

Dirigent proteins in conifer defense: gene discovery, phylogeny, and differential wound- and insect-induced expression of a family of DIR and DIR-like genes in spruce (*Picea* spp.)

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Abstract

The outer stem tissues of conifers provide a durable constitutive and inducible defense barrier consisting of suberized or lignified periderm, sclereids, a network of terpenoid-filled resin ducts, and phenolic phloem parenchyma cells. Microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) bark attacked by stem-boring weevils (*Pissodes strobi*) or through mechanical wounding demonstrated significant accumulation of transcripts resembling dirigent protein (DIR) genes. To investigate this gene family and its spatial and temporal patterns of expression in conifer defense, we isolated cDNAs representing 19 unique DIR and DIR-like genes from Sitka spruce, white spruce (*P. glauca*), and interior spruce (*P. glauca* × *engelmannii*). Sequence alignments also identified a large number of DIR-like proteins in other plant species, which share several conserved protein motifs with known DIR proteins. Phylogenetic analysis of 72 DIR and DIR-like proteins suggests five distinct subfamilies, DIR-a and four DIR-like subfamilies (DIR-b, DIR-c, DIR-d and DIR-e). Previously characterized members of the DIR-a subfamily direct stereoselective phenolic coupling reactions in the formation of lignans and possibly lignins. The spruce genes identified here are members of the DIR-a and DIR-b subfamilies. Using gene-specific quantitative real-time PCR we measured constitutive expression for six DIR-a genes and three DIR-like genes in different stem tissues, green shoot tips, and roots of Sitka spruce. DIR-like genes revealed ubiquitous high expression in all tissues. In contrast, the six DIR-a genes showed a gradient of transcript abundance in stem tissues with highest levels in the outer cortex and lowest levels in the inner xylem. Gene-specific transcript profiling of six DIR-a genes confirmed rapid and strong accumulation (up to 500-fold) in wound- and weevil-induced stem bark and xylem. These findings suggest a role for spruce DIR genes in constitutive and induced phenolic defense mechanisms against stem-boring insects.

Abbreviations: DIR, dirigent; EST, expressed sequence tag; ORF, open reading frame; PAL, phenylalanine ammonia lyase; PP cells, phloem parenchyma cells

Introduction

Genes encoding dirigent proteins (DIR) were first described as rapidly induced transcripts in a variety

of crop species including pea (*Pisum sativum*) (Fristensky *et al.*, 1985; Riggelman *et al.*, 1985), wheat (*Triticum aestivum*) (Görlach *et al.*, 1996), and barley (*Hordeum vulgare*) (Lee *et al.*, 1996).

Davin *et al.* (1997) demonstrated that a DIR protein isolated from *Forsythia suspensa*, in the presence of a phenol oxidase, could direct stereoselective bimolecular phenoxy radical coupling to form the lignan (+)-pinoresinol from *E*-coniferyl alcohol. Based on this activity and its associated cellular localization in *Forsythia intermedia*, Burlat *et al.* (2001) proposed that DIR proteins could also function in the formation of lignin. In recent years, a large number of DIR gene homologues have been detected in various plant species (Davin and Lewis, 2000). In western red cedar (*Thuja plicata*), a family of nine DIR genes was shown to direct *E*-coniferyl alcohol coupling to produce (+)-pinoresinol (Kim *et al.*, 2002). It has been proposed that (+)-pinoresinol is the lignan precursor to plicatic acid, which is highly abundant in the heartwood of western red cedar (Johansson *et al.*, 2000), and may play a role in defense against opportunistic pathogens. Furthermore, lignans have been shown to exist in most plant tissues (Ayres and Loike, 1990), and possess antimicrobial and antiviral activities as well as substantial antioxidant and cytotoxic capacity (MacRae and Towers, 1984; Pauletti *et al.*, 2000; Rahman and Gray 2002). In order to evaluate a possible role of DIR genes in the constitutive and induced defense response of conifers against wounding or stem-boring insects we explored the newly developed spruce cDNA microarray, full-length cDNA, and expressed sequence tag (EST) resources (www.treenomix.com) for DIR genes and their expression.

In contrast to plant responses to abiotic stress or pathogens, relatively little is known about how plant genomes respond to attack by herbivorous insects. Insect-induced defense responses identified by large-scale gene expression profiling have recently been described for two herbaceous angiosperm species, the wild tobacco *Nicotiana attenuata* (Hui *et al.*, 2003; Voelckel *et al.*, 2004) and *Arabidopsis thaliana* (Reymond *et al.*, 2000, 2004). Very little is known about insect-induced gene expression in long-lived conifer trees, which belong to the gymnosperms. Although conifers are resistant against most herbivorous insects, some insect species such as certain bark beetles among the *Scolytidae*, shoot and root weevils (*Curculionidae*), sawflies (*Hymenopterae*), or budworms (*Lepidopterae*) are known to cause substantial damage. For instance, the white pine weevil (*Pissodes strobi*) is a major pest of Sitka spruce

(*Picea sitchensis*), white spruce (*P. glauca*) and hybrid interior spruce (*P. glauca* × *engelmannii*) in the Pacific Northwest, and often devastates planted Norway spruce (*P. abies*) forests in eastern Canada (Alfaro *et al.*, 2002). Adult weevils feed on the phloem of living trees and, in May or June, lay their eggs into the bark of the very tip of the apical shoot. The developing larvae then feed downwards into the phloem, cambium, and outer xylem, and consequently destroy the tree's apical leader as well as previous years growth.

Conifer defenses against insects are best characterized in Sitka spruce and Norway spruce, and a few other species of the *Pinaceae* (Huber *et al.*, 2004). The bark of spruce trees provides a durable constitutive defense barrier, containing suberized or lignified periderm, sclereids, phenolic phloem parenchyma (PP) cells, and a system of resin ducts filled with terpenoid resins (Raffa and Berryman, 1982; Franceschi *et al.*, 1998; Franceschi *et al.*, 2000). In addition, species of spruce display inducible defense responses upon insect attack, mechanical wounding, or fungal inoculation. These induced defenses include the activation and *de novo* formation of resin duct systems in bark and xylem (Franceschi *et al.*, 2002; Martin *et al.*, 2002; Hudgins *et al.*, 2003). Induced terpenoid resin defenses in Norway and Sitka spruce have been well characterized at the anatomical, biochemical, and molecular levels (Franceschi *et al.*, 2002; Martin *et al.*, 2002, 2003, 2004; Fäldt *et al.*, 2003). For example, it has been shown that traumatic resin accumulation in Sitka spruce bark and xylem is induced by weevil feeding, and involves the induction of a family of terpenoid synthase genes (Byun McKay *et al.*, 2003; Miller *et al.*, 2005). The induced activation of phenolic PP cells in Norway spruce has also been well characterized at the anatomical level (Franceschi *et al.*, 1998, 2000; Krokene *et al.*, 2003; Nagy *et al.*, 2004), with concomitant enrichment of the phenylalanine ammonia lyase (PAL) enzyme at the plasma membrane (Franceschi *et al.*, 1998). PAL is associated with the initiation of phenolic metabolism including biosynthesis of lignans and lignins. As well, Nagy *et al.* (2004) have demonstrated increased expression of chalcone synthase, an enzyme catalyzing the first step in flavonoid biosynthesis, in Norway spruce bark inoculated with the pathogenic fungi *Ceratocystis polonica*. However, little else is known about

induced gene expression for the formation of phenolic compounds in spruce defense.

Microarray expression profiling of Sitka spruce challenged by mechanical wounding or by feeding white pine weevils identified a family of DIR and DIR-like genes among the most strongly up-regulated transcripts in induced bark tissues. Here we describe EST and full-length cDNA discovery of 19 spruce DIR and DIR-like genes, detailed quantitative expression analysis in constitutive as well as wound- and insect-induced Sitka spruce tissues, and present a comprehensive phylogeny of the plant DIR gene family. Our results indicate a role of DIR genes in the wound- and insect-induced defense response in Sitka spruce.

Materials and methods

Plant material and insects

Seedlings of Sitka spruce (*Picea sitchensis* [Bong] Carriere; clone FB3-425) were provided by Dr. David Ellis (CellFor Inc., Vancouver, Canada) and grown outside at UBC for 2 years, in 3:1 (v/v) peat:vermiculite (with 2.4 g l⁻¹ Dolomite, 0.5 g l⁻¹ NutriTrace micronutrients, and 3.6 g l⁻¹ Osmocoat) in 656 ml cone-tainers (Stuewe & Sons Inc., Corvallis, USA), and watered daily. Adult white pine weevils (*Pissodes strobi* Peck) were provided by Dr. Rene I. Alfaro (Pacific Forestry Centre, Canadian Forest Service, Victoria, Canada) and maintained as previously described (Miller *et al.*, 2005). Two weeks prior to inoculation with weevils (May 2003), trees 60–70 cm in height were moved inside the greenhouse where a constant 16/8-h photoperiod was provided by high-pressure sodium lamps. Average greenhouse temperature during May was 22.8 °C (19.8 °C minimum and 24.9 °C maximum), with an average humidity of 31.4%. Unless otherwise mentioned, all other reagents and solvents were from Fischer Scientific (Pittsburgh, USA), Sigma–Aldrich (St. Louis, USA), EM Science (Darmstadt, Germany) or Invitrogen (Carlsbad, USA).

Treatment of trees with weevils, wounding, and tissue harvest

Weevils were kept without food on moist filter paper for 60 h prior to experiments. Ten weevils per tree were caged with mesh bags on the upper

two-thirds of the saplings. Mechanical wounding was done by piercing the bark of each tree with a 26.5 gauge needle at 5 mm intervals on opposite sides along the entire length of the stem. Corresponding untreated controls for each time point were represented by trees covered with mesh bags. Treatment and controls were each replicated with five trees per time point. Bark and xylem tissue from five trees for each treatment and time point was separately harvested at 2, 6 and 48 h after the onset of treatment. The bark of the upper two-thirds of the tree, excluding the uppermost green tip, was cut longitudinally with a razor blade and peeled away from the woody inner stem tissues, with the majority of phloem tissue remaining attached to the bark. The outer immature xylem was harvested with a razor blade by gently scraping the soft tissue from the outside of the woody inner stem tissues. Bark tissue was harvested individually from each tree and separately frozen in liquid nitrogen and stored at –80 °C prior to RNA isolation. Immature xylem tissue from each group of five trees per treatment/time point was pooled prior to RNA isolation. Tissue harvest was completed within 5 min per tree. For the tissue profiling of DIR expression, cortex, phloem, cambium and immature xylem were rapidly harvested under a dissecting microscope (15 min per tree), along with young lateral shoots and roots, from 10 saplings harvested in May 2004 and pooled by tissue. All tissues were frozen in liquid nitrogen.

RNA isolation, microarray hybridization and analysis

Total RNA was isolated, quantified, and checked for integrity and purity by spectrophotometer, agarose gel, and reverse-transcription with Superscript II reverse transcriptase (Invitrogen) with an oligo d(T₁₈) primer and αP³² dGTP (Amersham-Pharmacia Biotech, Buckinghamshire, UK) incorporation according to Kolosova *et al.* (2004). For each treatment and time point, equal amounts of total RNA were combined from each tree prior to cDNA microarray analysis. All microarray experiments were designed to comply with MIAME guidelines (Brazma *et al.*, 2001). All scanned microarray TIF images, an ImGene grid, the gene identification file and ImGene quantified data files are available at <http://doug-las.bcgsc.bc.ca>. A complete description of the

manufacture of the spruce 16.7K array will be reported elsewhere (Ralph *et al.*, in prep.). Methods describing the cDNA microarray hybridizations and data analysis are provided as supplemental information (Supplemental Materials and Methods). For each time point, total RNA from insect-treated and mechanically wounded bark was compared to RNA from untreated control bark using 4 technical replicate hybridizations for each comparison with dye flips (24 slides total). Total RNA from insect-treated and mechanically wounded bark were also compared within treatments across the three time points, as well as between treatments at each time point, using two technical replicate hybridizations for each comparison with dye flips (18 slides total).

Isolation of spruce full-length DIR cDNA clones

A TBLASTN search of the Treenomix spruce EST database (www.treenomix.com) containing 77 160 3'-end sequences was performed using publicly available plant DIR sequences (Kim *et al.*, 2002) to identify 96 spruce putative DIR ESTs. CAP3 (Huang and Madan, 1999) was used to assemble ESTs into singletons and contigs (40 bp overlap, 95% identity). DIR clones in the pBluescript II SK(+) vector were identified in our cDNA library glycerol stocks, insert sizes determined using PCR with -21 M13 forward (5'-TGTAACGACGG-CCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primers, and sequenced from both ends using the same primers. In this manner 15 unique spruce DIR full-length cDNAs and four partial cDNAs were obtained. To obtain full-length clones for two partial cDNAs, the 5'RACE system (Ambion, Austin, USA) was employed using *Pwo* PCR polymerase (Roche, Mannheim, Germany). The resulting PCR products were cloned into a PCR-Blunt cloning vector (Invitrogen) for sequence analysis using the M13 forward and reverse primers.

Sequence and phylogenetic analyses

Additional plant DIR genes were identified in a comprehensive search of GenBank using BLAST tools and the set of plant DIR previously described (Kim *et al.*, 2002). This process was repeated with each newly identified set of plant DIR genes until no further sequences with significant similarity

were identified. Predictions for pI and molecular mass were made using the entire ORF and the pI/Mw tool at Expasy (www.expasy.org/tools/pi_tool.html). Amino acid alignments of all identified gymnosperm and select *Arabidopsis* and rice DIR proteins (Figure 1) were made with ClustalW (www.ebi.ac.uk/clustalw/) and Boxshade (<http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html>). Since transit peptides are not well conserved, these were truncated prior to phylogenetic analysis using SignalP 3.0 with a neural network model (Bendtsen *et al.*, 2004; www.cbs.dtu.dk/services/SignalP/). All plant DIR sequences were aligned using Dialign (threshold = 0) (Morgenstern *et al.*, 1998; <http://bioweb.pasteur.fr/seqanal/interfaces/dialign2-simple.html>). Multiple sequence alignments were manually adjusted prior to maximum likelihood analysis using Phylml v2.4.1 (Guindon and Gascuel, 2003) with the JTT (Jones *et al.*, 1992) amino acid substitution matrix. The proportion of invariant sites as well as the alpha shape parameter were estimated by Phylml. Trees were generated using BIONJ (Gascuel, 1997), a modified neighbor joining algorithm. SEQBOOT of the Phylip v3.62 package (Felsenstein, 1993; <http://evolution.genetics.washington.edu/phylip.html>) was used to generate 100 bootstrap replicates, which were then analyzed using Phylml and the previously estimated parameters. CONSENSE, also from Phylip, was used to create a consensus tree. Treeview (Page, 1996) was used to visualize all trees. Bootstrap values above 50% were added to the maximum likelihood tree generated from the original data set. Conserved motifs were identified using the Consensus tool (www.bork.embl-heidelberg.de/) with the manually optimized Dialign alignment. The subcellular localization of spruce DIR proteins was predicted using the TargetP v1.01 (Emanuelsson *et al.*, 2000; www.cbs.dtu.dk/services/TargetP/) and PSORT (Nakai and Horton, 1999; <http://psort.nibb.ac.jp/>) software programs.

Real-time PCR

cDNA was synthesized using the superscript II first-strand synthesis system (Invitrogen) in a 20 μ l reaction containing 1 μ g of DNase I-treated total RNA with 0.5 μ g oligo d(T₁₂₋₁₈). An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA. Real-time PCR was conducted on a MX3000P™

PCR system (Stratagene, La Jolla, USA) using SYBR green QRT-PCR reagents (Stratagene) according to the manufacturer's instructions. Reaction mixtures contained 0.5 μ l of cDNA as template, 0.5 pmol of each primer and 12.5 μ l of SYBR green reagent in a final volume of 25 μ l. A master mix of cDNA and 2 \times SYBR green reagent was used to ensure that each reaction contained an equal amount of cDNA. Gene-specific primers for each DIR were designed within the 3'UTR sequence (Supplemental Table VI). Primer specificity (single product of expected length) was confirmed by analysis on a 2% agarose gel and by melting curve analysis (data not shown). Primers for spruce β -actin were designed (GenBank accession number CO232865) and served as a quantification control. The program for all PCR reactions was: 95 $^{\circ}$ C for 10 min; 40 cycles of 15 s at 95 $^{\circ}$ C, 1 min at 55 $^{\circ}$ C, and 30 s at 72 $^{\circ}$ C. Data were analyzed using MX3000P™ Real-Time PCR system software (Stratagene). All PCR reactions consisted of at least two technical replicates. Transcript abundance of each DIR gene was normalized to β -actin by subtracting the Ct value of β -actin from the Ct value of each DIR gene, where Δ Ct = Δ Ct_{DIR_{iso}-form} - Δ Ct _{β -actin}. Transcript abundance of the DIR genes in control and treated samples were obtained from the equation $(1 + E)^{-\Delta$ Ct}, where E is the PCR efficiency, as described by Ramakers *et al.* (2003), which is derived from the log slope of the fluorescence versus cycle number in the exponential phase of each individual amplification plot, using the equation $(1 + E) = 10^{\text{slope}}$.

Results

Discovery of wound- and insect-induced DIR genes by microarray analysis

We initially investigated a possible role for DIR proteins in the wound- and insect-induced defense response of Sitka spruce as part of a larger microarray study of transcriptional changes in spruce defense against the white pine weevil (the complete microarray analysis will be published elsewhere; S. Ralph and J. Bohlmann unpublished results). In brief, 2-year-old Sitka spruce saplings were subjected to either feeding by adult white pine weevils or mechanical wounding. Transcript profiles were monitored 2, 6 and 48 h after the onset of

treatment, and compared to untreated control trees using a cDNA microarray printed with 16 756 unique elements derived from 11 spruce cDNA libraries. At the peak response (48 h) to weevil herbivory, 2393 or 14.2% of array elements were differentially expressed (fold change > 1.5 \times , $P < 0.05$ relative to untreated control trees), compared to only 582 or 3.4% differentially expressed after 2 h of insect feeding. The response to mechanical wounding was considerably more rapid, peaking at 2 h with 2348 or 14.0% of array elements differentially expressed, and this diminished to 1203 or 7.1% by 48 h. Six array elements (IS0011_E22, IS0013_K10, IS0013_G20, WS00913_G16, WS00914_H24 and WS01011_J07) with high similarity to plant DIR genes demonstrated substantially increased hybridization signals with RNA from spruce bark in response to both the mechanical wounding and insect feeding treatments (Table 1). Up-regulation of transcripts hybridizing with several of the DIR elements on the microarray was evident after as little as 2 h of insect feeding, at which point feeding damage on sapling stems was limited to fewer than 5 feeding holes per tree. The magnitude of induction continued to increase up to 48 h, by which time the number of feeding holes had increased to > 20 per tree. In contrast, the response to mechanical wounding was more pronounced and rapid, with peak induction of transcripts hybridizing to DIR elements at 6 h post-treatment, and a diminishing response to 48 h. The differing temporal response to insect herbivory and mechanical wounding treatments likely reflects the slow, but continuous feeding by weevils compared with a one time wounding event by needle at inception. It is noteworthy that at least one DIR element (i.e. WS0083_C01) on the microarray showed significantly down-regulated transcript levels in response to weevil feeding and mechanical damage, while the remaining 10 DIR elements were mostly unresponsive to these treatments.

cDNA cloning of a family of 19 DIR and DIR-like genes from spruce

A TBLASTN search of the Treenomix spruce EST database (www.treenomix.com) containing 77 160 3'-end sequences was performed with publicly available plant DIR sequences (Kim *et al.*, 2002) and led to the identification of 96 putative spruce

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Figure 1. Amino acid sequence alignment of the DIR family from gymnosperms and selected *Arabidopsis* and rice proteins generated using ClustalW (blosum matrix, gap open and gap extension penalties of 5 and 0.5, respectively) and Boxshade. Conserved similarity shading is based on 50% identity (black) and 50% similarity (gray). Amino acid motifs characteristic of the entire plant DIR family (see 'Materials and methods') are marked by red bars. The beginning of N-terminal transit peptides predicted using SignalP 3.0 are highlighted in red. DIR nomenclature is as follows: P, *Picea glauca*, *P. sitchensis* or *P. glauca* × *engelmannii*; Th, *Tsuga heterophylla*; Tp, *Thuja plicata*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*. For additional details of each gene see Table VI, supplemental information. The following proteins were not included due to their large ORFs: AtDIR9, AtDIR10, AtDIR16, AtDIR18, AtDIR24 and OsDIR1.

DIR ESTs. Additional sequencing, 5'-rapid amplification of cDNA ends (RACE), and sequence comparisons indicated these ESTs represent 17 new full-length DIR and DIR-like cDNAs, based on overall similarity to other plant DIR cDNAs and the presence of a starting methionine, and two new partial cDNAs, *PDIR4* and *PDIR19* (Figure 1 and Table 2). Pairwise sequence similarities among predicted amino acids of the 19 spruce DIR cDNAs range from 98.4% identity between *PDIR3* and *PDIR7*, to 19.5% identity between *PDIR11* and *PDIR18* (Table 3). The high amino acid identity (98.3% and 96.4%, respectively) between *PDIR3* (*P. glauca* × *engelmannii*) and *PDIR7* (*P. glauca*), and between *PDIR5* (*P. glauca* × *engelmannii*) and *PDIR15* (*P. sitchensis*) suggests these cDNAs may represent species-specific alleles of the same genes. The predicted open reading frames (ORFs) (Table 2) for the 17 full-length cDNAs range from 178 (*PDIR17*) to 224 (*PDIR18*) amino acids, and have predicted pI values ranging from 6.20 (*PDIR2*) to 8.86 (*PDIR18*). The predicted molecular masses range from ca. 19.3 (*PDIR17*) to 25.3 (*PDIR18*) kDa.

The 19 spruce DIR and DIR-like genes clearly separate into two distinct groups based on sequence relatedness. The predicted amino acid sequences of *PDIR2*, *PDIR5*, *PDIR6*, *PDIR8*, *PDIR13*, *PDIR15*, *PDIR16*, *PDIR18* and *PDIR19* are most similar to the other gymnosperm DIR proteins reported in western red cedar (Kim *et al.*, 2002) and western hemlock (*Tsuga heterophylla*) (Gang *et al.*, 1999) (Figures 1 and 2). In contrast, the predicted amino acid sequences of *PDIR1*, *PDIR3*, *PDIR4*, *PDIR7*, *PDIR9*, *PDIR10*, *PDIR11*, *PDIR12*, *PDIR14* and *PDIR17* share greatest similarity to

a newly identified group of DIR-like proteins in *Arabidopsis* (Figures 1 and 2). Between the spruce DIR and DIR-like subfamilies of proteins, there are several amino acid sequence motifs that are conserved in both groups (Figure 1).

Using the TargetP v1.01 (Emanuelsson *et al.*, 2000) and PSORT (Nakai and Horton, 1999) subcellular localization software, we predict that all 17 full-length and one near full-length (*PDIR4*) spruce DIR proteins are targeted to the secretory pathway, either through the default pathway for extracellular release, or possible final localization in the endoplasmic reticulum membrane, microbody or vacuole (data not shown). The subcellular location of *PDIR19* could not be predicted because this clone likely lacks at least 20–30 amino acids at the N-terminus.

Phylogeny of the plant DIR family including DIR-like proteins

In an attempt to decipher the evolutionary relationships within the spruce DIR and DIR-like family and relative to other plant DIR genes, we performed a comprehensive search of GenBank for DIR genes and identified 83 putative full-length cDNAs or genomic ORF sequences (Table V, supplemental information). These include known and putative DIR from the gymnosperms western red cedar (nine genes) and western hemlock (two genes), and the angiosperm monocots (41 genes) and dicots (31 genes). No DIR genes were identified outside of the seed plant division.

To group plant DIR proteins according to sequence similarity we performed maximum likelihood analysis using a subset of 72 protein sequences for which a supporting full-length cDNA was available (ORFs predicted solely from genomic DNA or short ESTs were not included). Alignments were performed using the Dialign software (Morgenstern *et al.*, 1998), which is capable of finding local similarities among divergent sequences, and then manually adjusted to define a conserved sequence of ca. 160 amino acids. Using the neighbor joining algorithm we generated a phylogenetic tree (Figure 2), which suggests there may be five distinct subfamilies within the extended DIR family, tentatively named DIR-a, DIR-b, DIR-c, DIR-d and DIR-e. The ability of DIR proteins to direct stereoselective formation of lignans has been demonstrated with *in vitro* assays for several members of

Table 1. cDNA microarray analysis identifies DIR transcripts as differentially expressed in response to insect feeding and/or mechanical wounding.^a

Clone ID	AGI code	E value	DIR name	Weevil at 2 h			Weevil at 6 h			Weevil at 48 h			Mechanical at 2 h			Mechanical at 6 h			Mechanical at 48 h		
				FC	P		FC	P		FC	P		FC	P		FC	P		FC	P	
IS0011_E22	At1g64160	0.041	<i>PDIR5</i>	1.54	0.223	1.99	0.055	4.19	<0.001	3.06	0.003	11.61	<0.001	2.16	0.033						
IS0013_K10	At1g64160	4e-18	<i>PDIR6</i>	1.42	0.241	1.09	0.764	2.36	0.006	2.97	<0.001	3.88	<0.001	2.27	0.009						
IS0013_G20	At1g64160	2e-33	<i>PDIR8</i>	1.51	0.127	1.69	0.053	5.76	<0.001	5.61	<0.001	8.56	<0.001	3.88	<0.001						
WS00913_G16	At1g64160	6e-17	<i>PDIR8</i>	1.83	0.034	1.74	0.050	5.01	<0.001	5.33	<0.001	10.17	<0.001	2.41	0.002						
WS00914_H24	At1g64160	2e-07	<i>PDIR13</i>	1.41	0.138	1.71	0.022	2.57	<0.001	2.99	<0.001	7.98	<0.001	2.27	<0.001						
WS01011_J07	At1g64160	2e-24	<i>PDIR19</i>	1.29	0.398	2.08	0.018	3.75	<0.001	3.60	<0.001	8.27	<0.001	2.92	0.001						
WS0036_M20	At1g58170	6e-27	<i>PDIR1</i>	0.54	0.091	1.18	0.629	0.98	0.971	0.90	0.774	1.20	0.601	1.10	0.784						
WS0083_C01	At1g58170	0.16	<i>PDIR1</i>	1.11	0.761	0.21	<0.001	0.28	0.001	1.46	0.304	0.51	0.077	0.48	0.054						
WS0047_F19	At2g21100	5e-15	<i>PDIR7</i>	0.90	0.607	1.22	0.312	0.51	0.002	1.23	0.294	0.92	0.719	0.81	0.318						
WS0034_J11	At2g21100	3e-44	<i>PDIR7</i>	0.93	0.773	1.28	0.309	0.72	0.205	0.88	0.610	0.80	0.384	0.90	0.699						
WS0038_B23	No match	n.a.	<i>PDIR7</i>	2.17	0.010	1.27	0.397	0.52	0.032	1.13	0.654	1.06	0.815	0.84	0.566						
WS0038_G19	At2g21100	4e-43	<i>PDIR10</i>	1.07	0.875	1.36	0.479	1.09	0.834	1.11	0.802	1.60	0.288	0.61	0.276						
WS00110_N11	At2g21100	3e-30	<i>PDIR10</i>	1.06	0.857	1.25	0.516	1.34	0.395	0.99	0.994	0.84	0.630	1.37	0.370						
WS00102_K18	At2g21100	5e-44	<i>PDIR11</i>	0.89	0.574	1.15	0.432	0.98	0.917	0.98	0.951	1.04	0.821	1.03	0.844						
WS0037_K05	At2g21100	2e-37	<i>PDIR12</i>	1.41	0.298	1.16	0.643	1.16	0.646	0.95	0.898	2.37	0.012	0.36	0.004						
IS0014_N16	At1g58170	6e-23	<i>PDIR14</i>	1.33	0.236	1.06	0.801	1.00	0.988	1.62	0.053	1.08	0.726	1.26	0.341						
WS0045_M14	At1g58170	5e-28	<i>PDIR17</i>	1.06	0.756	1.63	0.015	1.18	0.376	1.70	0.008	1.81	0.003	1.16	0.431						

^a Spruce 16.7K cDNA microarray analysis of transcriptional changes in bark tissue from Sitka spruce trees subjected to weevil herbivory, mechanical wounding or left untreated, 2, 6 or 48 h after the onset of treatment. Data expressed as fold-change (FC) relative to untreated trees, and *P* values denote significant differences between treated and untreated trees (see supplemental methods). Best match annotation to *Arabidopsis* using BLASTX against the non-redundant division of GenBank is provided.

Table 2. Gene name, genotype, protein and transcript features of spruce DIR and DIR-like genes.

Clone ID	DIR nomenclature	cDNA library	Species (genotype)	cDNA length (bp)	ORF length (aa)	pI	MW (kDa)
WS0031_H14	<i>PDIR1</i>	Outer xylem	<i>Picea glauca</i> (PG29)	886	186	6.77	20.6
WS00911_I09	<i>PDIR2</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	903	195	6.20	22.2
WS0093_M13	<i>PDIR3</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	882	188	8.65	20.6
WS00927_L10	<i>PDIR4^a</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	784	184	9.10	20.4
IS0011_E22	<i>PDIR5</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	975	196	8.56	22.3
IS0013_K10	<i>PDIR6</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	964	195	7.39	22.1
WS0055_G05	<i>PDIR7</i>	Phloem	<i>Picea glauca</i> (PG29)	928	188	8.76	20.7
IS0013_G20	<i>PDIR8</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	982	196	6.30	22.2
WS00924_E04	<i>PDIR9</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	1043	192	6.42	21.2
WS0038_G19	<i>PDIR10</i>	Outer xylem	<i>Picea glauca</i> (PG29)	840	184	8.80	20.3
WS01012_K18	<i>PDIR11</i>	Roots	<i>Picea sitchensis</i> (Gb2-229)	700	186	8.26	20.8
WS02610_M19	<i>PDIR12</i>	Phloem	<i>Picea glauca</i> (PG29)	911	184	6.70	20.0
WS00914_H24	<i>PDIR13</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	871	195	8.30	22.3
IS0014_N16	<i>PDIR14</i>	MeJa/wounded bark with phloem	<i>Picea sitchensis</i> × <i>engelmannii</i> (Fal-1028)	918	184	6.83	20.3
WS01043_L01	<i>PDIR15</i>	Roots	<i>Picea sitchensis</i> (Gb2-229)	859	196	8.81	22.5
WS01041_G07	<i>PDIR16</i>	Roots	<i>Picea sitchensis</i> (Gb2-229)	903	196	6.59	22.1
WS0045_M14	<i>PDIR17</i>	Roots	<i>Picea sitchensis</i> (Gb2-229)	847	178	7.50	19.3
WS00825_K05	<i>PDIR18</i>	Outer xylem	<i>Picea glauca</i> (PG29)	910	224	8.86	25.3
WS01024_K15	<i>PDIR19^a</i>	Roots	<i>Picea sitchensis</i> (Gb2-229)	793	159	6.34	18.2

^a Partial cDNA.

Table 3. Sequence relatedness of spruce DIR and DIR-like proteins.^a

	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s
PDIR3 (a)	100																		
PDIR7 (b)	98	100																	
PDIR1 (c)	90	91	100																
PDIR10 (d)	83	84	87	100															
PDIR9 (e)	74	75	73	74	100														
PDIR14 (f)	82	83	80	80	80	100													
PDIR11 (g)	75	76	76	75	74	78	100												
PDIR4 (h)	74	75	73	75	74	78	80	100											
PDIR12 (i)	78	77	77	81	71	76	72	70	100										
PDIR17 (j)	52	52	51	53	49	53	50	50	52	100									
<i>PDIR2 (k)</i>	26	26	24	24	23	26	23	23	25	26	100								
<i>PDIR6 (l)</i>	26	26	24	23	23	26	24	23	24	23	91	100							
<i>PDIR8 (m)</i>	26	26	24	23	23	24	23	23	24	25	88	86	100						
<i>PDIR16 (n)</i>	26	26	24	24	23	25	23	23	24	25	89	88	93	100					
<i>PDIR13 (o)</i>	27	27	25	25	24	27	25	25	25	25	87	86	85	86	100				
<i>PDIR5 (p)</i>	24	24	22	21	23	24	21	22	23	24	85	84	81	81	81	100			
<i>PDIR15 (q)</i>	24	24	22	21	21	22	21	21	22	22	85	84	82	81	81	96	100		
<i>PDIR18 (r)</i>	21	21	19	20	20	21	19	20	20	22	67	65	65	65	65	66	65	100	
<i>PDIR19 (s)</i>	23	23	22	22	21	24	22	23	23	24	68	67	66	67	68	67	66	61	100

^a Results from pairwise amino acid sequence comparisons are shown as percent identity among members of the DIR-a subfamily (italics), the DIR-b subfamily (bold) and between DIR-a and DIR-b subfamily members (regular).

the DIR-a subfamily cloned from *Forsythia intermedia* (Davin *et al.*, 1997), *Podophyllum peltatum* (Xia *et al.*, 2000), and western red cedar (Kim *et al.*, 2002). Since biochemical functions for members of subfamilies DIR-b, DIR-c, DIR-d and DIR-e are not known we refer to these genes as DIR-like. It is important to note that overall sequence identity between and within subfamilies is in some cases very low, which could explain why a relatedness of known DIR proteins (DIR-a) with the DIR-like proteins was not previously recognized. However, when a minimum of 50% amino acid identity is applied as a criterion to identify conserved motifs common to all 72 plant DIR or DIR-like proteins in our analysis we found the following motifs (Figure 1) characteristic of this extended DIR- and DIR-like protein family: motif I, LsLYFHDAahG beginning at amino acid 43 (all positions relative to *PDIR1*); motif II, FGsasVhDDPaT beginning at amino acid 74; motif III, SssVGRAQGHY beginning at amino acid 92; motif IV, uTashsG beginning at amino acid 128; and motif V, RcaS VVGGTGcFhMARGaAsacT beginning at amino acid 143; where h = hydrophobic, p = polar, a = aliphatic, s = small, u = tiny, c = charged and x = any. These motifs clearly connect DIR and DIR-like proteins, and justify a broader view of this family and its sequence diversity.

Nine of the 19 spruce DIR genes group into subfamily DIR-a (Figure 2), along with all other known gymnosperm DIR sequences, and a collection of 11 DIR genes from several angiosperm monocot and dicot species. Within subfamily DIR-a predicted ORFs range from 182 (*AtDIR5*) to 224 (*PDIR18*) amino acids (Figure 1), and amino acid identity ranges from 12.3% to 99.5%. The DIR-a cluster of six rice (*Oryza sativa*) and *Arabidopsis* proteins is notably divergent from the other DIR-a members. When only the conserved portion of the protein sequence (ca. 160 amino acids) used for the phylogeny analysis is examined, amino acid identity ranges from 36.2% to 99.4%. The spruce DIR amino acid identity across the entire protein length ranges from 61.2% to 96.4%, with predicted ORFs ranging from 195 (*PDIR2*, *PDIR6* and *PDIR13*) to 224 (*PDIR18*) amino acids. The remaining 10 spruce DIR-like genes group into subfamily DIR-b, along with 9 DIR genes from several angiosperm monocot and dicot species. Despite previous work with DIR genes from western red cedar and western hemlock, no gymnosperm sequences of the DIR-b group have previously been reported. Within subfamily DIR-b amino acid identity is extremely divergent, ranging from 9.5% to 98.9%, with very little conservation within the N-terminal half of proteins. When only the

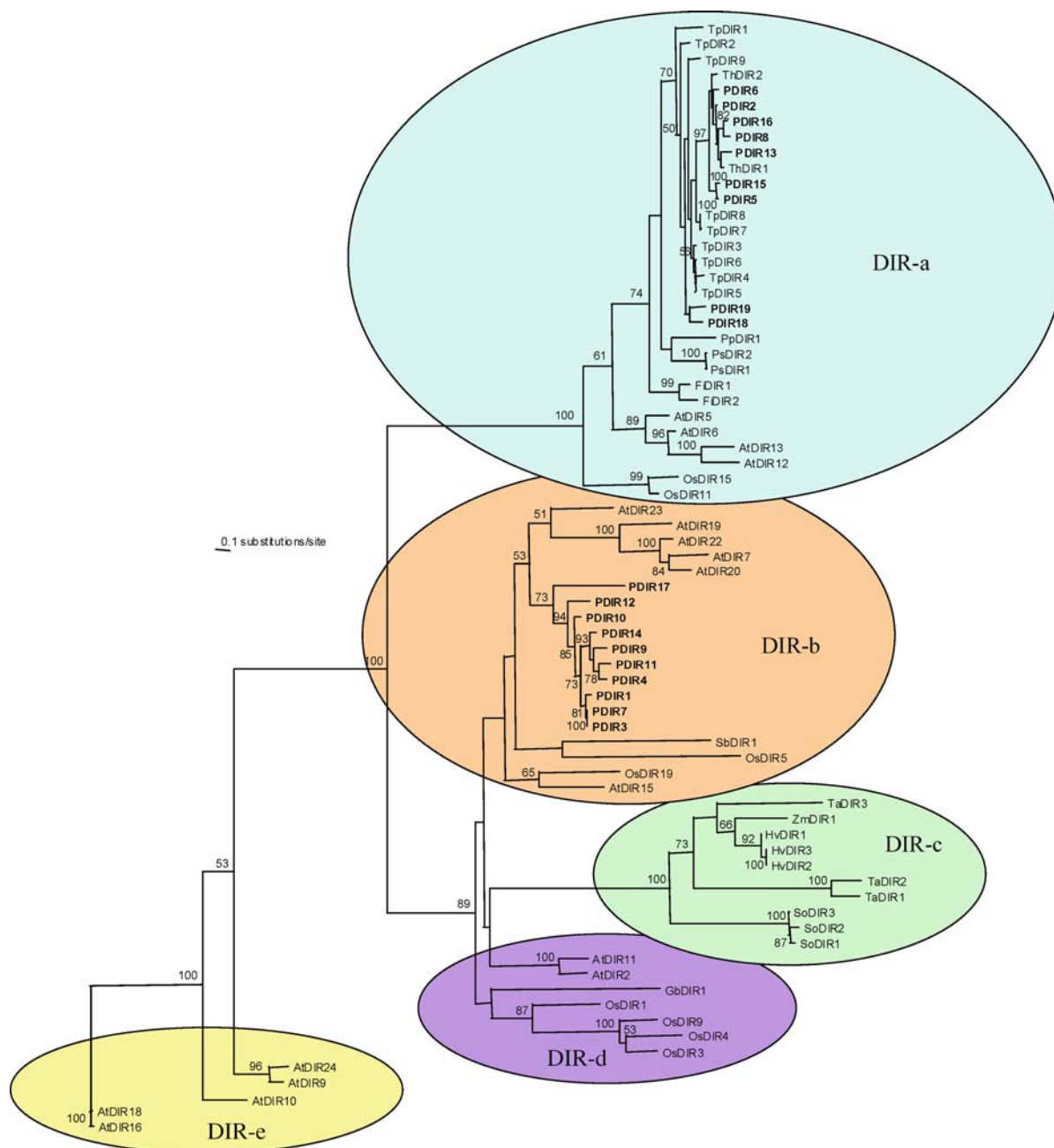


Figure 2. Phylogenetic tree of plant DIR and DIR-like amino acid sequences. Amino acids of 72 DIR or DIR-like proteins were analyzed by maximum likelihood using PhymI. Bootstrap values are only provided for nodes with greater than 50% support. Maximum likelihood values represent percentages of 100 gamma-corrected replicates ($\log L = -12\,751$). DIR nomenclature is as follows: P, *Picea glauca*, *P. sitchensis* or *P. glauca* × *engelmannii*; Th, *Tsuga heterophylla*; Tp, *Thuja plicata*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Hv, *Hordeum vulgare*; Ta, *Triticum aestivum*; So, *Saccharum officinarum*; Ps, *Pisum sativum*; Fi, *Forsythia intermedia*; Pp, *Podophyllum peltatum*; Sb, *Sorghum bicolor*; Gb, *Gossypium barbadense*; Zm, *Zea mays*. For additional details of each gene see Table VI, supplemental information.

conserved portion of the protein sequence used for the phylogenetic analysis is examined, amino acid identity ranges from 21.3% to 100%. In contrast,

amino acid identity across the entire length of spruce DIR-b members is high, ranging from 49% to 98.4%, with predicted ORFs ranging from 178

(PDIR17) to 192 (PDIR19) amino acids. Among the entire DIR-b subfamily, ORFs range from 125 amino acids for AtDIR22 to 192 amino acids for PDIR9 and SbDIR1. There is no information in the literature concerning expression patterns or proposed functions for any DIR-like genes in subfamily DIR-b, and this group, along with DIR-c, DIR-d and DIR-e, has not previously been recognized in the literature, although all four subfamilies are clearly related to the DIR-a family (Kim *et al.*, 2002).

No conifer genes have been identified in subfamilies DIR-c, DIR-d, and DIR-e, which are therefore described only briefly and for completeness of this analysis. Subfamily DIR-c consists exclusively of angiosperm monocot DIR-like genes including three representatives from sugarcane (*Saccharum officinarum*), and seven members from wheat, barley, and corn (Figure 2). ORFs range from 187 (SoDIR1, 2, and 3) to 345 (TaDIR2) amino acids. Within subfamily DIR-c amino acid identity is extremely divergent, ranging from 9.6% to 97.4% across the entire length of each protein, with very limited conservation within the N-terminal half of proteins. Within the conserved part of the protein sequence, identity ranges from 19.3% to 100%. The seven DIR-like genes from wheat, barley, and corn possess a unique ~140 amino acid extension at their C-terminus with high similarity to the jacalin-like domain common to lectin proteins (Sankaranarayanan *et al.*, 1996), with a proposed role in defense against insects (Blanchard *et al.*, 2001). Subfamily DIR-d consists of seven angiosperm genes from *Arabidopsis*, rice, and cotton (*Gossypium barbadense*) with amino acid identities ranging from 12.0% to 77.5% when the entire proteins are considered, and 17.1% to 78.1% when only the conserved part of each protein is considered. ORFs range in size from 185 (AtDIR2) to 229 (OsDIR1) amino acids. Subfamily DIR-e consists of only five *Arabidopsis* DIR-like genes, with amino acid identities ranging from 3.8% to 94.3% across the entire length of each protein, and from 33.3% to 98.1% when only the conserved region is considered. ORFs range from 243 (AtDIR16) to 447 (AtDIR10) amino acids, which is considerably longer than DIR or DIR-like proteins from the other four subfamilies. In particular, AtDIR10 is the largest DIR or DIR-like protein discovered to date, possessing ~150–200 additional amino acids at the N-terminus that

has similarity to other plant glycine-rich proteins, as well as weak similarity to jacalin-like domains from other *Arabidopsis* proteins.

Expression profiles of DIR and DIR-like genes in Sitka spruce in response to mechanical wounding and weevil attack detected by real-time PCR

To validate our initial observations of differential wound- and insect-induced gene expression from the cDNA microarray studies (Table 1), we applied quantitative real-time PCR to examine gene-specific transcript levels of five DIR-a and three DIR-like genes in Sitka spruce bark and xylem tissue subjected to mechanical wounding or weevil feeding. The data are presented as transcript abundance normalized to β -actin levels (Figure 3) and as fold-induction relative to untreated control tissues (Table 4). Gene-specific primers were designed based on full-length cDNA sequences. Gene expression analysis for members of the DIR-a group included *PDIR2*, *PDIR5*, *PDIR6*, *PDIR8* and *PDIR13*. Among members of the DIR-b subfamily, we analyzed transcript levels for *PDIR1*, *PDIR10*, and *PDIR12*. In general, results from real-time PCR analysis were in good agreement with the overall observations of microarray analyses. We found rapidly increased transcript levels (fold change >2) of the DIR-a subfamily genes *PDIR6*, *PDIR8* and *PDIR13* in bark tissue 2 h after the initiation of weevil feeding or after mechanical wounding. Peak induction for these genes in bark tissue occurred post mechanical wounding at 6 h for *PDIR8* (92.2-fold), and 48 h for *PDIR6* (21.8-fold) and *PDIR13* (115.6-fold). Similar to our microarray results, induction by weevil feeding was less rapid and of lower magnitude when measured by real-time PCR, with peak induction at 48 h for *PDIR8* (28.1-fold), *PDIR6* (9.1-fold) and *PDIR13* (27.1-fold). It is important to note, that damage caused by stem-boring weevils is a relatively slow and continuous process compared to the one-time, rapid mechanical wound treatment. Among other DIR-a subfamily genes examined, *PDIR2* was also induced, but with a slower initial response that peaked at 48 h after mechanical wounding (53.3-fold) and weevil feeding (46.9-fold). *PDIR5* was only moderately induced 48 h post-treatment with maximum responses of 14.4-fold (mechanical wounding) and 3.7-fold (weevil feeding). Addi-

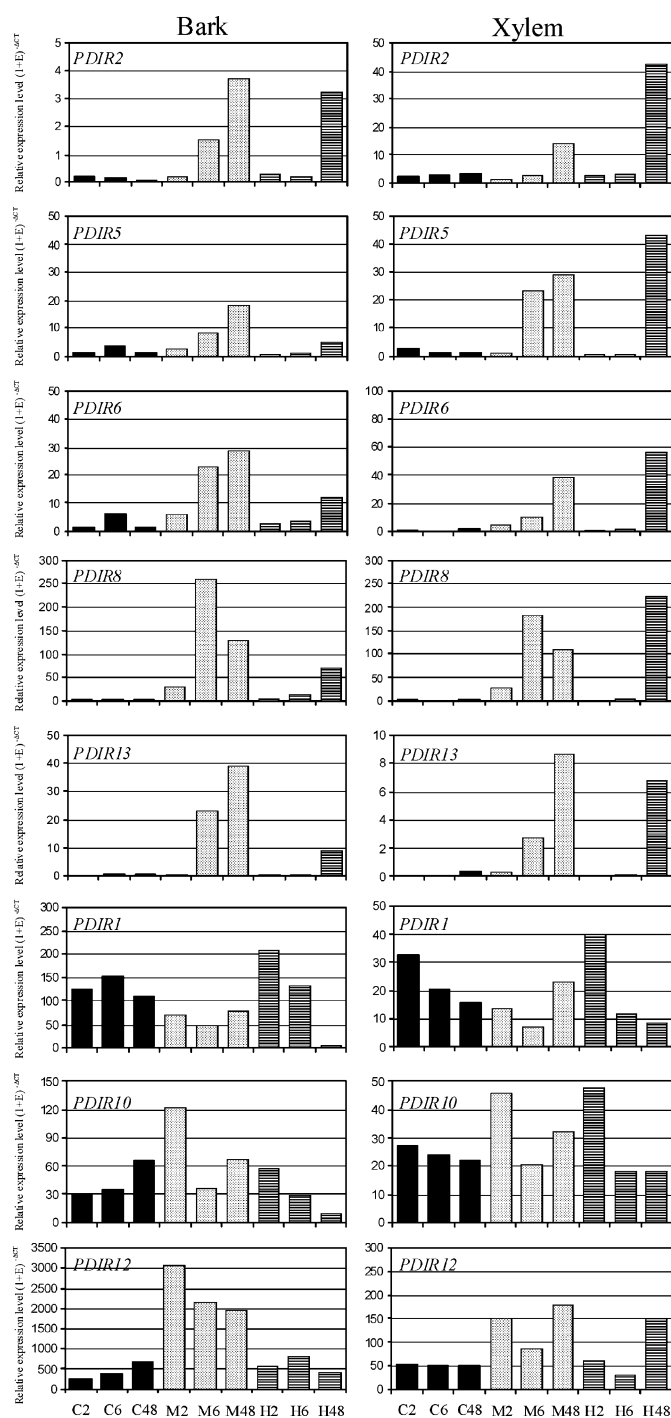


Figure 3. Relative abundance of mRNA transcripts of eight spruce DIR or DIR-like genes in bark and xylem tissue of Sitka spruce trees subjected to weevil herbivory (H), mechanical wounding (M) or left untreated as a control (C), 2, 6 or 48 h after the onset of treatment. Values represent the mean of two or more technical replicates. Transcript abundance of each DIR gene was normalized to β -actin by subtracting the Ct value of β -actin from the Ct value of each DIR gene, where $\Delta Ct = \Delta Ct_{DIR\ isoform} - \Delta Ct_{\beta\text{-actin}}$. Transcript abundance of the DIR genes in control and treated samples were obtained from the equation $(1 + E)^{-\Delta Ct}$, where E is the PCR efficiency, as described by Ramakers *et al.* (2003). A DIR transcript with a relative abundance of one is equivalent in transcript abundance to β -actin in the same tissue.

Table 4. Relative abundance of select DIR and DIR-like genes in response to weevil herbivory or mechanical wounding in spruce xylem and bark.^a

DIR Name	Bark						Xylem					
	Weevil at 2 h		Weevil at 6 h		Weevil at 48 h		Mechanical at 2 h		Mechanical at 6 h		Mechanical at 48 h	
<i>PDIR2</i>	1.28	1.44	46.92	0.99	10.49	53.36	1.19	1.06	14.08	0.56	0.93	4.59
<i>PDIR5</i>	0.64	0.34	3.70	2.19	2.47	14.45	0.19	0.51	43.32	0.30	21.71	29.31
<i>PDIR6</i>	2.24	0.60	9.14	4.77	3.58	21.82	0.90	7.92	39.76	10.34	54.13	27.25
<i>PDIR8</i>	2.87	5.22	28.19	20.90	92.24	51.84	0.61	19.66	77.13	22.46	511.06	38.15
<i>PDIR13</i>	5.48	2.48	27.15	3.37	90.39	115.66	1.07	3.25	20.47	4.06	71.67	25.86
<i>PDIR1</i>	1.66	0.86	0.06	0.56	0.31	0.71	1.22	0.58	0.52	0.42	0.36	1.46
<i>PDIR10</i>	1.98	0.85	0.13	4.22	1.06	1.03	1.77	0.78	0.80	1.69	0.87	1.44
<i>PDIR12</i>	2.34	2.10	0.60	12.07	5.66	2.90	1.16	0.61	2.98	2.82	1.77	3.53

^a Values were determined using real-time PCR and represent fold-change differences relative to transcript expression in untreated control tissues from the same time point (see Figure 3).

tional experiments have also shown that transcript levels for *PDIR2*, *PDIR5*, *PDIR6*, *PDIR8* and *PDIR13* remain elevated in bark tissue until at least 72 h after mechanical wounding when combined with a one-time application of the plant hormone methyl jasmonate (0.01% MeJA dissolved in 0.1% Tween; unpublished data).

Similar to these observations in bark tissue, all five Sitka spruce DIR-a genes were also induced in xylem tissue harvested from the same trees (Figure 3 and Table 4). Xylem tissue was not profiled by cDNA microarray due to limiting tissue quantities. The response of DIR-a genes was generally more rapid after mechanical wounding than weevil feeding in xylem, and was of similar magnitude to that observed in bark tissue with maximum responses to each treatment as follows: *PDIR2* – 14.0-fold, weevil 48 h; 4.5-fold, mechanical 48 h; *PDIR5* – 43.3-fold, weevil 48 h; 29.3-fold, mechanical 48 h; *PDIR6* – 39.7-fold, weevil 48 h; 54.1-fold, mechanical 6 h; *PDIR8* – 77.6-fold, weevil 48 h; 511.0-fold, mechanical 6 h; and *PDIR13* – 20.4-fold, weevil 48 h; 71.6-fold, mechanical 6 h.

In contrast to the induction by mechanical wounding or insect-attack of transcript accumulation of Sitka spruce DIR-a gene subfamily members, the DIR-b subfamily members *PDIR1* and *PDIR10* did not show increased transcript levels in response to weevil feeding or mechanical wounding in either bark or xylem tissues (Figure 3 and Table 4). Both genes demonstrated reduced transcript levels in bark tissue 48 h after initiation of weevil feeding (*PDIR1* – 16.6-fold reduced; *PDIR10* – 7.6-fold reduced), similar to results obtained with microarrays (Table 1). *PDIR12*, also from the DIR-b subfamily, was induced in response to mechanical wounding and to a lesser extent by weevil feeding, in both bark and xylem tissues. Peak induction to mechanical wounding was observed in bark tissue 2 h post treatment (12.0-fold), compared to a maximum of 2.9-fold in xylem after 48 h of weevil feeding.

Constitutive expression of selected spruce DIR and DIR-like genes

In an attempt to illustrate differences in spatial patterns of RNA expression, the relative constitutive abundance of six DIR-a and three DIR-like genes was quantified in total RNA isolated from different stem tissues (cortex, phloem, cambium,

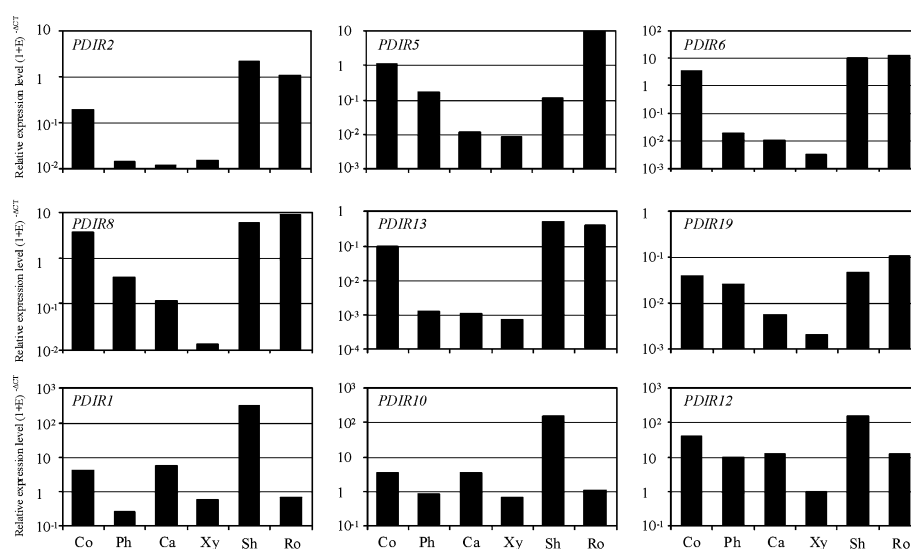


Figure 4. Relative abundance of mRNA transcripts of nine spruce DIR or DIR-like genes in tissues collected from 2 year old Sitka spruce trees harvested in May. Tissues examined include cortex (Co), phloem (Ph), cambium (Ca), xylem (Xy), lateral shoot tips (Sh) and roots (Ro). Values represent the mean of two or more technical replicates. Transcript abundance of each DIR gene was normalized to β -actin by subtracting the Ct value of β -actin from the Ct value of each DIR gene, where $\Delta Ct = \Delta Ct_{DIR\ isoform} - \Delta Ct_{\beta\text{-actin}}$. Transcript abundance of the DIR genes in control and treated samples were obtained from the equation $(1 + E)^{-\Delta Ct}$, where E is the PCR efficiency, as described by Ramakers *et al.* (2003). A DIR transcript with a relative abundance of one is equivalent in transcript abundance to β -actin in the same tissue.

and xylem), young lateral shoot and root tissues, and normalized to β -actin levels. All nine genes examined were ubiquitously expressed, often at levels considerably greater than β -actin (Figure 4). *PDIR1*, *PDIR10* and *PDIR12*, all from subfamily DIR-b, were ubiquitously expressed at high levels, with maximum expression in the lateral green shoot tips. In contrast, *PDIR2*, *PDIR5*, *PDIR6*, *PDIR8*, *PDIR13* and *PDIR19*, all belonging to subfamily DIR-a, were in general expressed at highest levels in cortex, young lateral shoots and roots, with significantly lower abundance in the inner stem tissues, phloem, cambium and xylem (Figure 4). Interestingly, for these six DIR genes there is a gradient of expression amongst the stem tissues: highest in the outermost tissue (cortex) to lowest in the innermost tissue (xylem).

Discussion

Discovery of induced DIR and DIR-like genes in Sitka spruce

In an effort to identify genes associated with defense against stem-boring insects in Sitka spruce we utilized cDNA microarrays to obtain tran-

script profiles of bark tissue from trees subjected to weevil feeding or mechanical wounding. An analysis of these profiles revealed among other differentially expressed genes a set of six array elements corresponding to DIR genes that were rapidly and strongly induced by both treatments (Table 1; the complete microarray analysis will be published separately). In contrast, 10 other array elements corresponding to DIR-like genes were largely unresponsive to these same treatments, and one element corresponding to *PDIR1*, was down-regulated. These initial observations of differential gene expression were confirmed by real-time PCR analysis. The improved quantification achieved with real-time PCR revealed induction of all five DIR-a subfamily members examined with peak responses in bark tissue ranging from 14.4-fold (*PDIR5*, mechanical 48 h) to 115.8-fold (*PDIR13*, mechanical 48 h) (Figure 3 and Table 4). In general, the response to mechanical wounding was more rapid and stronger than weevil feeding, and similar results were observed in both bark and xylem tissues. The more rapid response to mechanical wounding compared to weevil feeding was in proportion to the massive damage inflicted by mechanical wounding at time 0 h compared to the slow, but

continuous feeding by weevils over time. This temporal pattern was the most commonly observed trend among all differentially expressed array elements, regardless of predicted biological function (S. Ralph and J. Bohlmann, unpublished). Among DIR-b subfamily members examined, *PDIR1* and *PDIR10* were relatively unchanged except for reduced levels in bark tissue 48 h after the onset of weevil feeding (Figure 3 and Table 4). However, *PDIR12* was induced in both tissues examined and with either treatment, with a peak response of 12.0-fold in bark tissue 2 h after the onset of mechanical wounding.

Insect- and pathogen-inducible DIR and DIR-like genes

cDNA microarray and real-time PCR expression profiles indicate that spruce DIR genes, but not DIR-like genes, are induced in response to stem-boring insect attack or mechanical damage to the stem, suggesting that induction may be a characteristic of the DIR-a subfamily. A review of the literature confirms the inducible nature of DIR genes in plants in response to stress. Previous publications and public sequence repositories revealed a family of 72 DIR and DIR-like genes from a wide range of seed plants, often annotated as genes of unknown function, and for which several genes have been demonstrated to be inducible at the transcript level to a wide variety of stresses and treatments. For example, the pea *PsDIR1* gene (DIR-a subfamily), originally labeled as DRR206-d, was the first DIR gene demonstrated to be responsive to stress treatments. Riggelman *et al.* (1985) and Fristensky *et al.* (1985) demonstrated that *PsDIR1* mRNA is strongly induced within 4 h following treatment with compatible or, to a lesser extent, with incompatible forms of *Fusarium* pathogens, or chitosan. Similar responses were also observed with *Fusarium oxysporum* (Hadwiger *et al.*, 1992). *PsDIR1* was also inducible in several pea cultivars by a variety of *Pseudomonas syringae* races (Daniels *et al.*, 1987), or after treatment with the cell wall envelope of *P. syringae* or heat-killed cells (Daniels *et al.*, 1988). In addition *PsDIR1* was shown to be inducible by the DNA-damaging agents mitomycin C and actinomycin D, and when the *PsDIR1* promoter was heterologously expressed in transgenic tobacco a similar increase

in the reporter β -glucuronidase was observed when plants were treated with these or other elicitors (e.g. H₂O₂; Choi *et al.*, 2001). A possible role for *PsDIR1* in plant defense was further substantiated by the observation of resistance to the fungal pathogens *Leptosphaeria maculans* (Wang *et al.*, 1999), *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Wang and Fristensky, 2001) in transgenic canola (*Brassica napus*) expressing *PsDIR1*.

Several other DIR-like genes are also inducible by various stress treatments including the induction of *JRG1C34* (*HvDIR1*, DIR-c subfamily) with the plant hormone jasmonic acid in barley (Lee *et al.*, 1996). Treatment of wheat with benzothiadiazole, salicylic acid or 2,6-dichloroisonicotinic acid induces the *wheat chemically induced 1* gene (*WC11* or *TaDIR1*, DIR-c subfamily; Görlach *et al.*, 1996). Most interestingly, another wheat DIR-like gene, *Hfr-1* or *TaDIR2* (DIR-c subfamily), is induced in response to feeding by avirulent (incompatible) first-instar larvae of the Hessian fly (*Mayetiola destructor*), but not by virulent larvae (compatible) (Williams *et al.*, 2002). Although the number of observations of DIR and DIR-like genes induced by pathogens or insects is still relatively small, combined with our examination of the spruce DIR-a and DIR-b genes, these data suggest that induction of DIR or DIR-like genes as part of a plant's defense response to biotic stress may have evolved multiple times in different plant lineages or may have been a function of a common ancestor of the plant DIR family.

Relatedness of DIR- and DIR-like genes in spruce, other gymnosperms and angiosperms

Other than the DIR and DIR-like gene families mined from the *Arabidopsis* (25 members) and rice (30 members) genomes, the 19 newly identified spruce genes represent the largest set of DIR or DIR-like genes cloned from a closely related group of species, and the largest for a gymnosperm system. Overall, the predicted amino acid sequences of this gene family are divergent, ranging from a low of 19.5% to a high of 98.4%. However, there are several gene clusters with high similarity. For example, from subfamily DIR-b, *PDIR1*, *PDIR3*, *PDIR4*, *PDIR7*, *PDIR9*, *PDIR10*, *PDIR11*, *PDIR12* and *PDIR14* share a minimum

pairwise identity of 70%. Within the DIR-a group, PDIR2, PDIR6, PDIR8, PDIR13 and PDIR16 share a minimum pairwise identity of 85% (Table 3), suggesting that each of these gene clusters may have arisen from multiple gene duplications. The maximum likelihood analysis of 72 plant DIR and DIR-like genes groups the DIR family into at least five distinct clusters: DIR-a, DIR-b, DIR-c, DIR-d and DIR-e, with the gymnosperm DIR genes contained within the first two subfamilies (Figure 2). The 11 known gymnosperm DIR genes from western red cedar and western hemlock along with nine of the new spruce DIR genes, group into subfamily DIR-a, and the remaining 10 new spruce DIR-like genes group into DIR-b. The occurrence of spruce DIR and DIR-like genes in two clades, which also contain angiosperm genes, suggests that the bifurcation of these two DIR classes occurred before the separation of angiosperms and gymnosperms. The multiple spruce DIR genes, several of which are stress inducible, may provide these conifers with improved fitness to defend against pathogens and herbivores.

Constitutive expression of DIR and DIR-like genes in Sitka spruce

To provide further insight into the possible roles of members of the spruce DIR gene family, we examined the relative constitutive abundance of six DIR and three DIR-like transcripts in different stem tissues (cortex, phloem, cambium, and xylem) and in young lateral shoot and root tissues (Figure 4). It is possible that DIR transcripts may be localized with specialized defense cell types such as phenolic phloem parenchyma (PP) cells, which represent only a small proportion of the complex phloem tissue. The ubiquitous high expression of spruce DIR-b members is suggestive of a possible role for these genes in a primary process or in constitutive defense. However, at this time no other DIR-b genes from other plants have been characterized with respect to gene expression or biological function. The only other study of constitutive expression of multiple DIR transcripts in a single species was performed by Kim *et al.* (2002) who examined expression of seven genes from the DIR-a subfamily in western red cedar. In general agreement with the constitutive expression of spruce DIR-a subfamily genes, they observed

consistently low expression in xylem, cambium and phloem tissue from 2-year-old sapling trees, moderate expression in scales, needles, young stems and shoots, and occasionally high levels in roots, female flowers and callus for some DIR transcripts.

A role of DIR genes in Sitka spruce defense response

Based on (i) observations of wound- and insect-inducible DIR transcripts in stem tissues of Sitka spruce, (ii) highest constitutive expression of Sitka spruce DIR genes in outer stem tissues, (iii) the ability to direct stereoselective lignan formation with DIR proteins (Davin *et al.*, 1997; Xia *et al.*, 2000; Kim *et al.*, 2002), and (iv) based on our current understanding of defense mechanisms in conifers (Huber *et al.*, 2004), we propose two possible roles for spruce DIR and DIR-like genes in the defense against stem-boring insects: First, a role in constitutive and/or induced production of phenolic defense metabolites associated with specialized PP cells and/or resin ducts; and second, a role in generation of precursors for lignan/lignin biosynthesis to strengthen/repair damaged cell walls to serve as a physical barrier. In the same roles, spruce DIR genes could also function against fungal pathogens.

In Sitka spruce, Norway spruce, and several other species of the *Pinaceae*, constitutive and induced resin ducts in bark and xylem serve an important role in the defense against insect attack and pathogenic fungi (Martin *et al.*, 2002; Franceschi *et al.*, 2002; Hudgins *et al.*, 2003). The toxic resin mixture contained within resin ducts is largely composed of terpenoid compounds and the synthesis of these compounds has been well characterized (Martin *et al.*, 2002; Fäldt *et al.*, 2003; Miller *et al.*, 2005; Ro *et al.*, 2005). Interestingly, xylem parenchyma cells associated with traumatic resin ducts, along with the lumen of these ducts, stain strongly with periodic acid-Schiff, and PAL has been localized to the epithelial cells lining traumatic resin ducts, suggesting that in addition to terpenoids, phenolic defense metabolites may also contribute to composition and toxicity of conifer resin (Nagy *et al.*, 2000). Less well understood is the induced activation of specialized PP cells in these species, which has been characterized thus far primarily at the

anatomical level. A role for PP cells in conifer defense has been established in several studies examining the anatomical response to mechanical wounding, bark beetle attack, or fungal pathogens in Norway spruce. These treatments resulted in an increased number and size of PP cells, and an increased size and altered pattern of distribution of phenolic bodies in PP cells, with marked differences between trees resistant or sensitive to attack (Franceschi *et al.*, 1998, 2000; Krokene *et al.*, 2003; Nagy *et al.*, 2000, 2004). PP cells are proposed to be active in the synthesis and storage of phenolic defense compounds in response to wounding. This is based on autofluorescence under blue light of the contents of vacuoles of PP cells and the immunocytochemical localization of PAL to PP cells, primarily at the plasma membrane (Franceschi *et al.*, 1998). PP cells stain more darkly approximately 3 weeks after fungal inoculation, suggesting there may also be a change in phenolic content, possibly altering solubility or toxicity (Franceschi *et al.*, 2000). Although the composition of the phenolic content of the PP cells has not yet been characterized, and little is known concerning induced gene expression for the formation of phenolic defense compounds in spruce, the DIR family may play a role in this defense mechanism.

DIR proteins have been proposed to control the monolignol coupling reactions leading to the formation of lignin (Davin *et al.*, 1997). Several weeks after inoculation of Norway spruce bark and cambium with the bark beetle associated fungal pathogen *Ceratocystis polonica*, cells surrounding the inoculation site become partially or completely lignified (Franceschi *et al.*, 2000; Nagy *et al.*, 2000). This lignification likely serves to strengthen cell walls to help contain the spread of the fungal pathogen, possibly via DIR gene induction and/or DIR activity. According to the model of monolignol coupling proposed by Davin *et al.* (1997), stereoselective bimolecular coupling by DIR proteins must be preceded by one-electron oxidation of the monolignols by a peroxidase or laccase enzyme. In addition to induced levels of DIR genes, the Sitka spruce 16.7 cDNA microarray profiles of wound- or insect-induced bark tissues also revealed induction of several laccase genes with patterns of expression similar to DIR genes (S. Ralph and J. Bohlmann, unpublished results). The concomitant induction of numerous laccase-like array elements, in a

temporal fashion analogous to the DIR transcripts, provides further support for a relationship between phenol oxidases and DIR proteins in the induced defense response in stems of Sitka spruce.

In conclusion, we identified a large family of DIR proteins in the spruce transcriptome that resulted in the discovery of related clades of DIR-like proteins of this large family of unique plant proteins. Our data support a role of the DIR and DIR-like genes in constitutive and insect-induced defense against weevils in Sitka spruce, which may also function against other insects such as bark beetles or insect-associated pathogens. To our knowledge, the genome-wide discovery of spruce full-length and near full-length DIR and DIR-like cDNAs is the most comprehensive reported for any species to date. Together with the microarray and real time PCR expression analysis reported here, it will provide an important platform for further investigation of this gene family for phenolic defenses against insects, in particular its role in cell wall strengthening and lignan formation in spruce defense, and a possible association with PP cells.

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