Wound-Induced Terpene Synthase Gene Expression in Sitka Spruce That Exhibit Resistance or Susceptibility to Attack by the White Pine Weevil[1][W]

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We analyzed the expression pattern of various *terpene synthase* (*TPS*) genes in response to a wounding injury applied to the apical leader of Sitka spruce (*Picea sitchensis* Bong. Carr.) genotypes known to be resistant (R) or susceptible (S) to white pine weevil (*Pissodes strobi* Peck.) attack. The purpose was to test if differences in constitutive or wound-induced *TPS* expression can be associated with established weevil resistance. All wounding treatments were conducted on 9-year-old R and S trees growing under natural field conditions within the range of variation for weevil R and S genotypes. Representative cDNAs of the *monoterpene synthase* (*mono-TPS*), *sesquiterpene synthase* (*sesqui-TPS*), and *diterpene synthase* (*di-TPS*) classes were isolated from Sitka spruce to assess *TPS* transcript levels. Based on amino acid sequence similarity, the cDNAs resemble Norway spruce (*Picea abies*) (−)-linalool synthase (mono-TPS; PsTPS-Lin*l*) and levopimaradiene/abietadiene synthase (di-TPS; PsTPS-LAS*l*), and grand fir (*Abies grandis*) δ-selinene synthase (sesqui-TPS; PsTPS-Sel*l*). One other *mono-TPS* was functionally identified as (−)-limonene synthase (PsTPS-Lim). No significant difference in constitutive expression levels for these *TPSs* was detected between R and S trees. However, over a postwounding period of 16 d, only R trees exhibited significant transcript accumulation for the *mono-* and *sesqui-TPS* tested. Both R and S trees exhibited a significant accumulation of PsTPS-LAS*l* transcripts. An assessment of traumatic resin duct formation in wounded leaders showed that both R and S trees responded by forming traumatic resin ducts; however, the magnitude of this response was significantly greater in R trees. Collectively, our data imply that the induced resinosis response is an important aspect of defense in weevil R Sitka spruce trees growing under natural conditions.

The production of oleoresin, a complex mixture of monoterpenes, diterpene resin acids, and sesquiterpenes, is an important physical and chemical defense against stem-boring insects and pathogens in conifers (Berryman, 1972; Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001; Huber et al., 2004; Martin and Bohlmann, 2005). Many conifers store large amounts of preformed oleoresin in specialized resin ducts or blisters in the bark, sapwood, and needles. Attack by stem-boring insects severs these ducts and blisters and releases the oleoresin, which repel attacking insects by intoxication and deterrence. Mono- and sesquiterpenoids emitted as volatiles can contribute to indirect defenses against herbivores, while diterpenoids can provide direct protection through formation of lasting physical barriers at the point of insect attack (Martin et al., 2003; Mumm et al., 2003; Miller et al., 2005).

In addition to preformed oleoresin defenses, synthesis of oleoresin and new traumatic resin ducts (TRD) can be induced in conifers by biotic or abiotic stimuli such as insect attack, pathogen invasion, and mechanical wounding (Croteau et al., 1987; Lieutier and Berryman, 1988; Tomlin et al., 1998; Nagy et al., 2000; Byun-McKay et al., 2003). In the absence of mechanical trauma, methyl jasmonate (MeJA) or ethylene can induce the formation of TRD (Martin et al., 2002, 2003; Hudgins et al., 2003, 2004; Hudgins and Franceschi, 2004), implicating these hormones as important signals in coordinating wound-induced defense responses in conifers. In species of spruce (*Picea* spp.) the formation of TRD is accompanied by the production of oleoresin (Franceschi et al., 2002; Martin et al., 2002; Miller et al., 2005) and the

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simultaneous formation of phloem parenchyma cells and associated phenolics (Franceschi et al., 1998, 2000). Conifers can also respond to insect attack by altering volatile terpenoid emissions, which can serve as parasitoid attractants (Hilker et al., 2002; Martin et al., 2003; Mumm et al., 2003; Miller et al., 2005). Thus, induced TRD defenses and volatile terpenoid emissions can play an important role in limiting insect and pathogen destruction of conifers through the qualitative alteration of terpenoid composition profiles that may alter their toxic properties or activities as kairomones in multitrophic defenses (Martin et al., 2002, 2003; Huber et al., 2004; Miller et al., 2005).

Mono-, sesqui-, and diterpenes are derived from the precursors gernanyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate through the action of the mono-, sesqui-, and diterpene synthase enzymes, respectively (Bohlmann et al., 1998). The formation of conifer diterpene resin acids involves further oxidation of diterpenes by one or more cytochrome P450-dependent monooxygenases (Funk et al., 1994; Ro et al., 2005). Conifer terpene synthase (TPS) enzymes are encoded by a large gene family that collectively accounts for terpenoid diversity in oleoresin, which can consist of 50 or more different terpenoids in a given species. A number of TPS genes have been isolated and characterized from conifers including grand fir (*Abies grandis*), loblolly pine (*Pinus taeda*), and spruce species (Bohlmann and Croteau, 1999; Fäldt et al., 2003; Phillips et al., 2003; Martin et al., 2004). In Norway spruce (*Picea abies*) several genes encoding monoterpene synthases (mono-TPS), diterpene synthases (di-TPS), and sesquiterpenes synthases (sesqui-TPS) have been functionally characterized (Martin et al., 2004). In Sitka spruce (*Picea sitchensis* Bong. Carr.), just a single mono-TPS has been isolated and shown to encode (−)-pinene synthase (Byun-McKay et al., 2003). Expression of *TPS* genes is positively regulated by mechanical wounding (Steele et al., 1998; Byun-McKay et al., 2003), MeJA (Fäldt et al., 2003; Miller et al., 2005), and insect attack (Byun-McKay et al., 2003; Miller et al., 2005).

In the Pacific Northwest, the white pine weevil (*Pissodes strobi* Peck.) is the most serious insect pest of regenerating Sitka spruce (Ying, 1991). The white pine weevil initiates attack in early spring when adults disperse to the apical leaders of host trees where they feed, mate, and oviposit. The larvae hatch, consume the phloem, cambium, and developing xylem, and effectively girdle the leader. Pupation occurs in the xylem, and adults emerge during the late summer. Such activities kill or damage the leader, resulting in reduced growth, stem deformities, and a significant loss of revenue (Alfaro, 1995). Because efforts to control weevil attack are largely ineffective (Alfaro and Omule 1990; Hall, 1994; Rankin and Lewis, 1994), planting of Sitka spruce is currently not recommended in high weevil hazard zones and has been reduced in British Columbia from more than 10 million to fewer than 1 million seedlings per year despite its importance as a timber species.

Given the limited success of weevil control programs, heritable host resistance is an ideal form of pest control. In British Columbia, resistant (R) Sitka spruce genotypes have been identified through repeated provenance trials and out plantings, but a thorough understanding of the mechanisms by which these R genotypes are able to limit their vulnerability to the white pine weevil is lacking (Ying, 1991; King et al., 2004; Ying and Ebata, 1994). In this study we sought to establish whether the induced resinosis defense response was associated with resistance of Sitka spruce to weevil attack. To achieve this we (1) isolated cDNA representatives from Sitka spruce for the mono-, sesqui-, and di-TPS families; (2) examined changes in expression levels of mono-, sesqui-, and di-TPS genes in R and susceptible (S) Sitka spruce genotypes following a simulated weevil attack under field conditions; and (3) documented the traumatic resinosis response in the wounded trees. Our data suggest that expression patterns of mono- and sesqui-TPS genes are related to resistance status of Sitka spruce. Furthermore, the formation of TRD was more vigorous in R trees, indicating that the induced resinosis response plays a key defensive role against weevil attack in Sitka spruce. These data may provide a foundation upon which current weevil resistance breeding programs can be based and by which Sitka spruce resistance to the white pine weevil can be predicted and evaluated.

RESULTS

Cloning of Mono-TPS, Sesqui-TPS, and Di-TPS cDNAs from Sitka Spruce

Similarity-based PCR and RACE cloning strategies were used to isolate cDNA representatives of the mono-, sesqui-, and di-TPS for characterization of the wound-induced traumatic resinosis response in R and S Sitka spruce genotypes. Four different cDNA fragments were obtained, two resembled known conifer mono-TPS (PsTPS1 [Byun-McKay et al., 2003] and PsTPS3), one resembled a known conifer di-TPS (PsTPS4), and the last was most similar to a known conifer sesqui-TPS (PsTPS5). Sequences of these partial TPS cDNAs were used for 5′ and 3′ RACE to recover three full-length TPS cDNAs and a fourth cDNA with a truncated C-terminal end. Based on amino acid sequence comparisons, one cDNA (PsTPS-Lim) is most closely related to the mono-TPS (−)-limonene synthase of Norway spruce (PaTPS-Lim; Martin et al., 2004; Figs. 1A and 2; 1,266 bits, 97% identity, 98% similarity; e = 0.0). A second full-length cDNA (PsTPS-Lin*l*) is most closely related to (−)-linalool synthase of Norway spruce (PaTPS-Lin; Martin et al., 2004; Figs. 1A and 2; 1,098 bits, 89% identity, 92% similarity, e = 0.0). The third full-length cDNA (PsTPS-Sel*l*) was most similar to δ-selinene synthase from grand fir (Figs. 1B and 2; 894 bits, 79% identity, 88% similarity; e = 0.0). The partial cDNA PsTPS-LAS*l* is most closely related to Norway spruce levopimaradiene/abietadiene synthase (PaTPS-LAS; Martin et al., 2004; Figs. 1C and 2; 1,040 bits, 92% identity, 94% similarity).

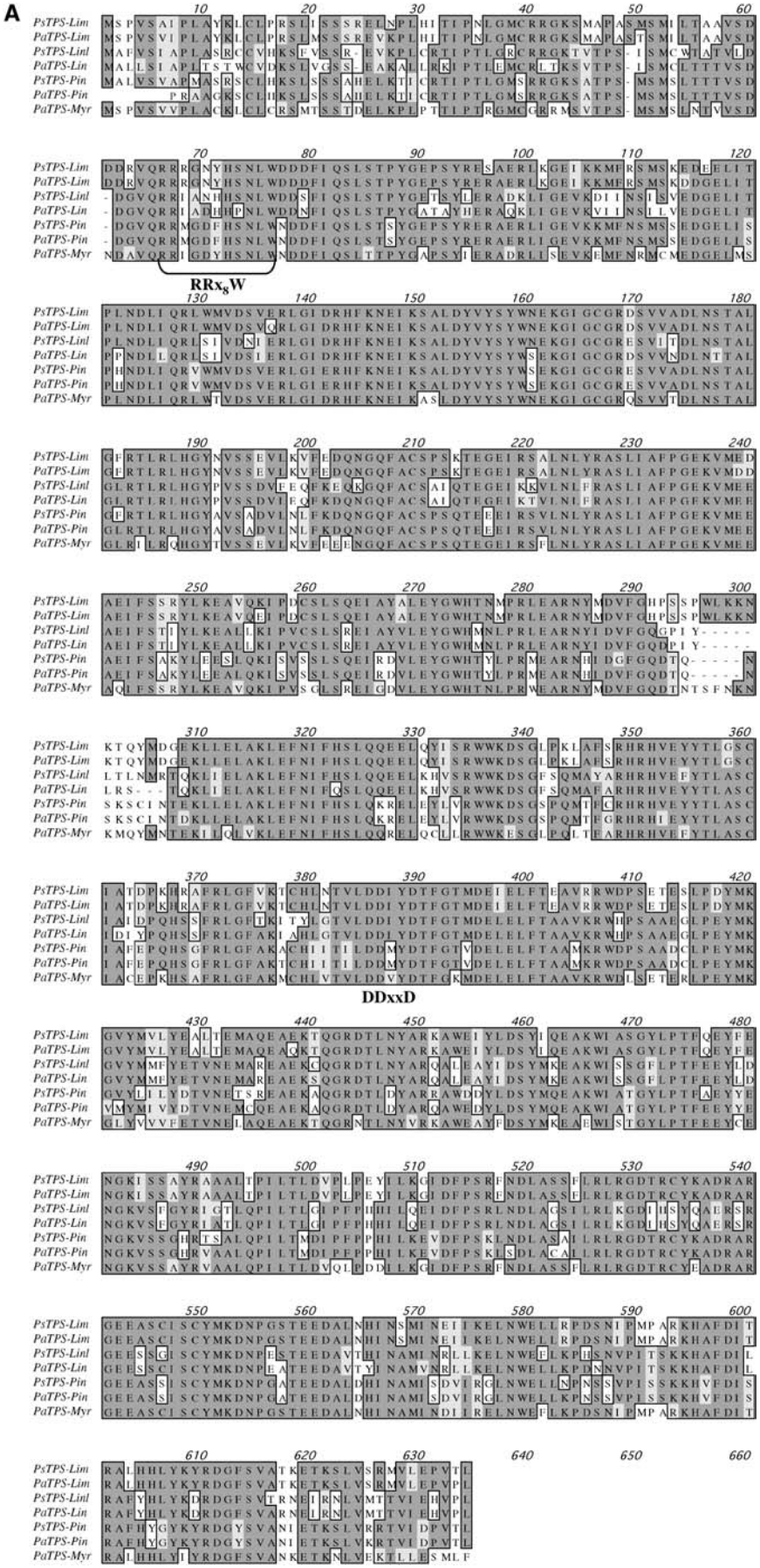
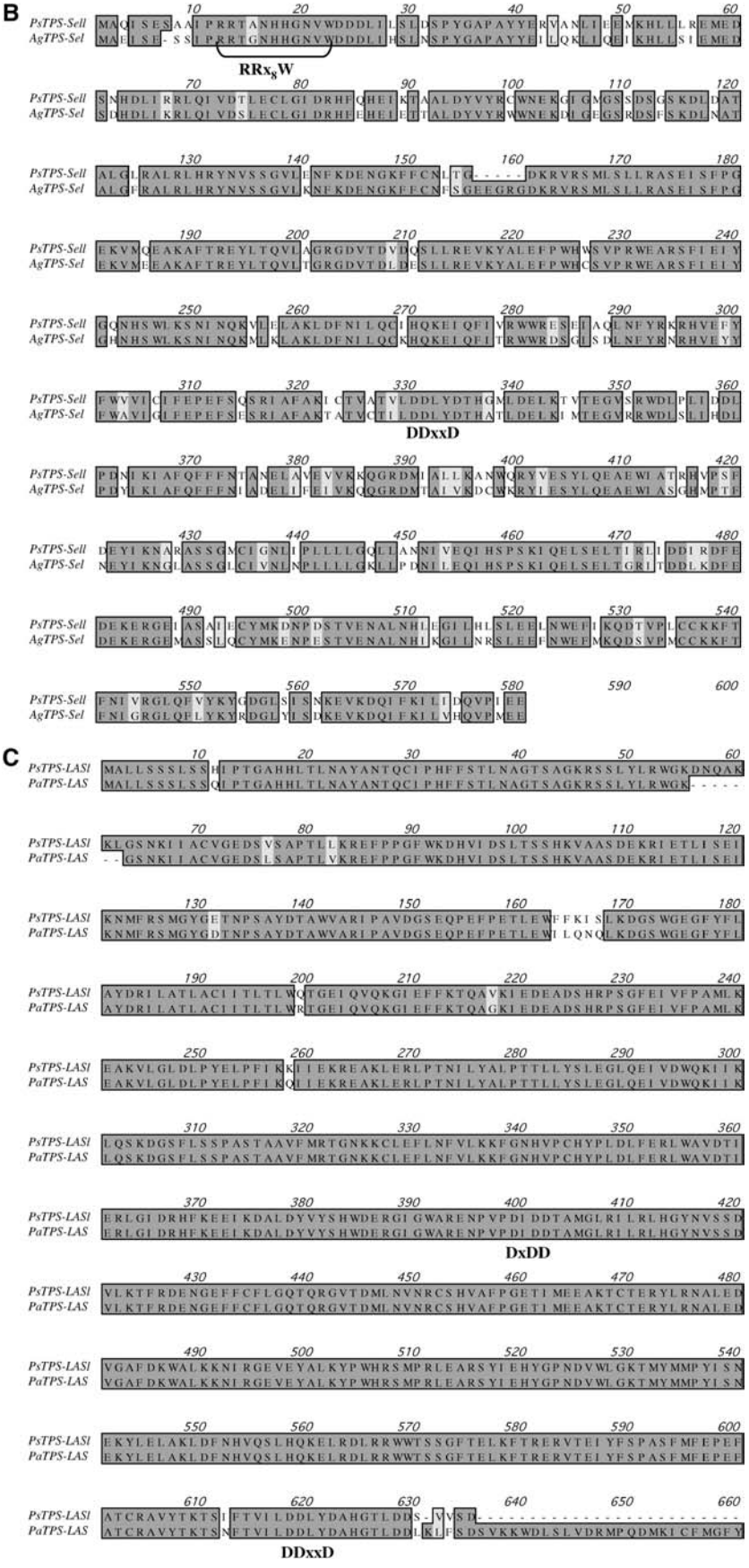


Figure 1. Multiple sequence alignment of the deduced amino acid sequences of Sitka spruce TPS with other conifer TPS. A, PsTPS-Lim and PsTPS-Lin*l* are aligned with mono-TPS from spruce species. B, PsTPS-Sel*l* is aligned with AgTPS-Sel from grand fir. C, PsTPS-LAS*l* is aligned with PaTPS-LAS from Norway spruce. Gray shading indicates amino acid residues that are identical in at least half of the sequences aligned. Light-gray shading indicates amino acid residues that are conservative replacements. GenBank accession numbers are as follows: PsTPS-Lim, DQ195275; PaTPS-Lim, AAS47694; PsTPS-Lin*l*, DQ195274; PaTPS-Lin, AAS47696; PsTPS-Pin is (−)-α,β-pinene synthase from Sitka spruce, AAP72020; PaTPS-Pin is (−)-α,β-pinene synthase from Norway spruce, AAS47692; PaTPS-Myr is myrcene synthase from Norway spruce, AAS47696; PsTPS-LAS*l*, DQ195273; PaTPS-LAS, AAS47691; PsTPS-Sel*l*, DQ195276; and AgTPS-Sel, AACO5727. Pa, Norway spruce; Ag, grand fir.

Figure 1. (*Continued.*)



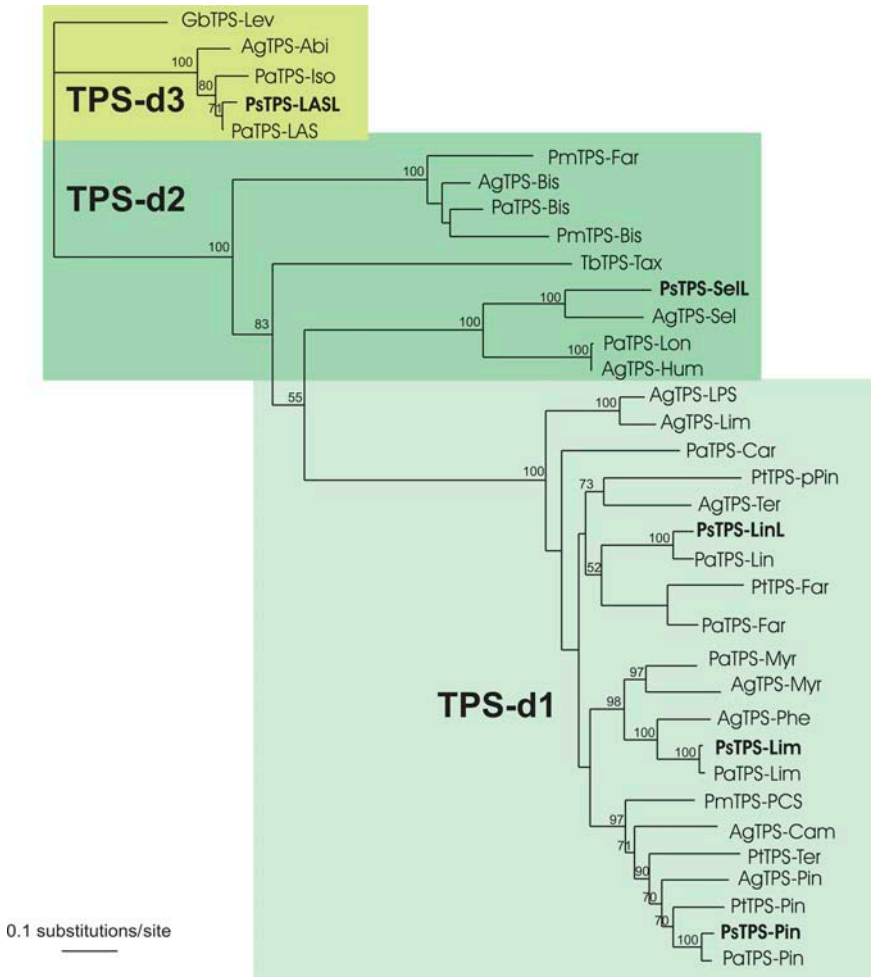


Figure 2. Phylogenetic tree of gymnosperm TPS amino acid sequences showing the Sitka spruce TPS (PsTPS) and their relationship to other conifer TPS. Sitka spruce TPS are shown in bold letters. From top to bottom, the following TPS included in the tree are: GbTPS-Lev is *Gingko biloba* levopimaradiene synthase, AgTPS-Abi is grand fir abietadiene synthase, PaTPS-Iso is Norway spruce isopimaradiene synthase, PmTPS-Far is *Pseudotsuga menziesii* (*E*)-β-farnesene synthase, AgTPS-Bis is grand fir (*E*)-α-bisabolene synthase, PaTPS-Bis is Norway spruce (*E*)-α-bisabolene synthase, PmTPS-Bis is *P. menziesii* (*E*)-γ-bisabolene synthase, TbTPS-Tax is *T. brevifolia* taxadiene synthase, PaTPS-Lon is Norway spruce longifolene synthase, AgTPS-Hum is grand fir γ-humulene synthase, AgTPS-LPS is grand fir (−)-limonene/(−)-α-pinene synthase, AgTPS-Lim is grand fir (−)-limonene synthase, PaTPS-Car is Norway spruce (+)-3-carene synthase, PtTPS-pPin is loblolly pine (+)-α-pinene synthase, AgTPS-Ter is grand fir terpinolene synthase, PtTPS-Far is loblolly pine α-farnesene synthase, PaTPS-Far is Norway spruce (*E*,*E*)-α-farnesene synthase, PaTPS-Myr is Norway spruce myrcene synthase, AgTPS-Myr is grand fir myrcene synthase, AgTPS-Phe is grand fir (−)-β-phellandrene synthase, PmTPS-PCS is *P. menziesii* (−)-α-pinene/(−)-camphene synthase, AgTPS-Cam is grand fir (−)-camphene synthase, PtTPS-Ter is loblolly pine α-terpineol synthase, AgTPS-Pin is grand fir (−)-α-pinene synthase, and PtTPS-Pin is loblolly pine (−)-α-pinene synthase. The other TPS included are also shown in Figure 1. Bootstrap values over 50% for maximum likelihood analyses are shown at nodes. Maximum likelihood values represent percentages of 100 gamma-corrected (log $L = -19{,}444.97$) replicates analyzed using Phyml.

The deduced open reading frames (ORFs) of PsTPS-Lim and PsTPS-Lin*l* are 636 and 631 amino acids long, respectively. PsTPS-Sel*l* encodes an ORF of 573 amino acids. PsTPS-Lim, PsTPS-Lin*l*, and PsTPS-Sel*l* all possess the conserved RRX_8W motif (Bohlmann et al., 1998). While PsTPS-Lim and PsTPSLin*l* each have an N-terminal extension associated with targeting of mono-TPS to plastids (Williams et al., 1998), PsTPS-Sel*l* lacks an N-terminal extension upstream of the RRX_8W motif, consistent with a cytosolic location for sesqui-TPS. PsTPS-Lim and PsTPS-Lin*l* are members of the gymnosperm TPS-d1 group and PsTPS-Sel*l* is a member of the TPS-d2 group (Martin et al., 2004; Fig. 2). PsTPS-LAS*l* encodes an ORF of 645 amino acids and contains an N-terminal putative transit peptide upstream of the sequence KREFPPGFW for targeting to plastids and also contains the approximately 200 amino acid sequence characteristic of members of the gymnosperm TPS-d3 subfamily (Martin et al., 2004). All Sitka spruce TPS sequences identified here have the DDXXD conserved motif involved in cofactor binding, with PsTPS-LAS*l* possessing an additional Asp-rich (DxDD) motif characteristic of a second active site of conifer di-TPS (Peters et al., 2001, 2003).

Functional Identification of PsTPS-Lim as (−)-Limonene Synthase

We have previously shown that the closely related TPS, PsTPS-Pin and PaTPS-Pin, of the related species Sitka spruce and Norway spruce are orthologous (−)-pinene synthase genes of nearly identical function (Byun-McKay et al., 2003; Martin et al., 2004). To further validate this model, we tested the function of Sitka spruce cDNA PsTPS-Lim, which most closely resembles the known Norway spruce (−)-limonene synthase PaTPS-Lim. PsTPS-Lim was expressed in the form of a truncated protein without the predicted plastid target sequence by introducing a starting Met immediately upstream of the RRX_8W motif as previously established for other conifer mono-TPS (Bohlmann et al., 1999; Martin et al., 2004). Recombinant PsTPS-Lim protein was active with geranyl diphosphate as substrate and produced the monoterpene (−)-limonene identified by gas chromatography-mass spectrometry (MS) using a Wiley MS standard library and by using authentic standards for comparison (Fig. 3). The recombinant PsTPS-Lim protein showed no activity when incubated with farnesyl diphosphate or geranyl-geranyl diphosphate as substrates.

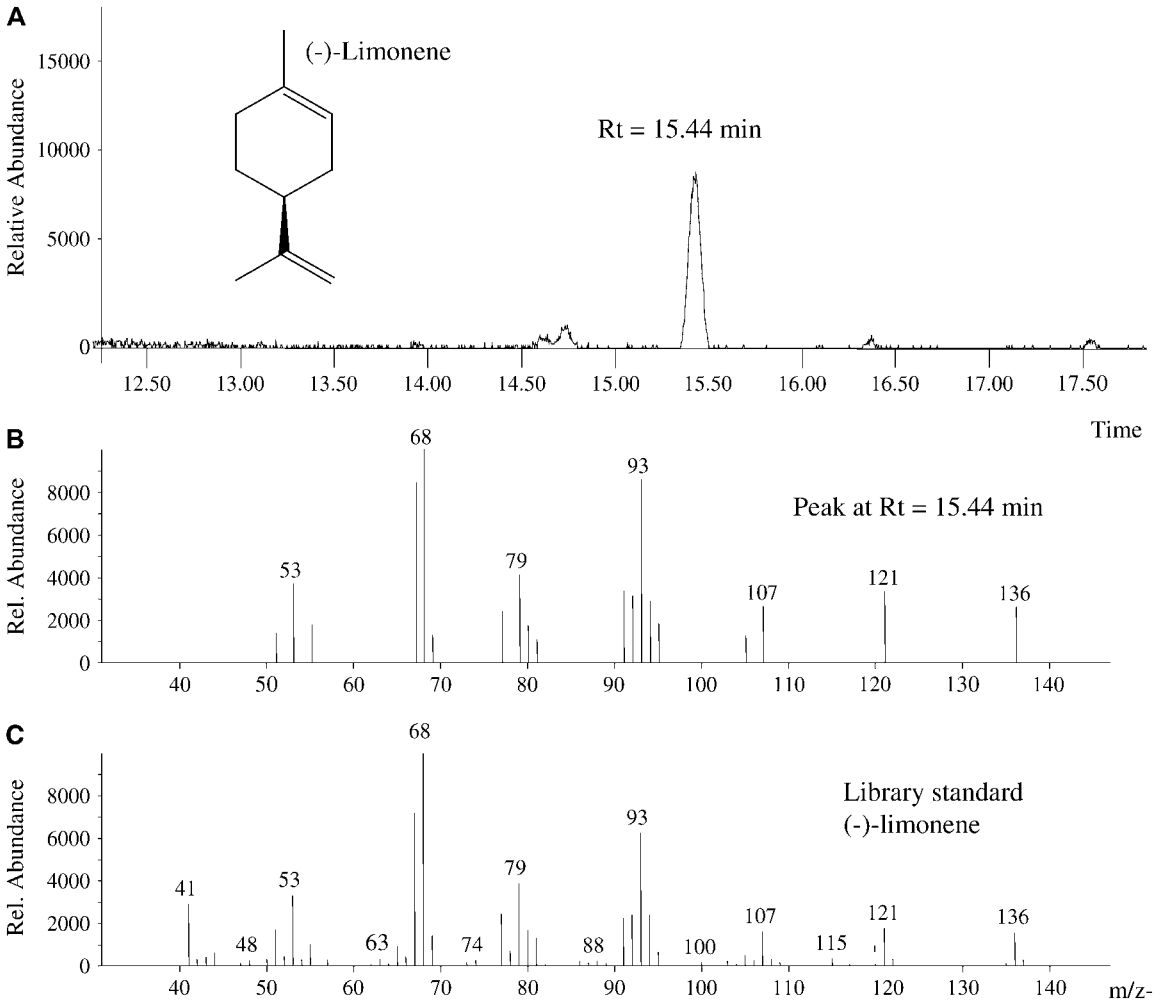


Figure 3. Functional identification of Sitka spruce PsTPS-Lim. A, Total ion chromatogram of the *PsTPS-Lim* product derived from geranyl diphosphate and separated on a HP-5 capillary column. B, Mass spectrum of major enzyme product identified as (−)-limonene. C, Mass spectrum of limonene library standard.

Evaluation of Cross-Hybridization between PsTPS cDNA Representatives

Conifer *TPS* genes display a high degree of nucleotide sequence relatedness among members of the TPS-d1, TPS-d2, and TPS-d3 subfamilies (Martin et al., 2004). Therefore, prior to initiating expression analyses with four different Sitka spruce mono-, sesqui-, and di-TPS probes we determined the level of cross-hybridization between them. Replicated applications of 1.0 ng DNA for each cDNA clone were applied to construct dot blots. Levels of cross-hybridization were determined by probing dot blots with each of the following probes: three TPS-d1 probes, PsTPS-Lin*l*, PsTPS-Lim, and PsTPS-Pin (Byun-McKay et al., 2003); the TPS-d2 probe PsTPS-Se*l*l; and the TPS-d3 probe PsTPS-LAS*l*. The extent of cross-hybridization we detected between and within TPS families was low (less than 10%). Results of a DNA dot blot obtained using the TPS-d1 PsTPS-Lim as probe is shown in Figure 4.

Wound-Induced Transcript Accumulation Detected with PsTPS-Lim in Apical Leaders of R and S Sitka Spruce

Transcripts hybridizing with the TPS-d1 mono-TPS probe PsTPS-Lim increased in response to wounding of apical leaders of Sitka spruce (Fig. 5A). Transcript levels increased 6 h after the wounding treatment and reached maximal levels 4 to 7 d after wounding. Transcript levels remained elevated thereafter to the 16-d postwounding time point. The overall response to drill wounding seen in R and S genotypes was similar. However, several differences were detected. Transcript accumulation in nonwounded trees (0 h) was higher in R trees, and overall wound-induced transcript accumulation levels were higher in R trees compared to S trees. The absolute difference in transcript level was significantly greater in R trees relative to S trees at the 7-d time point ($t_{\alpha = 0.05} = 0.019$). A significant increase in transcript level detected with PsTPS-Lim was apparent 1, 4, 7, and 16 d following wounding in R trees compared to the level in the nonwounded control

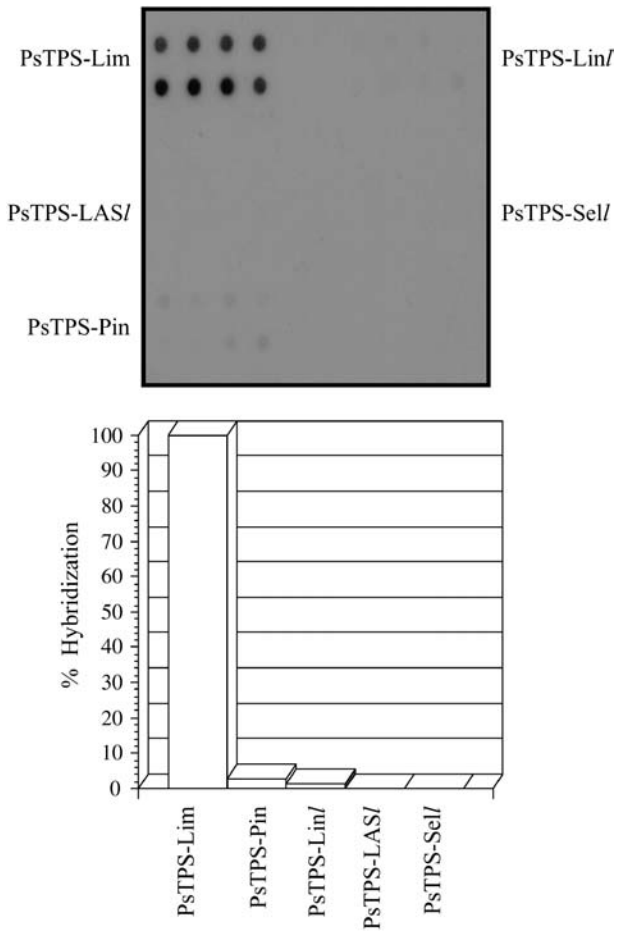


Figure 4. Cross-hybridization between the PsTPS-Lim cDNA and other Sitka spruce TPS cDNAs. Eight replicates of 1 ng DNA for each of the PsTPS cDNAs were blotted onto a nylon membrane. The plot shows the percent hybridization with the PsTPS-Lim probe.

($P_{\alpha = 0.05} = 0.0018$). In contrast, transcripts in unwounded S genotypes were lower than in R genotypes, and no significant change in transcript level detected by PsTPS-Lim was found for any postwounding time point examined.

Wound-Induced Transcript Accumulation Detected with PsTPS-Lin*l* in Apical Leaders of R and S Sitka Spruce

Transcripts hybridizing with the TPS-d1 mono-TPS probe PsTPS-Lin*l* increased in response to drill wounding in S and R genotypes (Fig. 5B). Increased transcript levels were detected at the earliest times examined and increased to a peak 1 d postwounding. Transcript levels had declined by 16 d after the wounding treatment. Expression in nonwounded leaders was lower in the R genotypes relative to the S trees, and expression increased to a peak 1 d postwounding in R trees. In the S trees absolute transcript levels were higher than in R trees; however, only at 12 h was this difference significant ($t_{\alpha = 0.05} = 0.014$). Significant increases in the transcript level detected with PsTPS-Lin*l*, relative to nonwounded trees were only detected in R genotypes 1 to 2 d following wounding ($P_{\alpha = 0.05} = 0.0006$). As with PsTPS-Lim, no significant difference in expression level was found in S genotypes at any time postwounding relative to the nonwounded trees.

Wound-Induced Transcript Accumulation Detected with PsTPS-Sel*l* in Apical Leaders

Transcripts hybridizing to the TPS-d2 PsTPS-Sel*l* probe increased in abundance in response to wounding in both R and S trees (Fig. 5C). Expression levels in nonwounded trees were greater in R trees relative to S trees, and in R trees the transcript level declined immediately after wounding. Relative to nonwounded trees, levels of RNA hybridizing to PsTPS-Sel*l* increased 1 d postwounding and reached their highest levels 4 d after wounding in R trees. In S genotypes, an increase in transcript level was not detected until 2 d postwounding although no drop in the transcript level at the earliest times after wounding was observed. Relative to the nonwounded trees, peak transcript levels occurred 4 to 7 d postwounding. A significant increase in RNA hybridizing to PsTPS-Sel*l* was observed 4 d postwounding in R genotypes only ($P_{\alpha = 0.05} = 0.0001$). In S genotypes, no significant increase in transcripts detected by PsTPS-Sel*l* was found at any of the time points examined.

Wound-Induced Transcript Accumulation Detected with PsTPS-LAS*l* in Apical Leaders of R and S Sitka Spruce

Transcript levels hybridizing with the TPS-d3 PsTPS-LAS*l* probe increased in apical leaders following a drill-wounding event in both R and S trees; however, the pattern of transcript accumulation differed between R and S trees (Fig. 5D). In R trees transcript levels were lower than in the nonwounded tree at the earliest time intervals after wounding. The transcript level increased at 2 d, peaked at 7 d postwounding and remained elevated to 16 d. In the S trees the transcript level did not drop after wounding and a significantly greater absolute transcript level was present at 6 and 12 h postwounding ($t_{\alpha = 0.05} = 0.036$ and 0.031, respectively) relative to the R trees. The transcript level increased 2 d postwounding to reach a peak at 7 d before declining by 16 d in S trees. For RNAs detected by PsTPS-LAS*l* a significant increase in transcript level was detected in both R and S genotypes relative to nonwounded trees at 7 d postwounding ($P_{\alpha = 0.05} = 0.0001$ and $P_{\alpha = 0.05} = 0.0076$, respectively). With the exception of the 16-d time point the absolute transcript level detected by PsTPS-LAS*l* was greater in S than in R genotypes.

Histochemical TRD

Increased abundance of RNA hybridizing to the Sitka spruce TPS-d1, -d2, and -d3 representative probes was detected in wounded Sitka spruce apical leaders. With the exception of the TPS-d3 PsTPS-LAS*l* probe, all TPS cDNA probes revealed a significant increase in transcript level in R genotypes but not in

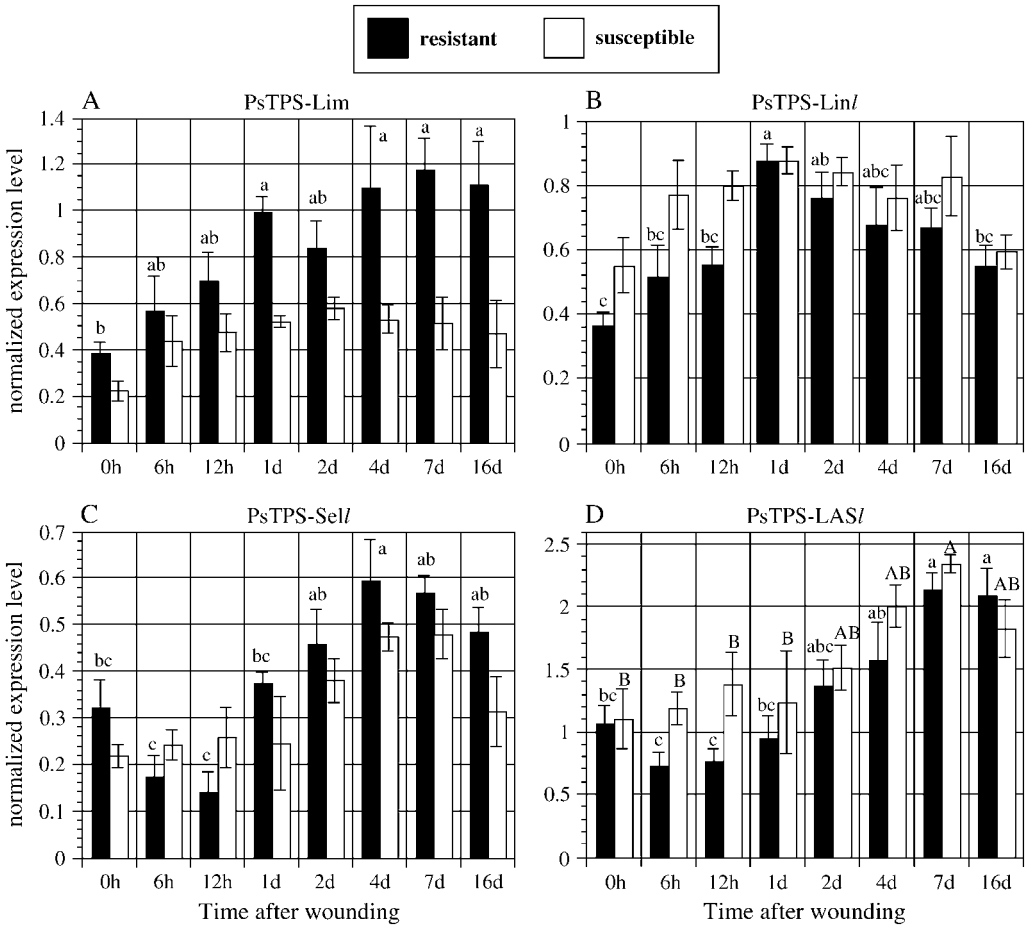


Figure 5. TPS gene expression in drill-wounded apical leaders of Sitka spruce. RNA was isolated from combined bark/xylem tissues derived from the apical leader of Sitka spruce that are R or S to attack by the white pine weevil. Samples for RNA were collected 6 and 12 h, and 1, 2, 4, 7, and 16 d after wounding; RNA was also isolated from nonwounded apical leaders (0 h). RNA was applied to dot blots and relative expression levels were obtained by dividing the log-transformed dot intensity areas obtained after hybridization with PsTPS-Lim (A), PsTPS-Linl (B), PsTPS-Sell (C), and PsTPS-LASl (D) probes with that obtained using the 18S-rRNA probe. SE bars are indicated. Histogram bars denoted with the same letter are not significantly different; uppercase letters denote S trees and lowercase letters denote R trees.

S trees. To determine whether increased expression of TPS genes was accompanied by a greater capacity for TRD formation in R trees, the presence of TRD was assessed in unwounded and drill-wounded leaders of both R and S genotypes (Fig. 6). R and S genotypes both showed a significant response to wounding ($P_{\alpha = 0.05} = 0.000031$ and $P_{\alpha = 0.05} = 0.0017$, respectively). In unwounded R and S genotypes, the presence of TRD was not significantly different ($P_{\alpha = 0.05} = 0.35$). However, drill wounding resulted in a significantly greater wounding response in R than in S genotypes ($P_{\alpha = 0.05} = 0.049$; Fig. 6).

DISCUSSION

Conifer defenses against insect attack contribute to tree resistance and fall into two broad categories: constitutive and induced (Tomlin and Borden, 1997; Huber et al., 2004). In both these defenses, oleoresin terpenoids play a prominent role (Phillips and Croteau, 1999; Trapp and Croteau, 2001; Huber et al., 2004; Martin and Bohlmann, 2005) and, in spruce species, are sequestered in specialized resin ducts. The constitutive resin duct system in Sitka and white spruce (*Picea glauca*) bark has been linked with resistance to the white pine weevil (Alfaro et al., 1996; Tomlin and Borden, 1997). Similarly, in white spruce, the wound-induced resinosis response involving the formation of xylem TRD appears to be more rapid and intense in R versus S families (Tomlin et al., 1998), and drill wounding induced a greater increase in terpenoid oleoresin accumulation in R trees (Tomlin et al., 2000). Recent work with Norway (Martin et al., 2002) and Sitka spruce (Byun-McKay et al., 2003; Miller et al., 2005) has established that increased terpenoid accumulation associated with TRD formation is the result of induced terpenoid biosynthesis and cannot be explained simply

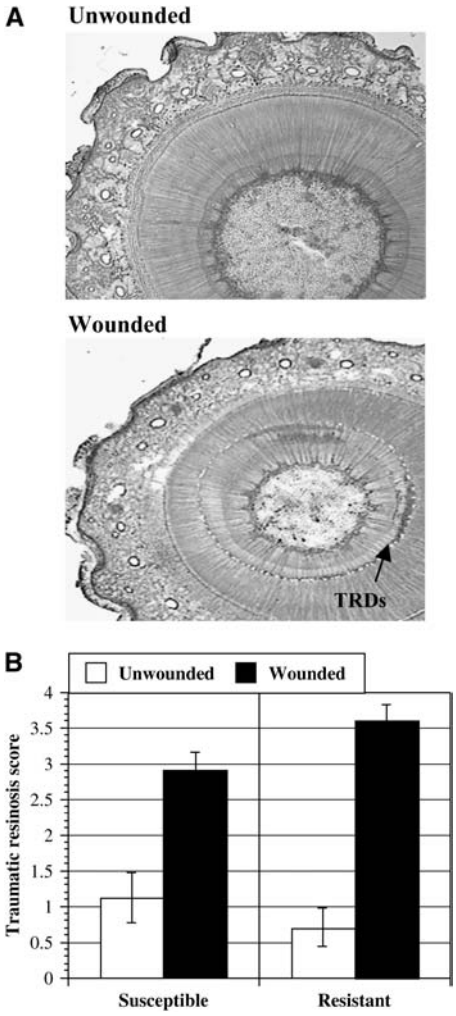


Figure 6. Induced TRD in developing xylem of drill-wounded and nonwounded apical leaders of Sitka spruce. A, Representative cross sections through a nonwounded and drill-wounded apical leader. Branches were harvested for histology approximately 2 months after drill wounding. TRDs, Traumatic resin ducts. B, Mean histochemical scores for drill-wounded and nonwounded apical leaders of R and S Sitka spruce. The criteria used to establish the scores are described in "Materials and Methods." SE bars are indicated.

by mobilization of constitutive resin to the wound site. The inducible oleoresin defense mechanisms of spruces and other conifers involve increased TPS and cytochrome P450 gene expression (Bohlmann and Croteau, 1999; Byun-McKay et al., 2003; Huber et al., 2004; Miller et al., 2005; Ro et al., 2005) and increased enzyme activities of TPS and prenyltransferases (Martin et al., 2002, 2003).

Genetic resistance against the white pine weevil, one of the most damaging pests of Sitka spruce, has been documented (Ying, 1991; King et al., 2004; Ying and Ebata, 1994) such that profound resistance has been noted in the Haney and Big Qualicum (BQ) provenances of coastal British Columbia. In these provenances R trees have a much-reduced attack rate with some genotypes exhibiting a 0% attack rate (Alfaro and Ying, 1990; Ying, 1991; King 1994; King et al., 2004). Although R trees have been used in weevil resistance breeding programs, a link between defense gene expression associated with traumatic resinosis and the resistance status of these trees has not yet been studied. We examined the association of constitutive or induced TPS defense gene expression and the induced formation of TRD with resistance and susceptibility of Sitka spruce to the white pine weevil. Induced TRD and changes in TPS defense gene expression were examined in response to wound-induced trauma delivered to leaders of S and R 9-year-old Sitka spruce trees growing under natural conditions in the field. We generated a set of four Sitka spruce TPS probes representing members of the three subfamilies (Martin et al., 2004) of conifer TPS: TPS-d1 (PsTPS-Lim, PsTPS-Lin*l*), TPS-d2 (PsTPS-Sel*l*), and TPS-d3 (PsTPS-LAS). These four TPS showed a minimal level of cross-hybridization and were used to examine expression of classes of TPS genes in wounded stem tissues of R and S Sitka spruce. The known Norway and Sitka spruce TPS are members of multigene families and share a high degree of nucleotide similarity within and between spruce species (Martin et al., 2004). Closely related pairs of Norway spruce and Sitka spruce TPS genes such as PaTPS-Pin and PsTPS-Pin or PaTPS-Lim and PsTPS-Lim are most likely orthologous genes of very similar or identical functions (Martin et al., 2004).

Expression of genes corresponding to the TPS-d1, TPS-d2, and TPS-d3 probes increased in drill-wounded apical leaders. TPS-d1 transcript levels increased rapidly and were elevated within 1 d of the wounding event. Transcripts related to PsTPS-Lin*l* peaked 1 to 2 d after wounding, whereas transcripts related to PsTPS-Lim peaked later at 4 to 7 d after wounding. Slower induction of TPS-d2 and TPS-d3 transcripts was noted with elevated levels evident 4 d following the wounding event. Transcripts related to PsTPS-LAS*l* peaked 7 d after wounding, while transcripts related to PsTPS-Sel*l* peaked 4 to 7 d after wounding. The slower induction of TPS-d2 and TPS-d3 transcripts is consistent with a slower wound-induced expression of sesqui- and di-TPS in stems of grand fir (Steele et al., 1998) and with weevil and MeJA TPS induction in stems of Sitka spruce (Miller et al., 2005). Increased TPS expression in the apical leaders of drill-wounded Sitka spruce was accompanied by a marked induction of TRD formation in secondary xylem tissues.

Important differences in both the magnitude and kinetics of TPS gene induction were noted between R and S genotypes of Sitka spruce. Transcript levels related to PsTPS-Lim were higher in R versus S trees and, relative to the nontreated trees, significant up-regulation was detected only in the R trees. Similarly, with the PsTPS-Lin*l* and PsTPS-Sel*l* probes significant increases in transcript level were detected in R but not in S trees. The pattern of rapid up-regulation of transcripts was similar for both mono-TPS probes, PsTPS-Lim and PsTPS-Lin*l* (TPS-d1), but differed for the

sesqui-TPS (PsTPS-Sel*l*, TPS-d2) and di-TPS (PsTPS-LAS, TPS-d3) probes, for which transcript levels initially declined immediately after the wounding event in R trees. Lowered di- and sesqui-TPS transcript levels were not detected in S trees. For transcripts related to PsTPS-LAS*l* significantly elevated levels were observed in both R and S trees. Changes in TPS expression in R versus S genotypes was accompanied by a significantly greater capacity for wound-induced formation of TRD in R trees, suggesting that induced resinosis is important for the resistance to weevil attack noted in Sitka spruce growing under natural conditions in the field.

Based on these results, it is possible that TPS-d1 genes that generate transcripts that hybridize with PsTPS-Lim could be developed into useful expression markers associated with resistance. While we found no significant cross-hybridization with the TPS gene probes tested here, including a previously characterized PsTPS-Pin gene (Byun-McKay et al., 2003), it is possible that PsTPS-Lim may hybridize to transcripts of other mono-TPS members not yet cloned or identified in Sitka spruce. Monoterpene products of the TPS-d1 synthases are major components of the constitutive and induced traumatic oleoresin of Sitka spruce stems (Miller et al., 2005). We have also identified major quantitative differences in individual monoterpenes, including limonene in Sitka spruce R and S genotypes, both under constitutive and wound-induced conditions (B. Miller and J. Bohlmann, unpublished data), which further support a function for mono-TPS expression in Sitka spruce resistance. We are currently using real-time qPCR techniques to establish whether the PsTPS-Lim gene transcript accumulates in response to wounding in R and S trees in a manner that reflects the expression pattern detected using the PsTPS-Lim hybridization probe.

The mono-TPS probe PsTPS-Lin*l* appears to be less valuable as an expression marker for resistance because clear differences between R and S genotypes were not detected with this probe. Although the level of amino acid identity between PaTPS-Lin and PsTPS-Lin*l* is too low to confidently assign enzyme function as (−)-linalool synthase for PsTPS-Lin*l*, it is nevertheless possible that the PsTPS-Lin*l* probe hybridizes with transcripts derived from the gene that encodes (−)-linalool synthase in Sitka spruce. As such, an inability to develop a robust expression marker from PsTPS-Lin*l* is not necessarily surprising, as (−)-linalool (the product of PaTPS-Lin; Martin et al., 2004) is at best only a minor component of spruce oleoresin (Martin et al., 2002, 2003; Miller et al., 2005). In contrast, linalool is a major component of MeJA- and weevil-induced volatile emissions in both Sitka spruce (Miller et al., 2005) and Norway spruce (Martin et al., 2003). Thus, spruce TPS-Lin genes may not contribute directly to resistance against stem-boring insects, although an indirect role in defense cannot be excluded (Martin et al., 2003; Miller et al., 2005). Sesquiterpenes formed by the TPS-d2 type enzymes represented in this study by the PsTPS-Sel*l* probe are also minor components of constitutive and induced Sitka spruce oleoresin (Miller et al., 2005). The apparent lack of association between resistance and expression of genes of the TPS-d3 group (PsTPS-LAS*l*) could be due to additional gene expression required for diterpene resin acid accumulation. While most mono- and sesquiterpenes in the oleoresin of Sitka spruce and other members of the Pinaceae are the immediate products of TPS enzyme activities, diterpenes formed by di-TPS are commonly further oxidized to the corresponding diterpene resin acids, which accumulate in constitutive and traumatic oleoresin. In future work, we will investigate a possible association between the expression of cytochrome P450 genes involved in diterpene resin acid formation (Ro et al., 2005) and resistance.

MATERIALS AND METHODS

Plant Material

Six-year-old Sitka spruce (*Picea sitchensis* Bong. Carr.) clonal trees derived from somatic embryos out planted at the University of British Columbia Malcolm Knapp research forest, Maple Ridge, British Columbia, Canada were used in 1997 for wound induction to obtain RNA for isolation of partial *TPS* cDNAs. Nine-year-old clonal Sitka spruce trees planted in 1992 as a British Columbia Ministry of Forest progeny trial at Sayward, Vancouver Island, Canada, were used for a drill-wounding time course experiment in 2001. Three weevil-R Haney (clones: 1139, 1209, and 1210) and BQ (clones: 855, 1241, and 1243) genotypes were available for study along with two S Haney (clones: 1212, 1215) and BQ (clones: 871, 1233) genotypes. These genotypes are derived from the Big Qualicum (east coast of Vancouver Island, British Columbia) and Haney (coastal mainland British Columbia) provenances in which outstanding resistance to white pine weevil (*Pissodes strobi* Peck.) attack was noted. Two-year-old R Sitka spruce trees (clone 898) from the British Columbia Ministry of Forest breeding program were used to obtain RNA for isolation of full-length cDNAs.

Simulated Weevil Attack Treatment

A drill-wounding treatment was used to simulate weevil attack and has been used for this purpose in spruce species (Tomlin et al., 1998, 2000; Byun-McKay et al., 2003). Drill wounding has the advantage of delivering a wound trauma of equal magnitude on R and S trees without being confounded by variation introduced by weevil feeding and oviposition activity. Thus, drill wounding permits assessment of a tree's capacity to respond with an induced defense. Wounding treatments occurred at field sites in the spring to coincide with natural weevil feeding activity on the British Columbia coast. Apical leaders were mechanically wounded using a Dremel minimite drill equipped with a 0.95-mm drill bit. Holes were drilled to a depth of 2 to 4 mm (depending on diameter) so that the bark was just penetrated. Wounding proceeded in two straight lines from the apex to the base along two sectors of the circumference with each wound spaced approximately 1 cm apart. Once the wound had been completed, leaders were covered in mesh bags to prevent insect damage until they were harvested. Leaders were harvested at the following times post wounding: 6 and 12 h, and 1, 2, 4, 7, and 16 d. For each genotype, a single tree was used per time point. Due to the limited number of available trees of the appropriate age of each genotype, time course experiments were independently replicated with multiple R and S genotypes. In addition, one tree per genotype was not wounded to provide a control. Harvested leaders were frozen on dry ice, transported to Simon Fraser University, and stored at −80°C.

Two additional trees were used for histochemical analyses. One tree served as a control while the other tree was wounded and harvested several weeks after wounding. The leaders were then transported to the Pacific Forestry Centre (Victoria, British Columbia, Canada) where they were prepared and analyzed for the presence of constitutive and induced TRD.

For full-length cDNA cloning, 2-year-old greenhouse-grown Sitka spruce trees (clone 898) were mechanically wounded as described above, sprayed with 0.01% (v/v) MeJA (Sigma Aldrich) as described in Martin et al. (2002) and Fäldt et al. (2003) and loosely covered with clear plastic bags. The leader and the previous year's internode were harvested 2 d later, frozen in liquid nitrogen, and stored at −80°C.

Histochemical and TRD Analysis

Samples were prepared for histochemistry as described in Byun-McKay et al. (2003). The wound response was quantified based on the extent of TRD formation and the number of stained cells, which are typically associated with TRD development (Byun-McKay et al., 2003). A relative scoring system was used similar to the one described in Byun-McKay et al. (2003). No apparent response in the early wood was rated as zero; cell staining restricted to a few, scattered cells was rated as 0.5; a complete ring of stained cells in the early wood was rated as 1.0; a ring of stained cells associated with scattered TRD was rated as 1.5; and an intense ring of stained cells along with a complete ring of TRD was rated as 2.0. The traumatic resinosis scores for each of the wounded and unwounded S and R genotypes were pooled and analyzed using a paired or two-sample equal *t* test.

RNA Isolation

Total RNA was isolated from 5 to 6 g of combined wood and bark tissue derived from drill-wounded or MeJA (Sigma Aldrich)-treated leaders of R and S Sitka spruce trees. RNA was isolated using a LiCl method optimized specifically for conifers (Wang et al., 2000).

Isolation of Partial and Full-Length TPS cDNAs

To isolate mono-, sesqui-, and di-TPS cDNAs, partial cDNA fragments were obtained using degenerate primers (Supplemental Table I) designed from nucleotide alignments of conifer TPS sequences (taxadiene synthase from *Taxus brevifolia*, and E-α-bisabolene, abietadiene, myrcene, (−)-α-pinene, (−)-limonene, δ-selinene, and γ-humulene synthases from grand fir (*Abies grandis*; Martin et al., 2004 and refs. therein). Isolation of partial mono-TPS cDNAs proceeded as described in Byun-McKay et al. (2003). To isolate sesqui- and di-TPS cDNAs RNA was isolated from Sitka spruce apical leaders that had been drill wounded and harvested 7 d later. First-strand cDNA was synthesized at 37°C for 1.5 h in an 80-μL reaction volume containing 140 μg total RNA, 8 mM dithiothreitol (DTT), 0.28 mM oligo $(dT)_{16}$, 140 units human placental ribonuclease inhibitor (Pharmacia Biotech), 0.63 mM dNTPs, 1,000 units Moloney murine leukemia virus reverse transcriptase (Gibco BRL), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. PCR was conducted using degenerate primers (Supplemental Table I) and 150 ng cDNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1 mM dNTPs, 0.5 μM each sense and antisense primer, 3 mM $MgCl_2$, and 2.5 units of Taq polymerase (Gibco BRL). PCR was carried out for 45 cycles using the following steps: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min. Each reaction was initially denatured at 94°C for 4 min and received a final extension at 72°C for 10 min. Partial cDNAs were subsequently cloned into pCR 2.1 TOPO-TA (Invitrogen) and the nucleotide sequence was determined.

Full-length cDNAs encoding mono-TPS were isolated using 5′ and 3′ RACE and an RNA template derived from drill-wounded/MeJA-treated leader and stem tissues from Sitka spruce clone 898. Sesqui- and di-TPS were obtained using the same method and RNA from Sitka spruce leader tissues sampled 7 and 16 d following drill wounding. Based on the nucleotide sequences of partial cDNA fragments, RACE primers were designed for mono-TPS, sesqui-TPS, and di-TPS (Supplemental Table I). The 5′ and 3′ TPS cDNA ends were amplified from reverse transcriptase reactions according to instructions outlined by the manufacturer (RLM-RACE; Ambion), cloned into pCR 2.1 TOPO-TA (Invitrogen), and the nucleotide sequence was determined. Full-length cDNAs were obtained using primers designed from the nucleotide sequences of the 5′ and 3′ RACE products and/or primers included with the RLM-RACE kit. Amplification was performed in a 50-μL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 units of SuperTaq Plus (Ambion), 0.5 μM each primer, 50 μM dNTPs, and 1 μL of the 5′ reverse transcriptase reaction. The following cycling conditions were used: 30 cycles of 94°C for 1 min, 55°C for 35 s, and 68°C for 2 min preceded by an initial denaturation at 94°C for 3 min and followed by a 7 min extension at 68°C. Full-length cDNAs were cloned into pCR2.1 TOPO-TA and purified using the Qiaprep spin miniprep kit (Qiagen) and the nucleotide sequence was determined.

Sequence and Phylogenetic Analyses

The deduced amino acid sequences of each full-length cDNA were aligned using ClustalW (version 1.4) in MacVector 7.2.3 using a 5.0 open gap penalty, 40% delay divergence, and Blosum similarity matrix to create sequence alignments shown in Figure 1. 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 anchored Dialign (Morgenstern et al., 2004; available at http://dialign.gobics.de/anchor/submission.php), 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 Jones-Taylor-Thornton (Jones et al., 1992) amino acid substitution matrix. The proportion of invariable sites as well as the α-shape parameter was estimated by Phyml. Trees were generated using BIONJ (Gascuel, 1997), a modified neighbor-joining algorithm. Phyml was used to generate 100 bootstrapped pseudo data sets from the original data set. These were then analyzed by Phyml using the previously estimated parameters. Treeview (Page, 1996) was used to visualize all trees. Bootstrap values above 50% for both the maximum likelihood analyses were added to the maximum likelihood tree calculated from the original data set.

Functional Identification of cDNA Clone PsTPS-Lim

Expression of recombinant TPS enzyme and functional characterization followed the general procedures described in Martin et al. (2004). A pseudo-mature version of the PsTPS-Lim full-length cDNA starting with a Met directly upstream of the conserved RRX_8W motif was generated by PCR. PCR was performed in a 50-μL volume containing 10 ng of template *pCR2.1-PsTPS3*, 3 units of high-fidelity Pwo DNA polymerase (Roche Diagnostics), and 5 μL of the provided PCR buffer with $MgSO_4$ [100 mM Tris-HCl, pH 8.85, 250 mM KCl, 50 mM $(NH_4)_2SO_4$, and 20 mM $MgSO_4$], 10 mM dNTPs, and 20 pmol of each primer (5′-CACCATGTCTCCTGTTTCTGCC-3′ and 5′-TTACAAAGTCACAGGTTCAAGGAC-3′). PCR conditions were 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, 2 min at 72°C, and ending with a 7-min final extension at 72°C. PCR products were cloned into the pET101/D-TOPO expression vector (Invitrogen) following the manufacturer's instructions and recombinant plasmid was transformed into *Escherichia coli* TOP 10 F′ cells (Invitrogen). Plasmid DNA *pET101/D-TOPO-PsTPS3* was purified and transformed into *E. coli* BL21-CodonPlus (DE3) cells (Stratagene) for expression. Positive clones were screened by PCR (same conditions as above) using T7 Forward and T7 Reverse vector-based primers (Invitrogen).

Bacterial strain *E. coli* BL21-CodonPlus (DE3) containing plasmid *pET101/D-TOPO-PsTPS3* was grown at 37°C in 100 mL of Luria-Bertani broth supplemented with 100 μg mL^{-1} ampicillin to A_{600} = 0.5. The culture was transferred to 20°C for 30 min, induced with 1 mM isopropylthio-β-galactoside, and then left to grow overnight. Cells were harvested by centrifugation and were resuspended and disrupted in 1 mL of monoterpene synthase buffer (25 mM HEPES, pH 7.2, 100 mM KCl, 10 mM $MnCl_2$, 10% [v/v] glycerol, and 5 mM DTT) by sonication (Branson Sonifier 250; AmTech) at 5W for 10 s. Lysates were cleared by centrifugation and assays were performed in 1-mL volumes with 5 μM DTT and 164 μM geranyl diphosphate (Echelon Research Laboratories) overlaid with 1 mL of pentane. Assay mixtures were incubated at 30°C for 2 h. Terpenoid products were collected with three consecutive pentane extractions (3 × 1 mL). The pooled pentane fraction was washed with water, dried over $MgSO_4$, and concentrated under nitrogen.

Products of monoterpene synthase assays were identified on a GC system (Agilent 6890 Series; Agilent Technologies) coupled to a Network Mass Selective Detector (Agilent 5973; Agilent Technologies). Monoterpenes were initially identified using a HP-5 capillary column (0.25 mm i.d. × 30 m with 0.25-μM film; Agilent Technologies) with an initial temperature of 40°C (2-min hold), which was then increased 3°C min^{-1} up to 140°C, followed by a 20°C ramp until 300°C (10-min hold). For differentiating enantiomers, a Cyclodex-B capillary column (0.25 mm i.d. × 30 m with 0.25-μM film; J & W Scientific) was used with an initial temperature of 60°C (15-min hold), which was then increased 0.5°C min^{-1} up to 200°C, followed by a 200°C ramp (10-min hold).

Compounds were identified using Agilent Technologies software and Wiley 126 MS library, as well as by comparing retention time with those of the appropriate enantiomerically pure standards (Aldrich Chemical).

RNA Dot-Blot Hybridization and Analysis

TPS gene expression was assessed with RNA dot blots. RNA from control and wounded trees was concentrated using microcon YM-100 tubes (Amicon, Millipore) and quantified using ribogreen fluorescence dye (Cedar Lane Labs). Eight micrograms of RNA was denatured, applied to a Hybond-N membrane (Amersham Biosciences Baie d'Urfe), and fixed by UV cross-linking followed by baking at 80°C for 1 h. Hybridization probes were prepared from Sitka spruce mono-TPS, sesqui-TPS, and di-TPS cDNA clones using 35 ng DNA with 1.85 MBq of [α-^{32}P]dCTP using a random labeling kit (Amersham Pharmacia). All probes were prepared from full-length cDNA clones with the exception of the di-TPS probe (PsTPS-LAS*l*), which was a partial cDNA. Membranes were prehybridized in 5% (w/v) SDS, 0.1% (w/v) bovine serum albumin, 1 mM EDTA, 500 mM NaH_2PO_4, and 50% (v/v) formamide for 1 h at 42°C and hybridized overnight at the same temperature with the [^{32}P]-labeled DNA probe. Membranes were subsequently washed at 42°C in 1× sodium chloride/sodium phosphate/EDTA (SSPE; 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA) buffer containing 0.3% (w/v) SDS, 1× SSPE/0.5% (w/v) SDS, and finally in 0.2× SSPE/1% (w/v) SDS. X-ray film (X-Omat blue XB-1; Eastman-Kodak) was exposed to membranes with an intensifying screen at −80°C. Consistency of RNA loading was assessed by hybridizing each dot blot with an 18S-rRNA Sitka spruce probe.

To assess cross-hybridization between different Sitka spruce TPS cDNAs, DNA dot blots were constructed containing eight replicate applications of 1.0 ng of each cDNA insert. The cDNA inserts were amplified using flanking M13 universal primers and DNA samples were denatured prior to applying to a Hybond-N membrane. DNA was fixed to the blot as described for the RNA blots above. Dot blots were individually hybridized with mono-TPS, di-TPS, and sesqui-TPS cDNA probes labeled as described above.

Dot intensity for DNA and RNA dot blots was determined using Scion image version 1.62c (macrofunction gel plot 2). For RNA dot blots, dot intensity was normalized by dividing the hybridization signal obtained for each *TPS* probe by that of the corresponding 18S-rRNA probe. To compare TPS expression levels by resistance status, average signal intensity for each time point was calculated by combining the normalized signal intensity data of the R and then the S Sitka spruce genotypes. The resulting data were log transformed to correct for heteroscedasticity and analyzed using ANOVA (generalized linear model; $\alpha = 0.05$; SAS Institute, 1990).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ195274 (PsTPS-Lin*l*), DQ195275 (PsTPS-Lim), DQ195273 (PsTPS-LAS*l*), and DQ195276 (PsTPS-Sel*l*).

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