to the terpenes, methyl salicylate (MeSA) was also found to be released after MeJA treatment in a diurnal rhythm at rates of less than 20 ng per hour per g needle fresh weight (Figure 4.7D). This compound was not detected in most measurements with control saplings.

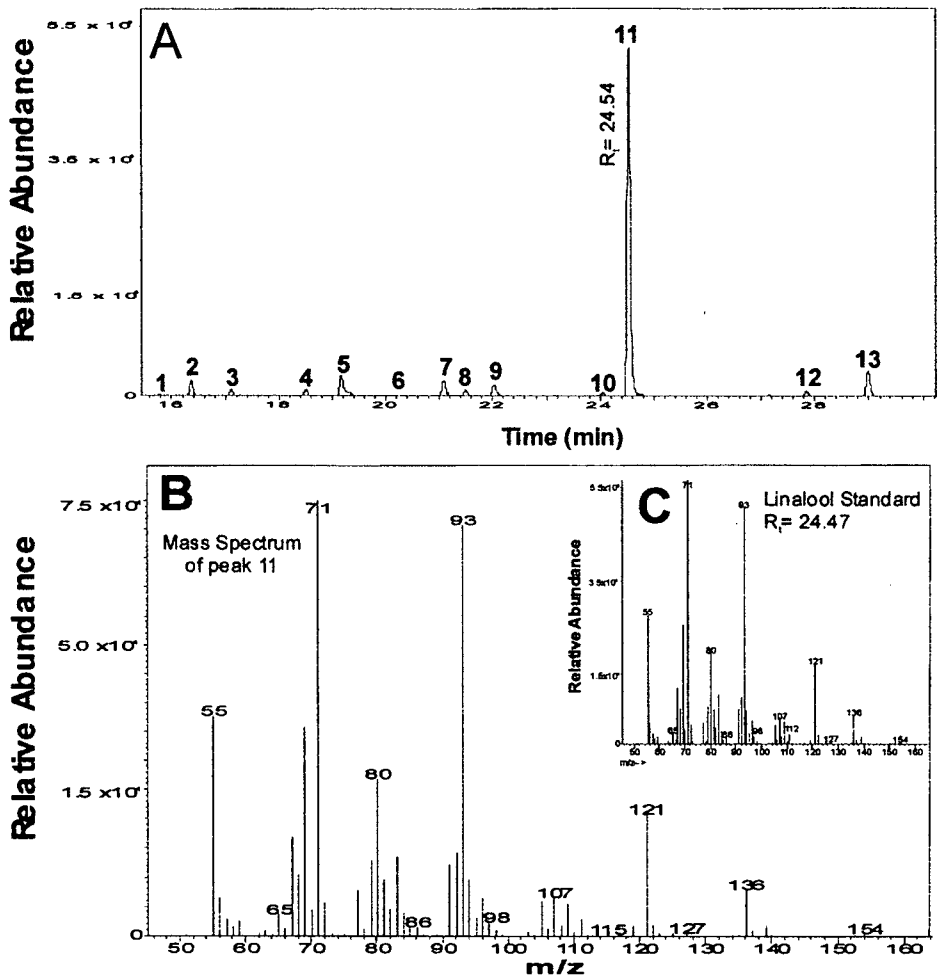

Figure 4.4: Identification of linalool as the major product of induced monoterpene synthase activity in clone II. Enzyme activity was assayed in extracts of needles harvested 30 h after MeJA treatment.

GC-MS total ion chromatograph of monoterpene synthase assay products (A) showing 13 monoterpene products (1) tricyclene (0.2%), (2) α-pinene (3.3%), (3) camphene (1.3%), (4) β-pinene (1.3%), (5) myrcene (5%), (6) 3-carene (0.2%), (7) limonene (3.6%), (8) *E*-β-ocimene (1.2%), (9) *Z*- β-ocimene (3%), (10) γ-terpinene (0.7%), (11) linalool (72.9%), (12) borneol (1.1%), and (13) α-terpineol (6.1%). Retention time and mass spectrum of peak 11 (B) match that of an authentic linalool standard (C).

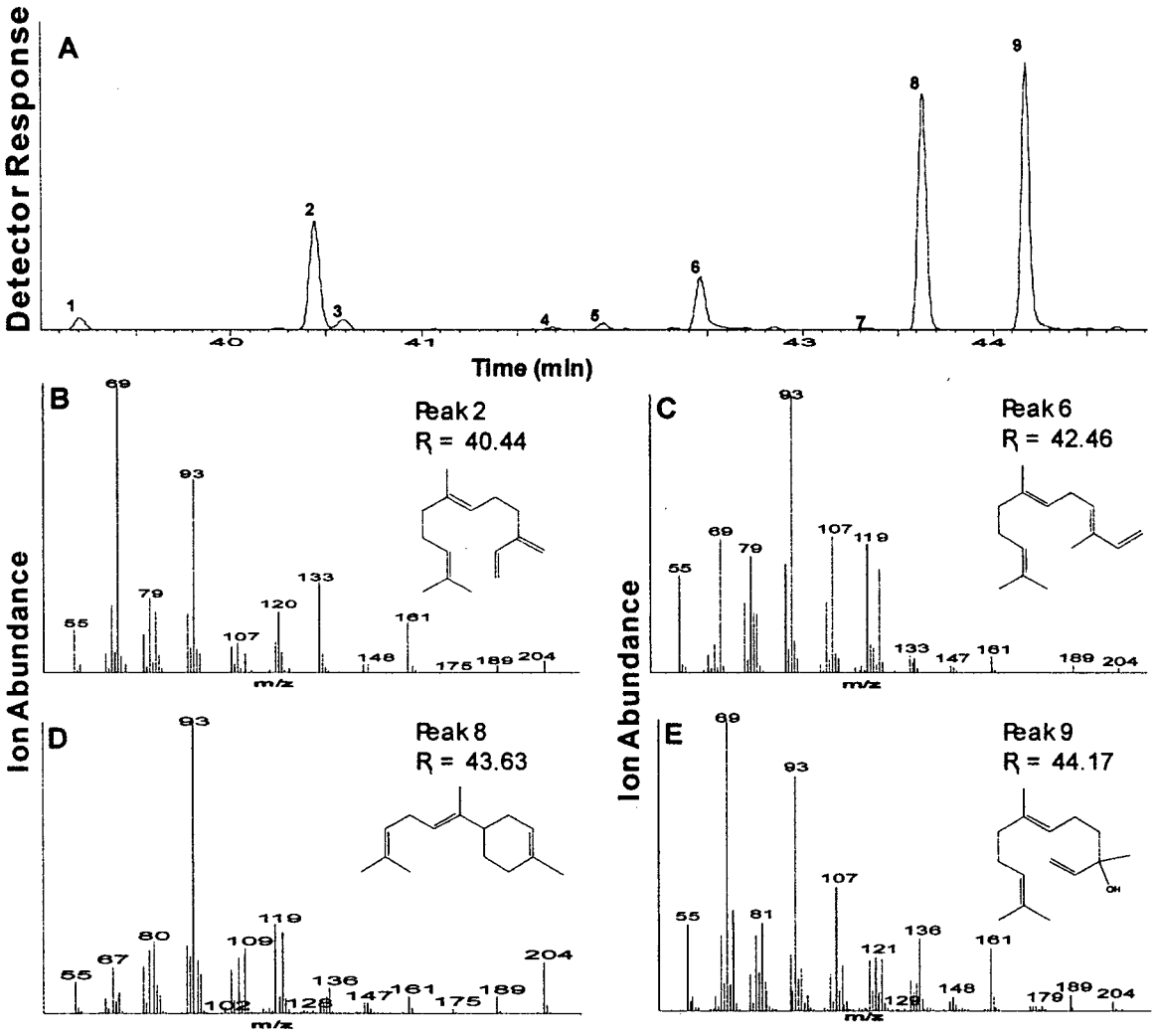

Figure 4.5: Identification of major products of induced sesquiterpene synthase activity in clone II.

Enzyme activity was assayed in extracts of needles harvested 30 h after MeJA treatment. GC-MS total ion chromatograph showing nine sesquiterpenes produced (A). Major products (peaks 2, 6, 8 and 9) were identified by comparison of retention time and mass spectra with authentic standards as *E*-α-farnesene (17.2%) (B), *E*,*E*-α-farnesene (8.1%) (C), *E*-α-bisabolene (30.3%) (D), and nerolidol (33.8%) (E).

DISCUSSION

Induced Terpene Accumulation in Needles is Much Lower Than in Wood

MeJA treatment of Norway spruce had profound effects on the synthesis, accumulation and emission of terpenes in foliage that were quite different from the effects of MeJA on the synthesis and accumulation of terpenes in the stems of this species. The increases in terpene accumulation in foliage were much less dramatic than in stems. In saplings sprayed with a 10 mM solution of MeJA, the wood tissue of the stems showed a 12-fold increase in monoterpene concentration and a nearly 40-fold increase in diterpene concentration, peaking 15-25 days after treatment, but no change in sesquiterpene levels (Martin et al., 2002). In contrast, the same concentration of MeJA caused only a 2-fold increase in monoterpenes and sesquiterpenes in needles and no increase in diterpenes, when measured 15 days after treatment. The increases in needles were of the same general magnitude as those seen in stem bark upon MeJA treatment (Martin et al., 2002). The large differences in the relative response of these organs can be attributed to the fact that induced terpene accumulation in wood tissue occurs in association with newly-formed traumatic resin canals in the xylem (Franceschi, 2002; Martin et al., 2002). Bark and needles also possess resin canals, but *de novo* formation of these structures after treatment with MeJA has not previously been observed. Thus, these tissues do not have storage space for large quantities of additional terpene oleoresin.

The relatively small increase in terpene accumulation in needles was not accompanied by any significant changes in terpene composition, unlike in stems. The major needle monoterpenes detected in this study were similar to those previously reported for Norway spruce (Holubova et al., 2000; Paule and Yazdani, 1992; Persson et al., 1993; Persson et al., 1996; Schönwitz et al., 1987) and correspond to compositional type 1b (high limonene, low myrcene, high bornyl acetate) as described by Schönwitz et al. (1990a). The elevated levels of monoterpenes and sesquiterpenes following MeJA spraying peaked 15 days after treatment, but declined to control levels rapidly after that, probably as a result of volatilization or catabolism. Seasonal declines of monoterpene levels in Norway spruce foliage have been previously noted (Schönwitz et al., 1990b).

Induced Terpene Synthase Activity in Needles Reflects Terpene Volatilization, Not Accumulation

Monoterpene and sesquiterpene synthase activities in Norway spruce foliage jumped five-fold after MeJA treatment. Increases in monoterpene synthase activity of a similar magnitude (up to 4-fold) have been measured in the foliage of other conifer species after real or simulated herbivory (Litvak and Monson, 1998). On the other hand, in conifer stems, much greater increases in induced monoterpene and diterpene synthase activity were recorded: 5 to 25-fold increases in monoterpene synthase activity in grand fir after mechanical wounding or fungal inoculation (Funk and Croteau, 1994; Lewinsohn et al., 1991a; Lewinsohn et al., 1993b), a ~400-fold increase in diterpene synthase activity in grand fir after mechanical wounding (Funk and Croteau, 1994), 20 to 30-fold increases in monoterpene and diterpene synthase activities in Norway spruce after MeJA treatment (Martin et al., 2002). However, only low increases in sesquiterpene synthase activities have been recorded in stems of grand fir, in keeping with the minor occurrence of this class in stem tissue (Steele et al., 1998b).

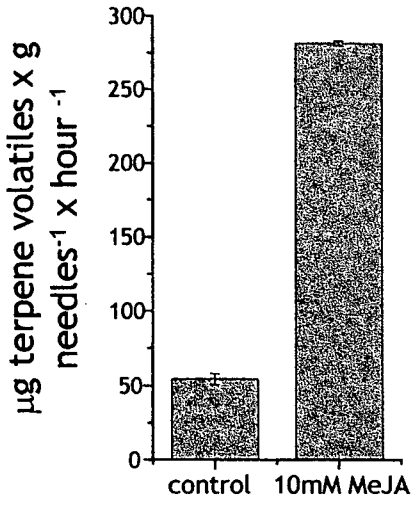

Figure 4.6: Emission rates of total monoterpenes and sesquiterpenes from Norway spruce in control and MeJA-treated trees.
Total volatiles were measured over a period of 93 h from two independent sets of treated and control trees of clone IB. Data represented are the mean quantities of volatiles emitted per hour and gram fresh weight needles. Standard error bars are shown.

Although the 5-fold increases in monoterpene and sesquiterpene synthase activities in Norway spruce foliage following MeJA treatment is sufficient to account for the 2-fold increase in monoterpene and sesquiterpene accumulation, the major products of MeJA-induced terpene synthase activity in Norway spruce needles (linalool and *E*-β-farnesene in clone IA; linalool, *E*-β-farnesene, *E,E*-α-farnesene and *E*-α-bisabolene in clone II) did not match the monoterpenes or sesquiterpenes accumulating in these organs. Instead, they correspond well to the major terpene volatiles emitted from needles of these plants after MeJA treatment. Treatment with MeJA led to a 5-fold increase in monoterpene and sesquiterpene emission and to a drastic shift in the composition of emitted terpenes, from predominantly monoterpenes to

predominantly sesquiterpenes. Nearly all of the major terpenes emitted after induction, including the monoterpene linalool, and the sesquiterpenes, *E*-β-farnesene, *E,E*-α-farnesene (clone II only) and *E*-α-bisabolene, were released only in trace amounts before MeJA treatment. The emission of volatile monoterpenes from Norway spruce has been documented in many previous studies (Kempf et al., 1996; Kesselmeier and Staudt, 1999; Janson, 1993), but prior reports of sesquiterpene emission come only from other conifer species, such as Scots pine (Heiden et al., 1999).

Terpenes Emitted After Induction are Synthesized De Novo and Released in a Diurnal Rhythm

Two lines of evidence indicate that the terpenes emitted in abundance after MeJA treatment are products of *de novo* synthesis and not released from storage reservoirs. First, these substances represent the principal products of induced terpene synthase activities in needles present after treatment and, second, they are nearly completely absent from stored needle oleoresin. This conclusion is corroborated by $^{13}CO_2$ feeding experiments with Norway spruce which showed that volatilized terpenes were rapidly labeled from newly assimilated photosynthate (Kesselmeier and Staudt, 1999; Schurmann et al., 1993).Several previous studies of terpene volatilization also attempted to distinguish between volatilization from preformed pools of resin terpenes and de novo synthesis and release. In some cases, both processes may be occurring. For example, in grand fir, volatilization of monoterpenes after stem wounding was accompanied by increased monoterpene synthase activity suggesting *de novo* synthesis (Lewinsohn et al., 1993b). However, unlike in Norway spruce, the composition of volatile monoterpenes was similar to that of the accumulated oleoresin, suggesting emission from stored pools. In addition, after wounding the monoterpene content of stems declined with respect to unwounded controls. In several other conifer species wounding of needles also reduced the pool of accumulated terpenes while increasing both monoterpene emission and monoterpene synthase activity (Litvak and Monson, 1998). In such cases, terpene synthesis may help replace compounds lost by wounding. However, in the present study, no net loss of terpenes from storage pools of needles was observed after induction, but rather a two-fold increase could be detected due to the use of MeJA that avoided inflicting physical damage on terpene storage sites. With MeJA treatment and radio-gas chromatography to identify terpene synthase assay products, the induced terpene synthase

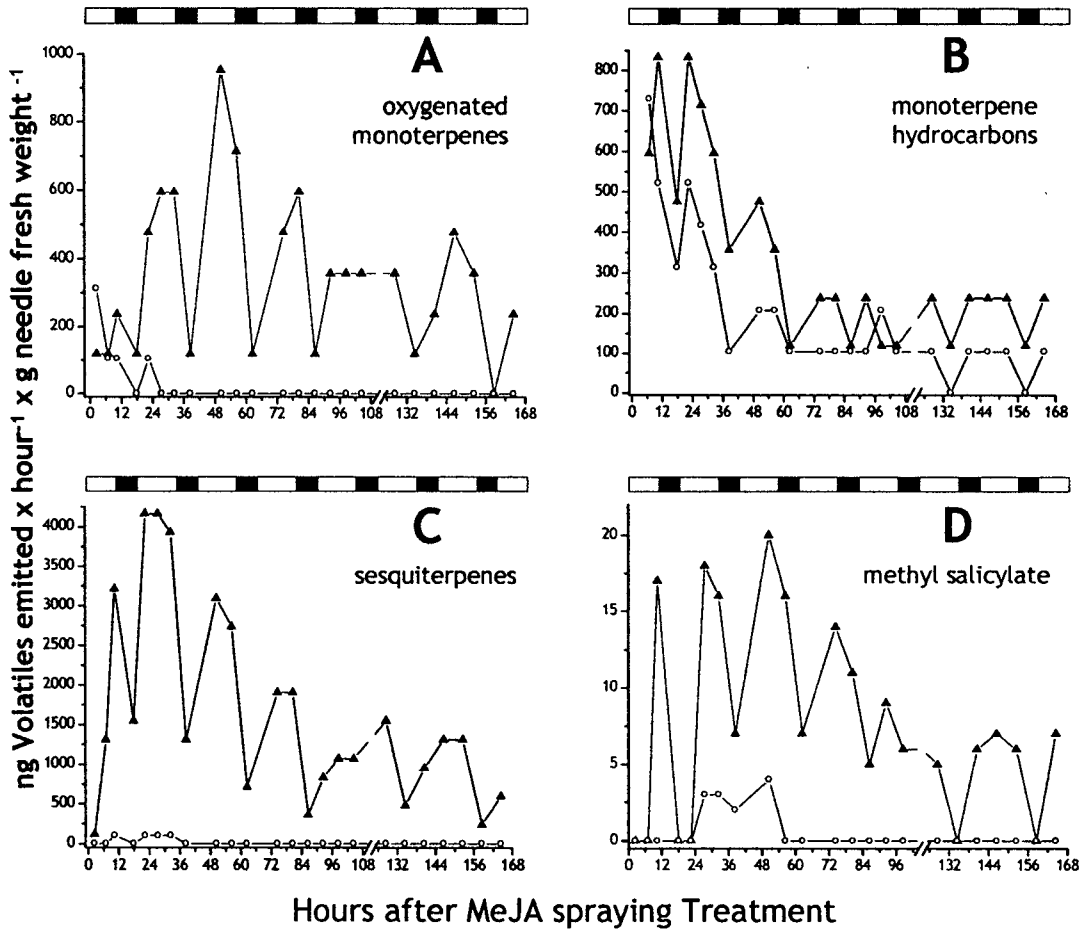

Figure 4.7: Time course of diurnal terpene emissions.

Oxygenated monoterpenes (A), monoterpene hydrocarbons (B), sesquiterpenes (C), and methyl salicylate (D) from Norway spruce saplings (clone IB) either treated with MeJA (▲) and or untreated (o). Volatiles were analyzed by GC-MS. Periods of light (white) and dark (hatched) phases are illustrated above each graph.

activity could be unequivocally associated not with the formation of terpenes accumulating in needles, but with *de novo* synthesis of the induced sesquiterpene-rich mixture of terpene volatiles. Emission of such mixtures may also occur in other conifers after wounding or other stresses, but sesquiterpene emission has not always been measured in previous studies of terpene volatilization. The biosynthetic machinery for *de novo* volatile formation may be localized separately from the sites of accumulated oleoresin formation in needles, which are thought to be associated with specialized resin cells or the epithelial cells of resin canals (Bouvier et al., 2000).

The volatilization of terpenes from Norway spruce saplings in our study followed a diurnal cycle, with total emission greater during the light periods than the dark periods regardless of treatment. Similar diurnal rhythms have been reported in previous studies of Norway spruce monoterpene emission (Bufler and Wegmann, 1991; Kesselmeier and Staudt, 1999; Janson, 1993), with peak nocturnal release only 50-75% as great as that at midday. Curiously, Iobserved much stronger rhythmic patterns of emissions for the two classes of highly induced compounds, sesquiterpenes and oxygenated monoterpenes, than for monoterpene olefins. Only very low amounts of sesquiterpenes and oxygenated monoterpenes were released during the dark period. For oxygenated monoterpenes, there is a precedent for this behavior. In stone pine (*Pinus pinea*), emission of the oxygenated monoterpenes, linalool and 1,8-cineole along with the olefin *E*-β-ocimene, occurred only in daylight, while emission of other monoterpene olefins occurred during both day and night periods (Staudt et al., 2000). Emission of oxygenated terpenes may be closely correlated with stomatal opening because of their relatively low gas/liquid phase partition coefficients (Niinemets et al., 2002).

The Biological Functions of Terpene Emission are Unknown

The emission of volatile terpenes by Norway spruce seems likely to have an important physiological or ecological function in this species since the rate of release after MeJA treatment is five-fold higher than before treatment with a vastly different, composition dominated by sesquiterpene and oxygenated monoterpenes and a strong diurnal rhythm. The major components of the volatilized blend, linalool, *E*-β-farnesene, *E,E*-α-farnesene and *E*-α-bisabolene, are commonly reported as volatiles from other plant species after herbivore damage where they serve to attract predators or parasitoids of the herbivores or repel herbivores directly (De Moraes et al., 2001; Hern and Dorn, 2001; Kessler and Baldwin, 2001; Paré and Tumlinson, 1997; Pichersky and Gershenzon, 2002). Similar roles can be envisioned for these components in Norway spruce. The release of herbivore-induced monoterpenes or sesquiterpenes from other species shares additional characteristics with the emission described in this study. It is often triggered by treatment with JA or MeJA (Dicke et al., 1999; Gols et al., 1999; Halitschke et al., 2000; Hopke et al., 1994; Kessler and Baldwin, 2001; Koch et al., 1999; Rodriguez-Saona et al., 2001; Schmelz et al., 2001), and found to occur with a distinct diurnal rhythm (De Moraes et al., 2001; Loughrin et al., 1994). Moreover, methyl salicylate, a non-terpenoid detected as a volatile in this study, has also been found to be

released after herbivore damage in other species, where it functions in the attraction of herbivore enemies (Dicke et al., 1999; Kessler and Baldwin, 2001; Ozawa et al., 2000).

In addition to (or instead of) an ecological role, the terpene volatiles of Norway spruce could have a physiological function that is fulfilled inside the plant prior to emission. Other plant terpene volatiles, such as the simple C_5 terpene, isoprene, and various monoterpenes, are thought to have internal roles also, either serving to increase the thermal tolerance of photosynthesis (Sharkey et al., 2001; Delfine et al., 2000), or protecting against oxidative damage (Loreto and Velikova, 2001). In order to learn more about the roles of the volatile as well as the non-volatile terpenes of Norway spruce, we are continuing to study the regulation of terpene biosynthesis in this species using molecular and biochemical approaches.

In summary, I have demonstrated that terpene metabolism in Norway spruce needles responds to MeJA induction very differently than terpene metabolism in the stems of this species. Although needles showed only relatively small increases in terpene accumulation compared to stems, MeJA triggered emission of a novel, blend of volatiles that is rich in sesquiterpenes and linalool, is synthesized *de novo* and released in a diurnal rhythm.

MATERIALS AND METHODS

Plant Materials

Young saplings of three clonal lines of Norway spruce (*Picea abies* Karst) were employed in these investigations. Clones IA (#3166-728) and IB (#273-728) were propagated in 1998 from lateral branches of current and previous year growth at the Niedersächsische Forstliche Versuchsanstalt, Escherode, Germany. These clones represent similar terpenoid chemotypes. Fully regenerated, two-year old, rooted saplings were grown and maintained in growth chambers as previously described (Martin et al., 2002). Two year-old trees of different genetic background, clone II (#924335 L11), were regenerated from somatic embryos obtained from the Canadian Forest Service, Laval, Canada.

Substrates, Standards and Reagents

Reagents were from Sigma-Aldrich (Steinheim, Germany) or Roth (Karlsruhe, Germany). Terpene standards were from Sigma-Aldrich, Roth, Bedoukian Research (Danburry, CT), and Helix Biotech (Richmond, BC) and were of the highest purity available. All solvents were GC grade. The substrates, [1-^{3}H]-geranyl diphosphate (GDP) (20 Ci mol^{-1}) and [1-^{3}H]-geranylgeranyl diphosphate (GGDP), were from Biotrend (Köln, Germany). [1-^{3}H]-Farnesyl diphosphate (FDP) (125 Ci mol^{-1}) was the gift of Rodney Croteau, Washington State University, Pullman. Unlabeled GDP and FDP were from Echelon Research Laboratories Inc. (Salt Lake City, Utah).

MeJA Treatment of Saplings and Harvest of Tissues

Saplings were sprayed with a 10 mM solution of MeJA (95% pure [w/w], Sigma-Aldrich) in distilled water as described previously (Martin et al., 2002). Saplings of approximately 40-50 cm in height with 2 whorls of branches were sprayed in a ventilated fume hood with 150 mL of 10 mM MeJA (95% pure [w/w], Sigma-Aldrich) in distilled water as described previously (Martin et al., 2002). Spraying was carried out over a period of 30 min to obtain a complete and even coating after which plants were left in a fume hood for 1 to 2 h until foliage was dry. Control saplings were sprayed with water. For measurements of terpene content and terpene synthase activities, four MeJA-treated saplings and four control saplings of clone IA were used at each time point. After the stems were cut off just above the ground, needles were removed from the stems and branches, frozen in liquid nitrogen and stored at -80°C.

Combined needles were used to make three replicate extracts for terpene analysis (0.1 g needles per extract) and one extract for terpene synthase assays (5 g needles per extract).

Volatile Collection

Dynamic headspace sampling was carried out with an automated collection system built by Analytical Research Systems (Gainesville, FL, USA) as described (Degenhardt and Gershenzon, 2000). The apparatus was installed in a controlled environment chamber set at 22°C constant temperature, 75% relative humidity, 350 µmol m^{-2} s^{-1} of photosynthetically-active radiation, and a 16 h photoperiod and. For each experiment, a pair of saplings, one control and one MeJA-treated, were placed in separate glass cylinders. Volatiles were collected only from the aerial portions of the saplings which were 40-50 cm high with 2 whorls of branches. The lower 2 cm of stem as well as pots, soil and roots were excluded from the volatile collection cylinder using a separation plate that closed loosely around the stem (see Röse et al. (1996) for a detailed illustration of the system). A stream of purified moistened air (50% relative humidity) was passed over each plant at a rate of 5 L min^{-1}. While the majority of the air passed through the plate opening around the stem thereby preventing influx of ambient air, a portion exited through one of a series of eight glass sampling tubes, arrayed around the base of the glass cylinder, each containing 75 mg Super Q (80/100 mesh). Collection was controlled with an automated port control system that drew efflux air for 2 hours at a rate of 1 L min^{-1} through individual glass sampling tubes and then switched to another tube four times in a 24 h period over the 165.5 h collection time course. Volatiles were eluted from sampling tubes with 0.5 mL dichloromethane containing 12.5 µg/mL isobutylbenzene as internal standard and were analyzed by GC-MS.

The time course was repeated three times with a separate pair of treated and control saplings each time with similar results. Results of two experiments are shown in figure 4 and one experiment is shown in detail in table 3. Plants of another clone (IB) were used because of insufficient material of clone IA was available. These two clones had nearly identical terpene profiles in their needles. For comparison with IB, an additional clone (II) was treated with MeJA and its volatiles monitored by solid phase microextraction (SPME) analysis over a 4-day period after treatment. Saplings were enclosed in an oven bag (Look™Terinex Ltd Bedford,United Kingdom) for a 30 min equilibration period. SPME fibers were inserted into the bag, left for 1 h and then analyzed immediately by GC-MS.

Extraction of Terpenes from Needles

Extraction of terpenes was essentially as previously described (Martin et al., 2002) based on a protocol by Lewinsohn et al. (1993). All steps of this procedure were carried out in 2 mL vials (glass with a teflon-coated screw cap, Hewlett-Packard, Palo Alto, CA). Needle samples of approximately 0.1 g (dry weight) were submerged into 1.5 mL tert-butyl methyl ether in a 2 mL vial containing 60 $\mu g\ mL^{-1}$ isobutylbenzene as an internal standard. Needles were extracted over 14 h with constant shaking at room temperature, and the extract was transferred to a fresh vial and washed with 0.3 mL of 0.1 M $(NH_4)_2CO_3$ (pH 8.0) to remove acidic impurities. The ether layer was then prepared for GC or GC-MS analysis by filtering through a Pasteur pipette column filled with 0.3 g silica gel (Sigma 60 Å) overlaid with 0.2 g anhydrous $MgSO_4$. The column was then eluted with 1 mL of diethyl ether to release bound oxygenated terpenes and both eluates were combined in a fresh vial. Finally, the sample was evaporated to an approximate volume of 100 µL and was stored at -20°C. The dry weights of each extracted tissue were determined after drying at 70°C for 20 h. Means and standard errors were calculated from three replicate extracts per time point per treatment.

Analysis of Monoterpenes and Sesquiterpenes

Super Q eluates from automated volatile collection and ethereal extracts from needles were analyzed using a Hewlett-Packard 6890 GC-MS system (70eV), with a DB-WAX column (0.25 mm x 0.25 µm x 30 m J&W Scientific). Split injections (1µL) were made at a ratio of 5:1 with an injector temperature of 220°C. The instrument was programmed from initial temperature of 40°C (3min hold) and increased at a rate of 1.5 °C min^{-1} until 45°C, 3°C min^{-1} until 80°C, 5°C min^{-1} until 180°C, followed by an additional ramp of 10°C min^{-1} up to 240°C (5min hold). Helium was used at a constant flow of 1 mL min^{-1}. For routine quantification, monoterpene and sesquiterpene analysis of ethereal extracts was also accomplished with a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID) fitted with a DB-WAX column as described above. The flow rate was 2 mL H_2 min^{-1} and the FID was operated at 300°C. One microliter of extract was introduced into the injection port at 220°C and a split ratio of 5:1. The GC was programmed with an initial oven temperature of 40°C (3min hold), a ramp of 3°C min^{-1} until 80°C, then a ramp of 5°C min^{-1} until 180°C, followed by a final ramp of 15°C min^{-1} until 240°C (5min hold).

For SPME analysis, the fiber was inserted directly into the injection port (200°C) of a GC-MS (6890 Agilent GC equipped with a 5973N quadrapole, 70eV), the program was

immediately started, and the SPME fiber was removed after a period of 45 sec. The GC was fitted with an HP-5 column (0.25 mm x 0.25 μm x 30 m, Hewlett Packard) and the He carrier gas flowed at 0.7 mL min^{-1}. The program had an initial oven temperature of 40°C (2 min hold), a ramp of 3°C min^{-1} until 160°C, then a ramp of 10°C min^{-1} until 200°C, followed by a final ramp of 20°C min^{-1} until 300°C (5 min hold). For analysis of chiral compounds a Cyclodex-B (0.25 mm x 0.25 μm x 30 m, J & W Scientific) column was used with 1μL splitless injection. The oven was programmed with an initial temperature of 40°C (2 min hold), a ramp of and 1°C min^{-1} until 80°C, a ramp of 5°C min^{-1} until 120°C, and a final ramp of 20°C min^{-1} until 200°C (5 min hold).

GC-FID- and GC-MS-generated peaks were integrated using Hewlett-Packard Chemstation software. Concentrations of monoterpenes and sesquiterpenes were calculated by comparing the integrated peak area to that of the internal standard isobutylbenzene. Identification of terpenes was based on comparison of retention times and mass spectra with authentic standards or with mass spectra in the Wiley library.

Enzyme Extraction

Terpene synthases were extracted from needles essentially as previously described (Martin et al., 2002). Using an analytical grinding mill (A10, IKA WORKS, Cincinnati) or a mortar and pestle, 5g of needles was ground to a fine powder in liquid nitrogen and combined with 50 mL extraction buffer containing50 mM MOPSO [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid] pH 6.8, 5 mM ascorbic acid, 5 mM sodium bisulfite, 5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (w/v) polyvinylpyrrolidone (M_r 10,000), 4 % (w/v) polyvinylpolypyrrolidone, 4 % (w/v) Amberlite XAD-4, and 0.1 % (v/v) Tween 20). The extract was allowed to shake at 4°C for 30 min and were then centrifuged at 10,000 *g* for 30 min. The supernatant was then filtered through two layers of no. 1 filter paper (Whatman, Kent, UK), divided into 4 mL aliquots, frozen in liquid nitrogen and kept at -80°C. Extracts were thawed only once before enzyme assay. Total protein concentration of each protein extract was determined with the Coomassie reagent and protocol of Bio-Rad Laboratories (Hercules, CA, USA).

TPS Enzyme Assays

TPS activities were determined according to published procedures (Lewinsohn et al., 1991b; Lafever et al., 1994; Martin et al., 2002; Bohlmann et al., 1997) with minor modifications. Before assaying enzyme activity, the frozen protein extracts were placed at 37°C until just thawed. The protein extracts were desalted in Bio-Rad Econo PacI0DG sizing columns pre-equilibrated with appropriate assay buffers. The mono-TPS assay buffer was 25 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)], pH 7.5, with 5 mM dithiothreitol, 10 % (v/v) glycerol, 1 mM $MnCl_2$ and 100 mM KCl. The sesqui-TPS assay buffer was 25 mM HEPES, pH 7.3, with 10 mM $MgCl_2$, 10 % (v/v) glycerol, and 10 mM dithiothreitol. The di-TPS assay buffer was 30 mM HEPES, pH 7.2, with 7.5 mM $MgCl_2$, 20 μM $MnCl_2$, 5 % (v/v) glycerol and 5 mM dithiothreitol. Enzyme activity was assessed with 1 mL of the desalted extracts with the addition of 10 μM GDP (with 1 μCi ^{3}H-GDP) for mono-TPS assays, 7 μM FDP (1 μCi ^{3}H-FDP) for sesqui-TPS assays, or 10 μM GGDP (0.5 μCi ^{3}H-GGDP) for di-TPS assays. Monoterpene and sesquiterpene synthase enzyme assays with unlabeled substrates were done with 318 μM GDP or 157 μM FDP. All enzyme assays were done in duplicate, overlaid with 1 mL of pentane to collect released volatiles, and incubated at 30°C for 1.5 h. To stop all enzyme activity, the extracts were immediately frozen. After thawing, the aqueous assay fraction was rapidly extracted with the pentane fraction by vortexing, and separation of the aqueous and organic fractions was achieved by centrifugation at 2,500*g* for 2 min. The 1-mL pentane overlay was removed and filtered through a Pasteur pipette filled with 0.4 g Silica gel (Sigma 60 Å) overlaid with 0.6 g $MgSO_4$ to remove non-specific substrate hydrolysis products and to dry the pentane extract. Each enzyme assay was extracted with an additional two portions of pentane, vortexed and centrifuged as before. These sequential extractions were also passed over the same column and pooled with the initial column eluent. Subsequently, the column was washed with pentane (2 X 1 mL) and the total volume was determined. The extracts were analyzed by liquid scintillation counting, 0.1 mL in 0.3 mL of Lipoluma (J.T. Baker, Deventer, The Netherlands) and GC. Pentane overlays were processed similarly for assays using non-labeled substrates, but the pentane was additionally washed with water to remove small organic acids (Peters et al., 2000).

Conditions for all enzyme assays, including pH optimum, incubation time, substrate concentration, and temperature optimum, were optimized for this system such that maximum activity was achieved in a linear range of product generation. In addition, the possibility that enzyme activities in induced tissues might have been inhibited by additional resin or phenolic substances was ruled out by experiments in which extracts from different stages of the time

course were mixed together. In all cases, the resulting enzyme activity was additive, implying that additional compounds found in induced tissues had no effect on enzyme activity.

Analysis of Terpene Synthase Assay Products

The extracts of enzyme assays were combined and evaporated to approximately 50 μL. From this, 2 μL were analyzed by GC-MS (6890 Agilent GC equipped with a 5973N quadrupole, 70eV). The GC was fitted with an HP-5 column (0.25 mm x 0.25 μm x 30 m, Hewlett Packard), the injection port was operated splitless at 200°C and the He carrier gas flowed at 0.7 ml min^{-1}. Both monoterpene and sesquiterpene synthase assays were analyzed by the same program. The oven temperature was programmed from 40°C (2 min hold) at 3°C min^{-1} until 160°C, then at 10°C min^{-1} until 200°C, followed by 20°C min^{-1} until 300°C (5 min hold). Chiral analysis of the monoterpene synthase assays was also carried out on GC-MS using a Cyclodex-B (0.25 mm x 0.25 μm x 30 m, J & W Scientific) column with 1 μL splitless injections. The oven temperature was programmed from 40°C (2 min hold) at 1°C min^{-1} until 80°C, then at 5°C min^{-1} until 120°C, followed by 20°C min^{-1} until 200°C (5 min hold). Products were identified by comparing retention times and mass spectra to those of authentic standards.

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5. FUNCTIONAL CHARACTERIZATION OF NINE NORWAY SPRUCE *TPS* GENES AND EVOLUTION OF GYMNOSPERM TERPENE SYNTHASES OF THE *TPS-d* SUBFAMILY[7],[8]

ABSTRACT

Constitutive and induced terpenoids are important defense compounds for many plants against potential herbivores and pathogens. In Norway spruce (*Picea abies*, L. Karst), treatment with methyl jasmonate (MeJA) induces complex chemical and biochemical terpenoid defense responses associated with traumatic resin duct development in stems and volatile terpenoid emissions in needles. The cloning of (+)-3-carene synthase was the first step in characterizing this system at the molecular genetic level. Here we report the isolation and functional characterization of nine additional terpene synthase (*TPS*) cDNAs from Norway spruce. These cDNAs encode four monoterpene synthases, myrcene synthase, (–)-limonene synthase, (–)-α/ß-pinene synthase, and (–)-linalool synthase, three sesquiterpene synthases, longifolene synthase, *E,E*-α-farnesene synthase, and *E*-α-bisabolene synthase, and two diterpene synthases, isopimara-7,15-diene synthase and levopimaradiene/abietadiene synthase, each with a unique product profile. To our knowledge, genes encoding isopimara-7,15-diene synthase and longifolene synthase have not been previously described and this linalool synthase is the first described from a gymnosperm. These functionally diverse TPS account for much of the structural diversity of constitutive and MeJA-induced terpenoids in foliage, xylem and bark and volatile emissions from needles of Norway spruce. Phylogenetic analyses based on the inclusion of these TPS into the TPS-d subfamily revealed that functional specialization of conifer TPS occurred before speciation of Pinaceae. Furthermore, based on TPS clades created by distinct branching patterns, the TPS-d subfamily is divided into groups according to sequence similarities and functional assessment. Similarities of TPS evolution in angiosperms and modeling of TPS protein structures are discussed.

[7] The work detailed here is currently in press in *Plant Physiology* (Martin et al., 2004)

[8] The cloning and phylogeny of nine TPS genes examined in this chapter (Martin et al., 2004) was the work of Dr. Jenny Fäldt and of the author. Dr. Jenny Fäldt cloned four of these genes and expressed and identified the product(s) of two. The author was responsible for the cloning of five of these genes, the expression and product identification of seven of these TPS. Furthermore, all the phylogenic analyses presented here are the work of the author. The author's work also involved all of the comparative modeling of TPS detailed in the discussion. Dr. Jörg Bohlmann acted as supervisor for this project. The writing of the manuscript was completed by Dr. Bohlmann and the author.

INTRODUCTION

Terpenoids are the largest class of specialized plant natural products (Croteau et al., 2000), which form an essential part of direct and indirect defense systems against herbivores and pathogens. Terpenoids can function in direct defense as toxins, feeding and oviposition deterrents, or insect hormone analogues. Low molecular weight terpenoids are often released as volatiles from plants during attack by herbivores. These emissions can attract predators and parasites of arthropod herbivores in certain forms of indirect defense (Kessler and Baldwin, 2002). Two classes of terpenoids, the monoterpenoids (10 carbon atoms) and diterpenoids (20 carbon atoms), provide the majority of constitutive and induced oleoresin defense metabolites in conifers (Bohlmann and Croteau, 1999; Seybold et al., 2000; Phillips and Croteau, 1999; Trapp and Croteau, 2001a; Martin et al., 2002) (Figure 5.1). Resin terpenoids are sequestered in specialized anatomical structures, such as resin ducts or resin blisters. Upon damage of resin ducts by stem boring insects, diterpenoids solvated with monoterpenoids exude creating a long-lasting chemical and physical barrier at the site of insect attack. Sesquiterpenoids (15 carbon atoms) comprise a smaller fraction of the oleoresin, yet these compounds are of enormous structural diversity in conifers, exceeding the already impressive diversity of the mono- and diterpenoids (Steele et al., 1998b; Martin et al., 2002). In Norway spruce (*Picea abies*) sesquiterpenoids, along with the monoterpenol linalool, become major constituents of induced volatile emissions following treatment with methyl jasmonate (MeJA) (Martin et al., 2003b). Whether emitted as volatiles or sequestered in resin ducts, conifer mono-, sesqui-, and diterpenoids occur commonly as complex blends. Their quantity as well as the quality and particular enantiomeric composition contributes to biological activities of terpenoid mixtures in protection against potential pathogens and herbivores.

The basic biosynthetic pathways of terpenoids have been elucidated in several plant systems (Croteau et al., 2000). Geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP) are the immediate precursors to mono-, sesqui- and diterpenes, respectively (Figure 5.1). These allylic prenyldiphosphates are utilized by terpenoid synthases (TPS) of three biochemical classes, monoterpene synthases (mono-TPS), sesquiterpene synthases (sesqui-TPS) and diterpene synthases (di-TPS) (Bohlmann et al., 1998b; Davis and Croteau, 2000). TPS form a wide range of structurally diverse cyclic and acyclic mono-, sesqui-, and diterpenes (Figure 5.1). TPS function through divalent metal ion dependent generation of enzyme bound carbocation intermediates (Cane, 1999; Wise and Croteau, 1999; Davis and Croteau, 2000). Various mechanisms of rearrangement and quenching of carbocations yield the large array of different terpenoid product profiles of the

single-product TPS and multiple-product TPS enzymes (Lesburg et al., 1997; Starks et al., 1997; Cane, 1999; Wise and Croteau, 1999; Davis and Croteau, 2000; Whittington et al., 2002). While many terpenoids exist as pairs of enantiomers (Figure 5.1), TPS exhibit preferential catalysis toward one stereoisomer resulting in mostly optically pure product profiles (Davis and Croteau, 2000; Phillips et al., 2003). The exact structural features that determine substrate specificity and the diverse product profiles of TPS enzymes are not known. Thus, biochemical functions of cloned TPS cannot be predicted accurately based on sequence similarity even for closely related enzymes (Bohlmann et al., 1999; Dudareva et al., 2003).

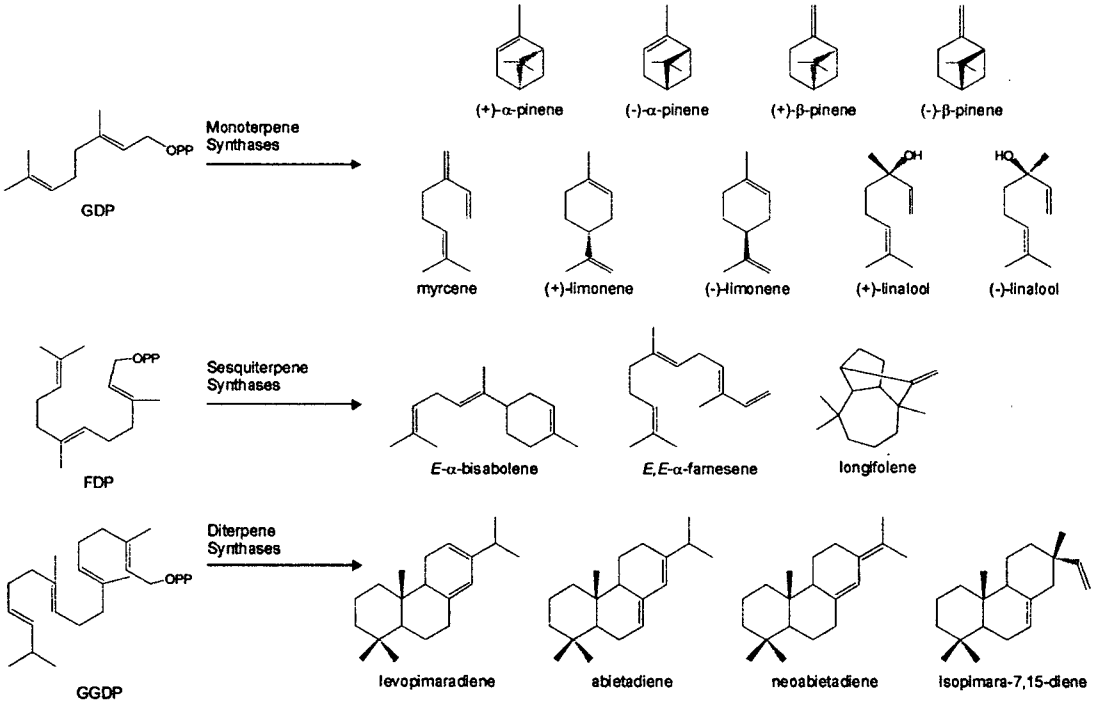

Figure 5.1: Major Norway spruce TPS products showing monoterpenes, sesquiterpenes and diterpenes produced by these enzymes.

Conifers are a rich source for a large array of TPS which have been characterized primarily from one species, grand fir (*Abies grandis*) (Bohlmann and Croteau, 1999). These studies showed that a family of *TPS* genes is central to structural diversity of terpenoid defenses (Stofer Vogel et al., 1996; Bohlmann et al., 1997; Bohlmann et al., 1998a; Steele et al., 1998a; Bohlmann et al., 1999). Recent work in loblolly pine (*Pinus taeda*) provided information about stereospecificity of cloned pinene synthases in this species (Phillips et al., 2003). However, the known grand fir and few loblolly pine *TPS* genes cannot account for all

oleoresin terpenoids in conifers. Nothing is known about *TPS* genes of induced volatile emissions in conifers (Martin et al., 2003b), and only few *TPS* genes of herbivore-induced terpene emission are known in other, angiosperm plants (Arimura et al., 2004; Fäldt et al., 2003a; Shen et al., 2000; Schnee et al., 2002). Species of spruce (*Picea spp.*), which are among the most important conifers for the forest industry, provide a very interesting system for molecular genetic and functional biochemical characterization of TPS and their roles in terpenoid defenses in gymnosperm trees (Alfaro et al., 2002). In Norway spruce (*Picea abies*), treatment with MeJA mimics insect attack in the induction of tissue-specific terpenoid responses. These responses include de novo formation of traumatic resin ducts in lieu of tracheids in the developing stem xylem (Franceschi, 2002; Martin et al., 2002), induced accumulation of terpenoid resin (Martin et al., 2002), and increased enzyme activities and elevated levels of transcripts of *mono-TPS* and *di-TPS* (Martin et al., 2002; Fäldt et al., 2003b). *Mono-TPS* transcripts were similarly induced in Sitka spruce (*Picea sitchensis*) by mechanical wounding or feeding by the spruce weevil (*Pissodes strobi*) (Byun McKay et al., 2003). MeJA treatment also induced a rhythmic, diurnal release of volatile sesquiterpenoids and oxygenated monoterpenoids, along with methyl salicylate, from needles of Norway spruce (Martin et al., 2003b).

Despite increasing knowledge of the anatomical and biochemical processes of induced terpenoid defenses in spruce, detailed molecular and biochemical dissection of these complex defense systems is currently limited by lack of identification and functional characterization of the *TPS* genes involved. Recently, a new *TPS* gene encoding (+)-3-carene synthase was isolated from Norway spruce (Fäldt et al., 2003b) and a (−)-α-pinene synthase gene was identified in Sitka spruce (Byun McKay et al., 2003). However most spruce *TPS* genes, their particular biochemical functions and their contribution to chemical defense remain to be discovered and functionally characterized. Here we describe cDNA cloning, biochemical characterization, and phylogenetic relatedness of nine *TPS* genes from Norway spruce, including four *mono-TPS*, three *sesqui-TPS*, and two *di-TPS*. The products of these TPS enzymes are major components of constitutive and MeJA-inducible terpenoid defenses in Norway spruce. I also present maximum likelihood and distance analyses where the inclusion of the ten Norway spruce *TPS* and five additional gymnosperm *TPS* from the literature made phylogenetic analyses of this subfamily considerably more robust and has provided sufficient evidence that gymnosperm *TPS* also form phylogenetic clusters based on function and sequence similarities.

RESULTS

cDNA Cloning of a Family of Nine TPS Genes from Norway Spruce

We cloned a set of nine *TPS* genes as cDNAs from Norway spruce (Table 5.1). Four of these genes (*PaTPS-Far, PaTPS-Lin, PaTPS-Bis, PaTPS-Pin*) were isolated by cDNA library filter hybridization as described in Fäldt et al. (2003b). Combination of similarity-based PCR strategies previously developed for isolation of conifer *TPS* genes (Bohlmann et al., 1997), mining of expressed sequence tags (ESTs), and RACE cloning strategies enabled the isolation of an additional five *TPS* cDNAs (*PaTPS-Lon, PaTPS-Lim, PaTPS-Iso, PaTPS-LAS, PaTPS-Myr*). Based on overall sequence similarity with known *TPS* genes from grand fir and loblolly pine and the Norway spruce 3-carene synthase, the nine *TPS* cDNAs represented a group of putative mono-TPS, sesqui-TPS and di-TPS enzymes. Pairwise sequence similarities among predicted amino acids of the nine new *TPS* clones and the Norway spruce 3-carene synthase (Fäldt et al., 2003b) are shown in Table 5.2.

Deduced amino acid sequences (Figure 5.2) of the open reading frames (ORF) of four *TPS* candidate genes, *PaTPS-Lim, PaTPS-Myr, PaTPS-Lin, PaTPS-Pin,* resembled most closely known conifer mono-TPS (Bohlmann and Croteau, 1999). Their predicted proteins contain in the range of 623 - 634 amino acids and have predicted isoelectric points (pI) in the range from 5.41 for PaTPS-Myr to 6.51 for PaTPS-Lim. PaTPS-Lin and PaTPS-Pin have very similar pIs at 5.71 and 5.79 respectively. cDNA clones of *PaTPS-Lim, PaTPS-Myr,* and *PaTPS-Lin* were full length and each included sequences for a putative N-terminal transit peptide of 62 - 64 amino acids upstream of a conserved RRX_8W motif for import of mature proteins into plastids, characteristic of mono-TPS (Aubourg et al., 2002; Bohlmann et al., 1998b; Williams et al., 1998). *PaTPS-Pin* is slightly truncated missing a starting methionine. The predicted amino acid sequence of PaTPS-Lim is most similar to β-phellandrene synthase (80% I, 89% S) from grand fir (Bohlmann et al., 1999) among the previously identified *TPS* genes. PaTPS-Myr is most similar to myrcene synthase (72% I, 80% S) from grand fir (Bohlmann et al., 1997). The third mono-TPS of this set, PaTPS-Pin, is most similar to (–)-pinene synthase (90% I, 91% S) from Sitka spruce (Byun McKay et al., 2003). The amino acid sequence of PaTPS-Lin is closest to the previously characterized terpinolene synthase (69% I, 81% S) from grand fir (Bohlmann et al., 1999).

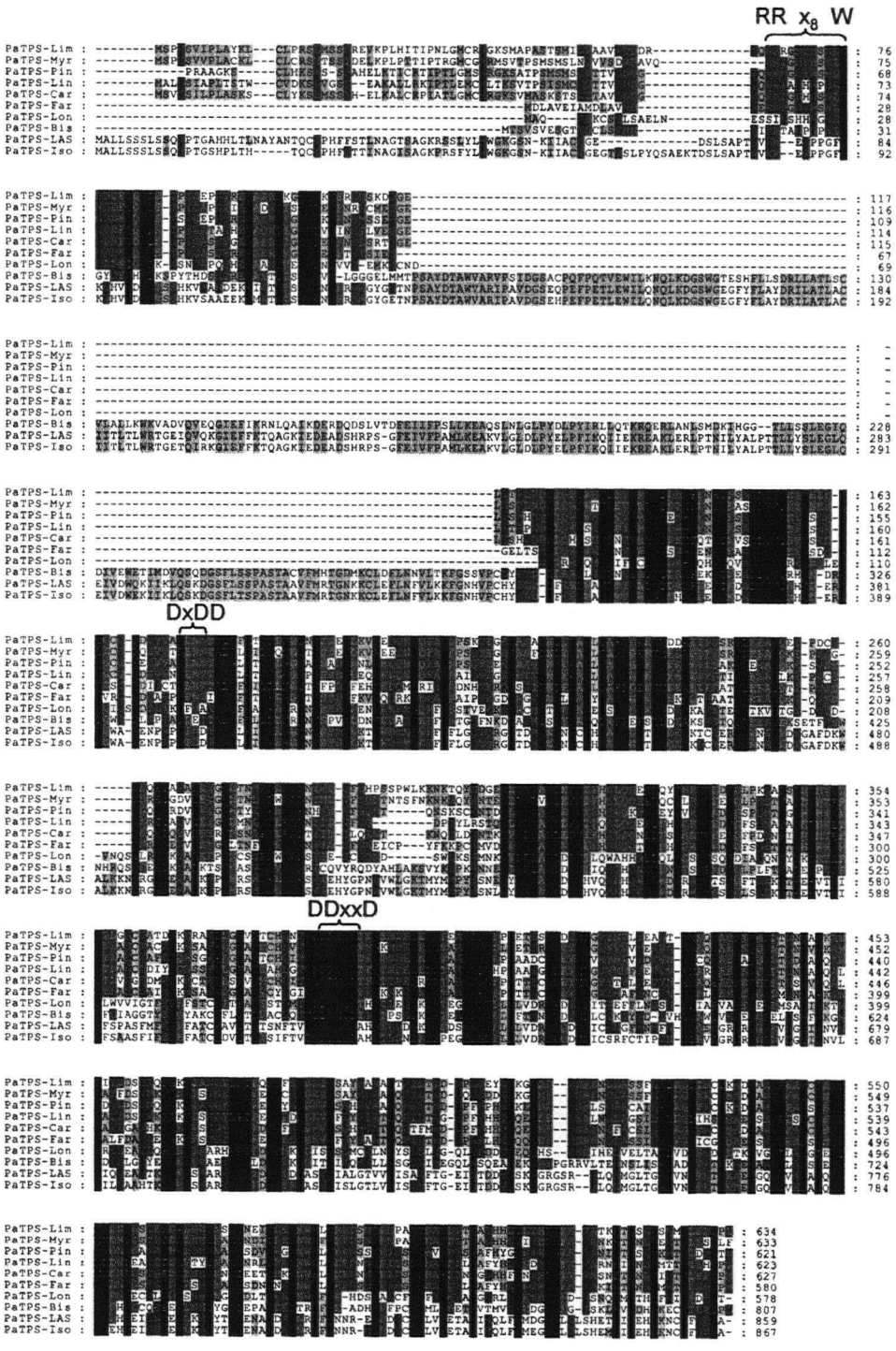

Figure 5.2: Amino acid alignments of Norway spruce (P. abies) mono-TPS, sesqui-TPS, and di-TPS generated by ClustalX and GeneDoc.

The RRX_8W is common to nearly all mono-TPS and is positionally preserved in some sesqui-TPS and di-TPS. Aspartate rich motifs, DxDD for di-TPS and DDxxD for mono-TPS, sesqui-TPS and di-TPS, necessary for the binding of cationic cofactors are shown. The ancestral conifer di-TPS motif of 200-215 amino acids is delineated by dashed/dotted line. Conserved similarity shading is based on 100% (black), 60% (dark grey) and 30% (light grey).

Three Norway spruce TPS clones, PaTPS-Far, PaTPS-Lon, PaTPS-Bis, resemble conifer sesqui-TPS, although they are quite different among each other in sequence similarity and length and represent the three different structural types of known conifer sesqui-TPS enzymes (Bohlmann et al., 1998a; Steele et al., 1998a; Phillips et al., 2003). All three predicted amino acid sequences lack long N-terminal sequences up-stream of the RRX_8W motif (Figure 5.2), consistent with cytsolic localization of plant sequi-TPS, and each had a predicted pI near 5.3. PaTPS-Far and PaTPS-Lon have predicted lengths of 580 and 578 amino acids. PaTPS-Bis is longer at 807 amino acids due to an additional sequence motif of approximately 200 amino acids similar to the ancestral di-TPS motif (Bohlmann et al., 1998a; Bohlmann et al., 1998b). Of these three putative sesqui-TPS, the amino acid sequence of PaTPS-Far has highest similarity with the loblolly pine α-farnesene synthase (81% I, 90% S) (Phillips et al., 2003), but surprisingly also has very high sequence similarity with the spruce mono-TPS described above (Table 5.2). PaTPS-Bis is most similar to grand fir *E*-α-bisabolene synthase (86% I, 91% S) (Bohlmann et al., 1998a). The third putative sesqui-TPS, PaTPS-Lon, is most similar with δ-selinene synthase from grand fir (62% I, 82% S) (Steele et al., 1998a).

Table 5.1: Gene name, accession numbers and functional annotation of Norway spruce TPS.

Gene	Clone	Accession	TPS class	Functional Annotation[a]
PaTPS-Car	PaJF67	AF461459	mono-TPS	(+)-carene synthase
PaTPS-Lim	PaDM743	AY473624	mono-TPS	(–)-limonene synthase
PaTPS-Myr	PaJB16	AY473626	mono-TPS	myrcene synthase
PaTPS-Lin	PaJF39	AY473623	mono-TPS	(–)-linalool synthase
PaTPS-Pin	PaJF104	AY473622	mono-TPS	(–)-α/β-pinene synthase
PaTPS-Far	PaJF71	AY473627	sesqui-TPS	*E,E*-α-farnesene synthase
PaTPS-Bis	PaDM03	AY473619	sesqui-TPS	*E*-α-bisabolene synthase
PaTPS-Lon	PaDM486	AY473625	sesqui-TPS	longifolene synthase
PaTPS-LAS	PaDM2420	AY473621	di-TPS	levopimaradiene/abietadiene s.
PaTPS-Iso	PaDM2425	AY473620	di-TPS	isopimara-7,15-diene synthase

[a] Functional annotation is based on the main terpenoid product of recombinant enzymes expressed in *E. coli*. Several PaTPS form multiple products which are described in the text and shown in the corresponding figures with GC-MS profiles.

Two TPS cDNAs, *PaTPS-LAS* and *PaTPS-Iso*, were very similar to the known conifer di-TPS, abietadiene synthase from grand fir (Stofer Vogel et al., 1996) and taxadiene synthase from *Taxus brevifolia* (Wildung and Croteau, 1996). Each contained the ancestral conifer di-TPS motif of 200 to 215 amino acids of unknown function (Bohlmann et al., 1998b) which

makes these proteins with approximately 860 amino acids substantially longer than most mono-TPS or sesqui-TPS (Figure 5.2). Both genes encoded a putative transit peptide upstream of the amino acid sequence KREFPPGFW (Figure 5.2). This motif is very similar in all known conifer di-TPS and is positionally and functionally conserved with the RRX_8W motif in mono-TPS (Bohlmann et al., 1998b).

Table 5.2: Sequence relatedness of Norway spruce TPS.

Results from pairwise amino acid sequence comparisons are shown as percent identity (upper line) and percent similarity (lower line) for mono-TPS (black, italics), sesqui-TPS (black, bold), and di-TPS (grey) from Norway spruce.

	a	*b*	*c*	*d*	*e*	f	g	h	i	j
(-)-limonene synthase (a)	*100*									
	–									
myrcene synthase (b)	*69*	*100*								
	80	–								
(-)-α/β-pinene synthase (c)	*68*	*65*	*100*							
	83	*80*	–							
(-)-linalool synthase (d)	*66*	*61*	*68*	*100*						
	82	*75*	*81*	–						
(+)-3-carene synthase (e)	*66*	*61*	*65*	*68*	*100*					
	81	*75*	*80*	*82*	–					
E,E-α-farnesene synthase (f)	***62***	***67***	***62***	***64***	***60***	**100**				
	75	***79***	***76***	***76***	***72***	–				
longifolene synthase (g)	***38***	***43***	***40***	***39***	***39***	**40**	**100**			
	57	***63***	***58***	***59***	***58***	**61**	–			
E-α-bisabolene synthase (h)	***29***	***29***	***29***	***28***	***28***	**29**	**30**	**100**		
	42	***43***	***42***	***42***	***42***	**43**	**44**	–		
levopimaradiene/abietadiene synthase (i)	*25*	*25*	*25*	*24*	*24*	**24**	**27**	**42**	**100**	
	38	*38*	*37*	*38*	*37*	**37**	**37**	**61**	–	
isopimara-7,15-diene synthase (j)	*24*	*25*	*24*	*24*	*24*	**23**	**26**	**41**	**90**	**100**
	37	*37*	*36*	*37*	*36*	**36**	**37**	**60**	**94**	–

Functional Expression and Biochemical Characterization of Cloned TPS Enzymes

Recombinant TPS enzymes were functionally characterized on the basis of substrate specificity, product profile and chirality of terpenoids formed. In order to determine the function of each of these *TPS* genes, all cDNAs were subcloned into expression vectors, expressed in *E. coli*, and cell-free extracts of all recombinant enzymes were tested for activity with the substrates, GDP, FDP, and GGDP (Fäldt et al., 2003b; Peters et al., 2000; Bohlmann et al., 1997). In some cases, it was necessary to try several different vectors to optimize expression of active protein. We used a set of four pET-derived vectors for

expression of spruce *TPS* cDNAs including the pSBET vector that codes for additional arginine tRNAs and was previously shown to enhance expression of active TPS enzymes in *E. coli* (Bohlmann et al., 1999), and the pET100/D, pET101/D and pQE50 vectors, which were shown previously to work effectively with *TPS* expression (Fäldt et al., 2003b; Miller et al., 2001). Terpenoid products formed by the recombinant TPS enzymes (Table 5.3) were analyzed using gas chromatography (GC) and mass spectrometry (MS) and GC-flame ionization detection (GC-FID) and identified by comparing retention times and mass spectra with those of authentic standards and library matches.

Functional Identification of Four Mono-TPS Genes

Four of the nine *TPS* cDNA clones, *PaTPS-Lim, PaTPS-Myr, PaTPS-Lin, PaTPS-Pin,* were confirmed after expression and functional characterization to encode mono-TPS enzymes (Table 5.3, Figure 5.6). The product profile of each mono-TPS was initially identified by GC-MS using an HP-5 or a DB-Wax column. Further analysis was performed to identify monoterpene enantiomers (Figure 5.3 - 5.4). Of the four mono-TPS, three enzymes encoded by *PaTPS-Lim, PaTPS-Myr,* and *PaTPS-Pin* form monoterpene hydrocarbons. PaTPS-Lim formed predominantly (–)-limonene (87.8%) along with four other minor products when incubated with GDP and was designated (–)-limonene synthase (Table 5.3, Figure 5.3). PaTPS-Myr catalyzed the formation of a single compound, myrcene, when incubated with GDP thereby designating this TPS as a myrcene synthase (Table 5.3, Figure 5.4). Chiral analysis of products formed by PaTPS-Pin with GDP as substrate revealed (–)-β-pinene (57.1%), (–)-α-pinene (27.4%) and (–)-β-phellandrene (11.0%) as the major monoterpenes produced along with five other minor constituents (Table 5.3, Figure 5.5). This TPS has been named (–)-α/β-pinene synthase. Assays with GDP and product analysis of PaTPS-Lin revealed a profile of two monoterpene alcohols, (–)-linalool (96.6%) and (+)-linalool (1.6%) (Figure 5.6), and an additional nine monoterpene hydrocarbons at very low abundance including *E*-β-ocimene, myrcene, α-terpinolene, and 3-carene (Table 5.3). This is the first linalool synthase isolated from a gymnosperm. The near optical purity of (–)-linalool synthase indicates that active site residues exert considerable control over the quenching of the carbocation by the addition of H_2O.

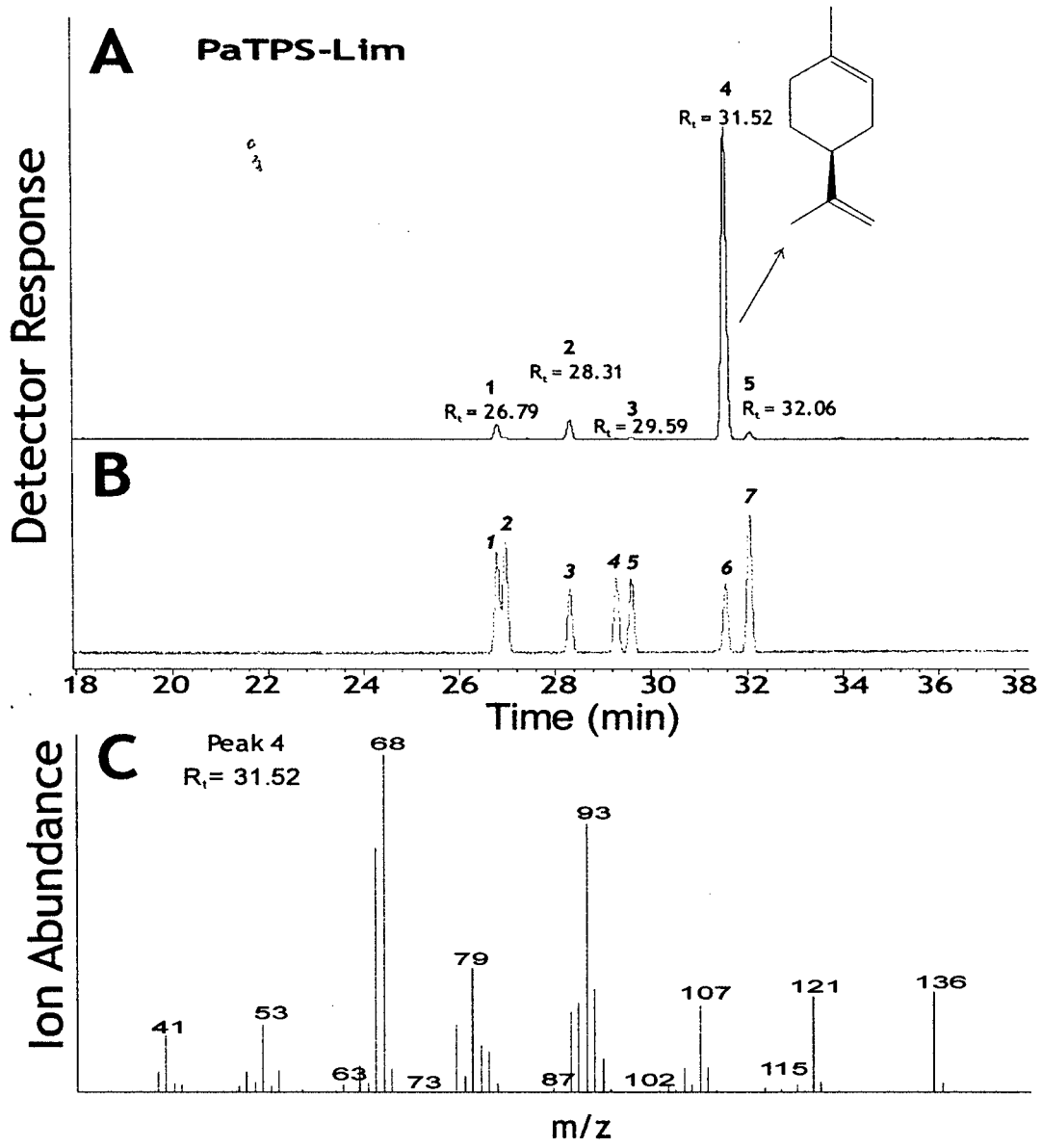

Figure 5.3: GC-MS chiral analysis of products formed by PaTPS-Lim, (–)-limonene synthase.

(A) Total ion chromatogram of the assay products (–)-α-pinene (1), myrcene (2), (–)-β-pinene (3), (–)-limonene (4), and (+)-limonene (5) along (B) with the chiral terpene standards (–)-α-pinene, R_t = 26.78 (1), (+)-α-pinene R_t = 26.95 (2), myrcene, R_t = 28.30 (3), (+)-β-pinene, R_t= 29.27 (4), (–)-β-pinene, R_t = 29.59 (5), (–)-limonene, R_t = 31.55 (6), and (+)-limonene, R_t = 32.05 (7). (C) Mass spectra of major peak identifies this compound as (–)-limonene.

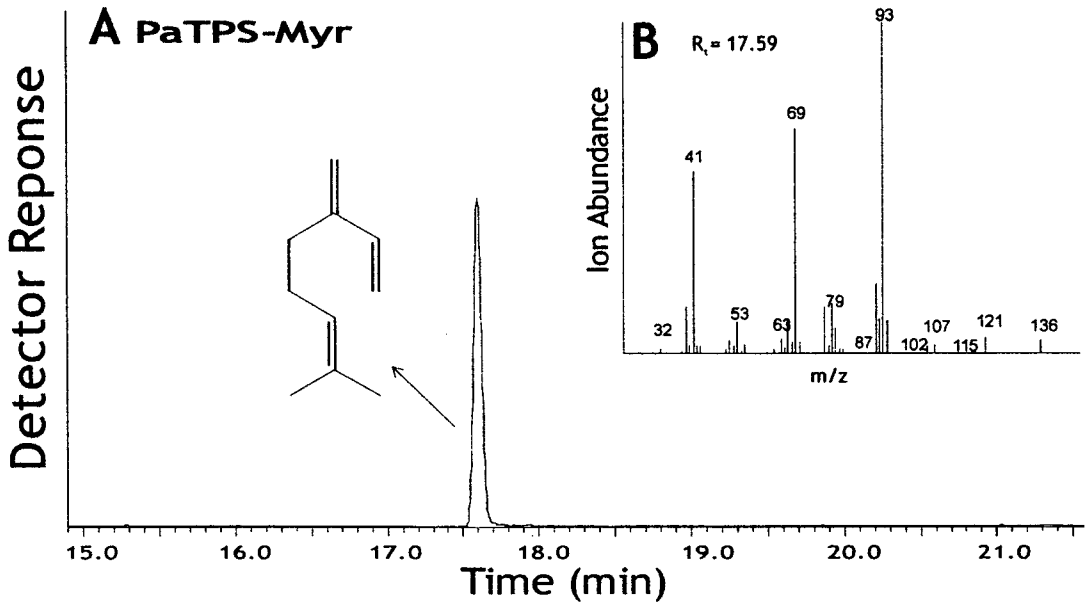

Figure 5.4: GC-MS analysis of products formed by PaTPS-Myr, myrcene synthase.

Total ion chromatogram of assay product (A). Mass spectra of major peak (B) identifies this product as myrcene.

Functional Identification of Three Sesqui-TPS Genes

Three cDNA clones, *PaTPS-Far*, *PaTPS-Bis*, *PaTPS-Lon*, were functionally identified as sesqui-TPS. After assaying PaTPS-Far with each of the three prenyl diphosphate substrates, GDP, FDP, and GGDP, activity was seen only with FDP. This enzyme produces *E,E*-α-farnesene as a single product (Table 5.3, Figure 5.7) confirming its identity as a sesqui-TPS. I have designated this TPS as *E,E*-α-farnesene synthase. Expression of PaTPS-Bis and enzyme assays revealed *E*-α-bisabolene as the single product of this enzyme identifying it as *E*-α-bisabolene synthase (Table 5.3, Figure 5.8). The third sesqui-TPS, PaTPS-Lon, produced mainly longifolene (60.6%) (Figure 5.9, Table 5.3) when assayed with FDP. This sesqui-TPS also produced multiple minor products including α-longipinene, α-longicyclene, *E*-β-farnesene, the sesquiterpene alcohol, longiborneol, cyclosativene, β-longipinene, and twelve other sesquiterpenes (each under 1.4% of total product). This enzyme was designated longifolene synthase for its major sesquiterpene product.

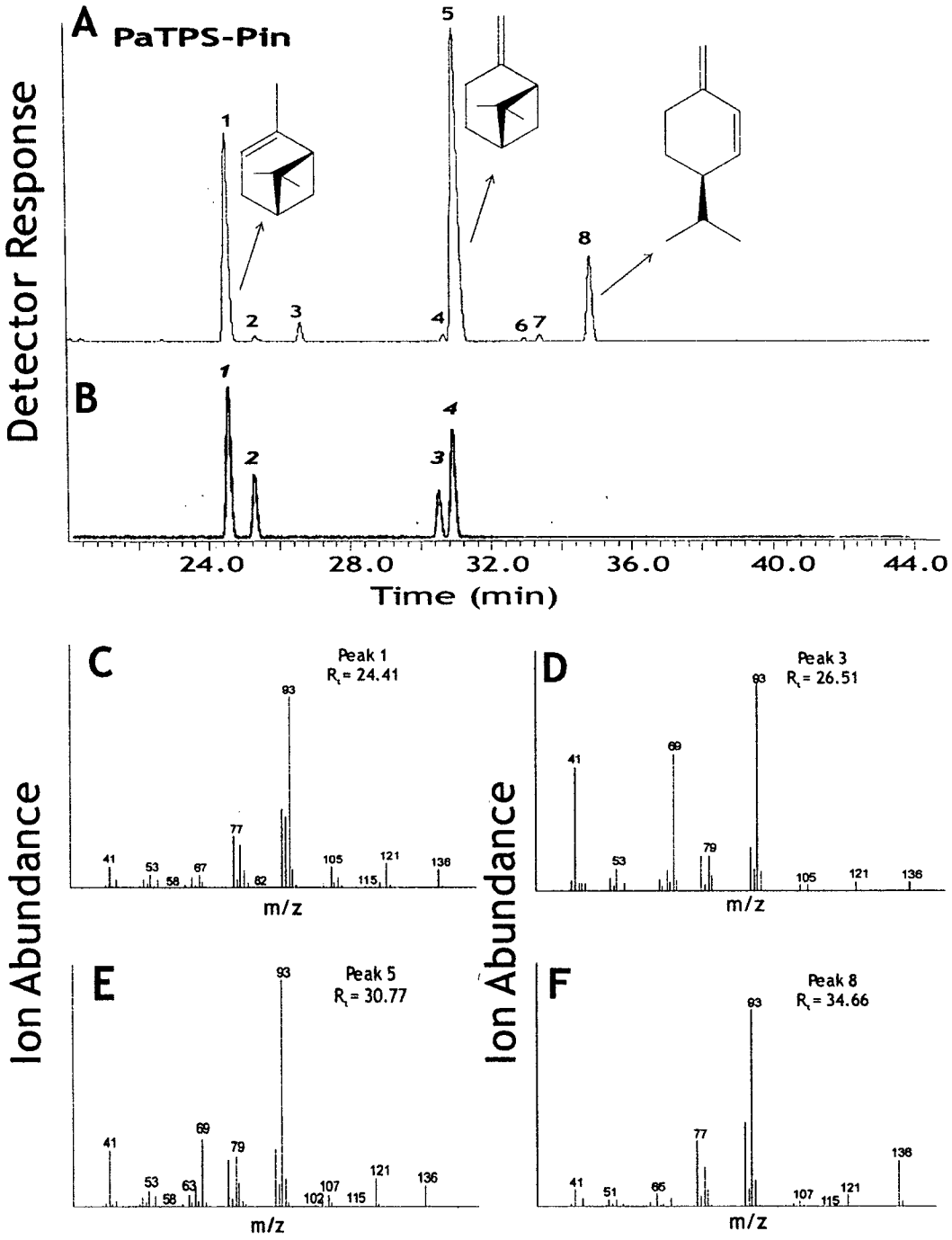

Figure 5.5: GC-MS chiral analysis of products formed by PaTPS-Pin, (–)-α/β-pinene synthase.

(A) Total ion chromatogram showing eight assay products (–)-α-pinene (1), (+)-α-pinene (2), myrcene (3), (+)-β-pinene (4), (–)-β-pinene (5), (–)-limonene (6), (+)-limonene (7), and (–)-β-phellandrene. (B) chiral terpene standards standards (–)-α-pinene, R_t = 24.40 (1), (+)-α-pinene R_t = 25.35 (2), (+)-β-pinene, R_t= 30.23 (3), (–)-β-pinene, R_t = 30.76 (4). Mass spectra of major peaks including (–)-α-pinene (C), myrcene (D), (–)-β-pinene (E) and (–)-β-phellandrene (F).

Table 5.3: Norway spruce mono-, sesqui- and diterpene synthases and the products that each produces.

TPS		Products	% Total
Monoterpene synthases			
	PaTPS-Lim	(–)-limonene	87.8
		myrcene	5.2
		(–)-α-pinene	4.4
		(+)-limonene	2.1
		(–)-β-pinene	0.5
	PaTPS-Myr	myrcene	100
	PaTPS-Pin	(–)-β-pinene	57.1
		(–)-α-pinene	27.4
		(–)-β-phellandrene	11
		(+)-β-pinene	0.6
		(–)-α-pinene	0.7
		myrcene	2.1
		(–)-limonene	0.4
		(+)-limonene	0.7
	PaTPS-Lin	(–)-linalool	96.6
		(+)-linalool	1.6
		E-β-ocimene	1.0
		myrcene	0.2
		α-terpinolene	0.1
		3-carene	0.2
		other monoterpenes	each < 0.07
Sesquiterpene synthases			
	PaTPS-Far	*E,E*-α-farnesene	100
	PaTPS-Bis	*E*-α-bisabolene	100
	PaTPS-Lon	longifolene	60.6
		α-longipinene	14.6
		longicyclene	5.9
		E-β-farnesene	3.4
		longiborneol	2.1
		cyclosativene	1.3
		β-longipinene	1.4
		other sesquiterpenes	each<1.4
Diterpene synthases			
	PaTPS-LAS	levopimaradiene	36.5
		abietadiene	32.1
		neoabietadiene	23.2
		palustradiene	8.1
	PaTPS-Iso	isopimara-7,15-diene	100

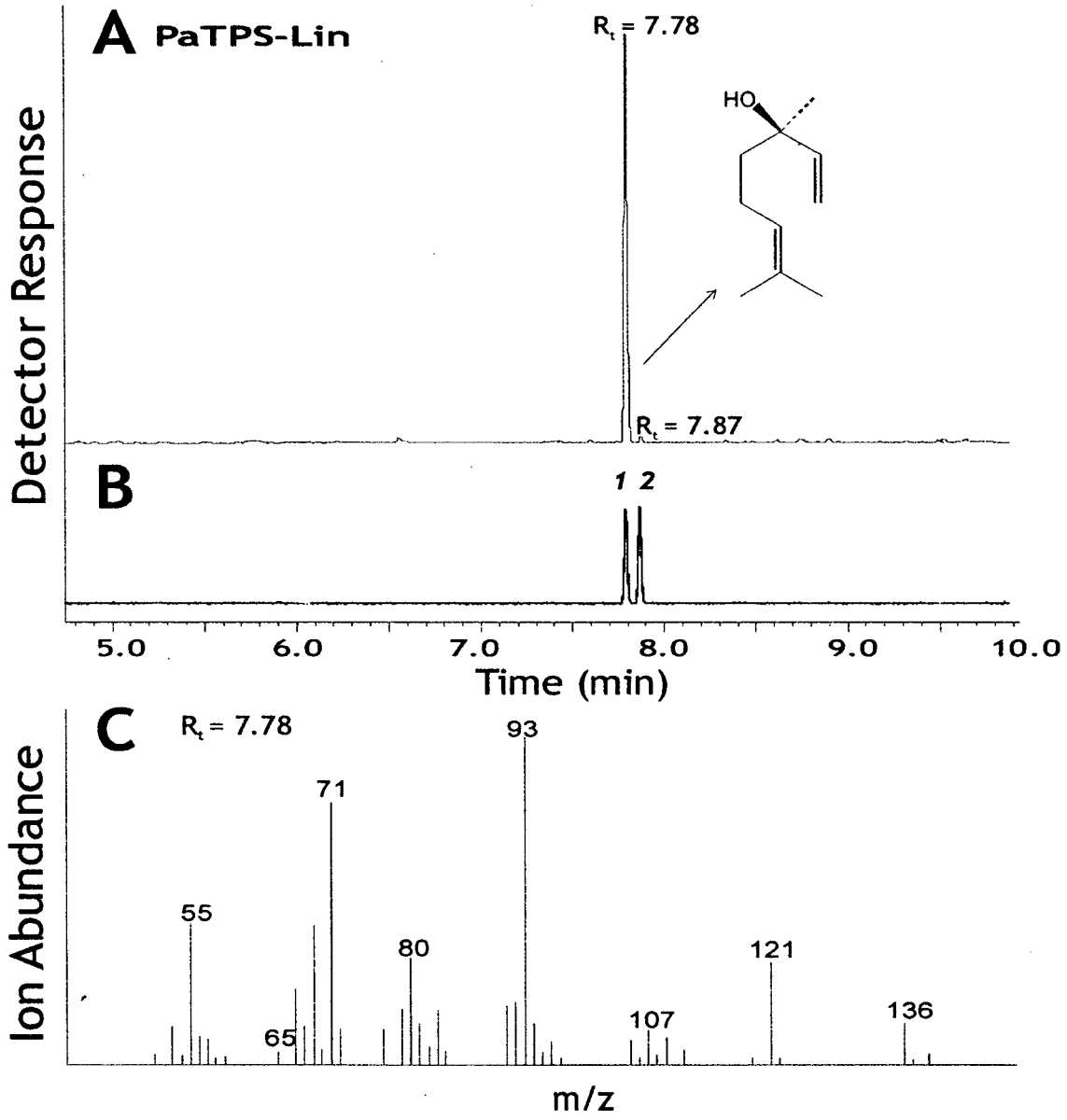

Figure 5.6: GC-MS chiral analysis of products formed by PaTPS-Lin, (–)-linalool synthase. **Total ion chromatogram of assay products (A) showing a major peak, Rt= 7.78 along with (B) chiral terpene standards showing (–)-linalool, Rt= 7.79 (1) and (+)-linaool, Rt= 7.87 (2). Mass spectra of major peak (C) identifies this peak as (–)-linalool.**

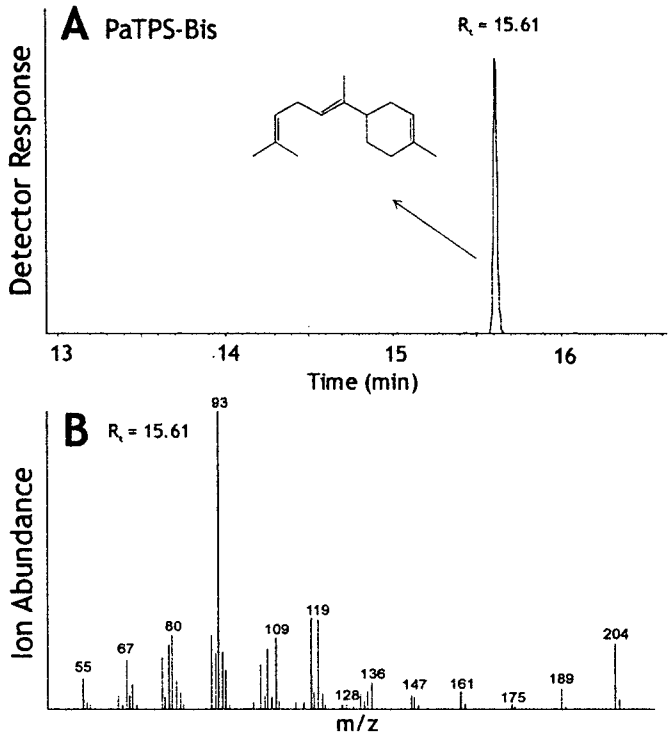

Figure 5.7: GC-MS analysis of product formed by PaTPS-Far, E,E-α-farnesene synthase.

Total ion chromatogram of a single assay product (A) along with mass spectra of the peak (B) identifying this product as E,E-α-farnesene .

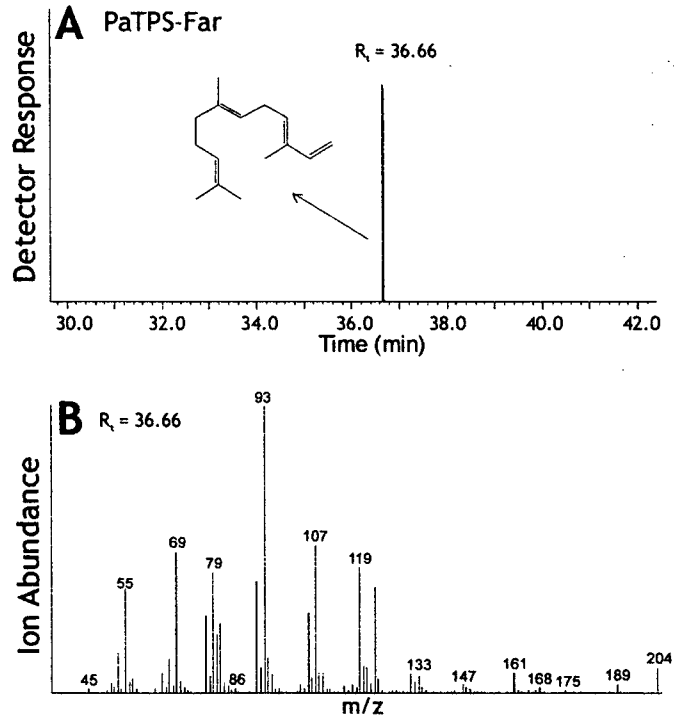

Figure 5.8: GC-MS analysis of the product formed by PaTPS-Bis, E-α-bisabolene synthase.

Total ion chromatogram of a single assay product (A) Mass spectra of this peak establishes this compound as E-α-bisabolene (B).

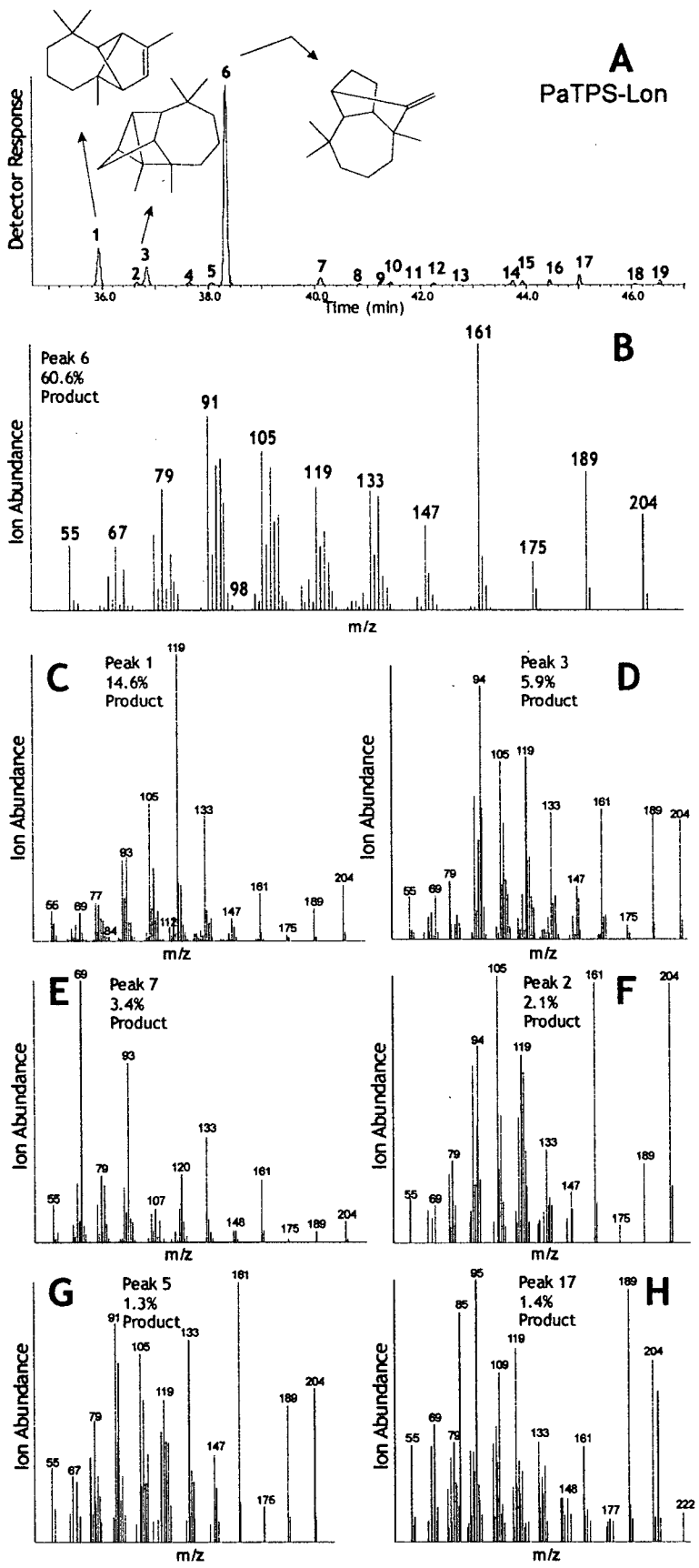

Figure 5.9: GC-MS analysis of the multiple-product forming PaTPS-Lon, longifolene synthase.

Total ion chromatogram of the assay showing 19 sesquiterpene products (A). Mass spectra of the major peak (B) and six additional products according to decreasing abundance including α-longicyclene (C), α-longipinene (D), *E*-β-farnesene (E), cyclostativene (F), β-longipinene (G), and longiborneol (H).

Functional Identification of Two Di-TPS Genes

The first of two Norway spruce *di-TPS* candidate cDNAs, *PaTPS-LAS*, encodes a predicted protein of 859 amino acids (Figure 5.2) and a pI of 5.52. Several truncations were made of this cDNA to determine if expression was improved with the removal of transit peptide (Peters et al., 2000), however the full-length gene gave the highest di-TPS activity. When incubated with GGDP, PaTPS-LAS formed the four diterpenes, levopimaradiene (36.5%, peak 1), abietadiene (32.1%, peak 2), neoabietadiene (23.2%, peak 3) and palustradiene (8.1%, peak 4) (Figure 5.10 A-D, Table 5.3). I used grand fir abietadiene synthase (Stofer Vogel et al., 1996; Peters et al., 2000) to compare product profiles of these two similar enzymes and found that product profiles of the two enzymes were nearly identical to each other and to published results with grand fir abietadiene synthase (Peters et al., 2000). I have designated this di-TPS levopimaradiene/abietadiene synthase after the major products it forms. The second di-TPS, PaTPS-Iso, is slightly longer than PaTPS-LAS at a predicted length of 867 amino acids (Figure 5.2). The predicted protein pI is 5.83. Heterologous expression of full-length PaTPS-Iso and enzyme assays with GGDP yielded a single product, isopimara-7,15-diene (Figure 5.10 E-F, Table 5.3). This di-TPS was hence designated isopimara-7,15-diene synthase. To our knowledge, an isopimara-7,15-diene synthase has not been previously described for any species.

The proposed reaction mechanisms for levopimaradiene/abietadiene synthase and isopimara-7,15-diene synthase proceed from *E,E,E*-GGDP to the bicyclic (+)-copalyl diphosphate intermediate (Figure 5.11). Studies in grand fir abietadiene synthase have demonstrated that this intermediate then freely diffuses to a second active site of the bifunctional enzyme where the sandaracopimarenyl intermediate is produced (Peters et al., 2001; Peters et al., 2003). Following the generation of sanadaracopimarenyl carbocation, the mechanisms of levopimaradiene/abietadiene synthase and isopimara-7,15-diene synthase diverge. To form the four products of LAS, a 1,2-methyl migration occurs to yield the abietane structure followed by deprotonation to give the different double bond configurations of levopimaradiene, abietadiene, neoabietadiene and palustradiene. The proposed reaction scheme of isopimara-7,15-diene synthase involves simple deprotonation of the sandaracopimarenyl carbocation (Figure 5.11).

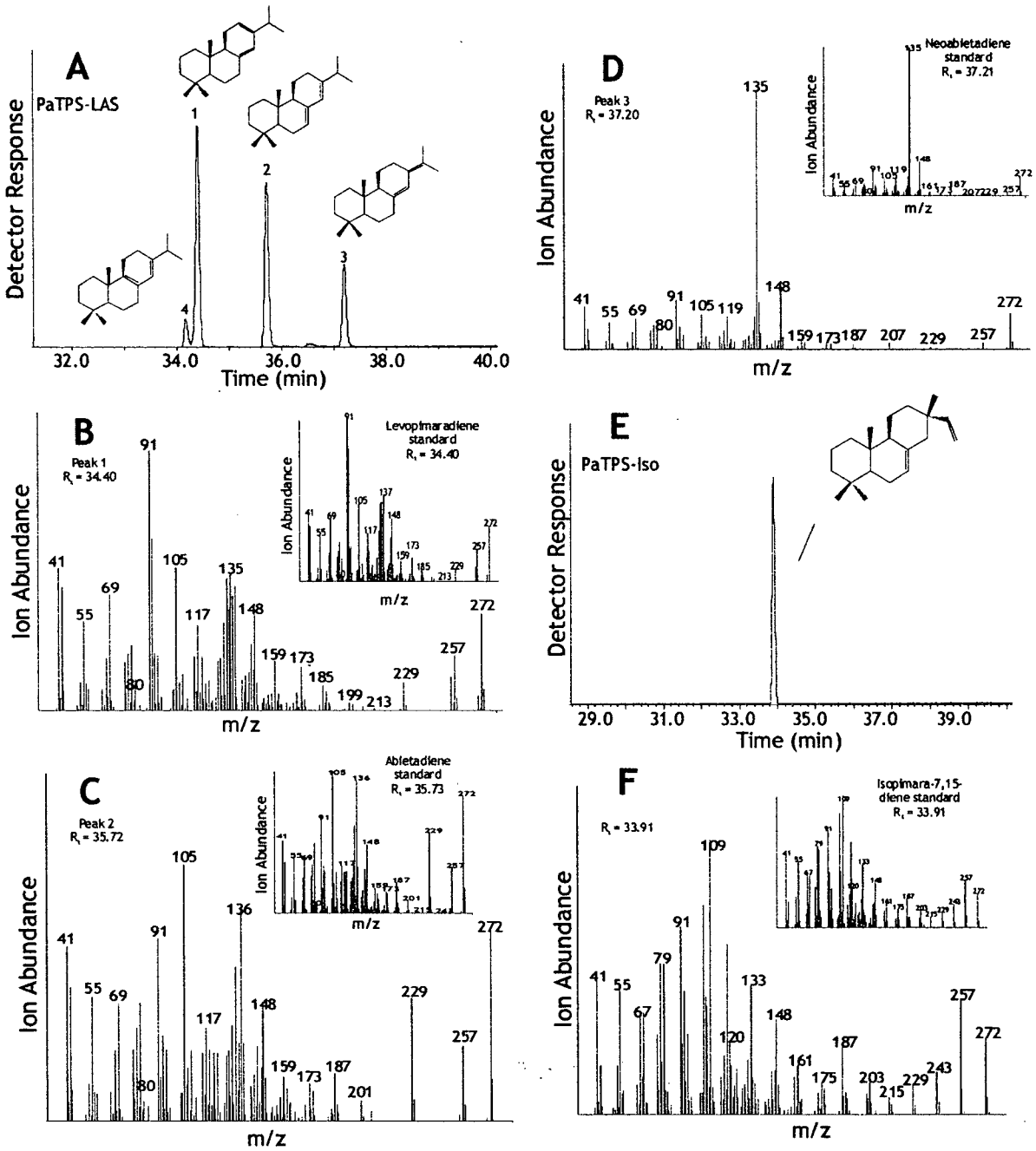

Figure 5.10: GC-MS analysis of products formed by PaTPS-LAS, levopimaradiene/abietadiene synthase and PaTPS-IS, isopimara-7,15-diene synthase.

Total ion chromatogram of multiple assay products of levopimaradiene/abietadiene synthase (A). Mass spectra are shown of peak one with insert of levopimaradiene standard (B), peak two with insert of authentic abietadiene (C) and of peak three with insert of neoabietadiene standard (D). Total ion chromatogram of the single product produced by isopimara-7,15-diene synthase (E). Mass spectra of this product are shown with an insert of authentic isopimara-7,15-diene (F).

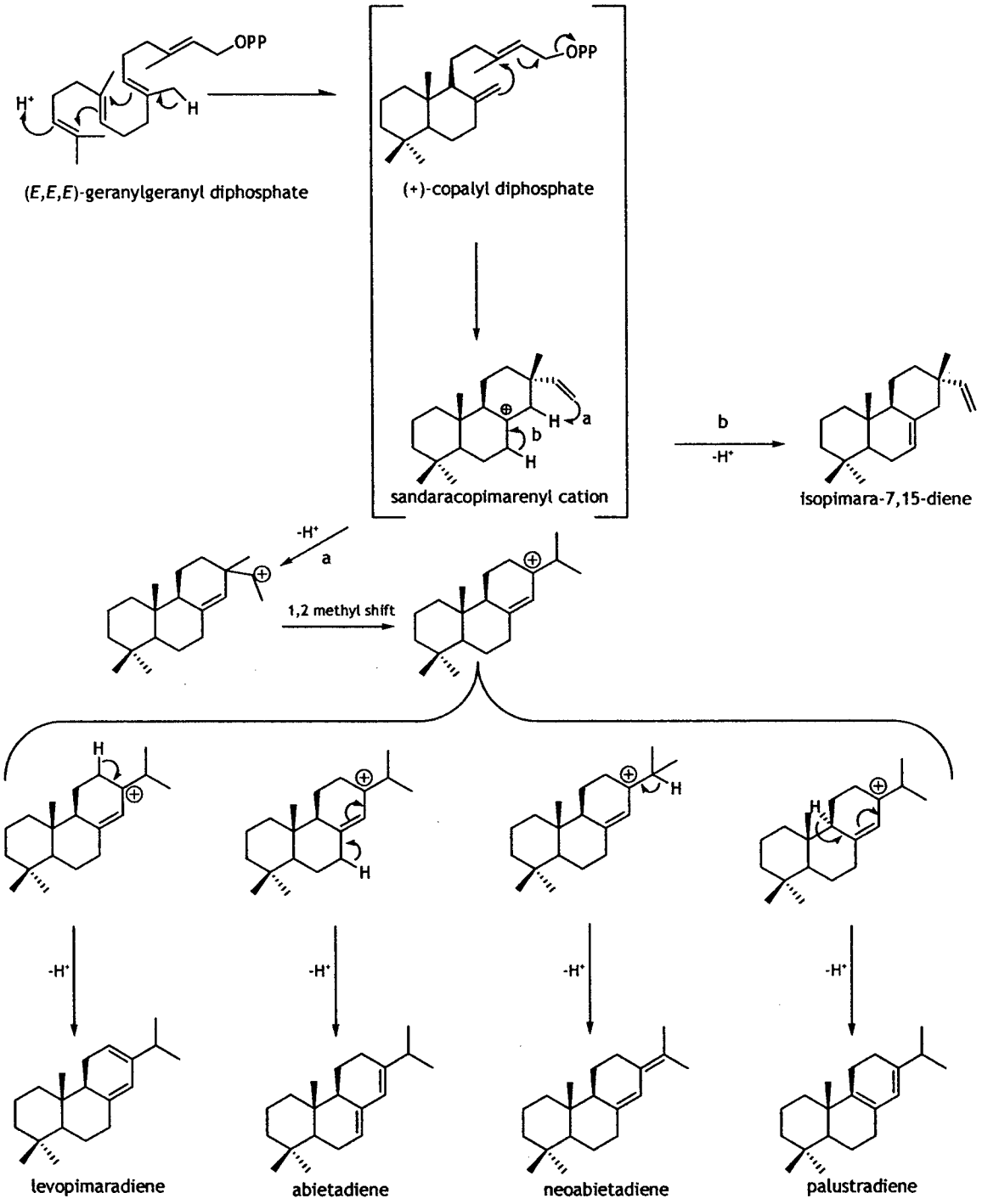

Figure 5.11: Proposed reaction mechanisms for diterpene synthases PaTPS-LAS and PaTPS-Iso.

The substrate GGDP undergoes initial cyclization reaction yielding (+)-copalyl diphosphate. In a second cyclization reaction, the intermediate sandaracopimarenyl carbocation is formed. The pathway to the left (a) reflects the reaction mechanism of levopimaradiene/abietadiene synthase

whereby a 1,2 methyl migration results in the abeitane skeleton. Final deprotonations from this skeleton yield the four detected products, levopimaradiene, abietadiene, neoabietadiene and palustradiene, each with different double bond configurations. The pathway towards the right (b) indicates the reaction mechanism of isopimara-7,15-diene synthase whereby the sandaracodimarenyl carbocation is deprotonated to yield isopimara-7,15-diene.

Phylogeny of the Gymnosperm TPS-d Subfamily

The monophyletic plant TPS family has been delineated into separate subfamilies (TPS-a through TPS-g) based on sequence relatedness as well as functional assessment [(Bohlmann et al., 1998b), Figure 5.13]. The extant TPS of plant secondary metabolism apparently arose from an ancestral *TPS* gene that was likely most similar in gene structure and sequence to the known gymnosperm di-*TPS* (Trapp and Croteau, 2001b; Bohlmann et al., 1998b). Such an ancestral *di-TPS* gene may have been involved in GGDP cyclization of gibberellic acid metabolism. While all extant *TPS* genes characterized to date seem to share a common origin, functional specialization of many TPS occurred after the separation of angiosperms and gymnosperms (Bohlmann et al., 1998b; Trapp and Croteau, 2001b).

All known gymnosperm TPS cluster in the TPS-d subfamily (Figure 5.12 and Figure 5.13) which contains mono-, sesqui- and di-TPS. Prior to the cloning of Norway spruce *TPS*, the gymnosperm TPS-d subfamily consisted mostly of TPS from grand fir (Bohlmann et al., 1998b), a member of the pine family (Pinaceae). Sufficient information on other conifer species was lacking thereby preventing a thorough comparison of TPS from different taxa of the pine family. Furthermore, an understanding of TPS evolution within the gymnosperm TPS-d subfamily and how this might compare with TPS evolution in angiosperms had also not been addressed. For instance, it was not known whether distinct branching patterns would further dissect the TPS-d subfamily based on function or whether the functional speciation of TPS preceded the speciation of the Pinaceae. However, with the inclusion of 10 new Norway spruce TPS as well as some gymnosperm TPS recently available from the literature, I was able now to reconstruct and further understand the phylogeny of the TPS-d subfamily. To this goal, I performed a maximum likelihood analysis of conifer TPS using PHYML (Guindon and Gascuel, 2003) and the JTT substitution matrix (Jones et al., 1992). The alpha shape parameter as well as the invariable site parameter were estimated prior to analysis of 100 bootstrap replicates. Phylogenetic trees were constructed using the modified neighbor joining program BIONJ (Gascuel, 1997). Bootstrap values are also included for distance analysis using PROTDIST (Felsenstein, 1993) and the Dayhoff PAM 001 matrix (Dayhoff, 1979)

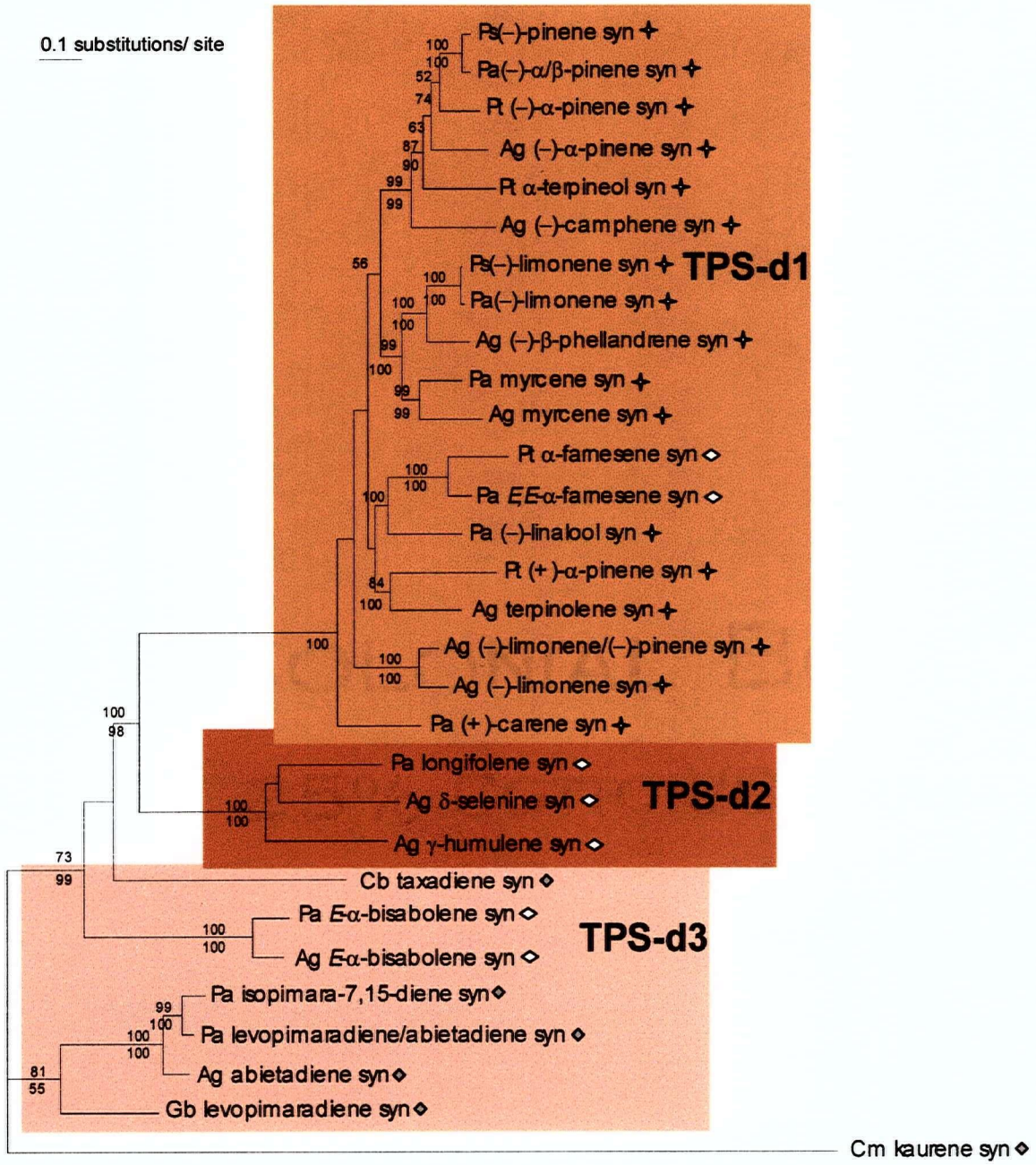

Figure 5.12: Phylogenetic tree of gymnosperm TPS amino acid sequences showing the TPS-d subfamily branching into three distinct groups of TPS involved in secondary metabolism.

Branching patterns seen in this analysis are cause for the further separation into TPS-d1 (primarily mono-TPS ✦), TPS-d2 (sesqui-TPS ◇) and TPS-d3 (primarily di-TPS ◆). Kaurene synthase from *Cucurbita maxima* shown as outgroup in this analysis. Bootstrap values over 50% for maximum likelihood (upper) and distance (lower) analyses are shown at nodes. Maximum likelihood values represent percentages of 100 gamma-corrected (log L = -52520.29) replicates analyzed using Phyml.

Table 5.4: Gymnosperm and angiosperm TPS represented in phylogenetic analyses

Species	TPS	Accession Number
Gymnosperms		
Picea abies (Norway spruce)	(+)-3-carene syn	AF461459
	(–)-limonene syn	AY473624
	(–)-linalool syn	AY473623
	(–)-α/β-pinene syn	AY473622
	myrcene syn	AY473626
	E,E-α-farnesene syn	AY473627
	longifolene syn	AY473625
	E-α-bisabolene syn	AY473619
	levopimaradiene/abietadiene syn	AY473621
	isopimara-7,15-diene syn	AY473620
Picea sitchensis (Sitka spruce)	(–)-limonene syn	unpublished
	(–)-pinene syn	AAP72020
Abies grandis (grand fir)	β-phellandrene syn	AAF61453
	myrcene syn	AAB71084
	α-pinene/limonene syn	AAF61455
	terpinolene syn	AAF61454
	(–)-limonene syn	AAB70907
	(–)-pinene syn	AAB71085
	(–)-camphene syn	AAB70707
	δ-selinene syn	AAC05727
	γ-humulene syn	AAC05728
	E-α-bisabolene syn	AAC24192
	abietadiene syn	AAB05407
Pinus taeda (loblolly pine)	(+)-α-pinene syn	AAO61228
	(–)-α-pinene syn	AAO61225
	α-terpineol syn	AAO61227
	α-farnesene syn	AAO61226
Taxus brevifolia	taxadiene syn	AAC49310
Ginkgo biloba	levopimaradiene syn	AAL09965
Angiosperms		
Mentha spicata	limonene syn	AAC37366
Schizonepeta tenuifoli	(+)-limonene syn	AAG01140
Salvia stenophylla	3-carene syn	AAG01140
Citrus limon	(+)-limonene syn 1	AAM53944
	(+)-limonene syn 2	AAM53946
	γ-terpinene syn	AAM53943
	(–)-β-pinene syn	AAM53945
Cinnamomum tenuipilum	geraniol syn	CAD29734
Populus alba x Populus tremula	isoprene syn	CA35696
Arabidopsis thaliana	linalool syn	AAO85533
	caryophyllene/humulene syn	AAO85539
	myrcene ocimene syn	AAG09310
Salvia officinalis	(+)-bornyl diphosphate syn	AAC26017
	(+)-sabinene syn	AAC26018
Ricinus communis	casbene syn	P59287
Malus x domestica	*E,E*-α-farnesene syn	AAO22848
Mentha x piperita	*E*-β-farnesene syn	AAB95209
Citrus junos	*E*-β-farnesene syn	AAK54279
Lactuca sativa	germacrene A syn	AAM11627
Gossypium arboretum	(+)-δ-cadinene syn	AAD51718
Artemisia annua	β-caryophyllene syn	AAL79181
	(–)-β-pinene syn	AAK58723
Mentha aquatica (citrate)	(–)-linalool syn	AAL99381
Clarkia breweri	(+)-linalool syn	AAC49395
Antirrhinum majus	myrcene synthase	AAO41726
	myrcene syn	AAO41727
	E-β-ocimene syn	AAO42614
Quercus ilex L.	myrcene syn	CAC41012
Nicotiana attenuata	5-epi-aristolochene syn	AAO85555
Stevia rebaudiana	copalyl diphosphate syn	AAB87091
	ent-kaurene syn KS22-1	AAD34294
	ent-kaurene syn KS1-1	AAD34294
Pisum sativum	copalyl diphosphate syn	AAB58822
Cucurbita maxima	ent-kaurene syn	T09672
Lycopersicon esculentum	copalyl diphosphate syn	BAA84918
Agastache rugosa	(+)-limonene syn	AAL17636
Capsicum annuum	5-epi-aristolochene syn	CAA06614

The inclusion of seventeen additional conifer TPS, including ten from Norway spruce, three mono-TPS and one sesqui-TPS from loblolly pine, two mono-TPS from Sitka spruce and one di-TPS from *Ginkgo biloba* (Table 5.4), fleshed out the resulting tree such that I can now discern three discrete groups within the gymnosperm TPS-d subfamily (Figure 5.12). As the identity of the TPS-d subfamily should be maintained, I propose the labeling for these groups to be TPS-d1, TPS-d2, and TPS-d3, based on sequence relatedness and functional assessment. Each group is dominated by TPS of one of the three biochemical classes, mono-TPS (TPS-d1), sesqui-TPS (TPS-d2) and di-TPS (TPS-d3). This grouping is analogous to the overall delineation of angiosperm TPS where each subfamily is composed of TPS with similar sequence structures and, in most cases, angiosperm TPS subfamilies contain members of the same biochemical class (Bohlmann et al., 1998b).

Considering the extremely long evolutionary divergence of extant gymnosperms, it is a striking feature of this analysis that most of the known gymnosperm TPS still segregate within the tree by common overall function. For example, all conifer mono-TPS fall into the TPS-d1 cluster and all di-TPS cluster in the TPS-d3 group (Figure 5.12). In contrast to mono-TPS, fewer sesqui-TPS have been identified in gymnosperms. While conifer sesqui-TPS form the TPS-d2 group, other sesqui-TPS cluster with the mono-TPS or di-TPS suggesting multiple origins of sesqui-TPS functions (see below).

The fact that all di-TPS cluster closely together, including the levopimaradiene synthase from *Ginkgo biloba*, taxadiene synthase from *Taxus*, and several conifer di-TPS, infers that the initial duplication event and inception of di-TPS of specialized gymnosperm secondary metabolism occurred before the separation of Coniferales and Ginkgoales probably more than 250 to 290 million years ago. Another important point gathered from this analysis includes the presence of intra-species gene duplications followed by divergent functional evolution. This is illustrated by the paralogs isopimara-7,15-diene synthase and levopimaradiene/abietadiene synthase from Norway spruce (Figure 5.12). While these two proteins are 94% similar and 90% identical, they produce very different, non-overlapping diterpenoid profiles (**Figure 5.10**, Table 5.3), which makes this pair of proteins an interesting target for future, biochemical structure-function analysis.

Another distinct result from this phylogeny of gymnosperm TPS is that a large suite of TPS with specific biochemical functions and substrate preferences apparently arose before the speciation of members of the Pinaceae and remained surprisingly stable since then. Examples of this include myrcene synthases, *E*-α-bisabolene synthases, and the pair of levopimaradiene/abietadiene synthase and abietadiene synthase, each represented by orthologous gene pairs of the exact same biochemical function in Norway spruce and grand fir

(Figure 5.12, Table 5.5), and all supported by high bootstrap values. Similarly, loblolly pine and Norway spruce α-farnesene synthases also seem to be a very old and functionally conserved orthologous gene pair since pines and spruces represent taxa of the Pinaceae that separated around 140 million years ago (Wang et al., 2000). Yet other TPS appear to have independently evolved further following speciation of members of the pine family such that the product profiles of these enzymes have more or less diversified though these genes are certainly homologous. The (–)-α-pinene synthases from Norway spruce, Sitka spruce, loblolly pine and grand fir are good examples of how some TPS have evolved to produce additional new products or different product ratios (Figure 5.12, Table 5.5). The (–)-pinene synthases of Norway spruce and grand fir both produce a greater proportion of (–)-β-pinene than (–)-α-pinene (Bohlmann et al., 1997), whereas in the closely related (–)-α-pinene synthase of Sitka spruce, (–)-α-pinene is the major product (Byun McKay et al., 2003) and in loblolly pine(–)-α-pinene is the only product (Phillips et al., 1999) (Table 5.5). Notably, each of these still forms (–)-α-pinene as a major product and they all group closely together within the TPS-d1 cluster (Figure 5.12). A similar situation exists for the (–)-limonene synthases from Norway spruce (this study), grand fir (Bohlmann et al., 1997) and Sitka spruce (Bohlmann et al, unpublished). These TPS homologs are all examples of how TPS can evolve to produce new functional variations on overall similar product profiles (Table 5.5). The phylogeny of these homologs is also supported by high bootstrap values (Figure 5.12). For some TPS such as α-terpineol synthase, 3-carene synthase, and (–)-camphene synthase, additional genes need to be identified before we begin to understand their evolution within the gymnosperms better. Finally, some TPS functions (e.g. (–)-limonene synthase) seem to have evolved more than once within the TPS-d1 subfamily.

Despite the much refined analysis of the gymnosperm TPS-d family presented here, the phylogenetic position of a gymnosperm di-TPS of gibberellic acid phytohormone metabolism relative to di-TPS of secondary metabolism remains enigmatic until a gymnosperm (–)-copalyl diphosphate synthase or kaurene synthase is cloned. It is indeed surprising that these gene functions have not yet been discovered in a gymnosperm despite considerable EST resources from pines and spruce and efforts of targeted screening for conifer di-TPS.

Comparison of Phylogeny of TPS from Angiosperms and Gymnosperms

After visualizing the placement of the newly identified Norway spruce TPS within the TPS-d subfamily, I wanted to further understand how these TPS related to the larger family comprised of both angiosperm and gymnosperm TPS. It was previously established that extant

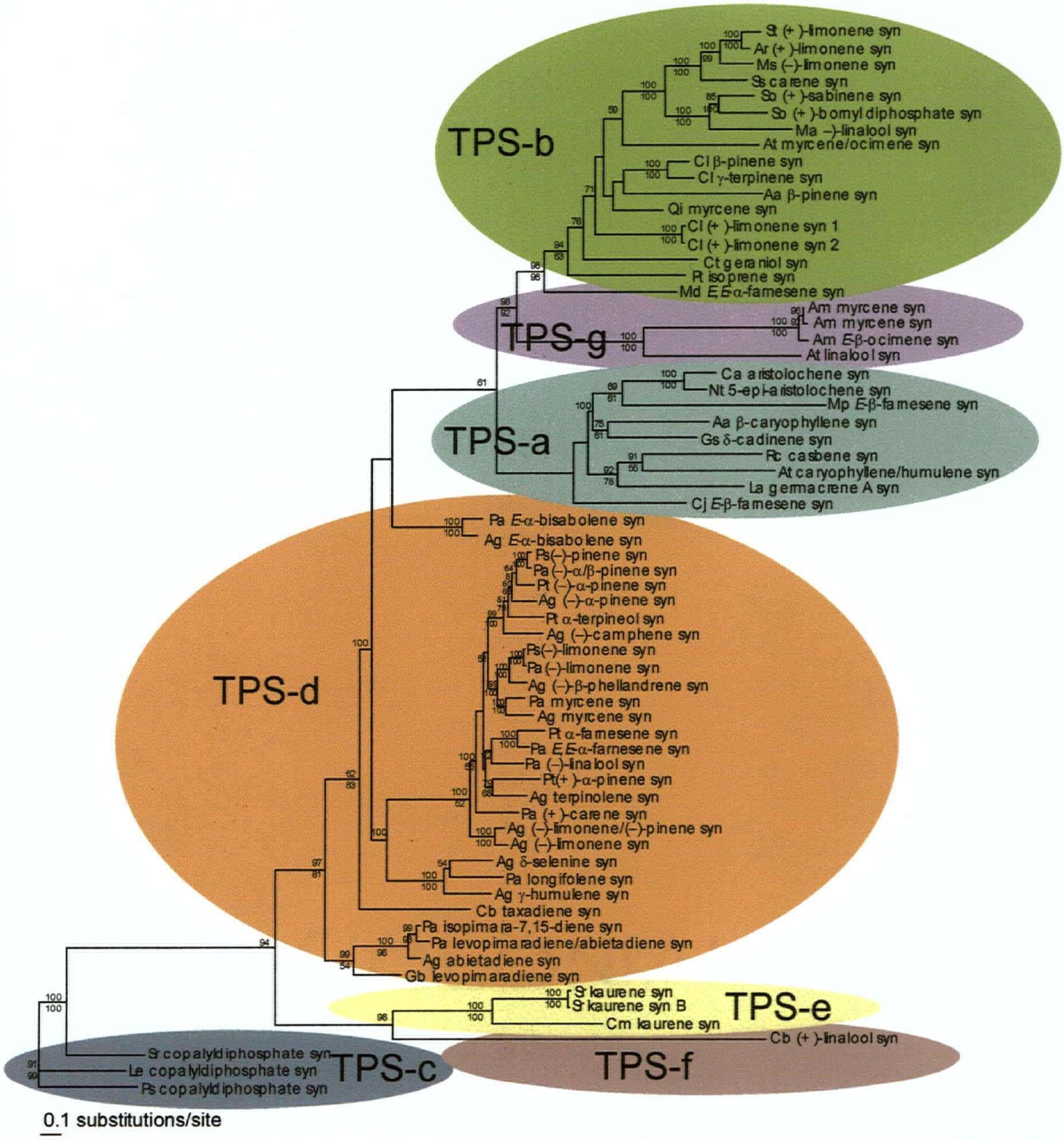

Figure 5.13: Phylogenetic tree illustrating the relationship of TPS involved in primary and secondary metabolism from angiosperms and gymnosperms.

Amino acids sequences (Table 5.5) of 67 TPS were analyzed by maximum likelihood and distance methods. Bootstrap values over 50% present at nodes for maximum likelihood (upper) and distance (lower) analyses. Maximum likelihood values represent percentages of 100 gamma-corrected (log L = -20452.16) replicates.

TPS functions evolved independently in gymnosperms and angiosperms (Bohlmann et al., 1998b). The numerous known angiosperm TPS of both primary and secondary metabolism are located within six subfamilies (Bohlmann et al., 1998b; Dudareva et al., 2003), that are distinct from the gymnosperm TPS-d cluster. Angiosperm TPS subfamilies are delineated into

Table 5.5: Product comparisons between conifer TPS

Conifer	TPS	Products	%	Reference
P. abies	(–)-α/β-pinene syn	(–)-β-pinene	57.1	*
		(–)-α-pinene	27.4	
		(–)-β-phellandrene	11	
		(+)-β-pinene	0.6	
		(–)-α-pinene	0.7	
		myrcene	2.1	
		(–)-limonene	0.4	
		(+)-linomene	0.7	
A. grandis	(–)-pinene syn	(–)-α-pinene	42	(Bohlmann et al., 1997)3}7)
		(–)-β-pinene	58	
P. sitchensis	(–)-pinene syn	(–)-α-pinene	77.8	(Byun McKay et al., 2003)4}3)
		(–)-β-pinene	22.2	
P. taeda	(–)-α-pinene syn	(–)-α-pinene	100	(Phillips et al., 1999)5}9)
A. grandis	myrcene syn	myrcene	100	(Bohlmann et al., 1997)3}7)
P. abies	myrcene syn	myrcene	100	*
P. abies	(–)-limonene synthase	(–)-limonene	87.8	*
		myrcene	5.2	
		(–)-α-pinene	4.4	
		(+)-limonene	2.1	
		(–)-β-pinene	0.5	
P. sitchensis	(–)-limonene synthase	(–)-limonene	100	Bohlmann et al, unpub
A. grandis	(–)-limonene synthase	(–)-limonene	major	(Bohlmann et al., 1997)3}7)
		β-phellandrene	lesser	
		β-pinene	lesser	
		α-pinene	lesser	
A. grandis	*E*-α-bisabolene syn	E-α-bisabolene	100	(Bohlmann et al., 1998a)6}a)
P. abies	*E*-α-bisabolene syn	E-α-bisabolene	100	*
P. abies	levopimaradiene/abietadiene syn	levopimaradiene	36.5	*
		abietadiene	32.1	
		neoabieatadiene	23.2	
		plaustradiene	8.1	
A. grandis	abietadiene syn	levopimaradiene	34	(Peters et al., 2000)5}0)
		abietadiene	31	
		neoabieatadiene	28	
		plaustradiene	2	
		sandaracopimaradiene	2	
		pimara-8(14),15-diene	3	
P. abies	isopimara-7,15-diene syn	isopimara-7,15-diene	100	*

sesqui-TPS (TPS-a), mono-TPS (TPS-b), copalyl diphosphate synthases (TPS-c) and ent-kaurene synthases (TPS-e) of gibberellic acid formation and secondary metabolism, the TPS-f subfamily with *Clarkia breweri* linalool synthase, and the newly identified TPS-g cluster which consists of a group of mono-TPS that produce acyclic compounds. I chose representative TPS from each of the angiosperm TPS subfamilies (Table 5.4) and the TPS-d subfamily and analyzed them as described above. Individual TPS subfamilies (TPS-a through TPS-g) are clearly demarcated within this analysis (Figure 5.13). In agreement with the analysis of genomic TPS sequences (Trapp and Croteau, 2001b), the di-TPS of the gymnosperm TPS-d subfamily cluster near the angiosperm di-TPS of gibberellic acid metabolism and the Clarkia linalool synthase. The close proximity of these sequences is largely influenced by the presence of the ancestral ~ 200 amino acid motif, an attribute that is shared among all these di-TPS sequences, Clarkia linalool synthase and several sesqui-TPS of the TPS-d subfamily, but apparently has been lost in the TPS-a, TPS-b and TPS-g subfamilies as well as in all conifer mono-TPS and most conifer sesqui-TPS known to date (Trapp and Croteau, 2001b; Bohlmann et al., 1998b).

An intriguing new discovery from comparative analysis of gymnosperm and angiosperm involves the presence of a cluster of unusual TPS that form either acyclic sesquiterpenes, oxygenated monoterpenoids or the hemiterpene isoprene within the TPS-d1 and the TPS-b subfamilies which are otherwise dominated by regular mono-TPS (Figure 5.13). Surprisingly, the two angiosperm and gymnosperm mono-TPS subfamilies both contain farnesene synthases. The independent characterization of ortholog farnesene synthases from Norway spruce (this study) and from loblolly pine (Phillips et al., 2003) provides good evidence that these unexpected sesquiterpene synthase activities are not the result of cloning- or other artifacts. However, this finding indicates that a relatively simple sesqui-TPS enzyme mechanism for the formation of acyclic farnesene from FDP likely arose from mono-TPS precursor enzymes in both angiosperms and gymnosperms. At least two necessary events were required for this to happen. Exons coding for transit peptides must have been eliminated, and modifications of the original mono-TPS active site enabling the acceptance of FDP over GDP must have occurred. Norway spruce farnesene synthase PaFar was not active with GDP, suggesting that this is not the result of simple extension of substrate specificity but involves the more difficult to explain loss of activity with the smaller GDP substrate. Within the TPS-d1 subfamily, the newly characterized (–)-linalool synthase from Norway spruce also clusters closely to these farnesene synthases from Norway spruce and loblolly pine. Similarly, in the TPS-b subfamily this cluster is comprised of *E,E*-α-farnesene synthase from *Malus x domestica* (AAO22848), the hemi-TPS isoprene synthase from *Populus alba* x *Populus tremula* (Miller et

al., 2001), and geraniol synthase from *Cinnamomum tenuipilum* (CAD29734) and likely the geraniol synthase from *Ocimum basilicum* (Iijima et al., 2004) (not shown). The presence of sesqui-TPS in these mono-TPS subfamilies (and a hemi-TPS in TPS-b) was supported by high bootstrap values and demonstrates a similar evolution of these TPS within both angiosperms and gymnosperms.

Another interesting feature of the gymnosperm and angiosperm TPS gene family is the presence of multiple clusters of limonene synthases (TPS-d1 and TPS-b), myrcene synthases (TPS-d1, TPS-b, and TPS-g), and linalool synthases (TPS-d1, TPS-b, TPS-f and TPSg) (Figure 5.13). The phylogenetic distance between these TPS suggests multiple gene duplication events both in gymnosperms and angiosperms were followed by multiple events of convergent evolution to arise at the same function. The ability of distantly related TPS of secondary metabolism to produce similar or identical products is a puzzling feature of the TPS gene family and again makes the case for experimental assessment of gene function over in silico comparative assignment of function.

DISCUSSION

In an effort to identify genes of direct and putative indirect terpenoid defenses in Norway spruce (Martin et al., 2002; Martin et al., 2003b) and in order to provide a better foundation for phylogenetic analysis of the gymnosperm TPS family, I have cloned and functionally characterized a set of nine new *TPS* genes from this conifer species. Along with the previously described (+)-3-carene synthase (Fäldt et al., 2003b), the ten Norway spruce *TPS* genes represent one of the largest and functionally most diverse sets of characterized *TPS* from a single species. This suite of genes contains members encoding for mono-TPS, sesqui-TPS and di-TPS each with a distinct product profile of terpenoids known to occur in Norway spruce resin defense and volatile terpenoid emissions (Martin et al., 2003b; Martin et al., 2002). Among the *TPS* genes described here are two of biochemical functions that have not previously been described in any species, namely the multi-product sesqui-TPS, longifolene synthase, and the new di-TPS, iso-7,15-pimaradiene synthase. In addition, we identified the first linalool synthase, a monoterpenol synthase, from a gymnosperm.

Sequence Relatedness of Norway Spruce TPS

As with TPS from grand fir (Bohlmann et al., 1999), the mono-TPS proteins from Norway showed in the range of 61 to 69% identity and 75 to 83% similarity among each other (Table II). Among the Norway spruce mono-TPS we discovered a gymnosperm linalool synthase which is only very distantly related in sequence to known angiosperm linalool synthases from *Clarkia breweri* (14% I, 25% S) (Dudareva et al., 1996) and *Arabidopsis thaliana* (23% I, 44% S) (Chen et al., 2003) (Figure 5.13), but rather resembles conifer TPS that form monoterpene hydrocarbons suggesting that linalool synthase activity evolved independently in gymnosperms and angiosperms. Furthermore, since this enzyme is also able to synthesize small amounts of cyclic monoterpenes, the production of linalool may be the result of recent amino acid changes in a TPS derived from a regular mono-TPS. Interestingly, the newly identified Norway spruce *E,E*-α-farnesene synthase (PaTPS-Far), a sesqui-TPS, is also very similar to the conifer mono-TPS (60 to 67% I; 72 to 79% S), however, the lack of a transit peptide was the first indication for a sesqui-TPS which was confirmed by functional characterization of the expressed protein. The mono-TPS and *E,E*-α-farnesene synthase are much less similar to the other two Norway spruce sesqui-TPS, longifolene synthase and *E*-α-bisabolene synthase (28 to 40% I; 42 to 63% S) and to the two di-TPS (24 to 25% I; 36 to 38%S).

Surprisingly, the three Norway spruce sesqui-TPS described here fall into three distinct groups of the gymnosperm TPS-d subfamily (Figure 5.12). *E,E*-α-farnesene synthase (PaTPS-

Far) is closest in sequence to mono-TPS (TPS-d1). The multi-product longifolene synthase (PaTPS-Lon) is most closely related to the previously characterized grand fir γ-humulene synthase and δ-selinene synthase (Steele et al., 1998a) of the newly defined TPS-d2 group. The third spruce sesqui-TPS, *E*-α-bisabolene synthase (PaTPS-Bis) is most similar to its ortholog in grand fir and clusters closely with conifer di-TPS of the TPS-d3 group containing a 200-215 amino acid motif of yet unknown function. This cluster association pattern suggests that three types of sesqui-TPS evolved several times independently within the conifer TPS-d family from ancestors shared with di-TPS or mono-TPS. For instance Norway spruce *E*-α-bisabolene synthase and *E,E*-α-farnesene synthase may have evolved from di-TPS and mono-TPS, respectively, after loss of exons coding for transit-peptides (Trapp and Croteau, 2001b) and specialization for FDP as substrate in the cytosol. Curiously, both *E*-α-bisabolene synthase and *E,E*-α-farnesene synthase catalyze relatively simple sesqui-TPS reactions, yielding each only a single acyclic or monocyclic product (Table 5.3). These functions may arise more easily than the complex sesqui-TPS cyclization mechanisms. The more complex cyclization reactions, in conifers, all seem to come with very elaborate multiple-product profiles as seen with members of the TPS-d2 subgroup in Norway spruce (this study) and grand fir (Steele et al., 1998a).

The two Norway spruce di-TPS, levopimaradiene/abietadiene synthase (PaTPS-LAS) and isopimara-7,15-diene synthase (PaTPS-Iso), are very similar (90% I, 94% S) to each other and much less similar to either sesqui-TPS (38 to 43% I, 57 to 63% S) or mono-TPS (24 to 42% I, 36 to 61% S). Remarkably, these two enzymes represent the two most similar TPS found in Norway spruce, however their product profiles are entirely different (Figure 5.10). It is also interesting to note, that despite very similar amino acid sequences, isopimara-7,15-diene synthase is a single-product enzyme whereas levopimaradiene/abietadiene synthase is a multi-product enzyme. Mechanistic differences between levopimaradiene/abietadiene synthase and isopimara-7,15-diene synthase most likely reside in a few amino acid differences within the active sites of these paralogous enzymes (see below).

Evolution of Gymnosperm TPS

The large TPS family of angiosperms has been divided into subfamilies based on sequence relatedness and functional assessment (Bohlmann et al., 1998b), and based on gene architecture (Trapp and Croteau, 2001b). Our phylogenetic analyses show that independent groups also exist within the gymnosperm TPS-d subfamily and that a further division based on these groups is justified. The inclusion of a large number of functionally characterized

Norway spruce TPS, described here, as well as several gymnosperm TPS from the literature enabled an expansion of the TPS-d tree into three groups based on sequence relatedness and biochemical classes. This analysis also indicated that some manifestation of biochemical functions of very similar di-TPS predated not only the separation of firs and spruces with the pine family, but in fact predated the separation of the Ginkgoales and the Coniferales. Since the Ginkgoales and the Coniferales evolved from a common progymnosperm ancestor (Chaw et al., 1997), this finding suggests that di-TPS of secondary metabolism existed prior to the division of these two orders. In this evolutionary model, we should expect to also find di-TPS of similar secondary metabolisms within other gymnosperm orders, such as the Cycadales. Our findings with enzymes of all three biochemical classes of TPS, mono-TPS, sesqui-TPS and di-TPS, show that similar conifer TPS do group together and therefore support an evolutionary model that suggests specialization of many TPS biochemical functions prior to conifer speciation. A number of TPS homologs in the three genera *Picea*, *Abies*, and *Pinus* produce the same product profile, while in other homologous TPS, some sequence mutations have resulted in slightly modified product profiles of these enzymes. With the isolation and phylogenetic analysis of an increasing number of gymnosperm TPS it will become increasingly feasible to pinpoint sequence mutations that lead to functional diversification and to discriminate those from functionally silent amino acid substitutions. Our new snapshot of the gymnosperm TPS-d family and comparison with the angiosperm TPS suggests that the TPS-d subfamily may have as many limbs as does the angiosperm tree of TPS (Figure 5.13). When analyzing the gymnosperm TPS-d subfamily within the larger family of known plant TPS, several important features were observed. Gymnosperm di-TPS cluster closely to the di-TPS primary gibberellic acid metabolism of the angiosperms (TPS-c and e) which all contain the ancestral ~ 200 amino acid di-TPS motif (Bohlmann et al., 1998b). This is similar to the result found when analyzing genomic TPS sequences (Trapp and Croteau, 2001b). However, the gymnosperm di-TPS (TPS-d3) still group cohesively with the rest of the TPS-d subfamily and members that do not contain the ancestral ~200 amino acid motif. Therefore, one important missing link between the gymnosperm and angiosperm TPS families, and potentially a shared root of these families, remains to be found, possibly with future discovery of a gymnosperm di-TPS for gibberellic acid formation.

Compelling similarities between angiosperm (TPS-b) and the gymnosperm (TPS-d1) TPS were found regarding simple sesqui-TPS which cluster closely with mono-TPS that produce oxygenated monoterpenes and with hemi-TPS (angiosperms). This demonstrates simple sesqui-TPS have evolved from mono-TPS in both gymnosperms and angiosperms. Likewise the presence of multiple rather distantly related limonene synthases, myrcene synthases, and

linalool synthases provide evidence that these TPS evolved more than once in angiosperms and gymnosperms.

Comparative Modeling of Norway Spruce TPS Active Site Structures

The cloning, functional characterization and phylogenetic analysis of a family of Norway spruce mono-, sesqui- and diTPS desribed in this study identified several interesting candidate enzymes for future structure-function analysis of TPS catalysis. In the absence of any authentic three-dimensional structure for a gymnopserm TPS, comparative protein modeling against more distant angiosperm TPS can reveal valuable information about amino acid positions in conserved proteins. The x-ray structures of 5-*epi*-aristolochene synthase (NtEAS) from *Nicotiana tabacum* (Starks et al., 1997) as well as bornyl diphosphate synthase (SoBDS) from *Salvia officialis* (Whittington et al., 2002) provide the necessary templates for the modeling of conifer TPS (Peters and Croteau, 2002; Phillips et al., 2003; Little and Croteau, 2002). Through comparative modeling I hope to pinpoint amino acids involved in catalysis and provide a starting point for future site directed mutagenesis of selected conifer TPS.

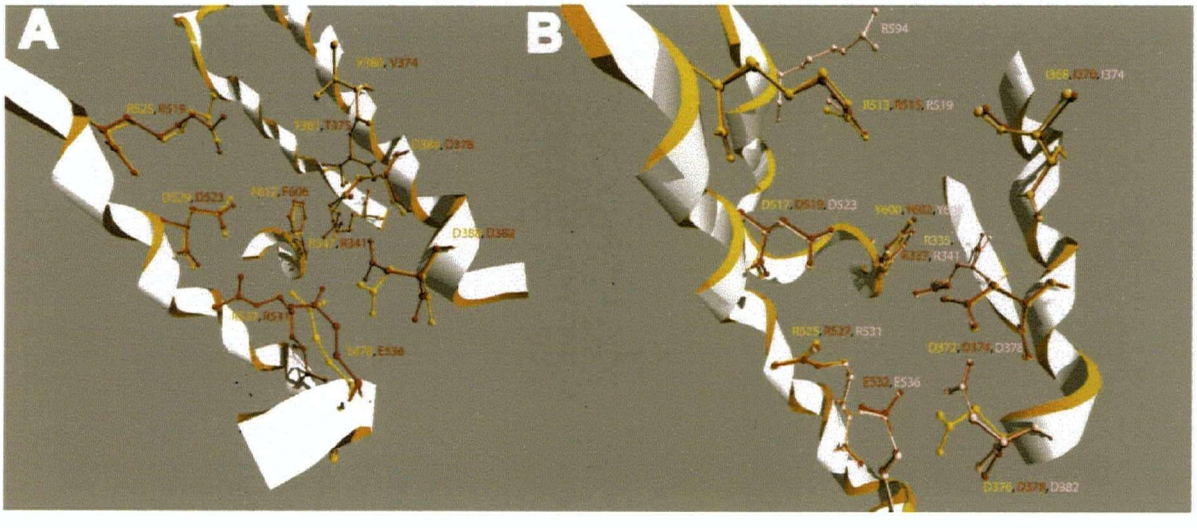

Figure 5.14: Comparative modeling of Norway spruce (P. abies) mono-TPS based on the crystal structure of bornyl diphosphate synthase from S. officialis.

(A) Models of P. abies myrcene synthase (orange) and grand fir (*A. grandis*) myrcene synthase (red) show identical amino acids present in the active site of these two homologous enzymes. (B) (−)-pinene synthase models from *P. abies* (orange), Sitka spruce (*P. sitchensis*) (pink) and *A. grandis* (red) show that these enzymes differ in the proximity of several amino acids with respect to the active site. Fewer amino acids seen in the *P. abies* model may help explain why this enzyme

produces many products as opposed to only two products formed from the other (−)-pinene synthases.

Initially, I selected two closely related myrcene synthases one from Norway spruce (PaTPS-Myr) and one from grand fir (AgMyr) to test the feasibility of comparative modeling of mono-TPS from two different species against the angiosperm SoBDS (Whittington et al., 2002). I found that these two conifer mono-TPS do have the same amino acids present in their modeled active sites (Figure 5.14A). Similarly, pinene synthases from Norway spruce (PaTPS-Pin), Sitka spruce (PsPin), and grand fir (AgPin) were also modeled. These mono-TPS are similar but all produce slightly different product profiles (Table 5.5). Models of the three pinene synthase enzymes were very similar (Figure 5.14B). Several amino acids seen in the AgPin (E532) and PsPin (E536 and R594) active site models were not seen within the PaTPS-Pin model in a 3 Å snapshot. I also chose to model two conifer sesqui-TPS, *E*-α-bisabolene synthases, from Norway spruce (PaTPS-Bis) and grand fir (AgBis) based on NtEAS (Starks et al., 1997). Comparative modeling placed the same amino acids within the active site of both of these enzymes (Figure 5.15A). These comparative models have painted a picture of some of the amino acids involved in the active sites of these TPS for future analysis and provided some level of confidence for modeling of conifer mono-TPS and sesqui-TPS, two biochemical classes of TPS for which three-dimensional structures exist with angiposperm TPS.

Recent work on abietadiene synthase (AgAS) from grand fir (Peters et al., 2001; Ravn et al., 2000; Peters et al., 2003; Ravn et al., 2002; Peters et al., 2000) has dissected the mechanism of this bifunctional conifer di-TPS. AgAS is very similar to Norway spruce PaTPS-LAS and PaTPS-Iso thereby making the recent findings relevant to the mechanisms of these two enzymes as well. While PaTPS-LAS and AgAS have nearly identical qualitative and quantitative muliple-product profiles, the single product of PaTPS-Iso is distinctively different. Yet, PaTPS-Iso is overall more similar in amino acid sequence to PaTPS-LAS, than PaTPS-LAS and AgAs are to each other. Modeling the active sites of PaTPS-LAS, PaTPS-Iso, and AgAS on NtEAS (Starks et al., 1997) revealed only two amino acid differences within this region (Figure 5.15B). One such difference is located within the conserved TPS active site of the PATPS-LAS and PaTPS-Iso models and is defined by an overlapping amino acid difference at Y686_PaTPS-LAS, Y696_AgAS and H694_PaTPS-Iso. The only other difference in these models is the presence of S721_PaTPS-Iso. In both PaTPS-LAS and AgAS, this position is occupied by A713 and A723 respectively (not shown). Levopimaradiene synthase from *Ginkgo biloba* (Schepmann et al., 2001), also capable of producing an abietane skeleton, has a Y700 and an A727 at these positions. These results suggest that comparative modeling of conifer di-

TPS against a distant angiosperm sesqui-TPS could pinpoint the relevant amino acid substitutions in otherwise highly conserved pairs of di-TPS. These modeling studies will direct future experiments which will analyze the involvement of these amino acids in catalysis.

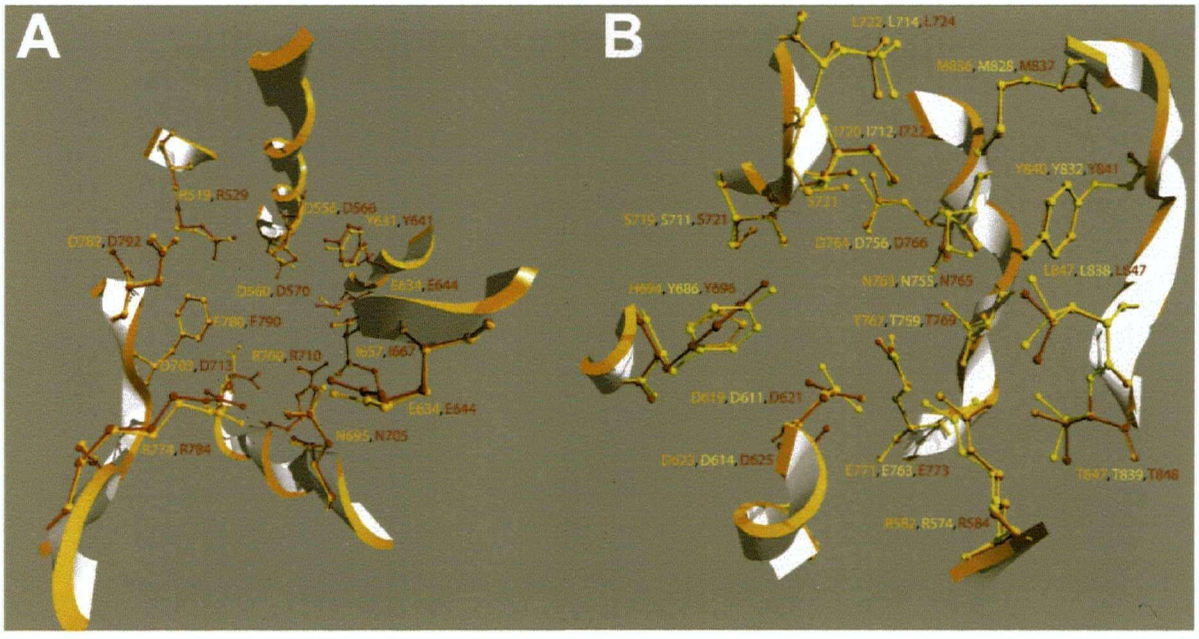

Figure 5.15: Comparative modeling of P. abies and A. grandis sesqui-TPS and di-TPS.

(A) E-α-bisabolene synthases from *P. abies* (orange) and *A. grandis* (red) demonstrate the same amino acids model in the active site of these homologs. (B) Models of isopimara-7,15-diene synthase PaTPS-Iso (orange) and levopimaradiene/abietadiene synthase PaTPS-LAS (yellow) from *P. abies* as compared to abietadiene synthase AgAS (red) from *A. grandis* demonstrate two main amino acid differences: H964_PaTPS-Iso versus Y686_PaTPS-LAS and Y696_AgAS; and S721_PaTPS-Iso versus A713_PaTPS-LAS (not shown) and A721_AgAS (not shown) between these three related proteins. Differences are unique to the functionally distinct PaTPS-Iso.

Conclusion

In summary, the family of 10 functionally characterized *TPS* genes from Norway spruce is critical to quality, quantity, and the structural diversity of terpenoid defenses in this species. The isolation of these genes has provided a means to better understand some of the phylogenetic relationships that exist in the TPS-d subfamily and the TPS family. Comparative modeling can provide further information beyond simple sequence alignments by allowing the identification of specific amino acids within the active sites of conifer TPS enzymes and will guide future structure-function analysis. Furthermore, the suite of 10 spruce *TPS* genes has provided the tools for transcript profiling of *mono-TPS*, *sesqui-TPS* and *di-TPS* in stem tissues and foliage in response to MeJA-treatment and in response to attack by stem boring insects

(*Pissodes strobi*) in species of spruce (Miller, Madilao and Bohlmann, unpublished results). These studies extend on previous TPS gene expression analysis in the spruce system (Fäldt et al., 2003b; Byun McKay et al., 2003) and show that *TPS* gene expression profiles closely match quantitative and qualitative changes in terpenoid metabolites in stems and terpenoid volatile emissions in foliage.

MATERIALS AND METHODS

Materials

Norway spruce seedlings (clone 244-932) used for RNA isolations and cDNA preparation were previously described (Fäldt et al., 2003b). RNA for RACE was prepared from 2-year-old Norway spruce seedlings grown under natural environmental conditions on the UBC campus. Substrates (2 mg/mL), GDP and FDP, were from Echelon Bioscience Inc. (Salt Lake City, UT). GGDP was from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Unless otherwise mentioned, all other reagents and solvents were from Fischer Scientific Ltd. (Pittsburg, PA), Sigma-Aldrich, or VWR International (West Chester, PA). All standards used in GC analysis were of the highest purity available.

General Molecular Biology Procedures

Routine PCR reactions were performed in volumes of 50 µL containing 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 200 µM of each dNTP, 0.1 µM of a forward primer and a reverse primer, one unit of Taq polymerase (Invitrogen, Carlsbad, CA), and 2ng of template DNA. PCR amplifications for subcloning of TPS cDNAs were performed in volumes of 50 µL containing 20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% Triton X–100, 10 µg BSA, 200 µM of each dNTP, 0.1 µM of each primer, 2.5 U of high fidelity Turbo Pfu polymerase (Stratagene, La Jolla, CA). All PCR reactions were performed using MJ PTC100-thermocyclers (Waltham, MA). QIAquick Gel Extraction kits (Qiagen, Valencia, CA) were used to extract DNA from agarose gels and plasmid DNA was isolated using QIAprep Spin Miniprep kits (Qiagen, Valencia, CA). DNA was sequenced using DyeDeoxy Terminator Cycle Sequencing (Applied Biosystems) at the Nucleic Acids Protein Services Unit (UBC, Vancouver, Canada). For hybridization experiments, DNA was labeled with [α-32P]dCTP (Easytide, Perkin Elmer, USA) using the Rediprime™ II random prime labeling system (Amersham-Pharmacia Biotech Inc., USA). Cloning vectors included the pCR-Blunt vector (Invitrogen), the pCR2.1-TOPO (Invitrogen), the pET100/D-TOPO and pET101/D-TOPO directional expression vectors (Invitrogen) and pSBET (Schenk et al., 1995).

cDNA Library Screening

A Norway spruce young shoot λ-ZAP phage library was screened for mono-TPS and sesqui-TPS exactly as described in Fäldt et al. (2003b). For isolation of di-TPS and sesqui-TPS

containing the 210 amino acid element (Bohlmann et al., 1998a; Bohlmann et al., 1998b) a portion of this region was amplified using the primers ASDL1 and ASDL2 (Table 5.6) and 2 µL of the λ-ZAPII cDNA library as a template. The resulting 540 bp amplicon was cloned into pCR2.1-TOPO (Invitrogen) and transformed into *E. coli* TOP10F' cells (Invitrogen). After single colony isolation and sequence verification from plasmid DNA, the 540-bp probe was amplified by PCR, purified by agarose gel electrophoresis, labeled with Rediprime procedure and used to screen by filter hybridization the Norway spruce cDNA library as described (Fäldt et al., 2003b). A total of 290 primary positive phage plaques yielded 72 pBluescript SK(-) phagemids isolated with the mono-TPS probe (Fäldt et al., 2003b) and 8 pBluescript SK(-) phagemids isolated with the di-TPS/sesqui-TPS probe after secondary screening. Phagemids were *in vivo* excised in *E. coli* XL1 Blue MRF' and transformed into *E. coli* SOLR. Plasmid DNA was prepared from individual transformants, inserts sequenced and compared to the GenBank database using blastx (www.ncbi.nlm.nih.gov/BLAST/). Bluescript plasmids identified as PaTPS-Far, PaTPS-Lin, PaTPS-Pin and PaTPS-Bis showed high sequence similarities to mono-TPS and sesqui-TPS and were subcloned into expression vectors. Sequences of partial plasmid clones similar to conifer di-TPS were used to design primers for RACE.

RACE

Norway spruce seedlings were sprayed with 0.1 mM MeJA in a 2% (v/v) Tween-20/H20 solution. Trees were harvested 2 days, 4 days, and 6 days after treatment. Samples of needles were combined over the three collection days and RNA extracted according to the method by Wang SX et al (2000). The FirstChoice RLM-RACE kit (Ambion, Austin, TX) was used to generate both 5'- and 3'-RACE PCR templates. Nested 5'-PCR reactions were carried out with 2 µL of the RACE template, 400 µM each dNTP, 200 mM Tris-HCl (pH 8.8), 20 mM MgSO4, 100 mM KCl, 100 mM (NH4)2SO4, 1% Triton X-100, 1 mg/ml nuclease-free BSA, 3.125 U of PfuTrubo, and 0.4µM each vector based forward primer and sequence based reverse primer. These conditions were held constant for all RACE PCR reactions. To isolate full-length di-TPS cDNAs, two sequential nested PCR reactions were performed using the outer Rc3-5c and inner Rc3-5a (Table 5.6) di-TPS primers. Products of this PCR reaction were cloned into the pCR-Blunt vector (Invitrogen) and transformed into E. coli TOP10F' cells (Invitrogen). Single colonies were isolated, plasmid DNA was sequenced and the resulting sequences were organized into contigs. Using the sequence information, RCAAS5-31 outer and RCAAS5-32 (Table 5.6) inner forward primers were designed for 3'-RLM-RACE PCR. Using cDNA-based forward primers and vector-based reverse primers sequential nested PCR reactions were

performed. Products were cloned into pCR-Blunt and sequenced as before. RACE methods produced overlapping sequences to discern the entire reading frame of di-TPS-like cDNA. Using this information, Pa24_05a forward and Pa2_3b reverse primers were designed and the 3' RLM-RACE template was used to amplify the full-length cDNA. Products of this reaction were cloned into the pCR-Blunt vector (Invitrogen) and sequenced. From this one template two different full-length cDNAs (PaTPS-LAS and PaTPS-Iso) were isolated. Primers designed to target mono-TPS genes were used to amplify full-length genes from the 3' RLM-RACE template. The primers 743_5'a and 743_3'stp were used in a primary reaction and 743_M5petTP and 743_3'stp in a secondary reaction, yielding the full-length cDNA, PaTPS-Lim. This PCR product was cloned directly into the pET101 (Invitrogen) expression vector and transformed into Top10 F'cells.

Table 5.6: Oligonucleotide primers used in this study

Primer Name	Sequence
ASDL1	GCIGTIGAIGCIGGIGAGCTIAIIAAIG
ASDL2	GAICTIAIIAAIGAICCATCITIIIITTG
Rc3-5c	CACTCGCGTTCTCTATGTATTC
Rc3-5a	TGTAAGAATCTCCCCAGTGAAAAG
RCAS5-31	GGTGGACATCATCGGGTTTCTCA
RCAS5-32	TGACGGGCATGGAACTTTAGACAA
Pa24_05a	ATGCTTTCGGCTCCTACACTCGTAA
Pa2_3b	CTAAGCAACGGGTTGGAAGAGGC
743_5'a	CATTATTTTGAGCTATCTCTTCAGGT
743_M5petTP	CACCATGTCTCCTGTTTCTGTCA
743_3'stp	TTACAAAGGCACAGGTTCAAG
cl39Pe5a	CACCATGGCTCTTCTTTCTATCGCACCGCT
cl39Pet5b	CACCATGAGACGCATAGCCGATCATCATCCCAAT
cl39Pet3a	TTATAAAGGCACATGCTCAATGACTG
5'71RR_pET	CACCATGAGACGCGTAGGCGATTATCATTCCAA
3'71_BAM3	CTTACATAGGTACAGGTTCAAGGAC
pET100pa03F100	CACCATGACTAGCGTTTCTGTTG
pET100pa03aR2502	GAGTGGGAGTGGTTCGATC
486_M5pet	CACCATGGCTCAGATTTCTAAATGTTCGT
486_3'stp	CTAAGTAAGTGGATCGATGAGAAT
245petM	CACCATGGCCTTGCTTTCGTCTTCA
Pa24_pet5b	CACCATGCTTTCGGCTCCTACACTCGTAA
25_L79_pet	CACCATGCTTTCGGCTCCTACACTCGTGA
JF104NDE1_103	TATGAGACGGATGGGCGATTTC
JF104BAM3_105	CTTACAAAGTCACAGGATCAATCACG
JF104NDE2_104	TGAGACGGATGGGCGATTTC
JF104BAM4_106	GATCCTTACAAAGTCACAGGATCAATCACG
JB016RE_152	GGTACCAGACGCATAGGTGACTACC
JB016RE_153	CTGCAGTTAAAAAAGCATAGATTC

Subcloning of TPS cDNAs for Expression in E. coli

Plastidial targeting peptides are present in monoterpene and diterpene synthases and delineated by the RRX_8W motif (Williams et al., 1998; Bohlmann et al., 1998b). For several TPS, deletion of plastidial targeting peptide was shown to improve expression of functional TPS enzymes (Williams et al., 1998; Bohlmann et al., 1999). For most mono-TPS and di-TPS cDNAs described here, two constructs were made, one with and one without the transit peptide. For subcloning, TPS cDNAs were amplified by PCR using high fidelity Turbo Pfu polymerase (Stratagene) with 2 µL of the amplified λ-ZAPII phage library (Fäldt et al., 2003b) or 2 ng of plasmid DNA. For PaTPS-Lin the primer combinations cl39Pe5a and cl39Pet3a or cl39Pet5b and cl39Pet3a were used for full-length and truncated constructs, respectively. Amplification of PaTPS-Far utilized the primer combination 5'71RR_pET and 3'71_BAM3. Primers for PaTPS-Bis were pET100pa03F100 and pET100pa03aR2521. PCR products were cloned into the pET100/D-TOPO expression vector. PaTPS-Lon was amplified using λ-ZAPII phage library as template and primers designed for GenBank accession AF369920, 486_M5pet and 486_3'stp. PCR product cloned into pET100/D-TOPO vector. According to Peters et al. (2000), the transit peptide of A. grandis abietadiene synthase is cleaved close to Leu79 since this truncation allows maximum enzyme activity. Similar to the A. grandis protein, PaTPS-LAS and PaTPS-Iso also have leucines at amino acids 72 and 80, respectively. cDNA inserts of PaTPS-LAS and PaTPS-Iso in the pCR-Blunt vector were amplified by PCR to generate full-length and truncated versions for subcloning into the pET100/D-TOPO vector. Since these two genes are highly similar, it was possible to use the same 3' primer, Pa2_3b, for amplification. The 5' primer for full-length constructs of both PaTPS-LAS and PaTPS-Iso was 245petM and for truncated constructs, Pa24_pet5b and 25_L79_pet, were used for PaTPS-LAS and PaTPS-Iso respectively.

The *PaTPS-Pin* cDNA insert of plasmid pBluescript-*PaTPS-Pin* was subcloned into pSBET expression vector. Introduction of *NdeI* and *BamHI* ends was accomplished by the sticky end PCR cloning strategy (Zeng, 1998). Primer combinations for insert PCR amplification were JF104NDE1_103 and JF104BAM3_105 as well as JF104NDE2_104 and JF104BAM4_106. The two amplified segments of *PaTPS-Pin* were denatured (2 min at 95°C), combined and re-annealed (30 min at room temperature), to create sticky ends (*NdeI* and *BamHI*), and subsequently ligated into *NdeI*/*BamHI*-digested pSBETa as described by Bohlmann et al., (1999). Restriction endonuclease sites, *KpnI* (5' end) and *PstI* (3' end), were introduced into the *PaTPS-Myr* cDNA insert of plasmid *PaTPS-Myr* by PCR amplification using the combination of JB016RE_152 and JB016RE_153 primers. PCR product of *PaTPS-Myr* was digested with *KpnI* and *PstI*, gel purified and ligated into *KpnI*/*PstI*-digested pQE50. In all cases, the recombinant expression plasmids

were rescued and maintained in *E. coli* TOP10 F' cells, analyzed by PCR using insert and/or vector based primers, sequenced. For expression, plasmids were transformed into *E. coli* BL21-CodonPlus (Stratagene) cells for the pET and pQE constructs or into BL21(DE3)Star (Invitrogen) cells for pSBET constructs.

Expression of TPS in E. coli and Enzyme Assays

Functional expression and enzyme assays have been previously described in Fäldt et al. (2003b). Briefly, cultures were grown in 5 mL LB and the appropriate antibiotic (either ampicillin or kanamycin), induced with 200 µM IPTG, centrifuged and resuspended in mono-TPS buffer (25 mM HEPES, pH 7.2, 100 mM KCl, 10 mM $MnCl_2$, 10% glycerol, 5 mM DTT), sesqui-TPS buffer (25 mM HEPES, pH 7.3, 10 mM $MgCl_2$, 10% glycerol, 10 mM DTT) or di-TPS buffer (25 mM HEPES, pH 7.2, 100 mM KCl, 10 mM $MgCl_2$, 10 µM $MnCl_2$, 5% glycerol, 5 mM DTT). Cells were homogenized by ultrasound treatment, extracts were cleared by centrifugation and the supernatant was removed and assayed for TPS activity with GDP, FDP, or GGDP. Substrate concentrations for assays were used at a final concentration of 137 µM GDP, 92.3 µM FDP and 40 µM GGDP. A 1 mL pentane overlay was used to trap terpene products during the assays and in all cases, after incubation at 30°C for 1 h, the reaction mixture was further extracted with pentane (3 x 1 mL). The combined pentane fraction was extracted with water or purified over silica/$MgSO_4$ as described (Bohlmann et al., 1997; Fäldt et al., 2003b; Lafever et al., 1994; Lewinsohn et al., 1991b; Martin et al., 2002; Peters et al., 2000). Pentane extracts were then evaporated to 50-100 µL and 1 or 2 µL were injected and analyzed by GC-FID and GC-MS as previously described (Fäldt et al., 2003b; Martin et al., 2002; Martin et al., 2003b). Controls for product formation independent of any cDNA were performed using extracts of *E. coli* BL21-CodonPlus (DE3) transformed with either plasmid without the insert.

Terpenoid Product Identification by GC-MS

Monoterpenes were identified and quantified by GC-MS analysis on an Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector (70 eV) using a DB-WAX (J&W Scientific, Palo Alto CA, USA) capillary column (0.25 mm i.d. x 30 m with 0.25-µm film) or an HP-5 (Agilent Technologies, Palo Alto, CA, USA) capillary columns (0.25 mm i.d. x 30 m with 0.25-µm film). An injector was used at 200°C and a column flow of 1 mL He min^{-1}. Using the DB-WAX column the following temperature program was used: the initial temperature of 40°C (4 min. hold) was increased to 150°C at 4°C min^{-1} followed by a 20°C

min^{-1} ramp until 230°C (5 min. hold). The temperature program for the HP-5 column began at 40°C (2 min hold) with an initial ramp of 3°C min^{-1} until 160°C followed by a 10°C min^{-1} increase to 200°C. A final increase of 20°C min^{-1} until 300°C (3 min hold) completed the program. A Cyclodex B (permethylated β-cyclodextrin in DB-1701, J&W Scientific) capillary column (0.25 mm i.d. x 30 m with 0.25-μm film) was used for separation of enantiomers of *PaTPS-Pin* monoterpenoid products. For separation enantiomers of α-pinene and β-pinene, sabinene, limonene, β-phellandrene, the following temperature program was used: initial temperature was 55°C (1 min. hold) which was then increased to 100°C at 1°C min^{-1} followed by a 10°C min^{-1} ramp until 230°C (10 min. hold). Analysis of enantiomers products of PaTPS-Lin and PaTPS-Lim were performed with a Cyclosil B column [30% hepatkis (2,3-di-O-methyl-6-O-t-butyl dimethylsilyl)-β-cyclodextrin in DB-1701] (Agilent Technologies) capillary column (0.25 mm i.d. x 30 m with 0.25-μm film). To separate linalool enantiomers, the following program was used on an Agilent 6890 GC fitted with an FID: 60°C (6 min hold) initial temperature then 3°C min^{-1} until 180°C followed by 20°C min^{-1} to 220°C (5 min hold). For the separation of limonene, α-pinene and β-pinene enantiomers, the program was as follows: 60°C (6 min hold) initial temperature then 3°C min^{-1} until 180°C followed by 20°C min^{-1} to 220°C (5 min hold). Sesquiterpenes and diterpenes were initially separated on an HP-5 capillary column (0.25 mm i.d. x 30 m with 0.25-μm film). Sesquiterpenes were separated with the following program of 40°C (1 min hold), then 5°C min^{-1} until 180°C, followed by 20°C min^{-1} until 300°C (1 min hold). Diterpenes were separated on the HP-5 column using the following program: 40°C (1 min hold), then 7.5°C min^{-1} until 210°C followed by 3°C min^{-1} until 280°C (5 min hold). Additional product analysis and confirmation of results obtained with the HP5 column were performed on the DB-WAX column (see above). The program used with the DB-WAX column was 40°C (3 min. hold), increased to 250°C at 5°C min^{-1} (5min hold). All terpenoid products were identified using authentic standards and/or library matches. Libraries utilized include the Wiley Library and *The Identification of Essential Oil Components by Gas Chromatography/Quadrapole Mass Spectroscopy* Library (Adams, 2002). Further confirmation of product ID was done by separation on more than one type of column combined with Kovat's index information (Adams, 2002).

Sequence and Phylogenetic Analyses

Predictions for pI were made using the entire open reading frame within EditSeq 5.00 (DNASTAR Inc.). Amino acid alignments were made with ClustalX (www-igbmc.u-strasbg.fr/BioInfo/) and GeneDoc (www.psc.edu/biomed/genedoc/). Since transit

peptides are not well conserved, these were truncated from mono-TPS and di-TPS prior to analyzing TPS for phylogenetic relationships. The sequences were then aligned using Dialign (Morgenstern et al., 1998) (available through MAGI at the HGMP www.hgmp.mrc.ac.uk), a program capable of finding local similarities in divergent sequences. Multiple sequence alignments were then hand corrected using GeneDoc (www.psc.edu/biomed/genedoc/). Maximum likelihood analyses using the data sets for the TPS-d subfamily as well as for the entire TPS family were analyzed by Phyml (Guindon and Gascuel, 2003) using the JTT (Jones et al., 1992) amino acid substitution matrix. The proportion of invariant sites as well as the alpha shape parameter was estimated by Phyml. Trees were generated using BIONJ (Gascuel, 1997), a modified neighbor joining algorithm. SEQBOOT of the Phylip 3.6 package (Felsenstein, 1993) (evolution.genetics.washington.edu/phylip.html) was used to generate 100 bootstrap replicates. These were then analyzed by Phyml using the previously estimated parameters. CONSENSE also from the Phylip 3.6 package (evolution.genetics.washington.edu/phylip.html) was used to generate a consensus tree. Distance analyses were completed on 1000 bootstrap replicated data sets (SEQBOOT) using PROTDIST and Dayhoff PAM 001 matrix (Dayhoff, 1979). NEIGHBOR and CONSENSE were used to generate the neighbor-joining consensus tree. All distance analyses were completed using programs of the Phylip 3.5c package (Felsenstein, 1993) available through UBiC Resources & Support at the UBC Bioinformatics Centre, University of British Columbia (www.bioinformatics.ubc.ca). Treeview (Page, 1996) was used to visualize all trees. Bootstrap values above 50% for both the maximum likelihood analysis and the distance analysis were added to the maximum likelihood tree calculated from the original data set.

Comparative Modeling

Comparative modeling of TPS was accomplished using the DeepView/Swiss-PDB Viewer and the SWISS-PROT Modeler (Guex and Peitsch, 1997). Energy was minimized by GROMOS96 (igc.ethz.ch/gromos) (Van Gunsteren et al., 1996) executed within the DeepView/Swiss-PDB Viewer interface. Snapshot views were examined by choosing amino acids with 3.25 Å (mono-TPS) 3.75 Å (sesqui-TPS) or 4.0 Å (di-TPS) of the substrate analog and Mg2+ ions from either bornyl diphosphate synthase from Salvia officialis (mono-TPS) or 5-epi-aristolochene synthase from Nicotiana tabacum (sesqui-TPS and di-TPS). Views were rendered using PovRay 3.5 (www.povray.org).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner under standard material transfer agreements for non-commercial research purposes.

ACKNOWLEDGEMENTS

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6. DISCUSSION

While each of the four previous chapters has had a detailed discussion pertaining to that chapter, this general discourse will relate the topics of induced and constitutive terpene biosynthesis with the activities of individual TPS, cloned and functionally characterized in this work. As the first three chapters have been published for over a year, I will also detail relevant studies in the field of conifer induced defense responses that have been published in the interim. Finally, a perspective for future directions in the study of conifer defenses will be provided.

BIOCHEMICAL FUNCTIONS OF CLONED NORWAY SPRUCE TPS AND CONSTITUTIVE AND INDUCED TERPENOID PROFILES

Studies presented in this dissertation describe the complex constitutive and MeJA-induced terpenoid defenses in Norway spruce at anatomical, chemical, and biochemical levels [Chapters 2 and 4, (Martin et al., 2002; Martin et al., 2003b)]. MeJA-induced responses in spruce resemble those induced by mechanical wounding, stem boring insects or fungal pathogens (Alfaro et al., 2002; Byun McKay et al., 2003; Nagy et al., 2000; Krekling et al., 2004; Franceschi, 2002). They include the well-orchestrated induction of TPS enzyme activities associated with the development of xylem traumatic resin ducts and the concomitant increases in terpenoid accumulation in wood and bark tissue [Chapter 2, (Martin et al., 2002)]. Following MeJA treatment, the volatile emission profile of Norway spruce saplings also dramatically changed producing many more oxygenated monoterpenoids, in particular the monoterpenol linalool, and an abundance of sesquiterpenoids [Chapter 4, (Martin et al., 2003b)].

The cloning and functional characterization of ten Norway spruce *TPS* genes allows an enhanced evaluation of induced changes in terpenoid metabolite profiles. Many of the products of the TPS described here [Chapters 3 and 5, and (Martin et al., 2004; Fäldt et al., 2003b)] correspond with the observed metabolite profiles after MeJA treatment or insect attack in species of spruce. My work on Norway spruce terpenoid responses to MeJA treatment (Martin et al., 2002; Martin et al., 2003b) is discussed here with an emphasis on those compounds that are products of the now characterized *TPS* genes [Table 6.1, Chapters 3 and 5, and (Martin et al., 2004; Fäldt et al., 2003b)].

While nearly all monoterpenoids produced by the cloned TPS are found constitutively in Norway spruce, increases in many monoterpenoids occurred after MeJA treatment in wood and bark, but to much lesser extend in needles [Chapters 2 and 4, (Martin et al., 2002; Martin et al., 2003b)]. The most prominent monoterpenes from Norway spruce are β-pinene and α-pinene. Accumulation of these compounds was substantially induced following MeJA treatment. Furthermore, analysis of enantiomers showed that following MeJA treatment the (—)-α- and (—)-β-pinenes increased relative to the (+)-α- and (+)-β-pinene enantiomers (Martin et al., 2002). This finding indicated the presence of enantiomer-specific pinene synthases. Here we identified a (—)-α/β-pinene synthase (*PaTPS-Pin*) (Chapter 5). Another highly abundant terpene in bark, wood and needles is myrcene. Concentrations of myrcene increased following MeJA treatment suggesting a role for myrcene synthase (*PaTPS-Myr*) in this response. Limonene is the fifth most abundant monoterpene in Norway spruce. Following MeJA treatment, concentrations of limonene increased dramatically in all three tissues. These findings indicate a role for (—)-limonene synthase (*PaTPS-Lim*). Accumulation of 3-carene increased dramatically in MeJA induced wood tissue (Table 6.1), although, 3-carene was also seen constitutively in bark, foliage, and volatiles, interestingly, 3-carene volatilization decreased from MeJA treated foliage [Chapter 3, Table 6.1 and (Fäldt et al., 2003b)]. Correspondingly, constitutive activities of 3-carene synthase are likely in bark, and foliage but an enhanced role is likely for 3-carene synthase in developing TDs.

Volatile terpenoid emissions in response to insect oviposition or MeJA treatment were recently described for Scots pine (*Pinus sylvestris*) (Hilker et al., 2002; Mumm et al., 2003) and Norway spruce [Chapter 4, (Martin et al., 2003b)]. Following MeJA treatment, monoterpenoids released by Norway spruce changed in composition from entirely hydrocarbons to mainly oxygenated monoterpenes. The most prominent monoterpene volatile released was linalool. A MeJA-induced linalool synthase activity was found in cell-free extracts of needles forming linalool in a ratio of 1:2 (*3R* : *3S*) [Chapter 4, (Martin et al., 2003b)]. Because TPS are normally highly stereospecific, there are likely two TPS responsible for the release of linalool in Norway spruce. The cloning and functional characterization of (—)-linalool synthase (*PaTPS-Lin*) was reported from this work (Chapter 5). The activities of this enzyme may be important in signaling predators or parasites of herbivores.

Table 6.1: Terpenoid metabolite analysis in tissues and headspace samples of Norway spruce.

Mono-, sequi-and diterpenoids are listed which match products of cloned *PaTPS* genes. Absolute amounts of metabolite are shown in Martin et al. (2002) and in Martin et al. (2003). In this summary, (+) indicates the presence of a metabolite; (++) indicates an increase of 1.4 to 3 fold after treatment with MeJA; (+++) indicates an increase of 3.1- 10 fold after MeJA; (++++) indicates an increase above 10 fold after MeJA treatment; (–) indicates a decrease of 1.4 to 4 fold after MeJA; and (0) indicates amounts below detection.

	Bark		Wood		Foliage		Volatiles	
	Control	MeJA	Control	MeJA	Control	MeJA	Control	MeJA
Monoterpenes								
α-pinene	+	++	+	++	+	++	+	++
ß-pinene	+	++	+	+++	+	++	+	++
myrcene	+	++	0	+++	+	++	+	+
3-carene	+	+	0	+++	+	+	+	-
limonene	+	+++	+	+++	+	++	+	+
ß-phellandrene	+	++	+	+++	+	++	+	-
α-terpinolene	+	++	+	+++	0	0	0	0
linalool	0	0	0	0	0	0	+	++++
Sesquiterpenes								
E,E-α-farnesene	0	0	0	0	0	0	+	+
E-α-bisabolene	+	++	+	-	0	0	+	++++
longifolene	+	++	+	-	+	-	0	0
longipinene	+	++	+	-	0	0	0	0
Diterpenes								
abietate	+	+	+	++	0	0	0	0
levopimarate	+	++	+	+++	0	0	0	0
neoabieatate	+	+	+	++	0	0	0	0
isopimarate	+	+	+	+	0	0	0	0

Although amounts of sesquiterpenoids in Norway spruce bark, wood, and needles are only a fraction of those of monoterpenoids and diterpenoids, there are profound increases in some sesquiterpenoids after MeJA treatment [Chapters 2 and 4, (Martin et al., 2002; Martin et al., 2003b)]. Most prominently, the release of the sesquiterpene volatile *E*-α-bisabolene was drastically increased after MeJA treatment [Chapter 4, (Martin et al., 2003b)]. *E*-α-bisabolene also increased in bark with MeJA treatment [Chapter 2, (Martin et al., 2002 and Table 6.1)].

Activities of the *E*-α-bisabolene synthase (*PaTPS-Bis*) found in this study are almost certainly involved in these responses. The sesquiterpenes longifolene and longipinene decreased in wood tissue following MeJA treatment (Table 6.1), but increased in bark tissue. It is likely that longifolene synthase (*PaTPS-Lon*) controls the levels of these two compounds. *E,E*-α-farnesene, the product of *PaTPS-Far*, was found only as a volatile emission in Norway spruce, but was not increased in response to elicitation [Chapter 4, Table 6.1, and (Martin et al., 2003)].

Diterpenes occur in Norway spruce mainly in the form of diterpene resin acids. The precursor of diterpenes is GGDP and is made by GGDP synthase. Activites of GGDP synthase were increased in MeJA treated wood [Chapter 2 (Martin et al., 2002)]. This suggests that a level of control of diterpene resin acids may reside partly in the flux of GGDP and the regulation of GGDP synthase. The four most abundant diterpene hydrocarbons that are produced by the two di-TPS of this study, levopimaradiene/abietadiene synthase *PaTPS-LAS* and isopimara-7,15-diene synthase *PaTPS-Iso*, are precursors for the major diterpene resin acids in Norway spruce bark and wood (Chapter 2, Table 6.1, and (Martin et al., 2002)]. Concentrations of resin acids abietate, levopimarate, and neoabietate increase in both bark and wood following MeJA treatment. This indicates a likely involvement of the levopimaradiene/abietadiene synthase described here. Isopimarate, the diterpene resin acid of isopimara-7,15-diene, was found in bark and wood, but was not increased after MeJA treatment. The finding of two separate *di-TPS* genes and identification of their product profiles suggests that coordinated up-regulation of diterpene resin acids abietate, levopimarate, and neoabietate but lack of increase of isopimarate is regulated by differential expression of these two *di-TPS*.

This correlative analysis of terpene accumulation, TPS activity, and functional characterization of cloned TPS should be used as a basis for future studies regarding TPS gene expression. The work presented here offers the tools and the means by which to further examine the multifaceted aspects of induced terpene defenses in spruce.

INFLUENCE OF THIS RESEARCH ON CONIFER DEFENSE STUDIES

An emerging picture of the multifaceted terpenoid defenses in Norway spruce has been constructed through studies of the MeJA induced defense responses and has emphasized the involvement of not just direct defenses, but also the possibility of tritrophic defenses. Research presented here on *P. abies* has highlighted the importance of MeJA inducible defenses in the differentiation of new cytohistological structures such as traumatic resin

ducts (TD) [Chapter 2, (Martin et al., 2002)], in the accumulation of oleoresin terpenoids and volatile release of terpenes which may play pivotal roles in signaling insect predators and parasites [Chapter 4, (Martin et al., 2003b)]. These results highlight the effectiveness of inducing traumatic resinosis in a dose dependent manner with MeJA and have led the way for investigations into other aspects of MeJA induced conifer defenses. Based on the findings presented in Chapter 2, Franceschi et al (2002) initiated field studies using mature trees and 2-year old saplings of Norway spruce and their results confirmed that MeJA treatment induces the formation of TD systemically. They also documented the MeJA induced development of polyphenolic parenchyma cells in the phloem. However, at this point, the role these cells play in defense has not been thoroughly analyzed. Similar to my studies, this research concluded that cytological changes induced by MeJA treatment were comparable to studies involving wounding (Nagy et al., 2000), fungal inoculations or bark beetle attacks (Christiansen et al., 1999; Franceschi et al., 2000; Krekling et al., 2004) or weevil damage (Alfaro, 1995). Additional studies examined the effects of MeJA on histological changes in *Larix occidentalis*, *Picea pungens*, *Pinus monticols*, *Pseudosuga menziezzi* and *Taxus brevifolia* (Hudgins et al., 2003). The results indicate that within the Pinaceae MeJA is a powerful elicitor of traumatic resinosis, however, this was not found true for *Taxus brevifolia*. While these studies observed only cytological changes, additional studies detailing qualitative and quantitative changes in MeJA induced terpenoid defenses among members of the pine family and other gymnosperms is under investigation (J.W. Hudgins, D.M. Martin, L. Madilao, V. Franceschi, and J. Bohlmann unpublished data).

MeJA was shown to induce *mono-TPS* and *di-TPS* gene expression in stem tissue and *mono-TPS* gene expression in foliage in Norway spruce [Chapter 3, (Fäldt et al., 2003b)]. A similar response was found to occur for *mono-TPS* gene expression in *P. sitchensis* following wounding and weevil attack (Byun McKay et al., 2003). The induction of terpene based defenses in Norway spruce has presented a rationale by which to make MeJA-induced RACE templates for the purpose of cloning genes involved in this response [Chapter 4, (Martin et al., 2004)]. One *mono-TPS*, *PaTPS-Lim*, and two *di-TPS*, *PaTPS-Iso* and *PaTPS-LAS*, were isolated from MeJA-induced templates. Future studies will address MeJA, wounding and herbivory induced gene expression of genes encoding these and the other seven TPS identified in the work presented here. The research unveiled in this thesis has also provided a rationale for the genomic and proteomic studies addressing conifer induced defense responses. MeJA as well as insect induced responses are currently topics addressed by the Forestry Genome Project in collaboration with Genome B. C.

The ten TPS cloned and functionally characterized (Chapters 3 and 5, (Martin et al., 2004; Fäldt et al., 2003b)] including two TPS, PaTPS-Lon and PaTPS-Iso, with unique functions and one TPS, PaTPS-Lin, with a function not seen before in a gymnosperm, enabled an expansion of the TPS-d subfamily and a further dissection of gymnosperm evolution. The phylogeny of the gymnosperm TPS has been re-constructed and the division of this subfamily based on sequence relatedness and biochemical function has been presented (Chapters 5). With the characterization of an increasing number of gymnosperm TPS genes we will likely see this family develop into a pattern suggestive of the six angiosperm subfamilies. At this point it may then be necessary for a further division of the gymnosperm TPS-d subfamily. Furthermore, isolation of TPS from the Cycadales or of gymnosperm TPS involved in gibberellic acid metabolism will enable a broader reconstruction of the origin and evolution of TPS.

SIMILARITIES OF MEJA INDUCED RESPONSES IN *P. ABIES* WITH THOSE FROM ANGIOSPERMS

Interestingly, the *de novo* formation of TD in *P. abies* after MeJA treatment is similar to the MeJA-induced development of terpene-filled trichomes in tomato (Li et al., 2004). Like TD, trichomes in this species both produce and store terpenes for use in defense against herbivores. These studies implicate that not only do JA and MeJA activate pathways involved in secondary metabolism, but they also regulate the formation of cells and structures involved in the synthesis and the storage of these molecules. Another important similarity between *P. abies* MeJA induced responses and those known from angiosperms included the identity of compounds released as volatiles. Induced volatiles from Norway spruce were dominated by sesquiterpenes including *E*-β-farnesene and *E,E*-α-farnesene, and oxygenated monoterpenes including linalool. These major components are also primary constituents of angiosperm volatiles that were released in response to JA induction or as a response to herbivore damage (De Moraes et al., 2001; Hern and Dorn, 2001; Kessler and Baldwin, 2001; Paré and Tumlinson, 1997; Pichersky and Gershenzon, 2002). The function of these compounds in indirect defenses has been well documented in angiosperms and it is likely that a similar mechanism is important for gymnosperms. Furthermore, it is possible that insect herbivores are better able to detect the acyclic and/or oxygenated metabolites as these compounds prevail in both of these systems.

FUTURE DIRECTIONS:

We are only beginning to understand inducible terpene-based defenses, the expression of *TPS*, and the activity of TPS, and how these defenses are integrated into histological changes, terpene accumulation and/or volatile production. Only a selected number of species, namely grand fir, sitka spruce and Norway spruce, have had terpene defenses examined on multiple levels and we are still some ways away from a complete picture of the development and induction of these defenses. The findings discussed here (Chapters 2 through 5) on the terpenoid defense mechanisms of Norway spruce and the cloning and characterization of corresponding TPS will help take conifer defense research in new directions.

Octadecanoids and Biological Elicitors

While The role of plant derived (endogenous) biological elicitors as well as elicitors present in herbivore saliva or excretions offers exciting avenues for future study in conifer defense. Currently, octadecanoid signaling, including jasmonate (JA) and MeJA, is still largely undescribed in conifers. It is not known whether octadecanoids function as endogenous elicitors in gymnosperms as they do in angiosperms (Kessler and Baldwin, 2002). To address this question, studies must be completed on gymnosperm species that monitor octadecanoid levels in vivo or MeJA emissions in response to herbivore or pathogen challenges. Much more can also be learned regarding the similarities of this response to that which is known from angiosperm species. Franceschi et al (2002) noted that MeJA did not activate a systemic response in mature trees as visualized by a decreasing intensity of TD development away from the MeJA treatment. The locality, translocation, and the very existence of MeJA and JA signaling in conifers calls for further investigation. Moreover, a comparison of MeJA induced responses versus herbivory induced responses will result in more thorough understanding of conifer chemical signaling and the role of JA in these responses. The role of other endogenous signal molecules such as auxin, ethylene and brassinosteroids should also be investigated with respect to the activity of these molecules in TD development.

Several chemical elicitors have been identified from herbivores of angiosperms (Kessler and Baldwin, 2002), however, insect derived elicitors from conifer herbivores have not yet been identified. It is likely that chemical compounds located in saliva or other excretions of conifer pests may play a pivotal role in mediating terpene defense responses. JA responsive transcription factors are known from several angiosperm species (Memelink et al., 2001). Therefore, the exploration of conifer transcription factors involved in *TPS* gene

expression and octadecanoid correlated gene activation in conifers presents a fascinating research opportunity. The discovery and characterization of conifer transcription factors involved in secondary metabolism may eventually enable the directed control of these pathways.

Effectiveness of Traumatic Resinosis and Volatile Emissions in Direct and Indirect Defense Interactions

An understanding of the role of traumatic resinosis in bark, wood and foliage has been initiated, however, it is still unclear how effective these defenses are against herbivores and pathogens. Future studies should concentrate on correlating induced traumatic resinosis with herbivore/pathogen mortality. Relating to my work with Norway spruce volatile emissions (Chapter 4), Mumm et al (2003) describe a similar volatile release for insect ovipositing as for jasmonate treatment and noted that these volatiles were effective in attracting egg parasitoids. The prominence of E-β-farnesene in both the emissions from Norway spruce as well as from *Pinus sylvestris*, requires additional investigation as this compound may be a key constituent in tritrophic signaling. Additional experiments are needed to evaluate how effective conifer volatile cues are in attracting an array of predators and parasites. Furthermore, the diurnal pattern of emission of spruce-induced volatiles begs a biological explanation and an exploration of how these emissions are possibly tailored to specific insects.

While much interest has been directed to the use of anti-aggregation phermones and other repellent chemicals to protect forests against herbivore attacks (Borden, 1984; Camacho and Borden, 1994; Miller et al., 1995; Poland and Borden, 1998), manipulating conifer induced terpene defenses has not been adequately addressed. MeJA treatments have been used to attract predators of tomato herbivores and the effectiveness of this treatment was dramatic (Thaler, 1999). Furthermore, MeJA applications have been shown to induce defense responses in Norway spruce (Martin et al., 2003b; Martin et al., 2002; Fäldt et al., 2003b; Franceschi, 2002). It is now time to address in field experiments whether pretreatment with MeJA will effectively 1) prevent herbivore attackes, 2) increase mortality of invading herbivores, 3) inhibit the growth of beetle associated pathogens, and/or 4) enhance tritrophic interactions and thereby control herbivore populations. Currently a study addressing the effectiveness of MeJA pretreatment on lodgepole pine (*Pinus contorta*) against the mountain pine beetle (*Dendroctonus ponderosae*) and blue stain fungus (*Ceratocystis polonica*) complex is underway. As we unravel the complex factors governing conifer resin

defenses, we may eventually be able to use induced resinosis to protect our trees and to manage damaging populations of conifer herbivores.

The Role of Induced Terpenes Against Fungal Pathogens

While MeJA treatment was shown to increase conifer seedling survival in response to the root pathogen *Pythium ultimum* (Kozlowski et al., 1999), the effects of induced terpene defenses have not been adequately addressed in root tissue nor have they been thoroughly explored in relation to the response of mature trees to pathogenic root fungi or root burrowing beetles. For instance, *Inonotus tomentosus* is a devastating root fungus of spruce trees and a median stage of this disease increases the likelihood of spruce beetle infestations (Lewis and Lindgren, 2002). The authors suggested that the presence of this fungus enhances the beetle's chance of colonization. It is possible that an *I. tomentosus* infection inhibits the tree's ability to mount a successful induced terpene response in this species. As mentioned above, an examination of the *P. contorta*, *D. ponderosae*, and *C. polonica* ecological triangle is expected to shed light on the interplay of beetle damage and associated fungal growth and how MeJA may protect this conifer. While the complex dynamics of tree/insect/fungus and tree/insect/predator interactions show both abiotic and biotic factors governing the health of forests (Eckhardt et al., 2002; Erbilgin and Raffa, 2002), understanding induced terpene defenses in these ecosystems will address one more aspect of this ecological system.

Structure-Function of Conifer TPS

More immediate outcomes of the characterization of ten *TPS* genes from Norway spruce include studies aimed at understanding structure-function relationships within individual TPS. Experiments aimed at determining which amino acids control the product profiles of the remarkably similar di-TPS, PaTPS-LAS and PaTPS-Iso, have been initiated and are based in part on the comparative modeling presented here. By identifying amino acids responsible for the differences in these di-TPS product profiles we will better understand the mode of catalysis of these enzymes. This information is particularly relevant since there is currently no crystal structure known for di-TPS nor for conifer TPS. The comparative modeling (Chapter 5) made a stronger case for the involvement of specific amino acids within these extremely similar TPS (90 % I, 94 % S) than for the less related myrcene synthases, pinene synthases and *E*-α-bisabolene synthases. Ultimately, we can expect the accuracy of comparative modeling of conifer TPS to be enhanced by the availability of a crystal structure

for a gymnosperm TPS. A deeper knowledge of the processes determining product specification may one day lead to tailor-made TPS with unique functions and enhanced biological activity for the biotechnological industry (Martin et al., 2003; Maharbiz et al., 2004).

Expression Analyses of TPS

The ten TPS identified from Norway spruce provided the tools to detail spruce *TPS* gene expression analyses in response to MeJA treatment, and in response to attack by stem boring insects (*Pissodes strobi*) (B. Miller, L. Madilao, and J. Bohlmann unpublished). The results of this research complement what is already know about the induced resinosis in Norway spruce [Chapters 2, 3, 4 and (Fäldt et al., 2003b; Martin et al., 2002; Martin et al., 2003b)] and provide a more thorough examination of insect induced TPS gene expression, than that published previously (Byun McKay et al., 2003). This analysis of TPS gene expression has enabled us to illuminate another facet of spruce terpene defenses and will certainly lead to a more comprehensive analysis of conifer induced gene expression.

Cellular Localization of TPS Gene Expression and TPS Activity

One of the most underrepresented areas of research on conifer defense responses includes the localization of TPS and *TPS* gene expression to specific tissues and/or cells. While it is believed that the epithelial cells surrounding the resin ducts both synthesize and secrete terpenes into the duct lumen, this has not been demonstrated experimentally. Furthermore, the coordination of *TPS* gene expression and the creation of new TPS protein with that of terpene accumulation should be addressed in a cell specific manner. In situ hybridization and immunolocalization of TPS class-specific probes provide a means by which to address this question. These methods have worked well in identifying cell types involved in synthesis of alkaloids in opium poppy (Facchini and De Luca, 1995; Bird et al., 2003). As the biochemistry of induced terpene defenses and the development of TD have been well described in spruce, this system is ideal for future studies in TPS localization.

Additionally, the advent of laser dissection microscopy techniques presents a fascinating means by which to further address RNA and protein analysis in a tissue and/or cell specific manner (Kehr, 2003). These techniques could be applied aptly to xylem mother cells and the epithelial cells that surround developing TDs and would allow a temporal and spacial analysis of cellular functions occurring in this response. We would expect such studies, at the

very least, to address receptors involved in monitoring signal molecules, the cell specific induction of *TPS* gene expression and other genes involved in this defense response, and to address terpene export proteins expected to secrete terpenes into the duct lumen for which ABC transporters could be candidates (Grec et al., 2003). Through cell-directed technologies, the processes governing TD initiation and development may soon come to light.

Genomic and Proteomic Analyses of Induced Terpene Defenses

While the understanding of induced conifer defenses has increased dramatically with much information now available from metabolite profiling of terpenoids and the study of associated terpenoid pathways, there is still much to be learned. Genomic and proteomic analyses are expected to provide answers to many lingering questions in this field. Forestry genomic and proteomic projects have already begun to tackle many of these issues. It is expected that this work will address in a tissue specific manner both a gobal gene expression profile as well as an expression profile targeted to *TPS* and other genes known to function in defense. This will result in a better understanding of which genes are involved in defense responses and will provide candidate genes for further characterization and in vivo analysis. Proteomic analyses will generate information about specific protein involvements and will also yield interesting protein candidates to target for an enhanced examination. Most importantly, both genomic and proteomic analyses will quite possibly offer information about genes and proteins not previously known or regarded to participate in plant defense. The synthesis of these upcoming results will enable us to conceptualize conifer defenses on many different levels and will eventually lead to a holistic understanding of these responses.

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8. APPENDIX

ROLE OF CO-AUTHORS

Chapter 2

The investigation of MeJA induced traumatic resinosis (Martin et al., 2002) presented in chapter two was exclusively done by the author, save for the one experiment involving prenyl transferases (Figure 2.8) that was completed by the co-author Dr. Dorthea Tholl. Drs. Jörg Bohlmann and Jonathan Gershenzon served as supervisors for this work. The writing of the manuscript was completed by Drs. Jörg Bohlmann and Jonathan Gershenzon and the author.

Chapter 3

The work detailing the cloning of 3-carene synthase (Fäldt et al., 2003b) was completed by the author, and Drs. Jenny Fäldt and Suman Rawat. The author was involved in teaching molecular biology to Dr. Fäldt. Furthermore, the author generated the probe and the cDNA library discussed in this work. The autor was also responsible for the phylogenetic analysis presented in Figure 3.8. Dr. Fäldt cloned, expressed and identified the products of this (+)-3-carene synthase. Dr. Suman Rawat performed the northern experiments and Dr. Jörg Bohlmann acted as supervisor for this project. The writing of the manuscript was completed by Drs. Bohlmann and Fäldt and the author.

Chapter 4

This work detailed in this chapter on MeJA induction of volatile terpenes (Martin et al., 2003b) was completed solely by the author. Again, Drs. Jörg Bohlmann and Jonathan Gershenzon served as supervisors for this work. The writing of the manuscript was completed by Drs. Bohlmann and Gershenzon and the author.

Chapter 5

The cloning and phylogeny of nine TPS genes examined in this chapter (Martin et al., 2004) was the work of Dr. Jenny Fäldt and the author. Dr. Jenny Fäldt cloned four of these genes and expressed and identified the product(s) of two. The author was responsible for the cloning of five of these genes, the expression and product identification of seven of these TPS. Furthermore, all the phylogenic analyses presented here are the work of the author. The

Dr. Jörg Bohlmann acted as supervisor for this project. The writing of the manuscript was completed by Dr. Bohlmann and the author.

It is not feasible for Drs. Fäldt, Gershenzon, and Tholl to sign this document as they reside in Europe. It is not feasible for Dr. Suman Rawat to sign this document as her whereabouts are not known.

I, Diane Marie Martin, verify this information to be accurate and fair.

_____

I, Dr. Jörg Bohlmann, verify this information to be accurate and fair.

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