

# Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome

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## ABSTRACT

Conifers are resistant to attack from a large number of potential herbivores or pathogens. Previous molecular and biochemical characterization of selected conifer defence systems support a model of multigenic, constitutive and induced defences that act on invading insects via physical, chemical, biochemical or ecological (multitrophic) mechanisms. However, the genomic foundation of the complex defence and resistance mechanisms of conifers is largely unknown. As part of a genomics strategy to characterize inducible defences and possible resistance mechanisms of conifers against insect herbivory, we developed a cDNA microarray building upon a new spruce (*Picea* spp.) expressed sequence tag resource. This first-generation spruce cDNA microarray contains 9720 cDNA elements representing *c.* 5500 unique genes. We used this array to monitor gene expression in Sitka spruce (*Picea sitchensis*) bark in response to herbivory by white pine weevils (*Pissodes strobi*, Curculionidae) or wounding, and in young shoot tips in response to western spruce budworm (*Choristoneura occidentalis*, Lepidopterae) feeding. Weevils are stem-boring insects that feed on phloem, while budworms are foliage feeding larvae that consume needles and young shoot tips. Both insect species and wounding treatment caused substantial changes of the host plant transcriptome detected in each case by differential gene expression of several thousand array elements at 1 or 2 d after the onset of treatment. Overall, there was considerable overlap

among differentially expressed gene sets from these three stress treatments. Functional classification of the induced transcripts revealed genes with roles in general plant defence, octadecanoid and ethylene signalling, transport, secondary metabolism, and transcriptional regulation. Several genes involved in primary metabolic processes such as photosynthesis were down-regulated upon insect feeding or wounding, fitting with the concept of dynamic resource allocation in plant defence. Refined expression analysis using gene-specific primers and real-time PCR for selected transcripts was in agreement with microarray results for most genes tested. This study provides the first large-scale survey of insect-induced defence transcripts in a gymnosperm and provides a platform for functional investigation of plant–insect interactions in spruce. Induction of spruce genes of octadecanoid and ethylene signalling, terpenoid biosynthesis, and phenolic secondary metabolism are discussed in more detail.

*Key-words:* dirigent proteins; full length cDNAs; herbivory; octadecanoids; phenylpropanoids; plant–insect interactions; spruce ESTs; terpenoids; tree and forestry genomics.

## INTRODUCTION

The pine family (Pinaceae) contains several gymnosperm tree species of major economic importance such as spruce (*Picea* spp.), pine (*Pinus* spp.), true firs (*Abies* spp.) and Douglas fir (*Pseudotsuga menziesii*). Conifers of the pine family dominate some of the largest ecosystems on earth and are critically important for global carbon fixation. Despite their ecological and economic importance, there

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has been relatively limited genomics research conducted with conifers compared to angiosperms, for which three complete genome sequences are available [*Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000); *Oryza sativa* (Goff *et al.* 2002; Yu *et al.* 2002); *Populus trichocarpa* (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>)], and for which more than 5 million expressed sequence tags (ESTs) have been deposited in GenBank as of 20 January 2006. Genetic and genomic research in conifers is hampered by their large size at maturity, slow growth rate, long generation times of up to 20 years, limited capacity for transformation as a research tool, lack of available mutant lines and a large genome (10–40 Gb) containing a high content of repetitive DNA.

Large-scale genomics of conifers was initiated with loblolly pine (*Pinus taeda*; Allona *et al.* 1998) and has recently been extended to species of spruce (Ritland *et al.* 2006). We have established a program of large-scale gene and protein discovery in spruce, which includes the generation of a suite of cDNA libraries, high-throughput EST and full length (FL)-cDNA sequencing, microarray development, genetic marker development (Rungis *et al.* 2004), and protein expression profiling (Lippert *et al.* 2005). A major emphasis of our program in conifer forest health genomics is to utilize these genomics and proteomics resources to investigate how conifer genomes respond to attack by forest insect pests and their associated pathogens. Insect-induced defence responses identified by microarray transcript profiling have recently been described for the wild tobacco (*Nicotiana attenuata*, Hui *et al.* 2003; Heidel & Baldwin 2004; Voelckel, Weisser & Baldwin 2004), *Arabidopsis* (Reymond *et al.* 2000, 2004; De Vos *et al.* 2005), sorghum (*Sorghum bicolor*, Zhu-Salzman *et al.* 2004) and poplar (*P. trichocarpa* × *deltoides*, Ralph *et al.* 2006a). In contrast, insect-induced responses in spruce, which provides a useful system to study genomics of plant–insect interactions in a conifer, have only been studied for a small number of genes involved in terpenoid and phenolic secondary metabolism and octadecanoid signalling (Byun-McKay *et al.* 2003, 2006; Miller *et al.* 2005; Ralph *et al.* 2006b).

Outbreaks of forest insect pests pose a challenge to the sustainability of both natural and plantation forests. The risk of forest insect pest epidemics, which cannot be addressed with short-term crop rotation or pesticide application, as is possible in agriculture, is increasing with the introduction of exotic pest species and with global climate change. Although conifers display resistance against most generalist herbivorous insects, some specialized insect species among bark beetles (Scolytidae), shoot and root weevils (Curculionidae), sawflies (Hymenopterae) or budworms (Lepidopterae) are known to cause substantial damage in conifer forests. For instance, the white pine weevil (*Pissodes strobi*) is a major pest of Sitka spruce (*Picea sitchensis*) in the Pacific Northwest of North America, and can devastate planted Norway spruce (*Picea abies*) forests in eastern Canada (Alfaro *et al.* 2002). As stem-boring insects, adult weevils feed on the phloem of living trees and,

in May or June, lay their eggs into the bark of the tip of the apical shoot. The developing larvae then feed downwards in the phloem, cambium and outer xylem, and consequently girdle the tree from the inside, destroying 2 or 3 years of growth. Another problematic insect, the foliage-feeding western spruce budworm (*Choristoneura occidentalis*), is the most widely distributed and destructive forest defoliator in western North America and is a major pest for Douglas fir, but will also feed upon Engelmann spruce (*Picea engelmannii*), white spruce (*Picea glauca*) and their hybrid interior spruce (*P. glauca* × *engelmannii*) (Alfaro *et al.* 1982). After overwintering as immature larvae, the early instar larvae become active in April and mine buds, feeding on expanding needles and the young green shoot tips. Mid- to late-instar larvae then strip current-year foliage from the upper crown before eventually forming webbing around the feeding site and emerging as adults in June or July. Repeated defoliation by western spruce budworms over several consecutive years results in top dieback, bole deformity and may result in an increased incidence of fungal disease and infestation by other insects.

The first lines of plant defence against insect herbivores are constitutive chemical and physical barriers; however, if these barriers are breached, inducible defences are of central importance in reducing herbivory (Karban & Baldwin 1997; Agrawal 1998). Conifer defences against insects are best characterized at the molecular genetic, biochemical and chemical levels in species of spruce, Sitka spruce and Norway spruce, and to some extent, also in a few other species of the Pinaceae family (Huber, Ralph & Bohlmann 2004). The bark of conifers provides a durable constitutive defence barrier, containing suberized or lignified periderm, sclereids, phloem parenchyma cells filled with phenolic secondary metabolites, and a system of resin ducts or resin blisters filled with complex mixtures of terpenoid resins (Raffa & Berryman 1982; Franceschi *et al.* 1998, 2000, 2005). In addition, many conifers possess defences that are inducible upon insect attack, treatment with methyl jasmonate (MeJA), mechanical wounding or fungal inoculation. These induced defences include the activation of existing resin ducts and *de novo* formation of traumatic resin duct systems in the bark and xylem (Franceschi, Krekling & Christiansen 2002; Martin *et al.* 2002; Hudgins, Christiansen & Franceschi 2003). Induced terpenoid resin defences in Sitka spruce and Norway spruce have been well characterized at the anatomical, biochemical and molecular levels (Franceschi *et al.* 2002; Martin *et al.* 2002; Fäldt *et al.* 2003; Martin, Gershenson & Bohlmann 2003; Martin, Fäldt & Bohlmann 2004). For example, traumatic resin duct formation and induced resin terpenoid accumulation in Sitka spruce bark and xylem is induced by weevil feeding, and involves the induction of a family of terpene synthase (TPS) genes (Byun-McKay *et al.* 2003, 2006; Miller *et al.* 2005).

Less is known concerning constitutive and inducible defences in conifer foliage tissues. Qualitative and quantitative compositions of mono-, sesqui- and diterpenoids have been characterized in Norway spruce needles (Schönwitz *et al.* 1990; Persson *et al.* 1996; Martin *et al.* 2003) and

in constitutive and MeJA- or weevil-induced terpenoid volatile emissions (Martin *et al.* 2003; Miller *et al.* 2005). Terpenoid volatiles emitted from conifers into the environment can serve as airborne signals that deter herbivores and attract predators and parasites of herbivores. For example, release of herbivore-induced volatiles from conifer foliage has been reported in Scots pine (*Pinus sylvestris*) in response to egg deposition by the pine sawfly (*Diprion pini*), which attracts an egg parasitoid (Hilker *et al.* 2002). Moreover, in Sitka spruce, weevil feeding has been shown to increase the accumulation of total mono-, sesqui- and diterpenoids in young needles, and MeJA treatment has been demonstrated to increase transcript levels in needles for a number of TPS genes (Miller *et al.* 2005).

Relatively few studies of induced defence responses beyond terpenoid metabolism have been conducted in species of spruce at the molecular level. To date, targeted studies have identified a small set of genes induced by biotic stress encoding octadecanoid biosynthesis enzymes (Miller *et al.* 2005), peroxidases (Fossdal, Sharma & Lönneborg 2001; Nagy *et al.* 2004a), a defensin (Fossdal *et al.* 2003), chitinases (Dong & Dunstan 1997; Hietala *et al.* 2004; Nagy *et al.* 2004a) a  $\beta$ -1,3-glucanase (Dong & Dunstan 1997), a dehydrin (Richard *et al.* 2000), a chalcone synthase (Nagy *et al.* 2004b) and a family of dirigent (DIR) proteins (Ralph *et al.* 2006b). We describe here the construction and application of a cDNA microarray consisting of 9720 ESTs representing *c.* 5500 unique genes, to study changes in transcript abundance in Sitka spruce bark and shoot tips in response to weevil and budworm herbivory, respectively, along with mechanical wounding. These large-scale transcript profiles combined with mining of our EST resource for sequence information for known plant defence pathways (i.e. terpenoid, phenylpropanoid and octadecanoid metabolism) provides the first genomic picture of insect-induced defence systems in a gymnosperm.

## MATERIALS AND METHODS

### Plant material and insects

Sitka spruce [*P. sitchensis* (Bong.) Carriere: clone FB3-425] seedlings were propagated by somatic embryogenesis and generously provided by Dr David Ellis (CellFor Inc., Vancouver, Canada). Prior to experimental treatment, trees were grown outside at the University of British Columbia for 1 or 2 years under natural light and environmental conditions to a height of 40–50 cm (1 year) or 60–70 cm (2 years). Trees were grown in 3:1 (v/v) peat: vermiculite, 2.4 g L<sup>-1</sup> Dolomite, 0.5 g L<sup>-1</sup> Nutritrace micronutrients and 3.6 g L<sup>-1</sup> Osmocote in 656 mL cone-tainers (1 year) or one gallon pots (2 years) (Stuewe & Sons, Inc., Corvallis, USA), and were watered daily. Western spruce budworms (*C. occidentalis* Freeman) were obtained from the Great Lakes Forestry Centre (NRCan, Sault Ste. Marie, Canada). Budworms were reared and maintained on artificial diet (Addy 1969) at 25 °C, 50–60% humidity and 16/8-h (light/dark) photoperiod. Groups of mid-instar larvae

were used in experiments. Two weeks prior to experiments with budworms and mechanical wounding, which commenced in September 2002, 1-year-old trees were moved inside a greenhouse with constant 16/8-h photoperiod provided by high-pressure sodium lamps. Average greenhouse temperature during September was 22.9 °C (20.2 °C minimum and 28.1 °C maximum), with an average humidity of 37.8%. Adult white pine weevils (*P. strobi* Peck) were generously provided by Dr Rene I. Alfaro (Pacific Forestry Centre, Canadian Forest Service, Victoria, Canada). Weevils were reared from larvae of infested Sitka spruce shoots collected at natural infestation sites in British Columbia in 2002 and maintained on fresh Sitka spruce shoots as food source. Two weeks prior to experiments with weevils, which commenced in May 2003, 2-year-old trees were moved inside a greenhouse with constant 16/8-h photoperiod 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%.

### Treatment of trees with budworms, weevils, or mechanical wounding, and tissue harvest

For the mechanical wounding treatment, the bark of each of three trees was cut horizontally at 5 mm intervals on opposite sides along the entire length of the stem. For insect treatments, budworms and weevils were kept without food on moist filter paper for 24 h and 48 h, respectively, prior to placing them on the trees. Ten insects (budworms or weevils) were added per tree under mesh bags on groups of five trees per time point. Insects were caged on the upper two-thirds of sapling trees. As controls, an equivalent number of Tween-treated trees (Martin *et al.* 2002; budworm and mechanical wounding treatments) and untreated trees (weevil treatment) were used. Control, mechanically wounded and insect-treated trees were kept in separate treatment groups in a well-ventilated greenhouse. For the budworm treatment, leaders were harvested 3 h and 52 h after treatment; for the weevil treatment, bark was harvested 48 h after treatment; for the mechanical wounding treatment, bark was harvested only 24 h after treatment to account for the difference in stress-response dynamics after rapid, single application of mechanical wounding compared with slow, continuous insect feeding. In each case, control trees were harvested at the same time point. To harvest shoot tips from budworm treatment experiments, only the young, light green portion of the shoot tip was collected (apical 10–15 cm section). To harvest bark tissue, the bark of the upper two-thirds of the tree, excluding the green shoot tip, was cut longitudinally with a razor blade and the outer tissue was peeled off the woody inner stem tissues. Tissues from five or three trees, respectively, from the budworm and mechanical wounding experiments were pooled by treatment and time point prior to RNA isolation, whereas tissues from the weevil experiment were harvested individually and separately flash frozen in liquid nitrogen and stored at –80 °C prior to RNA isolation following the protocol of Kolosova *et al.* (2004). For the weevil

experiment, equal quantities of total RNA were pooled from each tree according to treatment prior to cDNA microarray analysis.

### Microarray hybridization and gene expression data analysis

For complete details of cDNA microarray fabrication and quality control, along with a detailed hybridization protocol please see Appendix S1. All microarray experiments were designed to comply with MIAME guidelines (Brazma *et al.* 2001). All scanned microarray TIF images, an ImageGrid, the gene identification file and ImageGene quantified data files are available at <http://douglas.bcgsc.bc.ca>. Total RNA from budworm-treated shoot tips 3 h and 52 h after treatment, as well as mechanically wounded bark 24 h after treatment, were each compared with RNA from control trees harvested at the same time point using four slides each (12 total) with dye flips. Total RNA from weevil-treated bark 48 h after treatment was compared with RNA from control trees harvested at the same time point using six slides with dye flips.

Before data normalization, the lowest 10% of median foreground intensities was subtracted from the median foreground intensities to correct for background intensity. After quantification of the signal intensities, data were normalized to compensate for non-linearity of intensity distributions using the variance stabilizing normalization (vsN) method (Huber *et al.* 2002). In order to assess the biological responses to budworm and weevil herbivory, as well as mechanical wounding, models containing a dye effect and a treatment effect for budworm 3 h minus control 3 h, budworm 52 h minus control 52 h, weevil 48 h minus control 48 h or mechanical wounding 24 h minus control 24 h were fit using data from four to six microarray slides. Expression variance was derived from technical variance among slides. In retrospect, it would have been preferable to use independent biological replicates throughout array analyses, instead of technical replicates with pooled samples from multiple biological replicates. However, we have recently shown that technical variation exceeds biological variation when genetically identical trees derived from clonal propagation are used under greenhouse conditions in a microarray analysis of plant herbivore responses, where nearly identical results were obtained with technical or biological replicates (Ralph *et al.* 2006a). The ratio of each treatment parameter estimate to the standard error was used to calculate a *t* statistic, from which a *P*-value was obtained. The *q*-value for each effect and gene was calculated for each of the models to adjust for the false discovery rate (Storey & Tibshirani 2003). All statistical analyses were performed within the R statistical package (<http://www.r-project.org/>).

### Real-time PCR and gene expression data analysis

Prior to reverse transcription, 15 µg total RNA per treatment and time point was treated with DNaseI (Invitrogen,

Carlsbad, USA) digestion according to the manufacturer's instructions to remove genomic DNA. The resulting RNA was divided into three aliquots of 5 µg and independent cDNA synthesis reactions were performed using Superscript II reverse transcriptase (Invitrogen) with an oligo d(T18) primer according to manufacturer's instructions. The efficiency of cDNA synthesis was assessed by gel electrophoresis prior to pooling of the three reactions. Gene-specific primers were designed (Supplementary Table S1) using a stringent set of criteria including predicted melting temperature of  $64 \pm 2$  °C, primer lengths of 20–24 nucleotides, guanine-cytosine contents of 40–60% and PCR amplicon lengths of 100–350 bp. Primer specificity (single product of expected length) was confirmed by analysis on a 2% agarose gel, by melting curve analysis and for at least one PCR reaction per gene, by sequence verification of PCR amplicons (data not shown). Primers for spruce elongation factor 1 $\alpha$  (EF1 $\alpha$ ) were designed (GenBank accession number CO233925; spruce EST WS0058\_E23) and served as a quantification control. PCR was performed in optical 96-well plates with a DNA Engine Opticon2 continuous fluorescence detector (MJ Research, Waltham, MA, USA) using SYBR Green to monitor dsDNA synthesis. Reactions contained 7.5 µL DyNAmo HS SYBR Green qPCR kit master mix (Finnzymes, Espoo, Finland), 10 ng cDNA and 0.3 µM of each primer in a final volume of 15 µL. Reactions with the cDNA template replaced by nuclease-free H<sub>2</sub>O or 10 ng of non-reverse transcribed RNA were run with each primer pair as a control. To further evaluate the efficiency and amplification performance of each primer pair, a 10-fold dilution series of corresponding DNA plasmids ( $10^{-2}$ – $10^{-6}$  ng template) was analysed with a minimum of three independent technical replicates per dilution (data not shown). The following standard thermal profile was used for all PCRs: 95 °C for 15 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s; then 72 °C for 10 min. Fluorescence signal was captured at the end of each cycle and melting curve analysis was performed from 65 °C to 95 °C, with data capture every 0.2 °C during a 1 s hold. Data were analysed using the Opticon Monitor analysis software version 2.02 (MJ Research). Quantification of each transcript in each cDNA source consisted of at least three independent (different 96-well plates) technical replicates. To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal ( $\Delta R_n$ ) versus cycle number, baseline data were collected between cycles 3 and 10. All amplification plots were analysed with an  $R_n$  threshold of 0.003 to obtain *Ct* (threshold cycle) values. Transcript abundance was normalized to EF1 $\alpha$  by subtracting the *Ct* value of EF1 $\alpha$  from the *Ct* value of each transcript, where  $\Delta C_t = C_{t_{\text{transcript}}} - C_{t_{\text{EF1}\alpha}}$ . Transcript abundance in control and treated samples were obtained from the equation  $(1 + E)^{-\Delta C_t}$ , 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 amplification plot and the equation  $(1 + E) = 10^{\text{slope}}$ . A transcript with a relative abundance of one is equivalent to the abundance of EF1 $\alpha$  in the same tissue.

## RESULTS AND DISCUSSION

### Development of a spruce cDNA microarray

Based on the generation of spruce ESTs, we have developed the first spruce cDNA microarray composed of 9720 cDNA elements selected from five cDNA libraries representing shoot tips, xylem and roots of various developmental stages, and bark and phloem treated with MeJA and mechanical wounding (Table 1). Treatment with mechanical wounding and MeJA can simulate, at least in part, insect attack in spruce (Martin *et al.* 2002, 2003; Miller *et al.* 2005). Clones on the array were selected from a sequence assembly of c. 12 000 3'-end ESTs (CAP3 software, Huang & Madan 1999) and represent c. 5500 unique genes. Functional annotation of array elements has been assigned according to The Arabidopsis Information Resource (TAIR; <http://www.arabidopsis.org>) *Arabidopsis* peptide set [BLASTX expect value (E) < 1e<sup>-05</sup>]. Overall, only 5570 (57.4%) of 9720 spotted cDNAs have similarity to *Arabidopsis* known or predicted protein sequences, which could be a result of the long evolutionary distance between gymnosperm and angiosperm genomes, the expression of unique genes in conifers that are not found in the *Arabidopsis* genome, and/or the bias in the spruce EST dataset towards 3' non-coding regions. Similarity of spruce transcripts with previously annotated angiosperm genes, or the lack thereof, is the topic of a separate paper (S. Ralph & J. Bohlmann, unpublished data). Low similarity of many conifer genes with *Arabidopsis* was also previously found for loblolly pine (Kirst *et al.* 2003) and presents a challenge to interpreting the biological functions of differentially regulated transcripts in a conifer system.

### Microarray transcriptome profiling of Sitka spruce responses to insect herbivory and wounding

We have previously established that feeding by white pine weevils on Sitka spruce induces selected genes in the oleoresin terpenoid defence pathway, specifically TPS genes, a few genes in the octadecanoid signalling pathway and a

family of DIR proteins (Miller *et al.* 2005; Ralph *et al.* 2006b). To extend this analysis with global transcriptome profiling, we used the spruce 9.7K cDNA microarray to examine changes in gene expression in spruce in response to insect herbivory and mechanical wounding. We also extended our previous analysis with the inclusion of two insect species, larvae of the spruce budworm and adult white pine weevils, which have different feeding patterns and consume different spruce tissues. Spruce budworms feed on the very young, green shoot tips and foliage, while weevils are stem-boring insects that drill into the bark of apical leaders to feed on phloem tissues. Clonal Sitka spruce saplings were subjected to either mechanical wounding of bark by razor blade, feeding on bark by stem-boring weevils or feeding on green leaders by defoliating budworms, with insects caged on trees using mesh bags (Fig. 1). In this initial transcriptome analysis, we selected the time point of 2 d (48–52 h) after the onset of insect feeding, based on earlier observations of strong induction of TPS and DIR genes at this time point (Miller *et al.* 2005; Ralph *et al.* 2006b). For comparison, and to assess the overall dynamics of an insect-induced transcriptome response in spruce, we also measured gene expression at an early time point (3 h) after the onset of budworm feeding. In contrast to insects, which required at least 1 d of continuous feeding to cause substantial tissue damage in our experiments, mechanical wounding causes rapid tissue damage. We therefore used a one-day time point for the analysis of wound-induced responses.

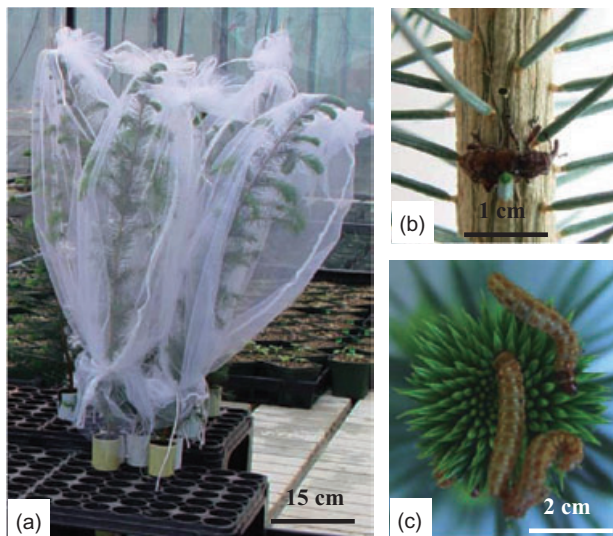
Differentially expressed (DE) genes were selected using two criteria: fold-change between treatment and control >1.5x and *P*-value < 0.05. To estimate the false discovery rate (FDR), we calculated *q*-values (Storey & Tibshirani 2003) and found the FDR for budworm feeding at 3 and 52 h, weevil feeding at 48 h and mechanical wounding at 24 h to be 19.0, 3.1, 5.1 and 2.4% at *P* = 0.05, diminishing to 10.7, 0.8, 0.7 and 0.3% at *P* = 0.001, respectively (Supplementary Table S2). For a complete list of expression data for all genes represented on the microarray see Supplementary Table S2. It should be noted that although microarrays measure transcript abundance, this could be the result of changes in the transcription rate or of changes in the rate

**Table 1.** cDNA libraries used for constructing the spruce 9.7K cDNA microarray

cDNA library	Tissue/Developmental stage	Species (Genotype)	No. on 9.7K array
WS-ES-A-1	Young shoots harvested from 25-year-old trees <sup>a</sup>	<i>Picea glauca</i> (PG-29)	3577
WS-PS-A-2	Flushing buds, young shoots and mature shoots harvested from 25-year-old trees <sup>a</sup>	<i>P. glauca</i> (PG-29)	1232
WS-X-A-3	Early (15 June), mid (10 July) and late (17 August) season outer xylem harvested from 25-year old trees <sup>a</sup>	<i>P. glauca</i> (PG-29)	2336
IS-B-A-4	Bark tissue (with phloem and cambium) harvested after razor blade wounding and treatment with 0.01% methyl jasmonate. Tissue was collected 0 (untreated), 3, 6 and 12 h post-treatment <sup>b</sup> .	<i>P. glauca</i> × <i>engelmannii</i> (Fal-1028)	1290
SS-R-A-5	Young growth (terminal 1–3 cm) and mature growth (distal to terminal 1–3 cm) roots <sup>b</sup>	<i>Picea sitchensis</i> (Gb2-229)	1269

<sup>a</sup>Field site located at Kalamalka Research Station in Vernon, British Columbia.

<sup>b</sup>1- or 2-year-old trees grown in potted soil under greenhouse conditions at the University of British Columbia.



**Figure 1.** Herbivory experiment set-up under greenhouse conditions. Insects were caged under mesh bags placed on Sitka spruce saplings (a). Weevils inflict damage by boring into bark of the stem and feeding on phloem (b), whereas budworms sever and consume young needles and green shoot tips (c). Scale bars indicate approximate size.

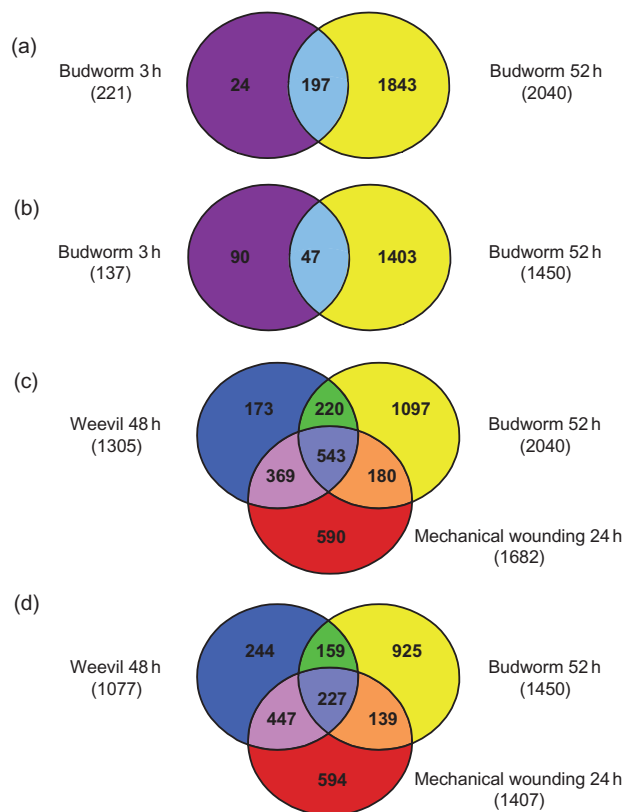
of transcript degradation. For brevity, these will collectively be referred to as changes in gene expression. The annotation of cDNA elements on the array as shown in Supplementary Table S2 is derived from annotation of each individual EST sequence, whereas annotation for genes described in Table 2 and Figs 4–6 is derived from a CAP3 sequence assembly of c. 80 000 3'-end spruce ESTs. The latter approach was chosen to improve annotation to *Arabidopsis* by using longer, contiguous spruce sequences. Figure 2 illustrates the overall transcriptional response in shoot tips 3 and 52 h after the onset of budworm feeding, as well as in bark tissue 24 h after mechanical wounding or 48 h after the onset of weevil feeding. After only 3 h of budworm feeding, 358 elements were DE (3.6% of the transcriptome analysed; 221 up-regulated, 137 down-regulated) compared to 3490 (35.9% of the transcriptome analysed; 2040 up-regulated, 1450 down-regulated) after 52 h, with 244 elements commonly DE at both time points. In comparison, 2382 (24.5% of the transcriptome analysed; 1305 up-regulated, 1077 down-regulated) and 3089 (31.7% of the transcriptome analysed; 1682 up-regulated, 1407 down-regulated) elements were DE in response to weevil feeding and mechanical wounding, respectively. These results demonstrate substantial overall changes of the host plant transcriptome at 2 d after onset of feeding for each of the two insect–spruce host interactions.

### Overall similarities and differences in the spruce host transcriptome response to insect herbivory and wounding

Considerable overlap was observed between the responses to weevil or budworm herbivory and mechanical wounding,

with 770 DE elements in common, despite the fact that different spruce tissues were compared (Fig. 2c & d). The overlap of DE elements was greatest between weevil feeding and mechanical wounding in bark tissue (1586 elements), with less overlap between the responses induced by the two insects feeding in different tissues, shoot tips and bark (1149 elements), or between mechanical wounding in bark and budworm feeding in shoot tips (1089).

To further analyse and illustrate the temporal patterns to budworm feeding in shoot tips and to compare weevil feeding and mechanical wounding in bark tissue, we generated scatter plots of  $\log_2$  treatment : control expression ratios. Array elements were classified as commonly or uniquely DE between treatments by dividing the plots into sub-planes and incorporating 95% confidence intervals for the expression of each element as a statistical measure of the likelihood that an array element belongs within a subplane (Fig. 3). Using a minimum fold-change criteria of 1.5x and 3x, respectively, for budworm herbivory at 3 and 52 h, we identified 64 array elements DE at both time points, 47



**Figure 2.** Overall changes of gene expression induced by weevils, budworms, or mechanical wounding. Venn diagrams showing distinct and overlapping patterns of genes induced or repressed by weevil feeding (48 h), budworm feeding (3 and 52 h), or mechanical wounding (24 h). All genes identified were differentially expressed (fold-change >1.5x and  $P$ -value < 0.05) between treatment and control RNA samples. (a & b) Intersection of genes that were up-regulated (a) or down-regulated (b) between budworm feeding after 3 and 52 h. (c & d) Intersection of genes that were up-regulated (c) or down-regulated (d) between weevil feeding, budworm feeding (52 h) and mechanical wounding.

**Table 2.** Selected herbivore- or wounding-responsive array elements

Clone ID	BLASTX versus <i>Arabidopsis</i>	AGI code	E-value	Mechanical at 24 h			Weevil at 48 h			Budworm at 3 h			Budworm at 52 h					
				FC	P	Q	FC	P	Q	FC	P	Q	FC	P	Q			
No significant match to <i>Arabidopsis</i>																		
IS00011_A15	No significant match	n.a.	n.a.	●	94.83	0.001	0.003	14.55	<0.001	0.003	●	2.50	0.009	0.143	13.45	0.006	0.011	
WS00044_F23	No significant match	n.a.	n.a.	●	0.53	0.020	0.013	0.77	0.064	0.061	●	0.30	0.026	0.163	0.17	0.001	0.008	
WS00034_E23	No significant match	n.a.	n.a.	●	0.29	0.006	0.006	0.30	0.006	0.015	●	0.91	0.159	0.286	6.52	0.019	0.018	
WS00044_G09	No significant match	n.a.	n.a.	●	7.09	0.006	0.006	1.77	0.023	0.032	●	1.38	0.002	0.116	0.01	<0.001	0.002	
Biological process unknown																		
WS00014_J09	Photoassimilate-responsive protein	At5g52390	e-10	●	1.10	0.019	0.012	0.80	0.083	0.072	●	0.80	0.005	0.126	10.26	0.012	0.014	
WS00018_A07	Plantacyanin	At2g02850	e-25	●	0.31	<0.001	0.002	0.28	<0.001	0.003	●	0.82	0.053	0.193	1.37	0.052	0.031	
WS00038_N22	Auxin-regulated protein	At2g33830	e-14	●	0.34	<0.001	0.003	0.40	<0.001	0.003	●	0.53	0.020	0.155	0.78	0.149	0.064	
WS00017_F02	Low temperature and salt responsive	At3g05890	e-13	●	0.28	0.011	0.009	0.30	0.001	0.008	●	0.96	0.503	0.467	1.50	0.083	0.043	
IS00014_J19	Lateral organ boundaries domain protein	At1g07900	e-20	●	0.89	0.109	0.043	8.43	0.002	0.009	●	1.55	0.074	0.213	4.18	0.041	0.027	
WS00015_F13	Phosphate-responsive protein	At5g64260	e-84	●	2.81	0.001	0.003	2.79	<0.001	0.005	●	2.44	0.009	0.143	7.58	0.001	0.008	
WS00016_C06	GDSL-motif lipase	At5g33370	e-77	●	1.08	0.634	0.175	1.11	0.416	0.226	●	0.99	0.993	0.610	18.56	0.010	0.014	
WS00039_N07	Gibberellin-regulated protein	At2g39540	e-16	●	0.82	0.063	0.029	0.57	0.090	0.076	●	1.78	0.055	0.195	13.37	0.013	0.015	
General metabolism																		
WS00012_O19	Arabinogalactan	At5g53250	e-05	●	0.68	0.045	0.022	4.43	0.007	0.017	●	1.68	0.031	0.170	8.45	0.011	0.014	
IS00012_L22	Haloacid dehalogenase hydrolase	At5g59480	e-28	●	3.00	0.032	0.018	2.40	<0.001	0.006	●	2.19	0.001	0.107	8.01	0.003	0.010	
WS000111_P23	Patatin	At2g26560	e-62	●	0.10	0.007	0.007	0.82	0.229	0.148	●	0.97	0.816	0.567	1.18	0.498	0.157	
WS00038_G12	Xyloglucan endo-1,4-β-D-glucanase	At4g25810	e-71	●	0.28	0.010	0.008	2.65	<0.001	0.004	●	2.10	0.001	0.116	18.50	0.017	0.017	
WS000110_E02	GAP3DH	At1g42970	e-23	●	0.48	<0.001	0.003	0.47	0.003	0.012	●	0.89	0.155	0.282	0.19	0.007	0.012	
WS00018_O18	Fructose-1,6-biphosphate aldolase	At4g38970	e-99	●	0.62	0.003	0.005	0.66	0.002	0.011	●	0.92	0.041	0.178	0.28	<0.001	0.002	
WS00013_N23	Galactinol synthase	At2g47180	e-81	●	0.99	0.856	0.221	0.63	0.015	0.025	●	1.05	0.289	0.369	0.25	0.002	0.009	
WS00019_N05	Fructose-1,6-biphosphatase	At1g43670	e-100	●	0.42	0.021	0.013	0.51	0.015	0.124	●	0.92	0.171	0.295	0.23	0.002	0.008	
WS00041_E18	Transketolase	At3g60750	e-127	●	1.37	0.016	0.011	1.19	0.210	0.139	●	1.03	0.740	0.545	0.27	<0.001	0.002	
WS00015_L24	Hydroxypyruvate reductase	At1g68010	e-55	●	0.81	0.021	0.013	0.94	0.121	0.094	●	0.99	0.971	0.605	0.28	<0.001	0.008	
WS00013_D08	Iron-sulphur complex protein	At4g22220	e-61	●	0.65	0.010	0.008	1.03	0.807	0.354	●	0.91	0.137	0.267	14.82	0.001	0.008	
WS00039_G18	Cellulose synthase	At1g55850	e-51	●	1.62	0.018	0.012	0.88	0.540	0.271	●	0.83	0.092	0.230	0.17	0.003	0.009	
WS00015_E01	Glutamine synthase	At5g37600	e-143	●	1.28	0.207	0.072	0.74	0.072	0.066	●	0.92	0.069	0.209	0.18	0.004	0.010	

Table 2. Continued

Clone ID	BLASTX versus <i>Arabidopsis</i>	AGI code	E-value	Fig.3b			Mechanical at 24 h			Weevil at 48 h			Fig.3a			Budworm at 3 h			Budworm at 52 h			
				class	FC	P	Q	FC	P	Q	FC	P	Q	FC	P	Q	FC	P	Q	FC	P	Q
WS0019_O08	Homocysteine S-methyltransferase	At1g17920	e-13	•	4.87	< 0.001	0.002	0.014	2.53	0.004	0.014	•	1.11	0.014	0.151	3.81	< 0.001	0.008				
WS00113_A19	VLCFA condensing enzyme	At1g68550	e-74	•	0.94	0.439	0.131	0.147	0.72	0.147	0.108	•	0.88	0.043	0.179	13.85	0.001	0.008				
WS0016_L07	Calcium-binding EF hand	At3g10300	e-52	•	0.98	0.927	0.235	0.620	0.90	0.620	0.299	•	0.97	0.610	0.507	13.97	0.006	0.011				
WS00110_A15	Carbonic anhydrase	At1g08080	e-39	•	15.83	0.001	0.003	< 0.001	15.30	< 0.001	0.006	•	1.27	0.005	0.128	9.41	0.001	0.008				
IS0013_C18	Expansin	At1g69550	e-51	•	5.90	0.002	0.004	0.024	0.66	0.024	0.032	•	0.84	0.342	0.398	2.75	0.012	0.014				
WS0015_F04	$\beta$ -galactosidase	At3g13750	e-55	•	5.26	< 0.001	0.002	0.746	0.92	0.746	0.337	•	1.12	0.370	0.410	0.64	0.038	0.026				
WS00113_D16	$\alpha$ -galactosidase	At5g08370	e-95	•	8.80	0.001	0.003	0.043	2.52	0.043	0.046	•	1.07	0.352	0.403	7.27	0.001	0.008				
WS00113_F20	Enoyl-CoA hydratase	At1g06550	e-43	•	4.30	0.002	0.004	< 0.001	3.34	< 0.001	0.004	•	0.90	0.095	0.234	5.16	0.003	0.009				
IS0011_B10	Acid phosphatase	At4g29270	e-54	•	24.76	< 0.001	0.003	< 0.001	11.45	< 0.001	0.004	•	3.59	0.019	0.153	25.88	0.002	0.009				
Photosynthesis																						
WS00111_J06	Photosystem II	At1g44575	e-49	•	0.63	0.002	0.004	0.002	0.39	0.002	0.010	•	0.86	0.003	0.126	0.35	0.010	0.013				
WS00113_A22	Photosystem I subunit VI	At1g52230	e-35	•	0.52	0.048	0.023	0.017	0.53	0.017	0.026	•	1.03	0.787	0.559	0.35	0.009	0.013				
WS0022_L16	Chlorophyll <i>a-b</i> binding protein	At1g29930	e-94	•	0.74	0.006	0.006	< 0.001	0.45	< 0.001	0.004	•	0.86	0.483	0.461	0.60	0.095	0.047				
WS0024_C24	Light-regulated protein	At3g26740	e-16	•	0.43	< 0.001	0.002	< 0.001	0.34	< 0.001	0.004	•	0.73	< 0.001	0.107	0.24	< 0.001	0.006				
WS0024_M05	Ferredoxin <sup>a</sup>	At1g60950	e-41	•	0.47	0.007	0.007	< 0.001	0.38	< 0.001	0.004	•	0.90	0.447	0.444	0.41	0.004	0.010				
Transport																						
WS0042_C02	Oligopeptide transporter	At1g22540	e-35	•	12.13	0.001	0.003	< 0.001	12.60	< 0.001	0.002	•	1.38	0.082	0.220	2.02	0.100	0.049				
IS0011_I18	ABC protein <sup>a</sup>	At3g28360	e-32	•	5.07	0.003	0.005	< 0.001	9.68	< 0.001	0.004	•	2.18	0.049	0.189	4.97	0.001	0.008				
WS0018_I09	Lipid transfer protein	At1g62500	e-25	•	4.71	< 0.001	0.002	< 0.001	3.75	< 0.001	0.005	•	1.36	0.005	0.126	6.61	0.014	0.015				
WS0015_C05	Lipid transfer protein	At5g48485	e-07	•	0.25	< 0.001	0.002	0.013	0.46	0.013	0.023	•	0.87	0.109	0.247	0.16	0.004	0.010				
WS0013_M24	Lipid transfer protein	At5g59310	e-23	•	0.88	0.287	0.093	0.502	1.12	0.502	0.258	•	0.85	0.133	0.264	34.02	0.003	0.009				
Transcriptional regulation																						
WS00111_M12	AP2 transcription factor <sup>a</sup>	At3g20310	e-09	•	5.23	0.011	0.009	0.013	2.83	0.013	0.023	•	1.26	0.056	0.196	3.09	0.014	0.015				
IS0013_F06	Basic helix-loop-helix transcription factor	At5g51780	e-05	•	11.89	0.004	0.005	0.009	9.78	0.009	0.019	•	0.98	0.497	0.465	1.75	0.002	0.008				
WS0042_F06	MADS-box transcription factor	At2g45660	e-14	•	0.79	0.193	0.068	0.873	0.97	0.873	0.372	•	1.03	0.409	0.429	5.57	0.013	0.015				
WS0044_J05	bZIP transcription factor <sup>a</sup>	At1g75390	e-20	•	10.15	0.001	0.003	0.003	6.36	0.003	0.012	•	1.12	0.058	0.197	1.07	0.729	0.210				

Table 2. Continued

Clone ID	BLASTX versus <i>Arabidopsis</i>	AGI code	E-value	Fig. 3b Mechanical at 24 h			Fig. 3a Weevil at 48 h			Budworm at 3 h			Budworm at 52 h					
				FC	P	Q	FC	P	Q	FC	P	Q	FC	P	Q			
Response to stress																		
IS0012_N03	Lectin	At1g52030	e-12	13.14	< 0.001	0.002	1.04	0.459	0.243	•	1.11	0.577	0.494	0.45	0.043	0.028		
WS0021_I16	$\beta$ -1,3-glucanase	At2g05790	e-114	1.12	0.134	0.051	1.07	0.184	0.127	•	0.95	0.361	0.408	11.44	0.002	0.009		
WS0017_B07	$\beta$ -1,3-glucanase	At2g01630	e-41	4.99	0.001	0.004	4.73	0.002	0.010	•	1.10	0.222	0.328	1.85	0.078	0.041		
IS0013_A20	Late embryogenesis abundant protein	At4g02380	e-05	13.94	0.002	0.004	24.98	< 0.001	0.075	•	4.24	< 0.001	0.126	20.02	< 0.001	0.007		
IS0013_J03	Class IV chitinase <sup>a</sup>	At3g54420	e-57	10.42	0.004	0.005	13.91	< 0.001	0.004	•	0.68	0.069	0.209	4.92	0.010	0.013		
IS0013_G04	Thaumatin	At1g19320	e-29	7.75	0.004	0.005	4.58	< 0.001	0.002	•	0.72	0.129	0.261	0.11	0.002	0.009		
WS0019_E03	Protease inhibitor	At5g43580	e-08	3.18	0.003	0.005	2.34	< 0.001	0.004	•	1.02	0.668	0.526	0.56	0.054	0.032		
IS0012_P21	Class II heat shock protein	At5g12020	e-30	0.17	< 0.001	0.003	0.37	< 0.001	0.003	•	0.76	0.061	0.201	0.92	0.286	0.104		
WS0023_A18	$\beta$ -glucosidase	At3g18080	0	1.21	0.037	0.020	1.91	0.005	0.014	•	1.25	0.026	0.163	6.25	0.009	0.013		
Detoxification, redox processes																		
WS0017_M23	lactoylglutathione lyase (glyoxalase I)	At1g80160	e-44	10.44	0.001	0.003	7.67	< 0.001	0.003	•	1.64	0.003	0.126	18.48	0.002	0.009		
WS00110_M24	Peroxidase	At5g05340	e-69	3.67	0.002	0.004	1.78	0.012	0.022	•	1.15	0.164	0.289	6.81	0.014	0.015		
WS0019_N09	Peroxidase	At1g71695	e-38	7.94	0.001	0.003	2.95	0.006	0.016	•	1.33	0.098	0.237	0.96	0.702	0.203		
WS0037_N15	Methylenetetrahydrofolate reductase	At3g59970	e-71	4.59	< 0.001	0.002	2.33	0.002	0.010	•	1.12	0.001	0.116	4.73	< 0.001	0.007		
WS0034_I08	Thioredoxin	At3g51030	e-27	1.90	0.005	0.006	1.64	0.001	0.008	•	1.12	0.268	0.357	6.78	0.005	0.011		
WS0016_L21	Thioredoxin	At3g02730	e-14	0.55	0.037	0.019	0.42	< 0.001	0.003	•	0.82	0.060	0.200	0.26	< 0.001	0.007		
WS0018_G23	Superoxide dismutase	At1g08830	e-64	0.91	0.060	0.028	0.89	0.093	0.078	•	1.04	0.142	0.271	0.25	< 0.001	0.008		
WS0023_I24	Glycolate oxidase	At3g14420	e-66	1.66	0.004	0.005	0.82	0.014	0.023	•	1.04	0.486	0.462	0.28	0.001	0.008		
WS0021_L21	Cytochrome b5	At5g25080	e-31	2.44	0.001	0.003	2.41	< 0.001	0.004	•	0.99	0.991	0.609	6.99	0.003	0.009		
IS0014_M04	Glutathione S-transferase	At1g10360	e-26	3.08	0.004	0.005	4.12	0.002	0.009	•	0.99	0.965	0.604	0.77	0.186	0.075		
WS0041_G15	Oxidoreductase	At4g23340	e-28	11.89	< 0.001	0.002	7.04	0.008	0.018	•	1.04	0.376	0.414	1.41	0.231	0.088		
IS0011_J03	Oxidoreductase	At1g23740	e-41	5.26	< 0.001	0.002	1.15	0.431	0.232	•	0.29	0.001	0.107	0.38	< 0.001	0.008		
WS0015_K19	Cytochrome P450	At1g33730	e-22	1.44	0.006	0.006	7.23	0.018	0.027	•	1.04	0.676	0.529	1.81	0.165	0.069		
IS0012_L15	Cytochrome P450	At4g22690	e-28	34.14	0.002	0.004	33.07	0.001	0.007	•	1.48	0.114	0.251	23.13	0.017	0.017		
Octadecanoid and ethylene signalling																		
IS0014_J20	Lipoxygenase <sup>a</sup>	At1g72520	e-111	4.24	0.005	0.006	4.47	0.009	0.019	•	1.72	0.062	0.202	5.61	0.005	0.011		
WS0023_N05	Allene oxide synthase	At5g42650	e-52	4.12	< 0.001	0.002	2.62	0.002	0.010	•	1.18	0.168	0.292	1.87	0.036	0.025		
WS0039_A09	Allene oxide cyclase <sup>a</sup>	At3g25770	e-62	4.36	< 0.001	0.003	8.30	< 0.001	0.002	•	5.11	0.002	0.123	18.78	0.002	0.009		
WS0035_O19	S-adenosylmethionine synthase	At4g01850	e-64	7.16	0.002	0.004	2.77	0.001	0.009	•	1.14	0.092	0.230	5.42	< 0.001	0.008		
WS00112_P16	ACC oxidase	At1g77330	e-50	6.57	0.001	0.003	7.95	0.002	0.009	•	1.24	0.042	0.178	3.50	< 0.001	0.008		

**Table 2.** Continued

Clone ID	BLASTX versus <i>Arabidopsis</i>	AGI code	E-value	Mechanical at 24 h			Weevil at 48 h			Budworm at 3 h			Budworm at 52 h				
				Fig.3b class	FC	P	Q	Fig.3a class	FC	P	Q	Fig.3a class	FC	P	Q		
Secondary metabolism																	
WS00112_G07	Shikimate kinase	At2g21940	e-54	•	3.17	0.014	0.010	2.53	0.002	0.009	•	1.34	0.013	0.149	5.69	0.006	0.011
WS0038_H08	DAHHP synthase <sup>a</sup>	At1g22410	e-106	•	2.61	0.005	0.006	3.31	0.001	0.008	•	1.37	0.013	0.151	14.16	0.001	0.008
IS0011_E16	Phenylalanine ammonia lyase <sup>a</sup>	At3g53260	e-43	•	43.05	0.001	0.003	12.56	<0.001	0.004	•	1.78	0.057	0.197	5.97	0.002	0.008
WS0031_M22	Cinnamate-4-hydroxylase <sup>a</sup>	At2g30490	e-46	•	12.89	<0.001	0.002	6.43	<0.001	0.004	•	1.43	0.028	0.164	15.49	0.001	0.008
WS0031_C20	4-coumarate-CoA ligase	At3g21240	e-78	•	3.84	0.002	0.004	3.27	<0.001	0.006	•	0.90	0.332	0.393	6.04	0.001	0.008
WS0041_M02	Caffeoyl-CoA O-methyltransferase	At4g34050	e-73	•	3.18	0.001	0.003	2.24	0.001	0.008	•	1.06	0.416	0.431	6.44	0.003	0.010
IS0014_O19	Caffeoyl-CoA O-methyltransferase	At4g34050	e-43	•	0.35	<0.001	0.003	0.38	<0.001	0.006	•	0.68	0.028	0.164	1.94	0.009	0.013
WS0021_F13	Caffeic acid O-methyltransferase	At1g51990	e-50	•	53.42	<0.001	0.002	19.10	<0.001	0.003	•	0.98	0.866	0.576	0.82	0.410	0.136
IS0011_E08	Laccase/diphenol oxidase <sup>a</sup>	At2g30210	e-50	•	63.86	<0.001	0.002	21.02	<0.001	0.002	•	2.77	0.109	0.248	19.09	<0.001	0.006
WS0016_N04	Laccase/diphenol oxidase <sup>a</sup>	At5g05390	e-64	•	55.20	<0.001	0.002	15.61	<0.001	0.005	•	3.28	0.066	0.206	24.87	0.001	0.008
WS0031_H14	Dirigent <sup>a</sup>	At1g65870	e-45	•	0.79	0.014	0.010	0.65	0.021	0.030	•	0.80	0.037	0.175	4.71	0.023	0.020
IS0013_K10	Dirigent <sup>a</sup>	At1g64160	e-48	•	68.19	<0.001	0.002	12.72	<0.001	0.003	•	1.33	0.021	0.155	3.28	0.065	0.036
IS0013_L11	Chalcone synthase <sup>a</sup>	At5g13930	e-174	•	1.99	0.005	0.006	2.71	0.002	0.010	•	1.07	0.441	0.442	2.28	0.017	0.017
WS0011_B22	Chalcone isomerase	At5g05270	e-55	•	2.67	<0.001	0.002	3.67	<0.001	0.005	•	1.13	0.274	0.361	3.59	0.001	0.008
WS00110_P10	Anthocyanin 5-aromatic acyltransferase	At5g23940	e-39	•	3.15	0.010	0.008	2.42	0.001	0.007	•	0.90	0.197	0.312	3.37	0.001	0.008
WS0022_G09	Flavonoid 3-hydroxylase	At5g07990	e-45	•	1.74	<0.001	0.002	2.21	0.004	0.013	•	0.92	0.106	0.245	4.69	0.001	0.008
WS0014_M03	Leucoanthocyanidin dioxygenase	At4g22880	e-88	•	1.46	0.006	0.006	1.69	0.003	0.012	•	0.91	0.089	0.226	12.72	<0.001	0.006
WS0016_K11	Dihydroflavonol 4-reductase <sup>a</sup>	At5g42800	e-67	•	3.35	0.001	0.003	2.46	<0.001	0.004	•	1.14	0.332	0.393	2.62	<0.001	0.007
WS00111_A06	Geranylgeranyl diphosphate synthase <sup>b</sup>	At4g36810	e-57	•	3.45	<0.001	0.003	1.62	0.004	0.014	•	1.12	0.312	0.384	13.07	0.001	0.008
WS00111_K11	Geranylgeranyl diphosphate synthase	At4g36810	e-56	•	0.45	0.004	0.005	0.46	<0.001	0.006	•	0.80	0.001	0.116	0.66	0.120	0.055
WS0017_E16	Terpene synthase <sup>b</sup>	At3g14490	e-06	•	16.76	<0.001	0.002	4.75	<0.001	0.003	•	3.13	0.021	0.155	8.83	0.004	0.010
WS00111_H11	Terpene synthase	At1g48800	e-07	•	12.69	<0.001	0.002	2.54	<0.001	0.004	•	1.88	0.006	0.134	1.66	0.031	0.023

A complete list of array elements is given in Supplementary Table S2.

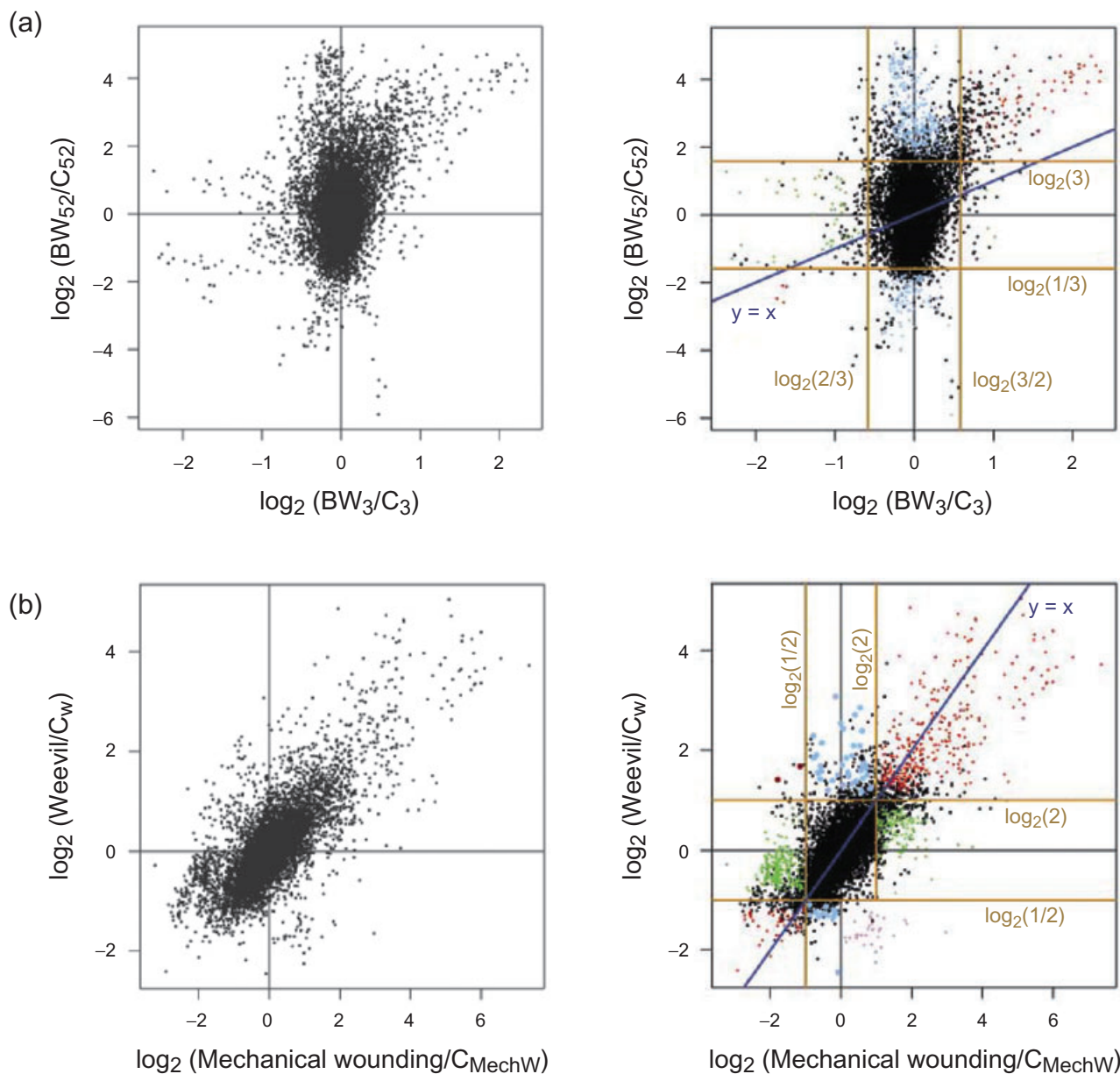
Colour scale from dark green to dark red correlates with fold-change expression.

<sup>a</sup>Expression determined by real-time PCR (Fig. 7 and Table 3).

ABC, ATP-Binding Cassette; AGI, Arabidopsis Gene Index; FC, fold-change; P, P-value; Q, q-value; GAP3DH, glyceraldehyde-3-phosphate dehydrogenase; VLCFA, very long chain fatty acid; bZIP, basic-leucine zipper; ACC, 1-aminocyclopropane-1-carboxylate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate.

Fold-change at least





**Figure 3.** Relative changes in gene expression (a) in green shoot tips 3 h and 52 h after the onset of budworm (BW) feeding and (b) in bark 24 h after mechanical wounding and 48 h after the onset of weevil feeding. Expression ratios are presented as  $\log_2$  fold-change between treated and control (C) plants (see Materials and Methods). The left and right scatter plots are identical, except that genes identified as differentially expressed (DE) between treatments are shaded in colour in the right-hand panels. In panel (a), red dots represent genes that are DE at both 3 h and 52 h; green dots represent genes DE after 3 h, but not after 52 h; and blue dots represent genes DE after 52 h, but not after 3 h. In panel (b), red dots represent genes DE after both mechanical wounding and weevil feeding; green dots represent genes DE after mechanical wounding, but not weevil feeding; blue dots represent genes DE after weevil feeding, but not mechanical wounding; and magenta dots represent a group of genes that are down-regulated by weevil feeding, and are contained within a distinct cloud of data points. The same colour coding is used in Table 2 to indicate the association of individual genes with expression groups identified here. Subplane divisions were selected to capture the upper ~15% of genes that are most DE relative to control trees for each treatment. In both panels, black dots on the right-hand scatter plot represent genes that showed no DE or whose unadjusted 95% confidence intervals were not completely contained within the subplane.

elements DE only at 3 h and 387 elements DE only at 52 h. To compare responses in bark to weevil feeding and mechanical wounding, we applied a twofold-change criteria and observed 339 array elements DE in response to either treatment, 361 elements DE only after wounding and 55 elements DE only after weevil feeding.

### Functional classification of insect- and wound-induced transcripts in Sitka spruce

Functional annotation of conifer genes is constrained by the often limited sequence similarity of genes from gymnosperms and angiosperms, and that the majority of annotated plant genes in public databases are from angiosperms. Nevertheless, *Arabidopsis* gene annotation databases (e.g. TAIR; <http://www.arabidopsis.org>) provide a useful common reference system for initial functional classification. Biotic stress- and wound-induced genes identified in our microarray analysis were classified into 10 functional groups based on annotation to *Arabidopsis*, and the most prominent genes are shown in Table 2. Viewed broadly, they include genes involved in general metabolism, photosynthesis, transport, transcriptional regulation, response to stress, detoxification and redox processes, octadecanoid and ethylene signalling, secondary metabolism, as well as cDNAs with no significant match to *Arabidopsis* and genes of uncharacterized biological processes. In the following sections we will briefly highlight some of the findings of insect-induced gene responses for each of the 10 functional categories. A specific emphasis will be on defence gene systems of special relevance for conifers, particularly signalling and responses in the terpenoid and phenolic secondary metabolite pathways. We will also briefly compare the transcriptome response to insect herbivory in this gymnosperm tree system with a very similar recent microarray analysis of plant-insect responses in an angiosperm tree species, poplar (*Populus deltoides* × *trichocarpa*), responding to foliage feeding forest tent caterpillars (Ralph *et al.* 2006a).

### Genes of uncharacterized biological function affected by insect herbivory or wounding

Examples of microarray cDNA elements with no similarity to *Arabidopsis* that represent insect- or wound-responsive genes include IS0011\_A15, WS0044\_F23, WS0034\_E23 and WS0044\_G09 (Table 2). These ESTs have high similarity to other spruce ESTs in our sequence database, as well as to gymnosperm EST sequences in the public domain, confirming that they represent true conifer genes. In addition, several spruce ESTs with similarity to *Arabidopsis* genes are annotated as uncharacterized biological function. Examples include a photoassimilate-responsive protein (WS0014\_J09) selectively induced after budworm feeding, a lateral organ boundaries (LOB) protein (IS0014\_J19) induced after weevil feeding, and a phosphate-responsive protein (WS0015\_F13) and a lipase containing the GDSDL amino acid motif (WS0016\_C06), both strongly induced by

budworm feeding (Table 2). Spruce EST WS0014\_J09 is weakly similar (TBLASTX;  $E\ 1e^{-07}$ ) to the PAR-1a and PAR-1b transcripts from tobacco that are induced in response to high soluble sugar levels in leaves, salicylate and after infection with potato virus Y (Herbers *et al.* 1995). LOB proteins are expressed in the boundary found between lateral organs and shoot apical meristems and are presumably involved in plant development (Shuai, Reynaga-Pena & Springer 2002). Similarly, spruce ESTs of uncharacterized function down-regulated in bark tissue in response to mechanical wounding or weevil feeding included plantacyanin (WS0018\_A07), belonging to the phytoeyanin family of blue copper proteins with a proposed role in reproduction (Dong, Kim & Lord 2005), and auxin-regulated (WS0038\_N22) and low temperature and salt responsive (WS0017\_F02) proteins.

### Genes of general metabolism affected by insect herbivory or wounding

We identified several genes involved in general metabolism that were consistently induced by wounding and both insect treatments including carbonic anhydrase (WS00110\_A15), an enoyl-CoA hydratase (WS00113\_F20), and an acid phosphatase (IS0011\_B10) (Table 2). During photosynthesis in  $C_4$  plants, carbonic anhydrase is involved in converting  $CO_2$  into bicarbonate for fixation by the primary carboxylating enzyme phosphoenolpyruvate carboxylase. A carbonic anhydrase in tobacco was shown to bind salicylic acid in chloroplasts, have antioxidant activity and function in the hypersensitive response in plant disease resistance (Slaymaker *et al.* 2002). Enoyl-CoA hydratase is potentially involved in a broad array of  $\beta$ -oxidation processes to degrade fatty acids.

Other array elements for genes involved in general metabolism were induced only in shoot tips after budworm feeding including an xyloglucan endo-1,4- $\beta$ -D-glucanase (WS0038\_G12), which catalyses the endo-hydrolysis of  $\beta$ -1,4-glucan linkages in cellulose and xyloglucan in plant cell walls; an iron-sulphur complex protein (WS0013\_D08), which are found in both mitochondria and plastids of plants and are essential for respiration and photosynthesis (Léon *et al.* 2003); a very long chain fatty acid (VLCFA)-condensing enzyme (WS00113\_A19), which controls the rate of VLCFA biosynthesis important for waxy cuticle formation (Millar *et al.* 1999); and a calcium-binding EF hand protein (WS0016\_L07), which have previously been demonstrated to be induced after insect feeding in leaves of *Arabidopsis* (Stotz *et al.* 2000), and are likely to have a regulatory role in the plant response rather than direct defensive function because members of this superfamily are involved in a wide variety of calcium-regulated cellular processes (Day *et al.* 2002).

There were also a large number of genes involved in energy and other primary metabolisms strongly down-regulated after budworm feeding that were generally less responsive to weevil feeding or wounding in bark (Table 2). These include two steps in glycolysis, glyceraldehyde-3-

phosphate dehydrogenase (WS00110\_E02) and fructose-1,6-biphosphate aldolase (WS0018\_O18); two steps in the Calvin cycle, fructose-1,6-biphosphatase (WS0019\_N05) and transketolase (WS0041\_E18); galactinol synthase (WS0013\_N23), responsible for raffinose family oligosaccharide production; hydroxypyruvate reductase (WS0015\_L24) of the photorespiratory carbon oxidation cycle; glutamine synthase (WS0015\_E01), which combines ammonium with glutamate to form glutamine during nitrogen fixation; and cellulose synthase (WS0039\_G18), which transfers a glucose residue to glucan chains in the cellulose microfibrils of cell walls. These differences between the response in induced bark and shoot tips could be a result of higher constitutive gene expression, combined with insect-induced redirection of energy and primary metabolisms in green shoot tips and young needles, which may be less important in the bark tissues.

### Photosynthesis genes affected by insect herbivory or wounding

In general, many genes associated with photosynthesis were down-regulated by budworm feeding in shoot tips as well as by wounding and weevil feeding in bark. Examples include photosystem I and II proteins (WS00113\_A22 and WS00111\_J06, respectively), chlorophyll *a-b* binding proteins (WS0022\_L16) and ferredoxin (WS0024\_M05) (Table 2). The inverse correlation between photosynthesis and defence-related gene regulation has also been observed in other large-scale studies of the response to insect herbivory in angiosperms (Hermsmeier, Schittko & Baldwin 2001; Zhu-Salzman *et al.* 2004; Ralph *et al.* 2006a). This response represents an example of redirection of gene expression to defence responses with reduced resource commitment to primary functions, and could be translated into induced reallocation of plant metabolism from primary processes to defence.

### Transport genes affected by insect herbivory or wounding

Plant defences against insects are likely to involve regulation of transport processes both for local and systemic defence signalling, as well as for the biosynthesis and local accumulation of defence chemicals. Among spruce genes associated with transport functions, we identified an oligopeptide transporter (WS0042\_C02) strongly induced in bark after wounding or weevil feeding (Table 2), which presumably translocates small peptides across cellular membranes in an energy dependent manner. The physiological role of peptide transport is still undefined; possible substrates for these transporters include glutathione,  $\gamma$ -glutamyl peptides, hormone-amino acid conjugates, peptide phytotoxins, and oligopeptides such as systemin with apparent signalling function in the induction of a systemic defence response to wounding by herbivores (McGurl *et al.* 1992; Stacey *et al.* 2002).

Likewise, an ATP-Binding Cassette (ABC) protein (IS0011\_I18) was also induced by both treatments in bark. In addition to their traditional role in detoxification processes in animal cells, ABC proteins in plants have been demonstrated to participate in chlorophyll biosynthesis, formation of Fe/S clusters, stomatal movement and ion fluxes (Martinoia *et al.* 2002). ABC proteins may also be directly involved in plant defence via transport of signalling molecules such as jasmonate (Theodoulou *et al.* 2005), transport of phytochemicals as has been shown for alkaloid (Shitan *et al.* 2003) and terpenoid (Jasiński *et al.* 2001) defence compounds, or reinforcement of cuticular waxes (Pighin *et al.* 2004). A large number of putative transport proteins, including several ABC proteins, were also induced in poplar in response to feeding by defoliating forest tent caterpillars (Ralph *et al.* 2006a), further supporting the importance of transport mechanisms in plant defence against insects.

One of the more abundant classes of DE genes involved in transport/general metabolism is the lipid transfer proteins (LTP). We observed some LTP genes to be induced by all three treatments, other LTPs were repressed by all three treatments, and others were selectively induced by budworm feeding only. LTPs are small, basic proteins synthesized as precursors that transfer phospholipids between membranes, bind fatty acids *in vitro*, and have been proposed to be associated with plant-insect interactions by contributing to cutin biosynthesis (Kader 1996), pathogen-defence reactions (Garcia-Olmedo *et al.* 1995), and the recognition of intruders in plants and in systemic resistance signalling (Blein *et al.* 2002; Maldonado *et al.* 2002). The interpretation of function is complicated by the fact that LTPs are represented by a large number of genes with several subfamilies, and thus far no systematic characterization of LTPs has been performed in any plant species.

### Transcriptional regulation affected by insect herbivory or wounding

Transcriptional regulation and intracellular signalling cascades for plant defence, including induced secondary metabolism, are poorly understood. Microarray expression profiling could be a useful approach to identify signalling and regulatory events in the induction of plant defence against insects as previously shown for *Arabidopsis* and poplar (De Vos *et al.* 2005; Ralph *et al.* 2006a). Weevil feeding and wounding in bark activated genes from the basic helix-loop-helix (bHLH; IS0013\_F06) and bZIP (WS0044\_J05) transcription factor families, and budworm feeding activated a member of the MADS-box transcription factor family (WS0042\_F06) (Table 2). Each of these transcription factor classes is represented by large gene families in *Arabidopsis* (i.e. bHLH, 139 genes; bZIP, 81 genes; MADS-box, 82 genes; Riechmann *et al.* 2000). To date most transcription factors linked to plant stress responses have been derived from the AP2/EREBP, WRKY, MYB and bZIP families (Stracke, Werber & Weisshaar 2001; Singh,

Foley & Oñate-Sánchez 2002). The fact that we identified only a small number of DE transcription factors in the present study likely reflects their paucity of representation on the microarray. To address this issue in future work, we have recently constructed a second, larger spruce cDNA microarray containing a larger number of transcription factors identified in normalized spruce cDNA libraries (S. Ralph & J. Bohlmann, unpublished data).

### General stress response genes affected by insect herbivory or wounding

Among genes commonly associated with plant responses to biotic stress, we observed several spruce lectin proteins to be selectively induced in bark after wounding (e.g. IS0012\_N03), and  $\beta$ -1,3-glucanase genes to be induced in either bark (WS0017\_B07) or shoot tips (WS0021\_I16) (Table 2). Lectins are carbohydrate-binding proteins, many of which have insecticidal activity (Peumans & Van Damme 1995).  $\beta$ -1,3-glucanases are pathogenesis-related (PR) proteins that are rapidly induced during fungal invasion and are proposed to contribute to plant defence by digesting cell wall components of the fungal pathogen. In white spruce,  $\beta$ -1,3-glucanase genes have previously been shown to be inducible in response to wounding, drying and flooding stresses, along with treatment with *Leptosphaeria maculans* fungal extract (Dong & Dunstan 1997). Chitinases were also induced in response to insect feeding and wounding in spruce bark (e.g. IS0013\_J03). Chitinases represent a large and diverse group of enzymes that catalyse the cleavage of internal  $\beta$ -1,4-glycoside bonds present in the biopolymers of *N*-acetylglucosamine found in chitin, a major component of fungal, bacterial and insect cell walls. Plant chitinases are classified as PR proteins and have been demonstrated in many plant systems, including spruce (Dong & Dunstan 1997; Hietala *et al.* 2004), to be transcriptionally induced in response to both biotic (e.g. viruses, bacteria, fungi, insect pests) and abiotic (e.g. drought, salinity, wounding, plant hormones) stress (Kasprzewska 2003). There is also emerging evidence that chitinase-like proteins may play a role in normal plant growth and development, as demonstrated by analysis of the *AtCTL1* chitinase-like gene responsible for the *elp1* mutation in *Arabidopsis* (Zhong *et al.* 2002). Several late embryogenesis abundant (LEA) genes, also known as dehydrins (e.g. IS0013\_A20) were also strongly induced by insects and wounding in Sitka spruce shoot tips and bark. LEA proteins are also induced in plant responses to other environmental stresses such as drought, salinity and freezing, and are found in high abundance during the late stages of embryogenesis; however, their physiological function is unclear (Wise & Tunnacliffe 2004). A LEA/dehydrin transcript from white spruce has previously been demonstrated to be induced in response to wounding, MeJA, drought, cold and abscisic acid (Richard *et al.* 2000). Glucanases, chitinases, lectins and dehydrins were also commonly induced in poplar leaves after feeding by forest tent caterpillars (Ralph *et al.* 2006a); however, the large number of strongly induced protease inhibitor class

transcripts observed in poplar were generally absent in spruce tissues, suggesting this class of proteinaceous defences may not be utilized in spruce.

### Detoxification and redox processes affected by insect herbivory or wounding

Tissue damage imposed by herbivore feeding is known to cause oxidative stress. Insect herbivory can expose plant cells to potentially toxic or otherwise biologically active metabolites, such as components of insect saliva or host secondary metabolites that are normally restricted to specialized cells or subcellular compartments. It therefore is not surprising that several genes potentially involved in protection of cells from oxidative stress or involved directly in detoxification were induced by one or more insect or wound treatments. These genes include, for example, lactoylglutathione lyase (WS0017\_M23), also known as glyoxalase I, peroxidases (e.g. WS00110\_M24), thioredoxin (e.g. WS0034\_I08), cytochrome b5 (WS0021\_L21), oxidoreductases (e.g. WS0041\_G15) and cytochrome P450s (e.g. IS0012\_L15) (Table 2). Lactoylglutathione lyase catalyses the first step in the glyoxalase system for the glutathione-based detoxification of methylglyoxal, which is formed primarily as a by-product of carbohydrate and lipid metabolism. In addition, this enzyme was demonstrated to be induced in response to drought and cold stresses in *Arabidopsis* (Seki *et al.* 2001). Peroxidase enzymes are associated with the oxidation of phenolic compounds in cell walls and the polymerization of lignin and suberin, and in spruce have been demonstrated to be induced in response to infection by the pathogen *Pythium dimorphum* (Fossdal *et al.* 2001). Not surprisingly, many of the same detoxification genes induced in spruce after insect feeding or wounding were also observed in poplar leaves after forest tent caterpillar feeding (Ralph *et al.* 2006a), suggesting there is a common set of genes involved in the oxidative stress response.

### Octadecanoid and ethylene pathway genes affected by insect herbivory or wounding

Plant responses to biotic and abiotic stresses are regulated locally and systemically by a complex network of signalling cascades including peptide signals (e.g. systemin), salicylic acid, ethylene, H<sub>2</sub>O<sub>2</sub>, and fatty acid-derived oxylipins such as the octadecanoid-derived jasmonic acid (JA) and MeJA (Howe 2004; Halitschke & Baldwin 2005). Earlier work has established that MeJA, mechanical wounding and insects can trigger similar traumatic resin defence responses in conifers (Martin *et al.* 2002, 2003; Hudgins *et al.* 2003; Miller *et al.* 2005). In addition, Miller *et al.* (2005) have shown up-regulation of putative allene oxide synthase (AOS) and allene oxide cyclase (AOC) transcripts in spruce after weevil feeding and MeJA application. Furthermore, Hudgins & Franceschi (2004) showed that MeJA-induced development of traumatic resin ducts is induced via ethylene signalling. We therefore evaluated representation of genes of the octadecanoid and ethylene pathways

on the spruce 9.7K cDNA microarray via similarity searches against known genes from *Arabidopsis* (Turner, Ellis & Devoto 2002; Stenzel *et al.* 2003) using a combination of BLASTX and TBLASTN searches of the TAIR *Arabidopsis* peptide set with a stringent threshold ( $E < 1e^{-20}$ ) (Table 2 and Supplementary Table S3). The formation of MeJA from membrane lipids in the octadecanoid pathway involves seven enzymatic steps [i.e. phospholipase, lipoxygenase (LOX), AOS, AOC, 12-oxo-phytodienoic acid reductase,  $\beta$ -oxidation enzymes and JA methyl transferase (Howe 2004)], of which six are represented on the spruce 9.7K microarray by 14 ESTs (Fig. 4; Supplementary Table S3). The first three steps in this pathway can also lead to 6-carbon alcohols, which require two additional enzymes (hydroperoxide lyase and alcohol dehydrogenase, the latter represented by two candidate ESTs on the array). Within the octadecanoid pathway several ESTs representing genes encoding proteins with high similarity to LOX, AOS and AOC (e.g. LOX, IS0014\_J20; AOS, WS0023\_N05; AOC, WS0039\_A09; Table 2) were up-regulated with both weevil and budworm feeding and with mechanical wounding treatments (Fig. 4 and Table 2). The induction of LOX, AOS and AOC transcripts in Sitka spruce in response to insect feeding and wounding, in different tissues, supports that these enzymes, and jasmonates in general, are important in activating and/or modulating the spruce defence response.

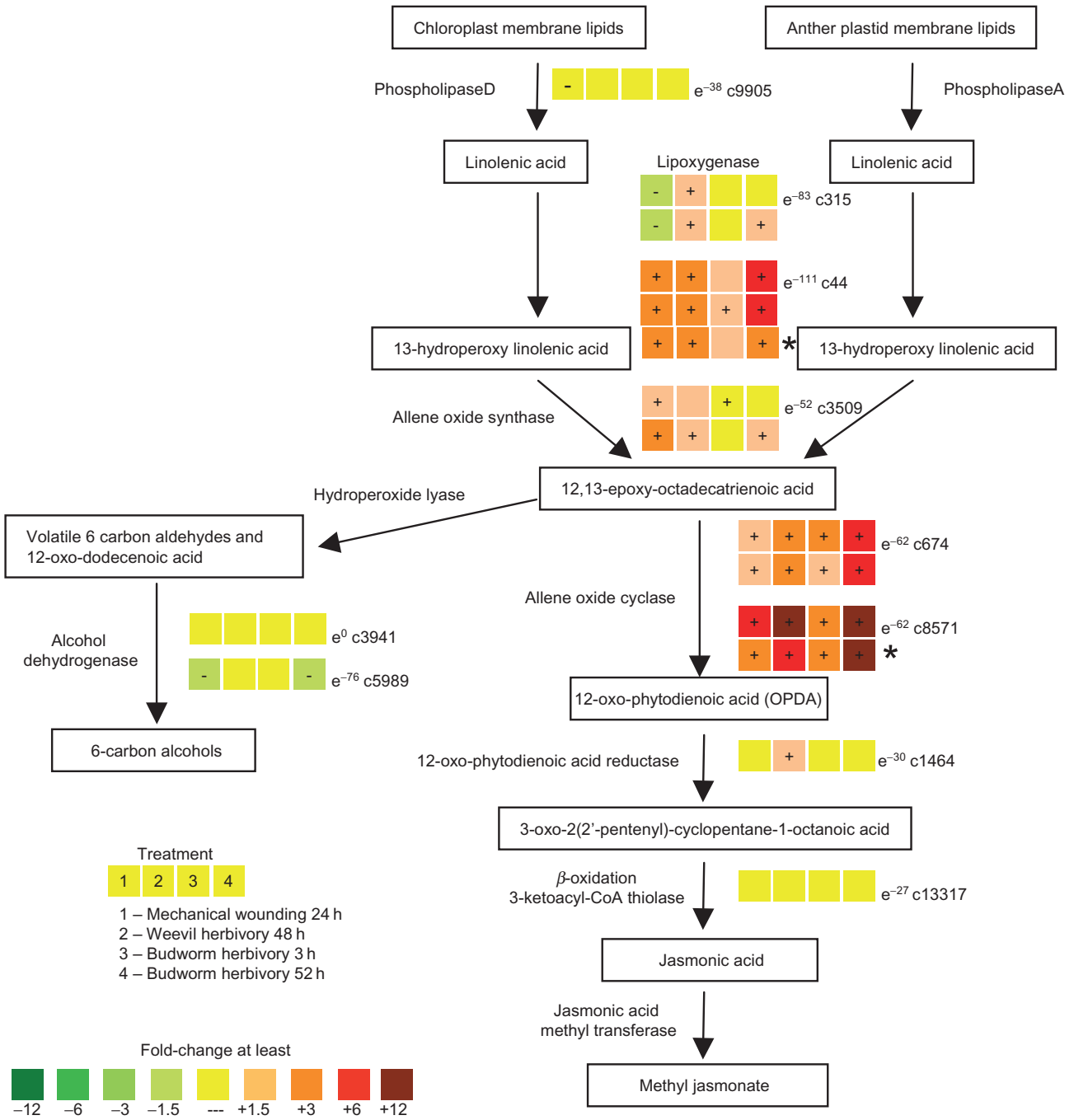
Two genes potentially involved in ethylene biosynthesis were also induced by both insect species and wounding in bark and shoot tissues, S-adenosylmethionine synthase (SAM synthase, WS0035\_O19) and 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase, WS00112\_P16) (Table 2). Ethylene is an important modulator in defence signal transduction (Feys & Parker 2000) that has been demonstrated to be induced in response to insect herbivory in several plant systems (Arimura *et al.* 2000; Winz & Baldwin 2001), as well as wounding in conifers (Hudgins *et al.* 2006), and has been demonstrated to regulate defence-oriented genes such as protease inhibitors (O'Donnell *et al.* 1996), defensin (Penninckx *et al.* 1998) and PR proteins (Díaz, ten Have & van Kan 2002). Building on the findings of Hudgins & Franceschi (2004), the present EST and microarray analysis of spruce candidate genes for ethylene and octadecanoid formation will allow a more detailed analysis of spatial and temporal signalling activities in spruce defence against insects.

### Terpenoid secondary metabolism genes affected by insect herbivory or wounding

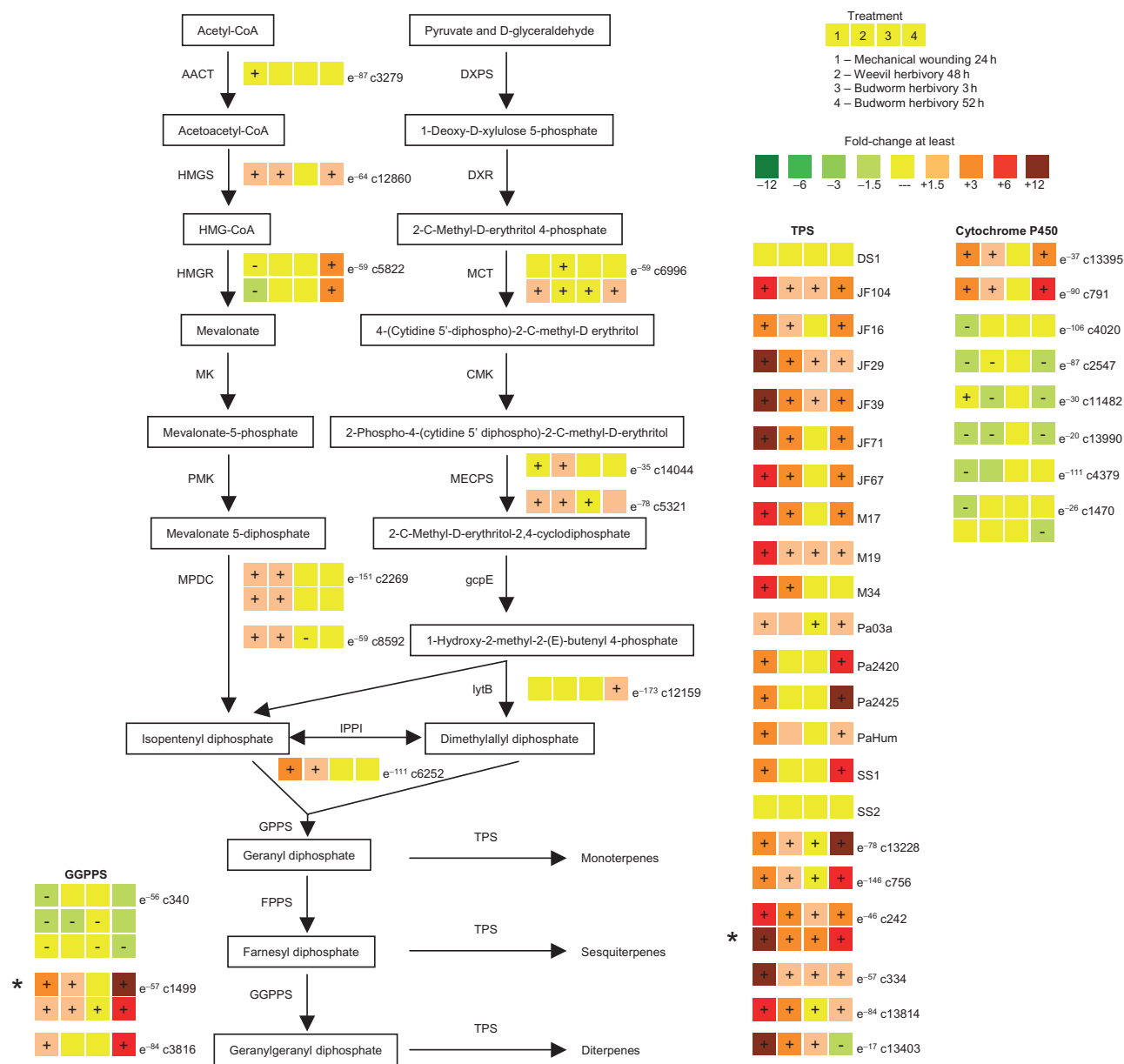
Terpenoids are formed in large volumes in conifer oleoresin and are perhaps the most prominent constitutive and inducible chemical defences (for recent reviews, Bohlmann & Croteau 1999; Phillips & Croteau 1999; Trapp & Croteau 2001; Huber *et al.* 2004; Martin & Bohlmann 2005). The pathways for isoprenoid biosynthesis lead to the production of hundreds of terpenoid compounds that are the major components of oleoresin defences and volatile emissions in conifers (Fig. 5). The formation of terpenoids

involves two pathways for the formation of isoprenoids, the mevalonic acid (MEV) and the methylerythritol phosphate (MEP) pathways, followed by a series of condensation reactions catalysed by prenyl transferases, namely geranyl diphosphate (GPP) synthase, farnesyl diphosphate (FPP) synthase and geranylgeranyl (GGPP) diphosphate synthase (Lange & Ghassemian 2003). GPP, FPP and GGPP are converted by TPS genes to the many basic mono-, sesqui- and diterpenoid structures found in conifers (Martin *et al.* 2004). Additional modification and structural diversification of terpenoids involves cytochrome P450 dependent monooxygenases, such as in the formation of diterpene resin acids (Ro *et al.* 2005).

Induced terpenoid resin defences and induced volatile emissions in spruce have been well characterized at the anatomical, biochemical and molecular levels for the role of a family of TPS (Martin *et al.* 2002, 2003, 2004; Byun-McKay *et al.* 2003, 2006; Fäldt *et al.* 2003; Miller *et al.* 2005), while the majority of other enzymes in these pathways have not yet been adequately investigated in insect-induced terpenoid defence responses in conifers. To address this issue, we evaluated representation of genes of the isoprenoid pathway on the spruce 9.7K cDNA microarray (Fig. 5; Supplementary Table S3) via similarity searches against known genes in *Arabidopsis* and conifers beginning from the first step of the MEP pathway (i.e. 1-deoxy-D-xylulose 5-phosphate synthase) to candidates for the final cytochrome P450 modification of terpenoid products (Lange & Ghassemian 2003; Martin *et al.* 2004; Ro *et al.* 2005) using a combination of BLASTX and TBLASTN searches of the TAIR *Arabidopsis* peptide set and the nonredundant (nr) division of GenBank with a stringent threshold ( $E < 1e^{-20}$ ). Among the 13 early steps of isoprenoid biosynthesis leading to the production of isopentenyl diphosphate or dimethylallyl diphosphate via the MEV pathway in the cytosol and endoplasmic reticulum or the MEP pathway in plastids, seven enzymatic steps are represented by 12 ESTs on the 9.7K array (Fig. 5; Supplementary Table S3). Of these, up-regulation was observed for 3-hydroxy-3-methylglutaryl-CoA synthase, mevalonate diphosphate decarboxylase and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase by weevil feeding and mechanical wounding in bark tissues, as well as for 3-hydroxy-3-methylglutaryl-CoA synthase and reductase, and the *lytB* ortholog by budworm feeding (Fig. 5). Prior to the action of the prenyl transferases there is up-regulation of an EST representing isopentenyl diphosphate isomerase by weevil feeding and mechanical wounding in bark (Fig. 5). Among the six putative GGPP synthase ESTs represented on the array (no ESTs with similarity to FPP or GPP synthases were identified), ESTs from two distinct contigs (1499 and 3816) were strongly up-regulated by both insect species and by mechanical wounding, suggesting some differential insect-induced expression for members of this GGPP synthase gene family (Fig. 5; also see GGPP synthase WS00111\_A06 and WS00111\_K11 in Table 2). A large number of known, as well as some putatively new TPS genes (23 ESTs) are spotted on the spruce 9.7K array and the vast majority are induced, often



**Figure 4.** Metabolic pathway scheme for octadecanoid biosynthesis. Arrows represent enzymatic reactions and boxes represent metabolic products. Each row of four coloured boxes adjacent to arrows represents an expressed sequence tag (EST) on the spruce 9.7K array, with individual boxes corresponding to the relative fold-change between a treatment and control for each type of treatment from left to right: mechanical wounding of bark 24 h, weevil feeding on bark 48 h, budworm feeding of green shoot tips 3 h, budworm feeding of green shoot tips 52 h. The colour scale indicates fold-change differences in gene expression between treated and control samples. Contig (c) numbers derived from a CAP3 sequence assembly of c. 80 000 3'-end spruce ESTs are provided adjacent to colour bars, along with expect values that represent BLASTX scores to The Arabidopsis Information Resource (TAIR) *Arabidopsis thaliana* peptide set. The '+' or '-' signs within boxes indicate statistical significance ( $P$ -value < 0.05) of up- or down-regulation, respectively. ESTs marked with an asterisk were also examined by real-time PCR (Fig. 7 and Table 3). A complete list of spruce ESTs with ID numbers listed in the same order (i.e. grouped by enzyme, top to bottom) as they appear in the figure is provided in Supplementary Table S3.



**Figure 5.** Metabolic pathway scheme for terpenoid biosynthesis. Arrows represent enzymatic reactions and boxes represent metabolic products. Each row of four coloured boxes adjacent to arrows represents an expressed sequence tag (EST) on the spruce 9.7K array, with individual boxes corresponding to the relative fold-change between a treatment and control for each type of treatment from left to right: mechanical wounding of bark 24 h, weevil feeding on bark 48 h, budworm feeding of green shoot tips 3 h, budworm feeding of green shoot tips 52 h. The colour scale indicates fold-change differences in gene expression between treated and control samples. Contig (c) numbers derived from a CAP3 sequence assembly of c. 80 000 3'-end spruce ESTs are provided adjacent to colour bars, along with expect values that represent BLASTX scores to The Arabidopsis Information Resource (TAIR) *Arabidopsis thaliana* peptide set or published conifer protein sequences [terpene synthases (TPS) and cytochrome P450s]. The '+' or '-' signs within boxes indicate statistical significance ( $P$ -value < 0.05) of up- or down-regulation, respectively. ESTs marked with an asterisk were also examined by real-time PCR (Fig. 7 and Table 3). A complete list of spruce ESTs with ID numbers listed in the same order (i.e. grouped by enzyme, top to bottom) as they appear in the figure is provided in Supplementary Table S3. AACT, acetyl-CoA acyl transferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MPDC, mevalonate diphosphate decarboxylase; DXPS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; MECPS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; gcpE, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; lytB, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IPPI, isopentenyl diphosphate: dimethylallyl diphosphate isomerase; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase.

strongly, in response to each type of insect feeding or wound treatment (Fig. 5, also see selected TPS in Table 2). Although subtle differences in expression among TPS genes is apparent, the high nucleotide similarity among spruce TPS family members (Martin *et al.* 2004) limits the ability to distinguish between gene-specific expression and cross-hybridization between closely related family members. Finally, among nine ESTs with similarity to four cytochrome P450 genes from loblolly pine, one of which catalyses a series of consecutive oxidation steps with different diterpenol and diterpenal intermediates (Ro *et al.* 2005), two ESTs were induced by weevil feeding or wounding in bark, as well as budworm feeding in leaders after 52 h. This first comprehensive microarray expression analysis of insect-induced conifer terpenoid pathway genes identified new targets for characterization both in the early and intermediate steps of the pathway, as well as new candidates for functional analyses of cytochrome P450 enzymes in terpenoid defences.

### Phenolic secondary metabolism genes affected by insect herbivory or wounding

In addition to terpenoids, phenolic secondary metabolites have been proposed to play a role in conifer defence against pathogens and potentially insects as well (Brignolas *et al.* 1995; Huber *et al.* 2004; Franceschi *et al.* 2005; Ralph *et al.* 2006b). However, much less is known about the molecular regulation of this pathway in conifer insect defence compared to the formation of terpenoids. Phenylpropanoid metabolism builds on the shikimate pathway, which links the metabolism of carbohydrates to the biosynthesis of aromatic amino acid precursors, phenylalanine and tyrosine, in the formation of phenolic compounds. In a series of seven metabolic steps, phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) are converted to chorismate, which is the precursor of the aromatic amino acids tryptophan, phenylalanine and tyrosine (Herrmann & Weaver 1999). Spruce genes representing two enzymatic steps within the shikimate pathway were induced by wounding and both types of insect feeding in bark and green shoot tips: 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (WS0038\_H08), the first step involving condensation of PEP and E4P; and shikimate kinase (WS00112\_G07), which is the fifth step of the pathway that catalyses the phosphorylation of shikimate to yield shikimate-3-phosphate (Table 2).

Following the formation of aromatic amino acids in the shikimate pathway, most secondary phenolic compounds, such as flavonoids, stilbenoids, condensed tannins and other polyphenolics along with the structural polymer lignin, are subsequently derived from phenylalanine via the action of a complex metabolic grid of enzyme activities (Dixon *et al.* 2001). We evaluated representation of the core phenylpropanoid pathway and select steps of branch pathways on the spruce 9.7K cDNA microarray (Fig. 6; Supplementary Table S3) via similarity searches against known genes from *Arabidopsis* (Costa *et al.* 2003; Hoffmann *et al.* 2003; Raes

*et al.* 2003) and conifers (Kodan, Kuroda & Sakai 2002; Ralph *et al.* 2006b) using a combination of BLASTX and TBLASTN searches of the TAIR *Arabidopsis* peptide set and the nr division of GenBank with a stringent threshold ( $E < 1e^{-20}$ ). There are presently 10 known enzymes for the monolignol biosynthesis pathway [i.e. phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), hydroxycinnamoyl CoA shikimate/quinic acid hydroxycinnamoyltransferase (HCT), *p*-coumarate-3-hydroxylase (C3H), ferulate-5-hydroxylase (F5H), 4-coumarate CoA ligase (4CL), caffeoyl-CoA O-methyltransferase (CCoAOMT), caffeic acid O-methyltransferase (COMT), cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD)], most of which are represented by small gene families in *Arabidopsis* that collectively encode 18 genes for which there is evidence of involvement in phenylpropanoid and lignin biosynthesis (Raes *et al.* 2003; Ehrling *et al.* 2005). In addition, as many as 45 additional genes annotated as related to phenylpropanoid genes encode enzymes of unknown specific function (Costa *et al.* 2003; Raes *et al.* 2003). On the spruce 9.7K cDNA microarray we identified 81 ESTs representing eight of the 10 enzymes (there were no representative ESTs for F5H or HCT).

At the entry point to phenylpropanoid metabolism the enzymes PAL and C4H are represented by 10 and seven ESTs, respectively, all of which showed up-regulation by at least one form of insect feeding or wounding (Fig. 6, e.g. PAL, IS0011\_E16; and C4H, WS0031\_M22 in Table 2). Among other genes from subsequent steps along the phenylpropanoid pathway, array elements for C3H, 4CL, COMT and CCoAOMT all demonstrate up-regulation for at least some members of each gene family (Fig. 6, e.g. 4CL, WS0031\_C20; COMT, WS0021\_F13; and CCoAOMT, WS0041\_M02 in Table 2). However, there are also notable differences among gene family members for several enzymatic steps such as among the CCoAOMT genes where ESTs representing contigs 7294 and 494 demonstrate consistent up-regulation in response to insect feeding and mechanical wounding (Fig. 6), whereas ESTs representing CCoAOMT contig 489 are down-regulated in the same bark tissues, and ESTs representing CCoAOMT contigs 8234 and 30 are only DE after budworm feeding for 52 h. In each case, the expression data among multiple ESTs representing each gene is remarkably consistent. Further annotation of spruce phenylpropanoid gene families encoding enzymes such as 4CL, COMT and CCoAOMT using FLcDNA sequences and comparative genomics will be required to determine if all such genes on the array encode *bona fide* phenylpropanoid enzymes (S. Ralph & J. Bohlmann, unpublished data). For example, of the seven *Arabidopsis* genes annotated as encoding enzymes related to CCoAOMT, only one has an expression pattern consistent with a function in monolignol biosynthesis (Ehrling *et al.* 2005). Thus, the spruce CCoAOMT ESTs with divergent expression patterns could represent CCoAOMT-like genes with functions distinct from true CCoAOMTs. Studies using gene-specific



primers and real-time PCR will also be required to dissect complex microarray profiles in gene families such as CCoAOMT, in which individual gene family members may be differentially regulated. Two other phenylpropanoid enzymes represented on the array, CCR and CAD, show limited differential expression except for ESTs representing CAD contig 1113 (Fig. 6), suggesting that carbon flux into monolignols themselves may not be a primary response to mechanical wounding and herbivory.

We also observed several other genes for enzymes of monolignol biosynthesis and other branches of phenylpropanoid metabolism to be induced after insect feeding or wounding, including laccases/diphenol oxidases (e.g. WS0016\_N04), DIR proteins (e.g. IS0013\_K10), and genes of flavonoid metabolism (e.g. chalcone synthase, IS0013\_L11; chalcone isomerase, WS0011\_B22; anthocyanin 5-aromatic acyltransferase, WS00110\_P10; flavonoid 3-hydroxylase, WS0022\_G09; leucoanthocyanidin dioxygenase, WS0014\_M03; and dihydroflavonol 4-reductase, WS0016\_K11) (Fig. 6 and Table 2).

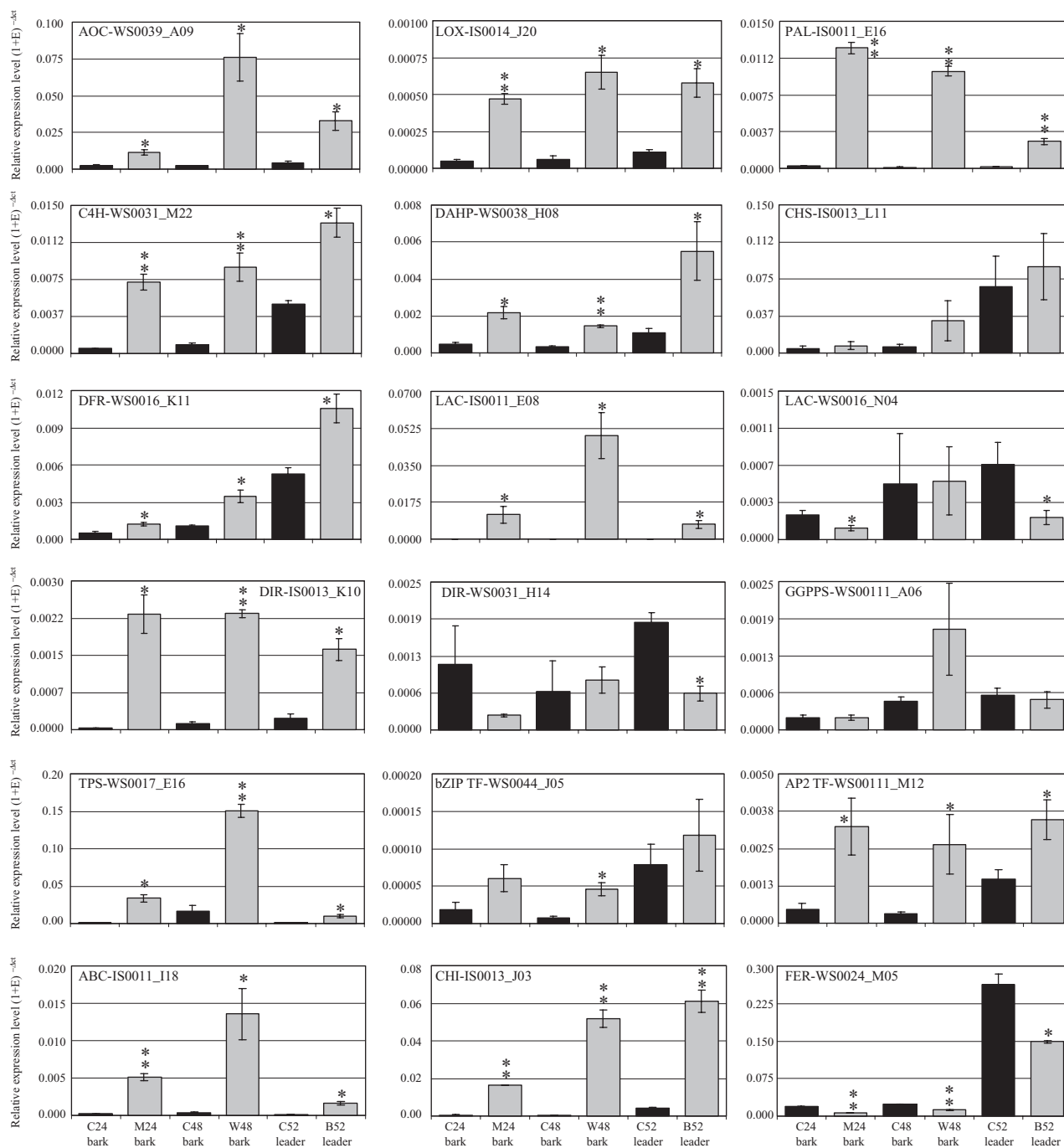
Laccases are proposed to be involved in the polymerization of monolignols to produce lignin and lignans based on their ability to oxidize monolignols and their close spatial and temporal correlation with lignin deposition (Boerjan, Ralph & Baucher 2003). Using a set of 17 *Arabidopsis* laccase (diphenol oxidase) search sequences collected from GenBank we identified 10 spruce ESTs spotted on the 9.7K cDNA microarray with high similarity ( $E < 1e^{-20}$ ; Fig. 6; Supplementary Table S3). ESTs from two contigs (6965 and 13 708) were strongly up-regulated in response to all treatments, whereas other laccase ESTs were only moderately up-regulated, if at all. Increased gene expression of laccases could lead to strengthening of cell walls during insect attack via increased lignin deposition and/or increased production of toxic lignans.

DIR proteins are proposed to direct, in the presence of a laccase or other oxidase, the stereospecific coupling of monolignols to form lignans, and possibly lignin (Davin *et al.* 1997). We have previously identified 19 putative DIR genes in spruce, several of which are rapidly inducible in bark in response to weevil feeding and wounding (Ralph *et al.* 2006b). A TBLASTN search using the 17 unique DIR FLcDNAs and two partial cDNAs from spruce identified 22 DIR-like ESTs spotted on the spruce 9.7K cDNA microarray ( $E < 1e^{-20}$ ; Fig. 6; Supplementary Table S3). The expression of these ESTs falls into two patterns, those up-regulated under all treatments (Fig. 6, i.e. contigs 7094, 128, 139 and 22) and those that are unresponsive to insect feeding or mechanical wounding in bark tissue, but that are up-regulated after budworm feeding for 52 h (Fig. 6, i.e. contigs 13 251, 2062, 6069, 720, 1223 and 8122). This is in agreement with our recent phylogenetic analysis and independent transcript expression analysis of spruce DIR gene family members, which suggest that these two groups of contigs belong to the DIR-a and DIR-b subfamilies, respectively, and only members of the spruce DIR-a subfamily appear to be induced in bark tissue after weevil feeding or mechanical wounding (Ralph *et al.* 2006b).

Finally, in the production of flavonoids/stilbenoids, the first step is catalysed by chalcone/stilbene synthase, which is frequently induced at the transcript level in various plant species, including spruce (Nagy *et al.* 2004b), in response to a variety of stresses (Dixon & Paiva 1995). Using chalcone and stilbene synthase search sequences from a variety of conifer species (Kodan *et al.* 2002; and additional sequences identified within GenBank) we identified 17 ESTs spotted on the spruce 9.7K cDNA microarray ( $E < 1e^{-20}$ ; Fig. 6; Supplementary Table S3); however, without functional testing it is not possible to reliably classify these ESTs as specifically representing a chalcone or stilbene synthase. A consistent pattern of up-regulation after insect feeding or wounding was observed among the majority of chalcone/stilbene synthase-like ESTs. The role of many of the insect-induced genes of the various branch points of spruce phenolic secondary metabolism remains to be investigated with regard to the enzymes' specific contributions to generate individual defence metabolites, for example those associated with phenolic parenchyma cells that have not yet been chemically profiled (Franceschi *et al.* 2005). The present gene expression profiling of insect-induced genes of these branch pathways contributes targets for functional characterization.

### Refined gene-specific expression using real-time PCR

In order to validate cDNA microarray expression profiles and obtain more refined gene expression data, we designed gene-specific primers for a subset of 18 transcripts selected from Table 2 because of their potential biological significance in plant defence and because collectively they exhibited both small and large fold-change DE between treatments (Fig. 7 and Table 3). Overall, very similar results (up- or down-regulation) were obtained for most transcripts between the two techniques of expression analysis, although the magnitude of the response was often greater with real-time PCR, likely as a result of the larger linear dynamic range of detection and use of gene-specific primers. Among the 18 transcripts examined, we observed excellent agreement between microarray and real-time PCR expression data for 15 transcripts including: AP2 (WS00111\_M12) and bZIP (WS0044\_J05) transcription factors, chitinase (IS0013\_J03), ABC transport protein (IS0011\_I18), ferredoxin WS0024\_M05, LOX (IS0014\_J20), AOC (WS0039\_A09), TPS (WS0017\_E16), PAL (IS0011\_E16), C4H (WS0031\_M22), chalcone/stilbene synthase (IS0013\_L11), dihydroflavonol 4-reductase (WS0016\_K11), DAHP synthase (WS0038\_H08), laccase (IS0011\_E08) and a DIR transcript (IS0013\_K10). For three of the transcripts examined we did observe differences in expression determined using these two methods (e.g. GGPPS, WS00111\_A06; DIR, WS0031\_H14; laccase, WS0016\_N04). This suggests that cross-hybridization between closely related gene family members may complicate our interpretation of microarray results in some cases.



**Figure 7.** Relative abundance of 18 representative mRNA transcript species in green shoot tips subjected to budworm herbivory (B) 52 h after the onset of treatment, or bark tissue subjected to mechanical wounding (M) or weevil herbivory (W) 24 h and 48 h, respectively, after the onset of treatment. Control (C) shoot tip tissue (52 h) and bark tissue (24 h) were treated with Tween (Martin *et al.* 2002), and control bark tissue (48 h) received no treatment. Values represent mean  $\pm$  SEM ( $n = 3$  or more independent technical replicates) normalized to elongation factor 1 $\alpha$  expression in each tissue. A Student's *t*-test (two-sample, unpaired, one-sided) was performed to test significance of up- or down-regulation of each transcript between treated and control tissues (\* $P < 0.05$ ; \*\* $P < 0.001$ ). AOC, allene oxide cyclase; LOX, lipoxygenase; PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; DAHP synthase, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase; CHS, chalcone/stilbene synthase; DFR, dihydroflavonol 4-reductase; LAC, laccase; DIR, dirigent; GGPPS, geranylgeranyl diphosphate synthase; TF, transcription factor; ABC, ATP-Binding Cassette protein; CHI, chitinase; FER, ferredoxin.

**Table 3.** Relative abundance of 18 mRNA transcripts determined by real-time PCR

Clone ID	BLASTX versus <i>Arabidopsis</i>	Mechanical at 24 h		Weevil at 48 h		Budworm at 52 h	
		FC	P	FC	P	FC	P
WS0039_A09	Allene oxide cyclase	4.58	0.003	34.11	0.005	7.38	0.005
IS0014_J20	Lipoxygenase	9.32	< 0.001	10.63	0.001	5.21	0.002
IS0011_E16	Phenylalanine ammonia lyase	44.56	< 0.001	78.12	< 0.001	18.07	< 0.001
WS0031_M22	Cinnamate-4-hydroxylase	13.53	< 0.001	9.33	< 0.001	2.62	0.001
WS0038_H08	DAHP synthase	4.43	0.001	4.34	< 0.001	5.00	0.016
IS0013_L11	Chalcone/stilbene synthase	1.76	0.277	4.99	0.097	1.30	0.341
WS0016_K11	Dihydroflavonol 4-reductase	2.31	0.004	3.12	0.002	1.99	0.003
IS0011_E08	Laccase	595.97	0.009	2638.46	0.006	564.74	0.020
WS0016_N04	Laccase	0.47	0.041	1.05	0.481	0.29	0.041
WS0031_H14	Dirigent	0.22	0.151	1.28	0.377	0.34	0.002
IS0013_K10	Dirigent	70.77	0.002	19.50	< 0.001	6.95	0.002
WS0011_A06	Geranylgeranyl diphosphate synthase	0.98	0.475	3.49	0.099	0.87	0.352
WS0017_E16	Terpene synthase	27.67	0.002	8.90	< 0.001	12.06	0.005
WS0044_J05	bZIP transcription factor	3.27	0.054	5.67	0.006	1.49	0.259
WS00111_M12	AP2 transcription factor	7.11	0.029	8.50	0.039	2.34	0.018
IS0011_I18	ABC protein	23.95	< 0.001	34.42	0.004	13.68	0.001
IS0013_J03	Chitinase	26.63	< 0.001	131.96	< 0.001	14.82	< 0.001
WS0024_M05	Ferredoxin	0.31	< 0.001	0.50	< 0.001	0.56	0.003

Values represent fold-change relative to associated control (see Fig. 7). A Student's *t*-test (two-sample, unpaired, one-sided) was performed to test significance (*P*) of up- or down-regulation of each transcript between treated and control tissues. ABC, ATP-Binding Cassette; DAHP synthase, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase.

## CONCLUSIONS

In conclusion, we have developed the first large-scale spruce cDNA microarray, which when applied to the study of insect feeding and wounding in bark and shoot tip tissues revealed several thousand DE genes. These defence gene sets contain several genes previously identified as components of the induced defence response to biotic and abiotic stress in spruce (e.g. endochitinases, TPS genes,  $\beta$ -1,3-glucanases, dehydrins, etc.), as well as a very large number of genes not previously associated with induced defence against insects in spruce or any other conifer species (e.g. ABC proteins, carbonic anhydrase, shikimate pathway genes, and transcription factors of the AP2/EREBP, bHLH, MADS-box and bZIP families). Our transcript profiling emphasizes the potential importance of jasmonates and ethylene in spruce defence signalling, as well as the role of complex terpenoid and phenolic secondary metabolite pathways. Many of the DE genes identified in processes such as signalling, transport or secondary metabolism are now available in our EST collection of over 180 000 spruce sequences as well as in the form of FLcDNAs for functional characterization. The present gene expression profiling will guide in the selection of genes for their further functional characterization.

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### SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

**Table S1.** Primer sequences used for real-time PCR (5' to 3' orientation).

**Table S2.** A complete list of expression data for all genes represented on the microarray.

**Table S3.** Gene name, clone ID, contig ID and sequence similarity to *Arabidopsis thaliana* peptide set or conifer proteins using BLASTX for steps in octadecanoid biosynthesis, phenylpropanoid metabolism and isoprenoid metabolism.

**Appendix S1.** Methodology for Supplementary Material.

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