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Global transcriptome analysis of constitutive resistance to the white pine weevil in spruce

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Running title: Transcriptome analysis of spruce resistance to weevil

Abstract

Constitutive defense mechanisms are critical to the understanding of defense mechanisms in conifers because they constitute the first barrier to attacks by insect pests. In Interior spruce, trees that are putatively resistant and susceptible to attacks by white pine weevil (*Pissodes strobi*) typically exhibit constitutive differences in traits such as resin duct size and number, bark thickness and terpene content. To improve our knowledge of their genetic basis, we compared globally the constitutive expression levels of 17825 genes between 20 putatively resistant and 20 putatively susceptible interior spruce trees from the British Columbia tree improvement program. We identified 54 up-regulated and 137 down-regulated genes in resistant phenotypes, relative to susceptible phenotypes, with a maximum fold change of 2.24 and 3.91, respectively. We found a puzzling increase of resistance by down-regulated genes, as one would think that "procuring armaments" is the best defense. Also, although terpenes and phenolic compounds play an important role in conifer defense, we found few of these genes to be differentially expressed. We found 15 putative small heat shock proteins (sHSP) and several other stress related proteins to be down-regulated in resistant trees. Down-regulated putative sHSP belong to several sHSP classes and represented 58% of all tested putative sHSP. These proteins are well known to be involved in plant response to various kinds of abiotic stress; however, their role in constitutive resistance is not yet understood. The lack of correspondence between transcriptome profile clusters and phenotype classifications suggests that weevil resistance in spruce is a complex trait.

Key words: gene expression levels, microarray, constitutive resistance, sHSP

Introduction

The white pine weevil (*Pissodes strobi*) is a major pest of North American forests (Drouin & Langor 1991; Alfaro 1994; Hamid et al. 1995). The weevil primarily attacks Sitka spruce (*Picea sitchensis*), white spruce (*P. glauca*), and Engelmann spruce (*P. engelmannii*), but it can also attack several other pine and spruce species and even Douglas fir (*Pseudotsuga menziesii*). Adults lay eggs in the bark below the terminal bud cluster, and larva feed on the terminal leader. Such attacks can lead to leader death and consequential stem deformation, which is an economic cost to the forest industry (Alfaro 1994). Knowledge of the genetic mechanisms of weevil resistance in spruce would aid in developing marker assisted breeding strategies for spruce, and add to our knowledge about the diversity of resistance mechanisms in the plant kingdom.

In conifers as in other plants, resistance to insect pests involves both constitutive (pre-existing) and induced defenses. Constitutive defense mechanisms are both mechanical (resin ducts, parenchyma cells and sclerenchyma) and chemical (oxalate crystals, and accumulation of toxic or repellant molecules) (Hall et al. 2011). Induced defenses form a second line of defense, operating during or after pest attack. They are generally more specific in their action, and include increases of resin flow and production of repellant or toxic chemicals, or even de novo defenses (formation of traumatic resin ducts, callus formation, synthesis of new chemicals that are possibly specific to a given pest). Most workers regard induced defensive mechanisms to be the most important component of insect defense, however constitutive resistance is less liable and easier to study and quantify in the context of quantitative genomics.

With regard to white spruce, several studies have identified constitutive features of resistance. Resistant trees possess a thinner bark, with a higher density of outer resin ducts and larger inner resin ducts (Tomlin & Borden 1994; Tomlin & Borden 1997b; Alfaro et al. 2004). In interior spruce (*Picea glauca-engelmannii* complex), resistance is positively correlated with tree growth (both height and trunk diameter), although weevils prefer to oviposit in longer leader shoots (Kiss & Yanchuk 1991; King et al. 1997). Gerson & Kelsey (2002) analyzed piperidine alkaloids contents of resistant and susceptible families of Sitka spruce, but they did not find any correlation with resistance to weevil ovipositing. With regard to terpenoids, Nault et al (1999) showed profiles to be good indicators of resistance in white spruce and Engelmann spruce. In Sitka spruce, resistant trees can show either a lower or a higher content of foliar terpenoids than susceptible trees, suggesting they can use either repellency strategy (the tree try to repel the insects) or stealth strategy (the tree try to be less attractive to the insects; Tomlin et al. 1997). However, higher levels of a diterpene (dehydroabietic acid) and two monoterpenes ((+)-3-carene and terpinolene) are associated with resistance in sitka spruce (Robert et al. 2010). Following this study, Hall et al. (2011) showed that the (+)-3-carene is produced by three different (+)-3-carene synthase genes. One was specific to resistant trees (*PsTPS-3car2*), one was specific to susceptible trees (*PsTPS-3car3*) and one is expressed in both phenotypes (*PsTPS-3car1*). They concluded that (+)-3-carene are explained by the variation in gene copy number, in gene sequence, in protein expression levels and in enzyme activity levels.

The development of 'omics' approaches and the development of several cDNA libraries within the Arborea I, II and Treenomix I, II spruce genome projects (http://www.arborea.ulaval.ca/; http://www.treenomix.ca; Pavy et al. 2005; Ralph, Yueh, et al. 2006; Ralph et al. 2008) opened insights into the nature of both constitutive and induced defense

mechanisms in spruce. To date, most published studies have focused on induced defenses (Ralph, Yueh, et al. 2006; Lippert et al. 2007; Lippert et al. 2009; Zulak et al. 2009; Robert et al. 2010; Hall et al. 2011). These studies compare the biological response to various types of induction (methyl jasmonate and chitosan elicitation; white pine weevil and western spruce budworm herbivory; mechanical wounding) at the transcriptome, proteome and/or metabolome levels. However, induced and constitutive defenses are complementary and distinct defense mechanisms. Induced defenses take place when constitutive defenses have been defeated by an insect attack. Their primary function is to reinforce the constitutive defense mechanisms and add new barriers against the insect attack. Consequently, we might expect induced and constitutive defenses to have a different genetic basis. The purpose of this study was to investigate these differences.

The comparison of resistant and susceptible trees at the global transcriptome level has not yet been conducted, and such a comparison can provide fundamental and perhaps unexpected findings about the basis of insect resistance in conifers. Here we present a comparative study of gene expression in interior spruce (*Picea glauca-engelmannii* complex) aimed to identify candidate genes involved in constitutive defense against white pine weevil. We used a set of 180 trees previously ranked for resistance to this weevil by breeders in the British Columbia Ministry of Forests. Using a 17825 member cDNA microarray, we compare gene expression levels between the 20 most resistant trees and the 20 most susceptible trees. Significantly upregulated and downregulated genes will identify a suite of genes involved in constitutive weevil resistance. Particular attention will be given to the putative small heat shock proteins (sHSP) that evidently play an important role in constitutive defense.

Materials and methods

Selection and sampling

As part of the British Columbia (BC) interior spruce tree breeding program (Experimental Project EP 670), 180 trees were selected in wild stands across the Prince George region of central BC (Figure 1). The parent tree selection criteria was largely height superiority, stem form, branch size and crown shape. Their ages varied from 100 to 200 years. Open-pollinated seeds were collected from each wild tree and test seedlings for each parent tree were grown in nursery beds near Prince George. Progeny tests of all families were established in 1972 at Aleza Lake, near Prince George, and in 1973 at three other sites: the Prince George Tree Improvement Station (PGTIS), Quesnel, and Barbie Lake. In the mid-1980s, the PGTIS and Aleza Lake sites began to suffer severe and repeated attacks of white pine weevils. In 1988, presence or absence of weevil damage was recorded for all trees on both sites. Kiss and Yanchuk (1991) reported that family damage was consistent between the two sites (r = .71) and had a moderately strong genetic basis ($h^2_{family} = 0.77$; $h^2_{individual} = 0.18$). King et al. (1997) reported similar results in other BC interior spruce populations. Based on these results, it appears that parental resistant scores can be readily estimated from weevil damage on their progenies. In 2003, all families on both sites were ranked according to the number of damaged trees and the observed damage was used to estimate resistance levels of the 180 parent trees. In this study, the 20 least and 20 most damaged families were chosen as the resistant and susceptible families.

In addition to collecting open-pollinated seed from the 180 parent trees in the wild, scionwood (i.e. shoot tips) was collected from each tree and all trees were cloned by grafting and established in clone banks at Vernon, Barnes Creek (near Enderby, B.C.) and PGTIS. Samples used for genetic analysis in this study were collected from parent tree grafts at the Barnes Creek

site. The use of cloned trees growing in the same location instead of wild trees located across a vast geographic area removes bias due to different environmental growth conditions.

RNA extraction and microarray profiling

Bark samples were collected from lateral shoots of the trees the Barnes Creek clone bank. Total RNA was extracted following Kolosova *et al*. (2004). RNA quantity and quality were assessed by measuring spectral absorbance between 200 and 350 nm and by visual assessment on a 1% agarose gel. cDNA synthesis was completed for each sample independently using Superscript II reverse transcriptase (Invitrogen) with an oligo dT12–18 primer. cDNA samples were hybridized using 3DNA Array 350 Expression array detection kit (Genisphere) onto the Treenomix Spruce cDNA microarray (21.8K version) comprising 18725 unique elements. A balanced design with dye swaps was used to make direct comparison of gene expression levels of resistant and susceptible trees. Each resistant tree was randomly contrasted with a susceptible tree.

Statistical analysis

Slides were scanned and spot intensity was quantified using ImaGene 6.0.1 software (BioDiscovery, Inc., El Segundo, CA, USA). To correct for background intensity, the lowest 10% of median foreground intensities per subgrid was subtracted from the median foreground intensities. Data were then normalized slide by slide, by variance stabilizing normalization to compensate for nonlinearity of intensity distributions (Huber et al. 2002). A linear mixed effects model was fit to the data taking account of both resistance/susceptibility and dye effects. Fold change, *P*- and *Q*-values were computed for all genes. Genes were considered to have a significant differential expression level when their *P*-value is below 0.05 and their fold-change above 1.5.

Heat map and cluster analysis were performed on genes with *P*-value < 0.05 and fold change > 1.5. Individuals and genes were clustered with Pearson correlation index and Spearman correlation index, respectively. Dendograms were drawn using the 'hclust' function in R Script.

To identify major themes appearing among the differentially expressed genes, we used the software Blast2Go (Conesa et al. 2005; Götz et al. 2008) to test for statistical overrepresentation of Gene Ontology terms (GO terms) among genes up- and down-regulated. A more detailed functional categorization was performed using Blastx and tBlastx search vs. viridiplantae database on NCBI. We considered only results with a E-value lower than 10^{-10}. Given the number of differently expressed putative small heat shock proteins, a particular emphasis has been given to this protein family. tBlastn searches using protein sequences of known sHSP of *Arabidopsis thaliana* and *Oryza sativa* (Scharf et al. 2001; Siddique et al. 2008; Sarkar et al. 2009) was performed over the whole microarray to identify putative members of the sHSP family. 61 representative sequences of the 16 known sHSP classes from *Arabidopsis thaliana* (Scharf et al. 2001; Siddique et al. 2008), *Populus trichocarpa* (Waters et al. 2008) and *Oryza sativa* (Sarkar et al. 2009) were added to this sequences data set. Sequences were first aligned using the online version of PROMALS3D (Pei et al. 2008) and then optimized manually. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling 1965). Phylogenetic relationships were inferred based on amino acid sequences using the Neighbor-Joining method to determine the exact class of each sequence. Only the conserved C-terminal sequences have been considered (see additional file). The reliability of the inferred tree was tested by bootstrap analysis with 1000 replicates (Felsenstein 1985).

Raw data and normalized data are uploaded to the Gene Expression Omnibus with accession number GSE27476 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27476).

Sequences for array clones are found in National Center for Biotechnology Information (NCBI) using the clone IDs given in Tables 2, 3 and Table S1 in Supplementary material.

Results

Resistance levels

The percentage of trees damaged by weevils was significantly higher among susceptible trees (68%) than among resistant trees (21%; $p < 2.2e\text{-}16$; Figure 2). No difference was found between susceptible and resistant trees neither in size nor in survival. Supplementary Table 1 summarizes the observed damages. These results show that we have a valid comparison of phenotypic differences between two classes of trees that differ in resistance to the weevil.

Gene expression profiles

Among the 18725 genes on our microarray chip, 2499 showed a *P*-value less than 0.05 for significant differences of gene expression between the two classes of trees that differ in resistance (Table 1). The highest *Q*-value observed among these genes was 0.282 but only one gene showed a *Q*-value less than 0.05. Fold changes (FC) were low with the maximums FC of 2.24 and 3.91 in up-regulated and down-regulated genes, respectively (Table 1 and Figure 3). Consequently, we considered gene expression to be significantly different if the *P*-value was less than 0.05 and FC was greater than 1.5. With such a rigorous criteria, we identified 54 genes as up-regulated, and 137 genes as down-regulated, in resistant trees compared to susceptible trees, for a total of 191 significant genes.

As a further verification of differential gene expression, we performed cluster analysis and heat map based on the 191 significant genes (Figure 4). The cluster analysis indicates two

groups, however, they do not match the resistant/susceptible classification; cluster #1 contained 11 susceptible trees, while cluster #2 contained 9 susceptible trees and 20 resistant trees. There is no evidence of a link between the resistance levels and the classification of susceptible trees in two distinct groups. The heat map (Figure 4) confirms the differences in gene expression profiles between the two clusters and suggests no difference between susceptible and resistant trees in cluster #2. Genes cluster in two main groups: (1) down-regulated genes and (2) up-regulated genes.

To find differences that might exist between resistant trees and susceptible trees of clusters #1 and #2 (Figure 4), we performed a complementary analysis. We fitted the data as previously described to a mixed linear model, but considered three groups of trees: group S1 = cluster #1 (S-157-162, S-154-135, S-163-166, S-160-176, S-164-163, S-165-65, S-162-111, S-174-128, S-159-43, S-155-62, S-169-72), group S2 = susceptible individuals of cluster #2 (S-170-107, S-176-133, S-161-60, S-156-103, S-158-131, S-167-95, S-173-117, S-179-105, S-166-130, see figure 4) and group R = resistant individuals (of cluster #2). This approach is not compatible with our experimental design as this analysis consists of three groups and the experimental design was made to compare two groups. Hence, individuals are not properly balanced over dyes and groups. Moreover, this statistical approach is not adequate as we predefine groups according to their gene expression profiles prior to the statistical comparisons based on the gene expression profiles. So results should be taken with caution. Only 30 genes are significantly differently expressed (FC up to 3.52) between group R and group S2 according to the criteria $P < 0.05$ and $FC > 1.5$ but with a *Q*-value of 1 (Table 2). This tends to confirm the low levels of difference between these groups. By contrast, the observed differences between

group S1 and group R are high with 274 up-regulated (FC up to 10.05) and 430 down-regulated genes (FC up to 3.40) in group S1.

Functional characterization

Using Blast2Go, we tested the occurrence of overrepresented GO terms among the set of significant genes arising from the comparison of resistant and susceptible trees, compared to the entire microarray. Among the biological processes, only a few categories were overrepresented (Figure 5): "response to hydrogen peroxide", "response to heat" and "response to high light intensity", and several higher categories. All belong to the wider category "response to stimulus". Among cellular components, the only overrepresented category is "microtubule associated complex". Among molecular functions, the two lowest overrepresented categories are "Rho guanyl-nucleotide exchange factor" and "microtubule motor". Although the trees were not stimulated, the overrepresented GO terms suggest that differentially expressed genes are involved in stress or stimulus responses, but their molecular functions remain obscure.

To complete analysis of the GO terms, Blastx and tBlastx searches were preformed against Viridiplantae on NCBI to deduce the functions of these putative genes, using E-values less than 10^{-10}. 106 clones gave no results or matched sequences with unknown functions. We did find 85 matches with annotations using either Blastx or tBlastx. Genes with significant blast results are presented in Table 3. Differentially expressed genes belong to various gene families with few apparent links, except for putatively stress related genes (including the putative small heat chock proteins, sHSP). Three genes were annotated as putative transcription factors and three genes are annotated as part of putative transposable elements, but their possible function here is unknown.

Of the 191 genes either up or down regulated between resistant and susceptible trees, we found very few differentially expressed genes to be putatively involved in phenylpropanoid and terpenoid metabolisms. Only four genes were putatively assigned to the terpenoid metabolism: one putative cytochrome P450 and two putative delta-selinene-like synthases that were down-regulated and one putative zeatin O-glucosyltransferase that was up-regulated. Eight to nine genes were putatively directly related to Phenylpropanoid metabolism: a putative UDP-glycosyltransferase, a putative laccase, two putative phenylcoumaran benzylic esther reductase, a putative zeatin O-glucosyltransferase, a putative caffeic acid O-methyltransferase, a putative Flavonol 4'-sulfotransferase and a putative cytochrome P450, and eventually the putative transcription factor (MYB16) that might be linked to phenylpropanoid or terpenoid metabolism (Bedon et al. 2007).

Differential expression of small heat shock proteins (sHSP) and stress related proteins

Of the 26 putative small heat shock proteins (sHSP) printed on our microarray chips, 15 were down-regulated in resistant trees. We compared their sequences with *Arabidopsis thaliana*, *Populus trichocarpa* and *Zea mays* sHSP sequences allowing class determination of the majority of these genes (Figure 6). The phylogeny is congruent with previous classifications of sHSP (Scharf et al. 2001; Siddique et al. 2008; Waters et al. 2008; Sarkar et al. 2009) with the exception of *Os21.8 ER,* which was previously characterized as a member of the endoplasmic reticulum group of sHSP but clustered here with *Os 18.8* of the cytoplasmic class X. Most of these spruce sHSP sequences cluster within the classes of sHSP previously identified in *Arabidopsis thaliana*, *Populus trichocarpa* and *Zea mays*. Those that failed to cluster might belong to new sHSP classes.

As in other species, the most diverse class of putative sHSP in spruce is the nucleocytoplasmic class I, represented by 7 putative clones (WS0052_F03, WS00923_A06, WS0061_N21, WS0262_N22, IS0014_L07, WS0261_O21, WS00823_L11; figure 6). Nucleocytoplasmic classes II and III are represented by two putative clones (WS0266_N22 and WS00825_O14) and one putative clone (WS00815_E02), respectively. WS0058_F08 putatively belongs to the peroxisomal class and WS0063_C15 and WS00919_I02 both putatively belong to the endoplasmic reticulum class. WS0087_J23, WS0058_B04 and WS00925_H13 do not cluster within any classes of either reference species. They may belong to a new class, specific to conifers. Six clones are found within a clade consisting of mitochondrial (group I) and chloroplastic sHSP. IS0014_C09 and WS0263_F23 unambiguously cluster within the mitochondrial group I of sHSP. Similarly, WS0063_G17 and WS00924_D21 unambiguously cluster within chloroplastic sHSP. Since WS0064_K01 and WS0061_H08 are branched between mitochondrial group I and chloroplastic sHSP within the large clade consisting of both mitochondrial and chloroplastic sHSP, they cannot be assigned with high confidence to either class. WS0092_E18, WS00826_O04 and WS0054_N08 do not match any known class of sHSP. Nevertheless, they are putatively related to the cytosolic classes V, VI and VII, respectively, and are tentatively assigned to these groups of sHSP. WS00930_B15 cannot be assigned to any sHSP class because the clone sequence is too short even though tBlastn and tBlastx searches place it as a putative sHSP.

The down-regulated putative sHSP belong to several classes working in different cellular compartments: nucleocytoplasmic (9 putative sHSP of class I-II-III-VI), endoplasmic reticulum (2 putative sHSP), peroxisome (1 putative sHSP) and chloroplast (1 putative sHSP). Two of the down-regulated putative sHSP could not be assigned to a particular class and operate in an

unknown cellular compartment and seem to belong to the new sHSP class. In addition to these putative sHSP, 14 putative stress related proteins of various gene families are differentially expressed (12 down-regulated and 2 up-regulated in resistant trees), including three putative Heat Shock Proteins and at least two putative universal stress proteins.

Discussion

Differences between resistant and susceptible trees

Our comparison gene expression for 18725 genes between 20 susceptible and 20 resistant trees found 54 up-regulated genes and 137 down-regulated genes in resistant trees, as compared to the susceptible trees. As presented in the introduction, several studies have shown that differences exist between resistant and susceptible phenotypes at the morphological, chemical and genetic levels Moreover, previous studies have shown several hundred genes are involved in induced defenses in both Sitka spruce and Norway spruce (Ralph, Yueh, et al. 2006; Lippert et al. 2009). Therefore, the number of differentially expressed genes (i.e. with FC higher than 1.5) was expected to be greater than 211 that found in this study (191 statistically significant).

Such a low number of differentially expressed genes suggest that differences between resistant and susceptible phenotypes are linked more to variation in gene sequences, and/or translation, and/or variation of catalytic efficiencies than to regulatory differences. Hall et al. (2011) showed that differences in (+)-3-carene levels can be explained by variation in: 1) the number of gene copies, 2) protein expression levels, 3) gene sequences and 4) catalytic efficiencies. Such differences can also be expected in other gene families and the observed differences of gene expression levels may not explain all of the observed phenotypic differences.

Another possible explanation for the low number of differentially expressed genes is that in conifers, several gene families are composed of a large number of closely related genes: terpenoid synthases (Martin et al. 2004; Keeling et al. 2008), cytochrome P450 monooxygenases (Hamberger & Bohlmann 2006), dirigent proteins (Ralph, Park, et al. 2006; Ralph et al. 2007), MYB transcription factors (Bedon et al. 2007; Bedon et al. 2010). Therefore, we can expect that some spots of the microarray hybridize with transcripts of two, or even several, similar genes. In these cases, the observed gene expression levels are the average of the respective gene expression levels (i.e. up-regulated genes cancel the effect of the down-regulated genes). The low number of differentially expressed genes can also be linked to the existence of disparate strategies of resistance (e.g. stealth or repellent). See part 4 of the discussion below.

Previous comparisons between resistant and susceptible trees have shown that resistant phenotypes in spruce are better "armed" to defend against weevils; however, these results are inconsistent. Tomlin and Borden (1994; 1997b) and Alfaro et al. (2004) found that resistant trees possessed more and larger resin ducts, while Tomlin et al. (1997) and Nault et al. (1999) reported no clear link between terpene profiles and resistance. Only one study suggested the existence of a stealth strategy (Tomlin et al. 1997). In the case that procuring "armaments" is the most common defense strategy, we might expect a majority of up-regulated genes in resistant phenotypes. However, most of the differentially expressed genes in this study were down-regulated (72%). This suggests that resistance could be linked more to a stealth strategy than to a repellent strategy. The silencing of certain genes may reduce the probability of detection and attack by weevils. Moreover, since resistance is useful only when weevils are present, the cost of a constant expression of genes involved in resistance might be higher than the associated benefit.

The comparison of resistant trees and the 11 susceptible trees of cluster #1 lead to a higher number of differentially expressed genes than the comparison of the 20 resistant and 20 susceptible trees. It suggests that more genes might show differences in constitutive expression levels. However, we cannot link the classification of the trees in three groups to a classification of phenotypes. Because this statistical approach is not adequate, we will not talk more about these results and we just mention them as further analyses.

Terpenoid and phenylpropanoid pathways: few genes were constitutively differently expressed in resistant spruces

Only three differentially expressed genes have been found across the terpenoid metabolic pathways. Only two putative Delta-selinene-like synthases are down-regulated in resistant trees. In grand fir, Delta-selinene synthase use farnesyl pyrophosphate as substrate to produce 34 different sesquiterpene olefins (Steele et al. 1998). The down-regulated gene annotated as putative abscisic acid 8'-hydroxylase belongs to the wide super family of cytochrome P450. This enzyme degrades abscisic acid into 8'-hydroxyabscic acid (Nambara & Marion-Poll 2005). Abscisic acid is an important terpenoid phytohormone involved in many plant developmental processes and plant responses to environmental stress and pathogens (Seo & Koshiba 2002). In particular, abscisic acid regulates the opening of stomates and thus the loss of water in cells. Pei et al. (2000) showed abscisic acid also triggers an increase in cytosolic calcium in guard cells. In *Pistia stratiotes*, the Ca^{2+} channels play an important role in calcium oxalate crystals formation (Volk et al. 2004). We might hypothesize that the reduced catabolism of abscisic acid is linked to an increase in the production of the toxic calcium oxalate crystals. However, more research is needed to confirm this hypothesis.

There are seven differently expressed genes that can be putatively assigned to phenylpropanoid metabolism. First, a putative caffeic O-methyltransferase (COMT) is down-regulated in resistant trees. This enzyme is known to be involved in methylation of precursors of both syringyl- and guaiacyl-lignin subunits in angiosperms (Do et al. 2007; Tu et al. 2010; Baucher et al. 2003; Vanholme et al. 2008). Several studies showed that down-regulation of COMT leads to syringyl/guaiacyl-lignin ratio change or event suppression of syringyl-lignin. COMT down-regulation also leads to the incorporation of 5'-hydroxy-guaiacyl units in lignin. However, syringyl-lignin does not exist in conifers and we found no studies that show an effect of COMT down-regulation on 5'-hydroxy-guaiacyl production in conifers. Because guaiacyl-lignin is the dominant lignin type in conifers, a decrease of COMT expression level could be associated with a decrease of lignin synthesis.

The up-regulated putative laccase enzyme belongs to the wide super family of the multicopper oxidase (Nakamura & Go 2005). In plants, some laccase enzymes are involved in lignin biosynthesis, although they have a large spectrum of substrates and form a large family of genes. In loblolly pine, eight laccase genes have been described and two of them have been functionally characterized (Sato et al. 2001; Sato & Whetten 2006). Both enzymes were able to oxidize coniferyl alcohol and produce dimers of coniferyl alcohol, and as a consequence are involved in lignin biosynthesis.

Two other up-regulated genes in our constitutive samples are annotated as putative phenylcoumaran benzylic ether reductase. Phenylcoumaran benzylic ether reductases are involved in phenolic secondary metabolism and convert 8'5'-linked lignin dehydrodiconiferyl alcohol into isodihydrodehydrodiconiferyl alcohol by the reduction of benzylic ether functionality (Gang et al. 1999). A previous study showed that a phenylcoumaran benzylic ether

reductase is involved in induced conifer defense following either mechanical wounding or weevil attack (Lippert et al. 2007).

The up-regulated gene annotated as putative UDP-glucosyltransferase plays an important role in lignin biosynthesis. After their biosynthesis, the monomers of lignin (*i.e.* p-coumaryl, coniferyl and sinapyl alcohols according to plant species) have to be translocated to the cell wall for the next oxidation step of lignin biosynthesis. The 4-O-β-D-glucosides of cinnamyl alcohols have been considered as the transport forms of coniferyl and sinapyl alcohols. A UDPG:coniferyl alcohol glucosyltransferase from *Pinus strobes* has been able to convert cinnamyl aldehydes as well as coniferyl and dihydroconiferyl alcohols into their corresponding O-β-D-glucosides in vitro (Steeves et al. 2001). However, because coniferyl and sinapyl alcohols might be able to freely diffuse through the plasma membrane, it has been suggested that these glucosides play no role in monolignol export for developmental lignin (Vanholme et al. 2008; Boija & Johansson 2006). Another noteworthy gene is annotated as putative MYB16, a member of the family of transcription factors. MYB16 belongs to the R2R3-MYB family and was shown to accumulate transiently in response to wounding in white spruce (Bedon et al. 2010)

At least two genes are annotated within the flavonoid metabolism. First, an up-regulated gene annotated as a putative flavonoid 3'-monooxygenase which belongs to the Cytochrome P450 superfamily. This gene is involved in central flavonoid metabolism, the leading precursors of flavones, anthocyanins and proanthocyanidins pathways (Winkel-Shirley 2001). Anthocyanins can play various roles, including the resistance mechanisms towards insect pests (Steyn et al. 2002). The second gene within the flavonoid metabolism is down-regulated and annotated as a putative flavonol 4'-sulfotransferase. Ralph et al. (2006) found that several genes of flavonoid metabolism, including a Flavonoid 3'-monooxygenase (=hydroxylase), are up-regulated after

white pine weevil herbivory, mechanical wounding, or western spruce budworm (*Choristoneura occidentalis*, Lepidoptera) feeding.

Many stress related proteins exist for weevil resistance

Our study shows that 15 of 26 putative sHSP and several other stress-related genes are down-regulated in resistant trees. sHSP belong to a large family of proteins. They are highly variable but they share a conserved α-crystallin domain of approximately 100 residues (Caspers et al. 1995; de Jong et al. 1998; Fu et al. 2006). sHSP are classified into at least eleven subfamilies localized in different cell compartments: cytosol, mitochondria, chloroplasts, endoplasmic reticulum, peroxisome (Helm et al. 1993; Vierling 1991b; Siddique et al. 2003; Waters et al. 1996; Scharf et al. 2001; Ma et al. 2006; Waters et al. 2008). The 15 down-regulated putative sHSP belong to class I, class II, class III, chloroplastic endoplasmic reticulum or cannot be assigned with confidence to a known class. The role of sHSP has been widely studied in plants. They are involved in plant response to various kinds of stress such as heat, cold, drought, heavy metals, salinity, oxidative and osmotic stress (Vierling 1991a; Waters et al. 1996; Wang et al. 2004; Sun & MacRae 2005; Haslbeck et al. 2005; Nakamoto & Vigh 2007). sHSP are also involved in normal development of plants, during embryo development, seed germination, somatic embryogenesis, pollen development and fruit maturation (Sun et al. 2002 and references therein). sHSP usually play a protection role (Haslbeck et al. 2005; Nakamoto & Vigh 2007). They can form stable complexes with denaturated proteins to prevent its aggregation. sHSP also form soluble aggregates with substrate proteins, creating a transient reservoir of substrates. Release and refolding of both complexes and aggregates need the cooperation of ATP-dependent chaperone systems. sHSP also play a role in membrane quality control and are potential membrane stabilizing factors.

Several sHSP were previously shown to be involved in conifer defense. Lippert et al. (2007) showed that weevil feeding induces the over expression of seven sHSP at the protein level (up to six-fold induction) in Sitka spruce. They also showed that transcript and protein expression levels are not correlated as six of the seven sHSP corresponding transcripts are not up-regulated following weevil feeding. The two-fold up-regulation of the seventh sHSP transcript (class I) is comparable to the up-regulation of the associated protein. Nevertheless, they observed that all the seven sHSP transcripts are constitutively expressed to high levels in bark tissue. Such constitutive expression of sHSP has also been observed in *Arabidopsis thaliana* (Siddique et al. 2008) but the constitutive role of sHSP remains unknown. The results of Lippert et al. (2007) suggest that sHSP transcripts accumulate in transient stocks and that sHSP expression is post-transcriptionally controlled. Recent studies have shown that RNA-binding proteins can regulate the stability, translation or localization of mRNA (Babitzke et al. 2009; Glisovic et al. 2008; Hogan et al. 2008). sHSP activity is also regulated at the protein level by phosphorylation or oligomer reorganization. As a consequence, the expression levels of sHSP transcripts do not necessarily correlate with the sHSP expression at the protein level. sHSP may not play a role in constitutive defense and, in fact, may be involved in induced defense, among other biological processes. However, the test of this hypothesis needs a time-series comparison of both the transcriptome and the proteome after induction (e.g. weevil feeding), based on both susceptible and resistant strains of spruce. Together with 15 putative sHSP, 12 putative stress related proteins are constitutively up-regulated in susceptible trees. Their potential role is yet to be discovered.

Phenotype prediction and efficiency of the approach

As in previous studies based on morphological features or terpene contents (Tomlin et al. 1997; Tomlin & Borden 1997a; Alfaro et al. 2004), our goal was to determine if the transcriptome profiling is able to predict resistance levels in Interior spruce. To determine whether the observed gene expression profiles corresponded to the observed phenotype (i.e. resistant/susceptible) we performed a hierarchical clustering (Figure 4). While the individuals clustered into two groupings, they did not match with the phenotype classification. One cluster contained 11 susceptible trees and a second cluster contained the remaining trees, i.e. both susceptible and resistant trees. The heat map clearly shows that 11 susceptible trees have a distinct profile of gene expressions compared to the other 29 trees. Therefore, it might be possible to identify certain susceptible phenotypes by analyzing the transcriptome profiles, but it will not be possible to identify resistant trees with a high degree of certainty using this approach. Four hypotheses could explain this pattern but at least three of them can be rejected.

First, the resistance levels might be inaccurately assessed for some progenies. The family size of all the examined trees varied between 14 and 175 trees (see additional table 1). Among the families used in the transcriptome comparison, 6 families (5 susceptible and 1 resistant) contained fewer than 80 individuals: S-165-65, S-161-60, S-166-130, S-170-107, S-179-105 and R-11-19 (respectively 42, 41, 30, 63, 14 and 42 trees). 4 of them are considered susceptible and clustered with resistant trees in the cluster #2. Consequently, the assessment of the resistance levels of these progenies might be questionable. However, this does not explain why susceptible progenies (with more than 80 individuals) S-176-133, S-156-103, S-158-131, S-167-95 and S-173-117 cluster with resistant trees. However, the original assessment of damage was based on natural levels of weevil attack. Attack patterns are rarely uniform in the wild and all trees do not

have the same probability of attack (He & Alfaro 1997). Therefore, some of the undamaged trees could have been "escapes" and never subject to attack, leading to some bias in the resistance levels assessment, particularly in the small progenies.

Second, the differences in the observed damages caused by weevils can be explained by environmental factors such as growth conditions. This hypothesis seems improbable because all the parent trees were collected within the same region (Prince Georges area) and the progenies were randomly mixed across several stands. All of them were grown in the same standard conditions. Moreover, as the trees used for gene expression profiling were grafted on the same rootstock, we do not expect high difference due to misadaptation to local soil conditions.

Thirdly, as the collected seeds were open-pollinated in the wild, we know only the mother and have no information about the fathers of the progenies used for resistance scoring. This may induce a bias if parents have very different levels of resistance. However, a previous study has shown a high family heritability ($h^2 = 0.70$) in a similar experiment design (King et al. 1997) and crosses between susceptible and resistant trees would lead to intermediate levels of resistance (Alfaro et al. 2004). As a consequence, a bias induced by the uncertainty of fatherhood of the progenies seems improbable.

Finally, the resistance or susceptibility may be based on several different strategies, involving different sets of genes. In this case, our experimental design does not allow us to identify genes involved only in rare strategies. If resistance can be associated with e.g. ten different profiles of gene expression, we can expect only a few trees for each strategy to be present in our sampling. In such a case, the differences in gene expression profiles will be

confused with individual variations because we did not classify the trees according to their strategy but according to their phenotype.

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Table 1: Summary of T-test comparisons between resistant and susceptible trees (=reference).

18725 analyzed genes	Up-regulated	Down-regulated
Genes with *P*–value < 0.05	1225 (FDR = 28.2%)	1274 (FDR = 28.2%)
Genes with fold change > 1.5	60	151
Maximum fold change	2.24	3.91
Significant genes (P < 0.05 and FC > 1.5)	54	137

Table 2: Summary of T-test comparisons between resistant and susceptible trees of clusters #1 and #2 (=references).

18725 analyzed genes	Resistant (20) vs. Group S1 (11)		Resistant (20) vs. Group S2 (9)	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Genes with *P*–value < 0.05	1778 (FDR = 18,9%)	1709 (FDR = 18,9%)	305 (FDR = 100%)	337 (FDR = 100%)
Genes with fold change > 1.5	326	482	79	56
Maximum fold change	3,39	10,04	2,84	3,22
Significant genes (P < 0.05 and FC > 1.5)	274	430	15	15

Table 3: Functional categorization of differentially expressed genes (*P*-value < 0.05 and fold change > 1.5). Colors green and red indicate down-regulation and up-regulation in resistant trees, respectively. gi#: GI number of spruce clone in NCBI database, FC: fold change, P: *P*-value, Q: *Q*-value.

gi #	Clone ID	Blastx	E value	tBlastx	E value	FC	*P*	*Q*
Cytochrome P450 family and Terpenoid metabolism								
gi|49040156	WS0101_H07	Abscisic acid 8'-hydroxylase cytochrome P450 [*Lactuca sativa*]	6E-53	cytochrome P450, putative [*Ricinus communis*]	8E-63	1,6	0,006	0,144
gi|49062217	WS00712_A10	delta-selinene-like synthase [*Picea sitchensis*]	6E-70	delta-selinene-like synthase [*Picea sitchensis*]	4E-101	1,55	0,024	0,221
gi|69352521	WS00929_B22	delta-selinene-like synthase [*Picea sitchensis*]	2E-119	delta-selinene-like synthase [*Picea sitchensis*]	7E-162	1,96	0,039	0,258
Phenylpropanoid metabolism								
gi|49057427	WS0263_L06	caffeic acid O-methyltransferase [*Pinus pinaster*]	2E-56	o-methyltransferase [*Ricinus communis*]	8E-61	1,76	0,007	0,154
gi|49014799	IS0012_L15	cytochrome P450 like protein [*Arabidopsis thaliana*]	5E-23	cytochrome P450 [*Populus trichocarpa*]	8E-24	1,75	0,001	0,102
gi|49059256	WS0071_C13	UDP-glucosyltransferase 3 [*Pueraria montana var. lobata*]	1E-25	glucosyltransferase-8 [*Vigna angularis*]	8E-27	1,72	0,034	0,248
gi|70636503	WS00730_B15	laccase [*Pinus taeda*]	4E-23	laccase (Lac7) [*Pinus taeda*]	1E-27	1,73	0,030	0,240
gi|49043266	WS01011_J14	phenylcoumaran benzylic ether reductase homolog TH6 [*Tsuga heterophylla*]	2E-13	phenylcoumaran benzylic ether reductase homolog TH6 mRNA [*Tsuga heterophylla*]	2E-15	1,95	0,004	0,126
gi|49056257	WS0058_F16	phenylcoumaran benzylic ether reductase -like protein [*Populus trichocarpa*]	2E-44	phenylcoumaran benzylic ether reductase -like protein [*Populus trichocarpa*]	4E-52	1,65	0,016	0,199
gi|49025769	WS00928_F16	steroid sulfotransferase 1 [*Brassica napus*]	3E-09	Flavonol 4'-sulfotransferase, putative [*Ricinus communis*]	2E-23	1,56	0,002	0,113
Small Heat shock proteins								
gi|49056795	WS0261_O21	chaperone [*Agave tequilana*]	1E-49	heat shock protein 18.2 [*Arabidopsis thaliana*]	9E-56	2,64	0,005	0,140
gi|49138681	WS00823_L11	small heat-shock protein [*Pseudotsuga menziesii*]	1E-69	low molecular weight heat-shock protein [*Pseudotsuga menziesii*]	9E-92	3,91	0,000	0,096
gi|49015440	IS0014_L07	heat shock protein 17.5 [*Malus x domestica*]	1E-54	small heat shock protein [*Malus x domestica*]	2E-62	2,01	0,008	0,158
gi|49054183	WS0052_F03	small heat-shock protein [*Pseudotsuga menziesii*]	1E-18	HSP18.2 gene for 18.2kDa heat shock protein [*Arabidopsis thaliana*]	4E-54	2,35	0,009	0,164
gi|49024180	WS00923_A06	small heat-shock protein [*Pseudotsuga menziesii*]	3E-54	heat shock protein 18.2 [*Arabidopsis thaliana*]	8E-60	2,25	0,012	0,182
gi|49140326	WS00825_O14	heat shock protein 17.0 [*Picea glauca*]	2E-12	heat shock protein 17.0 [*Picea glauca*]	8E-78	1,7	0,008	0,161
gi|49131870	WS00815_E02	small heat stress protein class CIII [*Lycopersicon peruvianum*]	5E-32	17.5 kDa class II heat shock protein mRNA [*Zea mays*]	2E-24	1,7	0,007	0,157
gi|49023311	WS00919_I02	heat-shock protein, putative [*Ricinus communis*]	7E-37	heat-shock protein, putative [*Ricinus communis*]	4E-39	1,5	0,001	0,096

gi|49016363	WS0063_C15	heat-shock protein, putative [*Ricinus communis*]	1E-29	small heat shock protein (hsp21.4) mRNA [*Cyclamen persicum*]	4E-31	2,51	0,004	0,123
gi|49056249	WS0058_F08	peroxisomal small heat shock protein [*Glycine max*]	3E-30	cytosolic class I small heat-shock protein HSP17.5 (hsp17.5 gene) [*Castanea sativa*]	4E-30	1,86	0,002	0,111
gi|49016448	WS0063_G17	heat shock protein [*Ammopiptanthus mongolicus*]	6E-54	heat shock protein (hsp) [*Ammopiptanthus mongolicus*]	4E-59	1,93	0,012	0,183
gi|49026225	WS00930_B15	class II cytoplasmic small molecular weight heat shock protein 17.1 [*Picea glauca*]	4E-09	class II cytoplasmic small molecular weight heat shock protein 17.1 (EMB29, SMW HSP17.1) mRNA [*Picea glauca*]	4E-11	3,63	0,001	0,102
gi|70654578	WS00826_O04	17.3 kDa class I heat shock protein [*Glycine max*]	1E-11	No match		3,31	0,001	0,102
gi|49056161	WS0058_B04	Hsp20.1 protein [*Solanum peruvianum*]	6E-40	small heat-shock protein of cytosolic class I [*Funaria hygrometrica*]	1E-44	2,02	0,006	0,145
gi|49025288	WS00925_H13	17.7 kDa heat shock protein [*Helianthus annuus*]	1E-32	17.7 kDa heat-shock protein gene [*Helianthus annuus*]	3E-32	1,78	0,034	0,248
Heat shock proteins family and other stress related proteins								
gi|49047895	WS01027_A08	Usp: Universal stress protein-like protein [*Astragalus sinicus*]	4E-20	ER6 protein (ethylene-inducible) [*Solanum lycopersicum*]	4E-21	1,71	0,003	0,117
gi|49021075	WS00914_D23	USP-like protein [*Astragalus sinicus*]	9E-27	USP-like protein mRNA [*Astragalus sinicus*]	3E-36	1,8	0,006	0,148
gi|49025415	WS00926_B20	stress-induced protein sti1-like protein [*Arabidopsis thaliana*]	6E-86	stress-inducible protein, putative [*Arabidopsis thaliana*]	1E-102	1,58	0,001	0,102
gi|49057553	WS0264_A17	Heat Shock 70kD protein [*Glycine max*]	4E-52	heat shock protein [*Ricinus communis*]	5E-54	2,39	0,001	0,102
gi|49015272	IS0014_D03	*No match*		Hsp90 mRNA for heat shock protein 90 [*Oryza sativa Japonica* Group]	3E-36	1,76	0,011	0,175
gi|49023568	WS00920_E06	Chaperonin CPN60-2, mitochondrial (HSP60-2) [*Cucurbita cv. Kurokawa Amakuri*]	2E-10	gland development related protein 19-like mRNA [*Gossypium hirsutum*]	1E-15	0,58	0,003	0,120
gi|49051850	WS0018_D18	hypothetic chloroplast chaperonin 21 [*Vitis vinifera*]	4E-82	cp10-like protein (CLP) mRNA [*Gossypium hirsutum*]	4E-92	0,57	0,001	0,100
gi|49056969	WS0262_G19	metallothionein-like protein [*Sesamum indicum*]	7E-15	seed-specific metallothionein-like protein (MT) gene [*Sesamum indicum*]	2E-15	1,78	0,027	0,230
gi|49017024	WS0091_I15	ethylene-responsive protein, putative [*Arabidopsis thaliana*]	1E-15	universal stress protein (USP) family protein [*Arabidopsis thaliana*]	4E-19	1,67	0,006	0,148
gi|49018081	WS00912_J05	jasmonate ZIM-domain protein 1 [*Solanum lycopersicum*]	2E-12	jasmonate ZIM-domain protein 1 mRNA [*Solanum lycopersicum*]	1E-10	0,63	0,023	0,220
gi|70621372	WS02610_H19	arsenite inducible RNA associated protein aip-1, putative [*Ricinus communis*]	4E-47	arsenite inducible RNA associated protein aip-1, putative [*Ricinus communis*]	4E-48	0,49	0,001	0,100
gi|68771533	WS0064_H09	alcohol dehydrogenase [*Pinus banksiana*]	1E-66	alcohol dehydrogenase [*Pinus banksiana*]	2E-86	1,59	0,003	0,118
gi|49023269	WS00919_G06	ATERDJ3A; oxidoreductase; putative DnaJ protein [*Arabidopsis thaliana*]	1E-31	heat shock protein binding protein, putative [*Ricinus communis*]	5E-35	0,58	0,005	0,130
gi|49132946	WS00816_J13	hypothetical water stress-induced protein [*Pseudotsuga menziesii*]	5E-39	galactinol synthase 1 [*Populus trichocarpa x Populus deltoides*]	2E-39	3,31	0,003	0,117

Transposable elements									
gi|49136084	WS00820_G23	copia-like retrotransposable element [Arabidopsis thaliana]	2E-40	genes for S-locus F-Box protein c, Sc-Rnase [Prunus dulcis]	7E-43	1,58	0,001	0,106
gi|49018914	WS0093_C16	copia-type polyprotein [*Arabidopsis thaliana*]	1E-21	retrotransposon gtd1-12e3-re-5 [*Glycine tomentella*]	2E-29	1,9	0,015	0,194
gi|49017619	WS00911_D13	integrase [*Boechera divaricarpa*]	2E-18	retrotransposon PpRT6 RNaseH-like gene [*Pinus pinaster*]	1E-42	1,6	0,002	0,108
Transcription factors									
gi|49123245	WS0032_G19	*No match*		R2R3-MYB transcription factor MYB16 [*Picea glauca*]	9E-55	2,69	0,001	0,096
gi|49016284	WS0062_O09	MBF1C (MULTIPROTEIN BRIDGING FACTOR 1C); DNA binding / transcription coactivator/ transcription factor [*Arabidopsis thaliana*]	3E-50	msh6-2 gene, exon 1 to 17 [*Arabidopsis thaliana*]	2E-56	1,58	0,017	0,203
gi|49015214	IS0014_A07	transcription initiation factor iib, putative [*Ricinus communis*]	5E-13	transcription initiation factor iib, putative [*Ricinus communis*]	3E-15	2,39	0,002	0,112
Other									
gi|49059326	WS0071_G04	AAA+-type ATPase (ISS) [*Ostreococcus tauri*]	2E-21	*No match*		1,53	0,000	0,050
gi|49021738	WS00915_F01	alpha-glucan phosphorylase [*Arabidopsis thaliana*]	5E-18	alpha-1,4-glucan phosphorylase L isozyme [*Cucurbita maxima*]	3E-41	1,57	0,012	0,180
gi|49025500	**WS00926_F17**	AT3G07090 [*Arabidopsis thaliana*]	1E-31	*No match*		0,62	0,001	0,100
gi|49042416	WS0108_M06	ATCNGC4 (CYCLIC NUCLEOTIDE-GATED CATION CHANNEL 4); calmodulin binding / cation channel/ cation transmembrane transporter/ cyclic nucleotide binding [*Arabidopsis thaliana*]	2E-22	putative ion channel, cngc4 [*Arabidopsis thaliana*]	1E-20	1,60	0,012	0,180
gi|49055173	WS0055_D11	ATPP2-A4 (Phloem protein 2-A4); carbohydrate binding [*Arabidopsis thaliana*]	8E-11	*No match*		0,53	0,014	0,190
gi|49022557	WS00917_H17	ATRBL14 (ARABIDOPSIS RHOMBOID-LIKE PROTEIN 14); zinc ion binding [*Arabidopsis thaliana*]	1E-35	ARABIDOPSIS RHOMBOID-LIKE PROTEIN 14; ATRBL14 [*Arabidopsis thaliana*]	8E-42	0,54	0,002	0,110
gi|49052167	WS00110_D02	cytoplasmic dynein light chain, putative [*Ricinus communis*]	2E-34	cytoplasmic dynein light chain, putative [*Ricinus communis*]	1E-40	1,5	0,006	0,142
gi|49025135	WS00922_M07	cytoplasmic dynein light chain, putative [*Ricinus communis*]	2E-29	cytoplasmic dynein light chain, putative [*Ricinus communis*]	2E-31	2,04	0,005	0,140
gi|49017248	WS00910_B07	cytoplasmic dynein light chain, putative [*Ricinus communis*]	3E-32	cytoplasmic dynein light chain, putative [*Ricinus communis*]	3E-38	1,7	0,006	0,142
gi|49025673	WS00928_B05	glycerophosphodiester phosphodiesterase [*Zea mays*]	4E-61	glycerophosphodiester phosphodiesterase [*Zea mays*]	6E-71	0,50	0,003	0,120
gi|49040869	WS0104_I05	hypothetical protein OsJ_14315 [*Oryza sativa Japonica* Group]	1E-12	*No match*		1,54	0,019	0,210
gi|69354546	WS00933_K09	IQ calmodulin-binding region; Apoptosis regulator Bcl-2 protein, BAG [*Medicago truncatula*]	2E-14	Bcl-2-associated athanogene-like protein [*Vitis vinifera*]	6E-19	0,55	0,021	0,210

gi|70634833	WS00724_G03	kinase, putative [*Ricinus communis*]	3E-40	receptor-like kinase [*Marchantia polymorpha*]	7E-42	1,57	0,006	0,140
gi|49045682	WS01018_L23	late embryogenesis abundant protein [*Picea glauca*]	2E-49	late embryogenesis abundant protein (EMB6) [*Picea glauca*]	8E-119	0,66	0,031	0,240
gi|49018587	WS0092_C13	metal ion binding protein, putative [*Ricinus communis*]	2E-41	metal ion binding protein, putative [*Ricinus communis*]	5E-49	0,43	0,015	0,200
gi|49019794	WS0097_C17	mitochondrial import inner membrane translocase subunit TIM14 [*Zea mays*]	1E-31	mitochondrial import inner membrane translocase subunit TIM14 mRNA [*Zea mays*]	9E-38	0,57	0,000	0,100
gi|69359064	WS00937_N04	monovalent cation:proton antiporter, putative [*Ricinus communis*]	1E-15	monovalent cation:proton antiporter, putative [*Ricinus communis*]	4E-18	1,50	0,034	0,250
gi|49023662	WS00920_J02	NADH:ubiquinone reductase subunit 2 [*Beta vulgaris subsp. vulgaris*]	5E-18	NADH dehydrogenase subunit 2 [*Cycas taitungensis*]	9E-32	0,63	0,002	0,120
gi|49024651	WS00924_F18	pirin, putative [*Arabidopsis thaliana*]	1E-12	pirin, putative [*Arabidopsis thaliana*]	1E-13	0,61	0,002	0,120
gi|49016662	WS0064_B23	protein phosphatase 2A regulatory A subunit [*Lolium perenne*]	2E-56	protein phosphatase 2A regulatory A subunit mRNA [*Lolium perenne*]	8E-67	0,66	0,028	0,230
gi|49018463	WS00913_L01	putative hexose transporter [*Vitis vinifera*]	1E-17	sugar transporter, putative [*Ricinus communis*]	1E-20	1,75	0,008	0,160
gi|49052548	WS00111_F08	putative neutral invertase [*Vitis vinifera*]	2E-19	ni1 gene for putative neutral invertase, exons 1-4, clone 48C19 [*Vitis vinifera*]	2E-18	1,74	0,020	0,210
gi|49016709	WS0064_F01	receptor-like protein kinase [*Glycine max*]	7E-26	receptor-like protein kinase 1 [*Glycine max*]	1E-34	0,36	0,001	0,100
gi|49017350	WS00910_G03	retinol dehydrogenase 12 [*Zea mays*]	9E-44	retinol dehydrogenase 12 [*Zea mays*]	1E-51	0,56	0,020	0,210
gi|49024211	WS00923_B17	retrotransposon protein [*Oryza sativa* (*indica* cultivar-group)]	2E-34	large subunit ribosomal RNA gene, partial sequence; chloroplast [*Abies homolepis*]	2E-178	0,50	0,009	0,160
gi|49045461	WS01018_B20	RING-H2 finger protein ATL5A, putative [*Ricinus communis*]	1E-21	RING-H2 finger protein ATL5A, putative [*Ricinus communis*]	2E-24	1,57	0,008	0,160
gi|49132959	WS00816_J18	Sor-like protein [*Ginkgo biloba*]	7E-08	galactinol synthase [*Coptis japonica*]	1E-40	0,56	0,000	0,100
gi|49027191	WS00930_L23	stem-specific protein TSJT1 [*Zea mays*]	1E-26	stem-specific protein TSJT1 [*Zea mays*]	9E-32	1,61	0,005	0,140
gi|49042539	WS0109_C08	sterol regulatory element-binding protein site 2 protease, putative [*Ricinus communis*]	2E-72	sterol regulatory element-binding protein site 2 protease, putative [*Ricinus communis*]	5E-82	1,52	0,006	0,148
gi|49140731	WS0081_N10	Transmembrane BAX inhibitor motif-containing protein 4 [*Zea mays*]	7E-12	Transmembrane BAX inhibitor motif-containing protein 4 [*Zea mays*]	2E-14	2,21	0,002	0,109
gi|49136155	WS00820_I09	Transmembrane protein TPARL, putative [*Ricinus communis*]	4E-45	Transmembrane protein TPARL, putative [R*icinus communis*]	2E-76	1,57	0,003	0,121
gi|49025187	WS00922_O17	UBX domain-containing protein, putative [*Ricinus communis*]	9E-07	UBX domain-containing protein, putative [*Ricinus communis*]	4E-20	1,57	0,031	0,240
gi|49024645	WS00924_F12	vacuole membrane protein, putative [*Ricinus communis*]	6E-65	vacuole membrane protein, putative [*Ricinus communis*]	2E-71	1,56	0,004	0,127
gi|49044389	WS01014_N23	zeatin O-glucosyltransferase [*Phaseolus lunatus*]	1E-57	zeatin O-glucosyltransferase (ZOG1) [*Phaseolus lunatus*]	1E-57	1,52	0,006	0,146
gi|49143022	WS0087_P05	zinc finger protein [*Populus euphratica*]	2E-21	NADH dehydrogenase [Cycas taitungensis]	2E-77	0,65	0,008	0,160
gi|49024616	WS00924_E06	Zn-dependent hydrolases, including glyoxylases	3E-29	metallo-beta-lactamase family protein [*Arabidopsis*	3E-28	0,45	0,000	0,100

		[*Zea mays*]		*thaliana*]					
gi|49055808	WS0056_A24	putative callose synthase catalytic subunit [*Gossypium hirsutum*]	1E-46	putative callose synthase catalytic subunit [*Gossypium hirsutum*]	3E-52	1,67	0,003	0,121
gi|70630512	WS0266_K23	dihydrofolate reductase-thymidylate synthase [*Populus trichocarpa*]	7E-31	bifunctional dihydrofolate reductase-thymidylate synthase, putative [*Ricinus communis*]	7E-35	0,59	0,002	0,120
gi|49020606	WS0099_J08	Adipocyte plasma membrane-associated protein, putative [*Ricinus communis*]	7E-34	Adipocyte plasma membrane-associated protein,putative [*Ricinus communis*]	1E-36	1,89	0,001	0,100
gi|49063070	WS0078_C13	germin-like protein [*Ananas comosus*]	3E-41	GLP5 (GERMIN-LIKE PROTEIN 5); manganese ion binding / nutrient reservoir [*Arabidopsis thaliana*]	8E-68	1,69	0,002	0,110

small Heat Shock Proteins were first identified using Blastx and tBlastx search and their class were further determined by phylogeny (see text).

Figure legends

Fig. 1: Parent trees origin within the Prince George area. The color scale (S-R) indicate the level of resistance of the trees, from highly susceptible to highly resistant, blue to red, respectively. Filled circles represent origin of the trees family used in the present microarray study. Open circle represent the origin of trees family not used in the microarray study, but used for the resistance ranking (map layers from MapPlace website http://www.empr.gov.bc.ca/MINING/GEOSCIENCE/MAPPLACE/Pages/default.aspx).

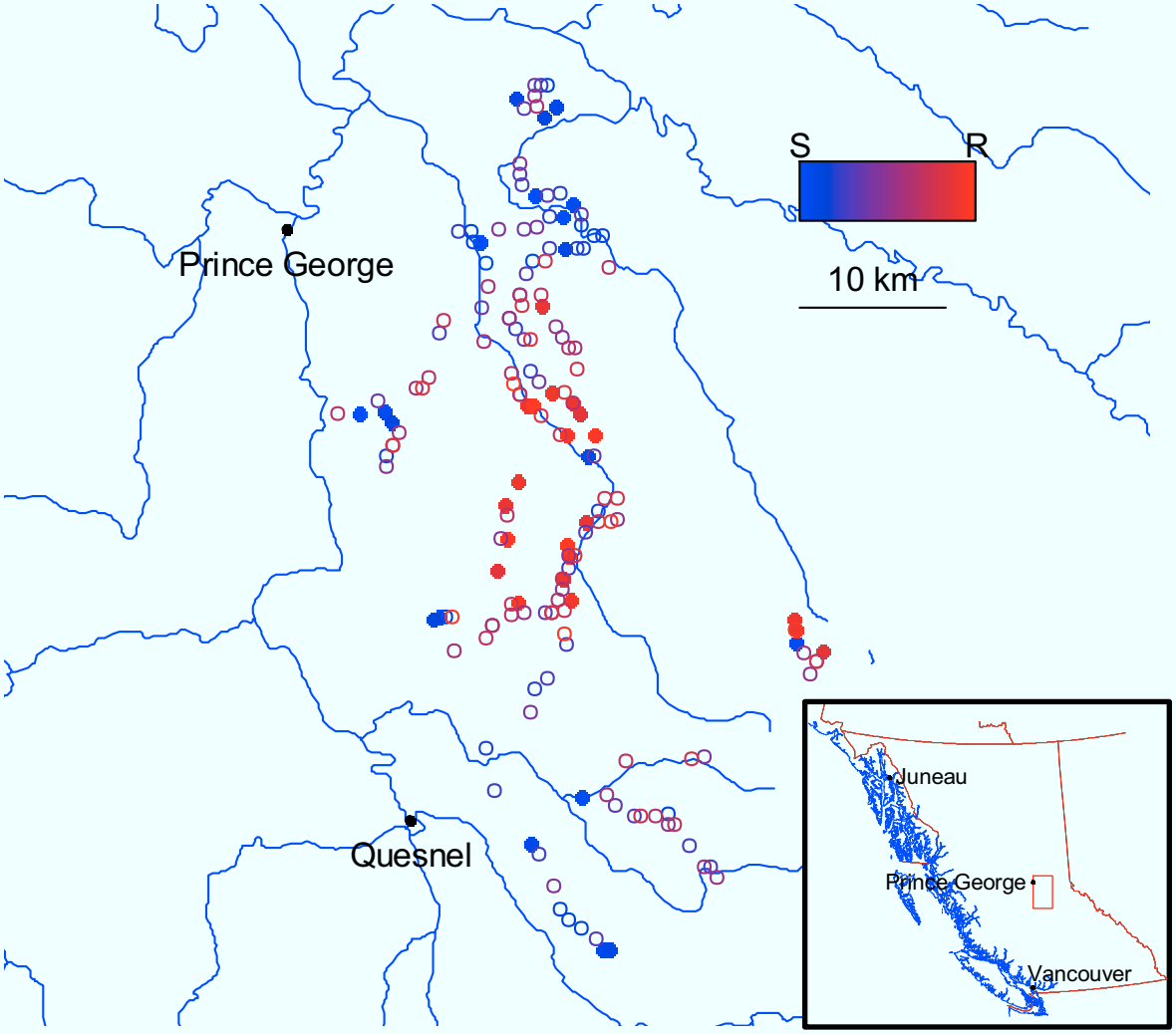
Fig. 2: Percentage of damage trees among progenies. Progenies are ordered from the less damaged to the most damaged. Resistant and susceptible families are located on the left and on the right, respectively. White bars and black bars show selected families for the present study.

Fig. 3: Smoothed densities color representation of volcano plot, showing the differential expression levels of 18725 genes between resistant and susceptible trees. Significant down-regulated and up-regulated genes are shown in blue and red respectively. *FC* = Fold change, *P* = *P*-value.

Fig. 4: Heat map of the 191 significantly differently expressed genes between susceptible and resistant trees to the white pine weevil. Blue and red squares at the top of the heat map indicate susceptible and resistant trees, respectively. Tree labels are indicated at the bottom as follow: the tree phenotype (R = resistant, S = susceptible), the family rank in progeny tests for resistance (1 = the most resistant; 179 = the most susceptible) and then the family number.

Fig. 5: Significantly overrepresented GO terms of genes among significant up-regulated or down-regulated genes between susceptible and resistant trees. Fisher's exact tests with multiple testing corrections were performed using Blast2GO software. Only Go categories with FDR lower than 0.05 are shown.

Fig. 6: Phylogenetic analysis of spruce sHSP. The tree was derived by Neighbor-joining method with bootstrap analysis (1000 replicates) from alignment of amino acid sequences of sHSP of rice, *Arabidopsis* and poplar. Bootstrap values higher than 50% are shown next to the branches. Phylogenetic analyses were conducted in MEGA4. EST clones ID of *Picea* are indicated in bold and underlined. Down-regulated sHSP are indicated by a closed black circle.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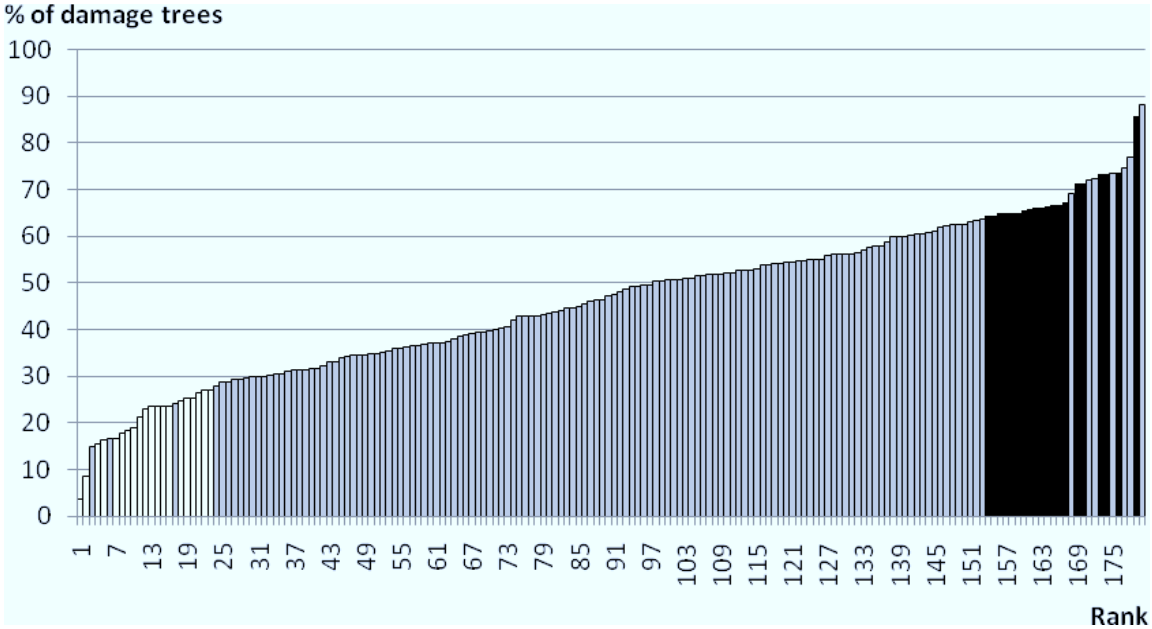
R
Prince George
10 km
Quesnel
Juneau
Prince George
Vancouver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49
55
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157
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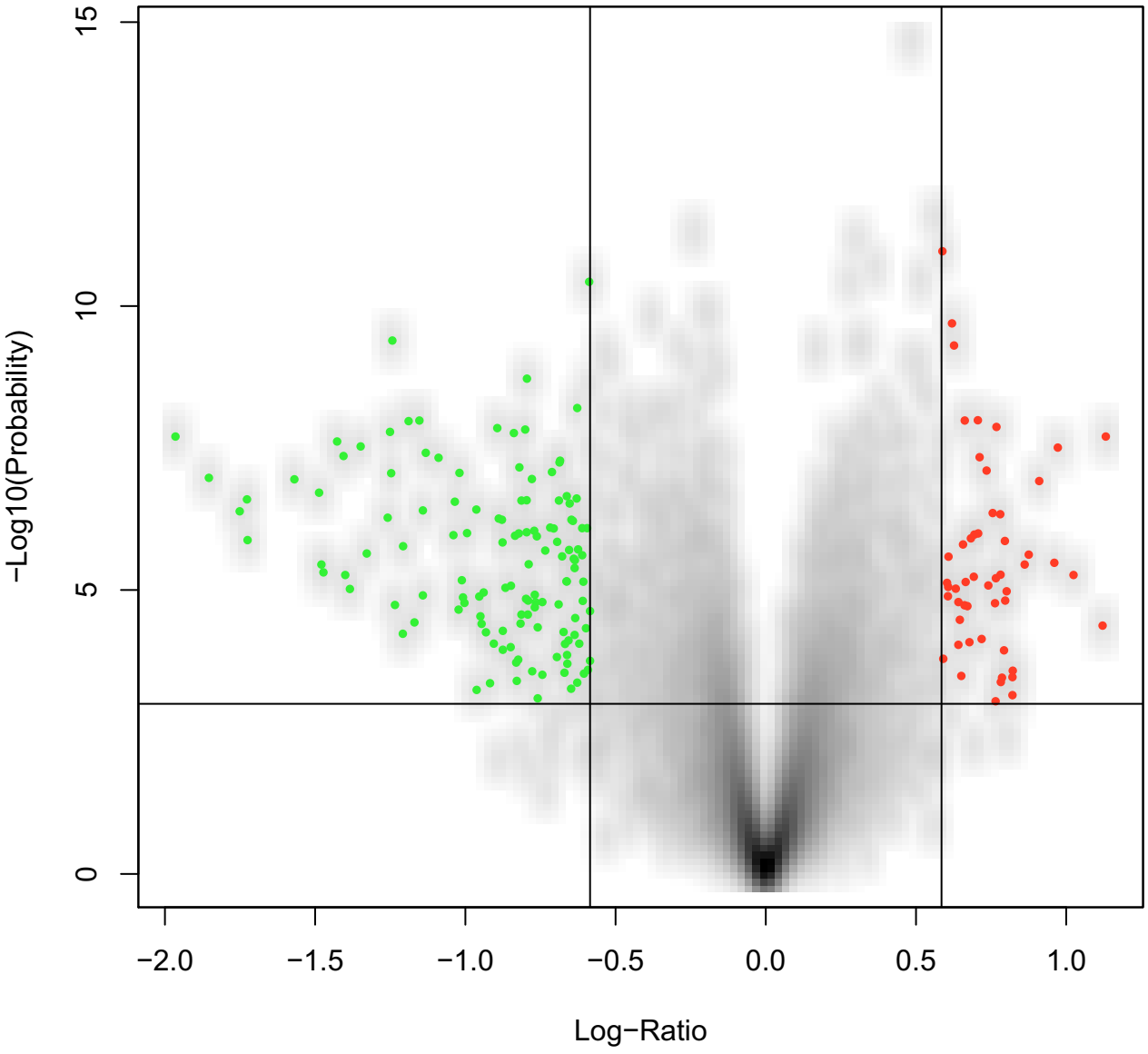
169
175
Rank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−1.0
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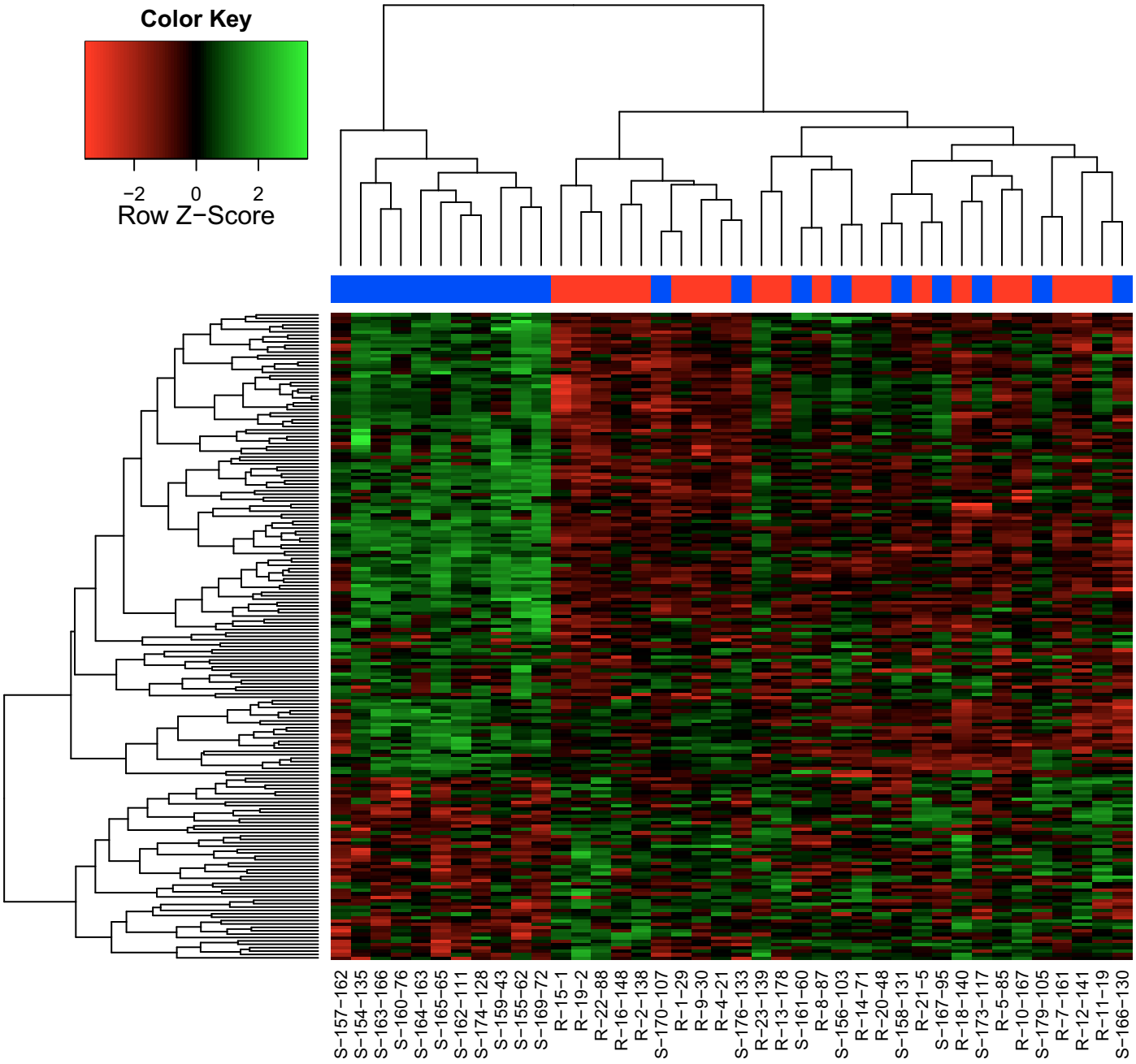
0.0
0.5
1.0
Log−Ratio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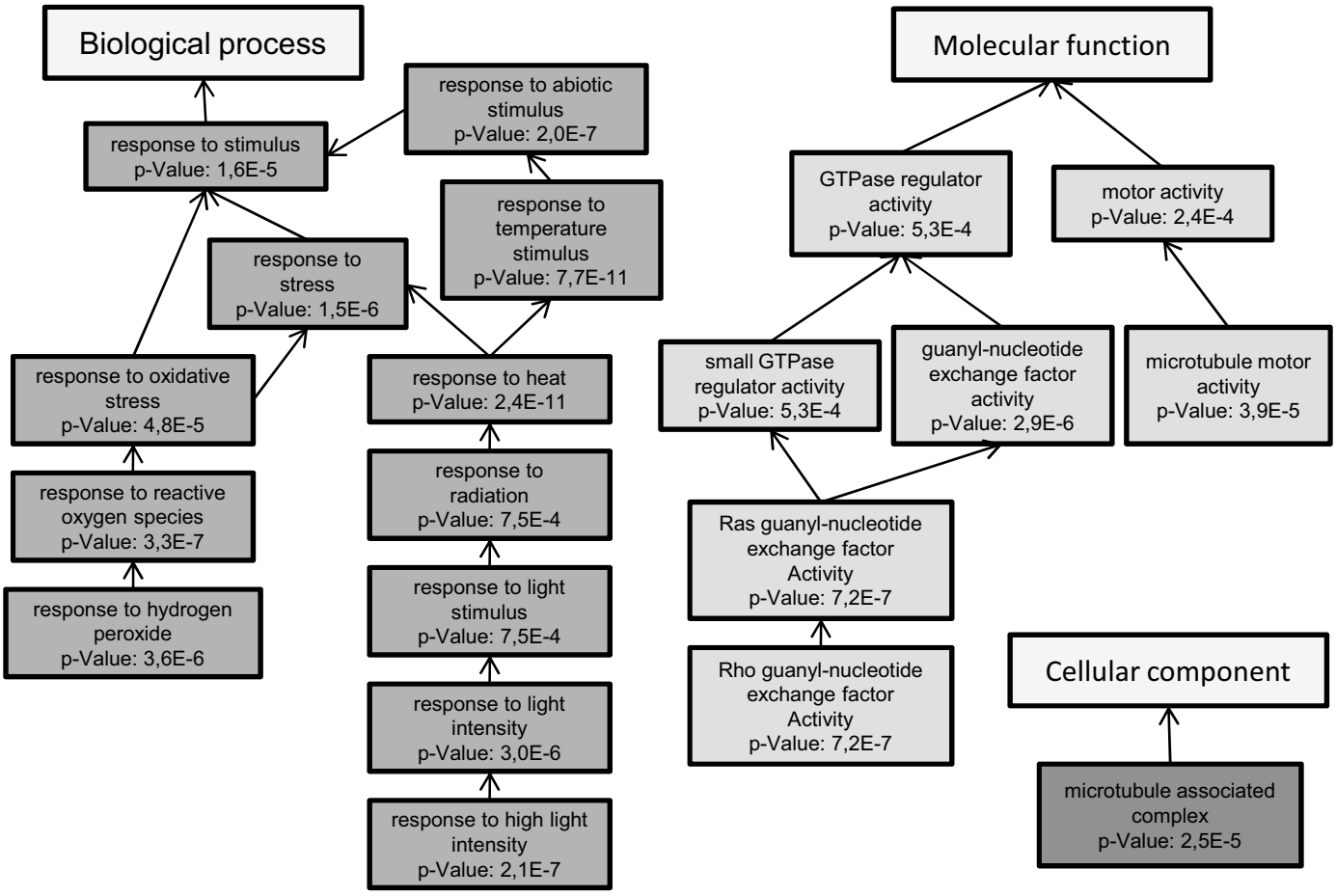
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response to light intensity
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response to high light intensity
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Cellular component
microtubule associated complex
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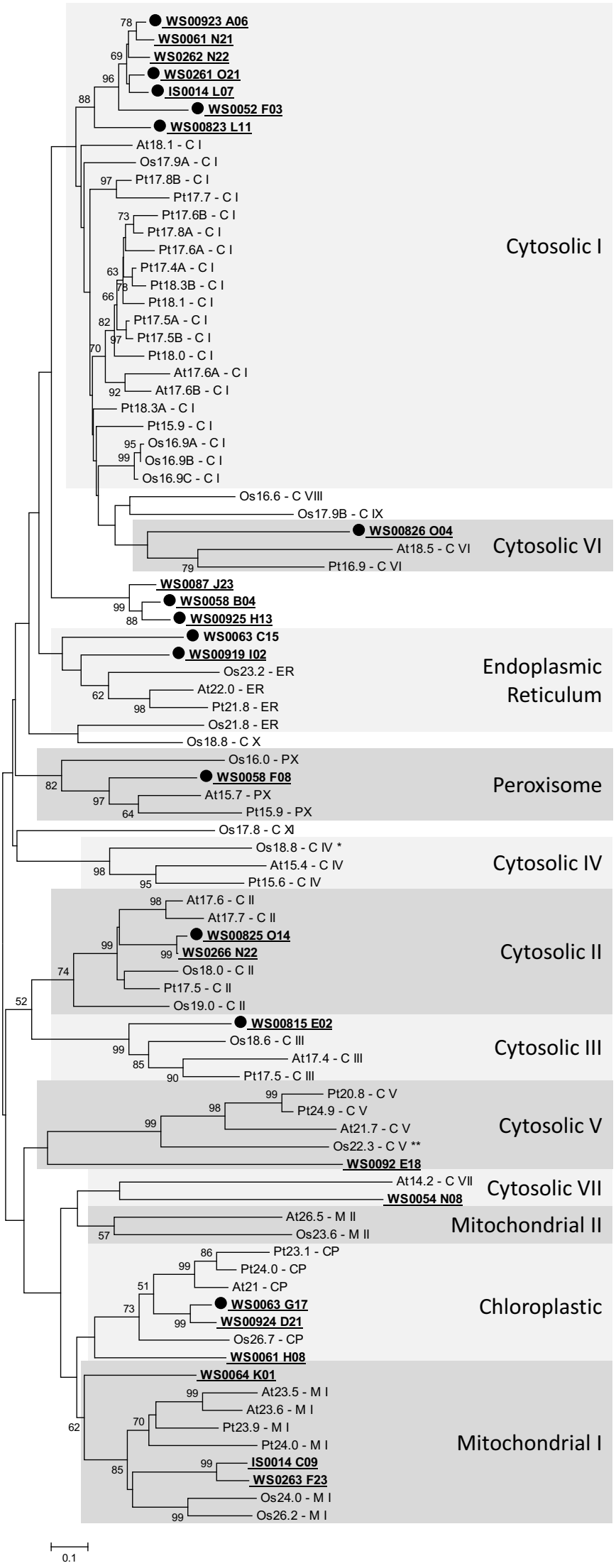
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Pt17.8B - C I
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Pt17.6A - C I
Pt17.4A - C I
Pt18.3B - C I
Pt18.1 - C I
Pt17.5A - C I
Pt17.5B - C I
Pt18.0 - C I
At17.6A - C I
At17.6B - C I
Pt18.3A - C I
Pt15.9 - C I
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Os16.9B - C I
Os16.9C - C I
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Os18.8 - C X
Endoplasmic Reticulum
Os16.0 - PX
WS0058 F08
At15.7 - PX
Pt15.9 - PX
Peroxisome
Os17.8 - C XI
Os18.8 - C IV *
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