ABSTRACT

EMERSON, JENNIFER LYNN. Gene Expression in Fraser Fir Related to Plant Defense and Infestation by the Balsam Woolly Adelgid. (Under the direction of Drs. John Frampton and Ross Whetten.)

Microarray technology was used to study gene expression changes in Fraser fir (*Abies fraseri* (Pursh) Poir.) following the application of chemicals known to be involved in plant defense signaling pathways, as well as changes related to infestation by the balsam woolly adelgid (*Adelges piceae* (Ratzeburg)).

Jasmonic acid, salicylic acid, sodium nitroprusside, and quercetin were applied to the foliage of Fraser fir seedlings, and gene expression was studied 24 hours after a single chemical application, one week after a single chemical application, and 24 hours after the last of seven daily applications. Cellular processes, metabolic processes, and response to stimulus were the functional categories with the greatest number of differentially regulated genes, as compared to the control. Many of the differentially expressed genes were known to be involved in general plant stress responses, with many having been found to be differentially regulated in *Arabidopsis thaliana* (L.) Heynh. in response to drought stress, abscisic acid treatment, or increased levels of ozone.

To examine gene expression changes related to balsam woolly adelgid (BWA) infestation, two studies were undertaken, a laboratory infestation study and a field study. In the field sampling study, the gene expression of trees naturally infested with BWA at varying levels, and showing various levels of response to BWA, were compared. Several genes known to be affected by abscisic acid treatment were differentially regulated, as well as one gene related to terpene production. For further study of the apical dominance loss that occurs with BWA infestation, the *A. thaliana* gene PIN3, which is related to plant tropism, may be

of interest. This gene is the best sequence match for a probe found to be differentially regulated due to infestation level and apical dominance loss in the field.

In the laboratory infestation study, gene expression differences between infested and uninfested seedlings of two fir species, Fraser fir and noble fir (*Abies procera* Rehder), were analyzed. Fraser fir is known to be very susceptible to BWA, showing high rates of mortality in natural stands, while noble fir is more resistant. A greater number of genes were found to be differentially regulated in Fraser fir than in noble fir, and multiple genes involved in the JA signaling pathway were found to be upregulated in Fraser fir. Several phenylpropanoid biosynthesis genes were found to be downregulated in Fraser fir, while one was upregulated in noble fir. Of particular interest may be the *A. thaliana* PAL1 gene, a phenylalanine-ammonia lyase related to plant defense, which had high sequence similarity to two probes on the array. One probe was upregulated due to infestation in noble fir, while the other was downregulated in Fraser fir.

Through this series of microarray experiments, a number of genes of interest have been identified. With further research, this information may be useful for future breeding of fir trees resistant to BWA.

Gene Expression in Fraser Fir Related to Plant Defense and Infestation by the Balsam Woolly Adelgid

by
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BIOGRAPHY

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DISSERTATION INTRODUCTION

Fraser fir (*Abies fraseri* (Pursh) Poir.) is found naturally only in a few stands at high elevation in North Carolina, Virginia, and Tennessee. It grows in cool, moist climates at elevations above 1372 m (4,500 ft) in the southern Appalachians (Beck 1990). At its highest elevations, it is found in nearly pure stands, while at lower elevations, it is commonly found growing with red spruce (*Picea rubens* Sarg.) (Beck 1990). It is also grown over a larger area as a Christmas tree species. North Carolina has a large Christmas tree industry, the wholesale value of which exceeds $100 million per year, and Fraser fir production accounts for over 96% of this industry (NC Cooperative Extension 2011).

One of the major pests of Fraser fir is the balsam woolly adelgid (BWA) (*Adelges piceae* (Ratzeburg)), which is an exotic pest introduced from Europe in 1908 (Kotinsky 1916; Balch 1952). Fraser fir has been found to be very susceptible to BWA infestation, and mortality rates as high as 95% have been seen in natural stands (Mitchell 1966; Mitchell and Buffam 2001). Control of BWA in Christmas tree plantations requires regular scouting to look for symptoms of infestation, and if found, the spraying of pesticides is required for control of BWA. Therefore, it would be very desirable to be able to breed for BWA resistance in Fraser fir. In order to do this, more must be learned about the response in Fraser fir to BWA infestation at the genetic level.

The objective of the research here was to begin to identify genes involved in BWA responses in Fraser fir, as well as genes involved in the major plant defense pathways in Fraser fir. Microarrays were used here to study gene expression changes in Fraser fir. The

first chapter is a literature review of BWA and the use of microarrays in plant defense research. The second chapter is a study of gene expression changes in Fraser fir with the application of various chemicals known to be involved in plant defense pathways. The third chapter is a field sampling experiment to study gene expression differences in field grown, naturally infested Fraser fir trees showing varying levels of infestation and symptoms of infestation. Chapter four is a laboratory infestation of Fraser fir and noble fir, to compare gene expression in infested and uninfested seedlings of these two fir species.

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Chapter 1

LITERATURE REVIEW

Balsam Woolly Adelgid

The balsam woolly adelgid, *Adelges piceae* (Ratzeburg), is native to central Europe and was first seen in the United States in Maine in 1908 (Kotinsky 1916). It attacks species of the genus *Abies* (Balch 1952). European fir species generally are not seriously affected by the balsam woolly adelgid (BWA), but for many North American fir species, BWA infestation results in crown dieback or tree death. In 1957, Fraser fir, *Abies fraseri* (Pursh) Poir., trees in the southern Appalachians, on Mt. Mitchell, were first found to be infested with BWA (Speers 1958). Since then, the adelgids have spread to all natural Fraser fir stands and have caused severe mortality. Fraser fir has been shown to be one of the most susceptible of the North American fir species (Mitchell 1966). Research has shown that by 1966, there was 95 to 98% Fraser fir mortality in the Mt. Mitchell area in North Carolina (Witter and Ragenovich 1986). But in some cases, a few Fraser fir trees have survived in areas of high infestation, suggesting that some individuals may possess resistance to BWA.

Most Adelgidae species alternate between primary and secondary hosts, but BWA in North America completes its entire life cycle on the secondary host, *Abies* species, and reproduces only by parthenogenesis (Balch 1952). Three subspecies of the BWA have been identified in North America, *A. piceae piceae* in British Columbia, the southeastern and northwestern United States, *A. piceae canadensis* in Quebec and the maritime provinces of

Canada and the northeastern United States, and *A. piceae occidentalis* in British Columbia (Foottit and Mackauer 1983). The life cycle of the adelgid consists of the egg stage, three larval instars, and the adult. The first instar, which is referred to as the 'crawler', is about 0.4 mm in length and purplish-brown in color. Once the crawler locates a suitable feeding site, it inserts its stylet into the bark, where it will remain permanently attached. It then transforms into a resting stage, which appears flattened, purplish-black in color, and fringed with waxy threads around the dorsal margin, down the mesial line, and outlining the segments. The adelgid will enter dormancy for at least three weeks before its first molt. This is the stage in which the adelgid overwinters and is referred to as the neosistens. The second and third instars are 0.45-0.55 and 0.6-0.67 mm in length, respectively, and are similar in appearance to the adult. The second and third instars and the adult are covered in long, waxy threads and appear to be a white wool mass, and are referred to as the sistentes (Balch 1952). There is also a rare form called the progrediens, which has been seen in Europe and maritime Canada and can be winged or wingless (Balch 1952; Mitchell et al. 1970). BWA are dispersed mainly by air currents, either in the crawler or egg stage (Balch 1952), or by human transport, such as on nursery stock. In Fraser fir stands in the southern Appalachians, BWA populations were found to complete two generations per year in most cases, with a third generation sometimes possible (Amman 1962; Arthur and Hain 1984).

The most important factor to crawler survival seems to be finding a feeding site where the cortical parenchyma is young, such as on callous growth or new shoots. On older stems, they settle in lenticels and crevices where the outer bark has split and the young

parenchyma tissue is near the surface (Balch 1952). The adelgids are mainly found either in the outer part of the crown or on the main stem and large branches. The area of the infestation will depend on the location and tree species (Greenbank 1970). Generally, crown infestations can only be maintained in warmer climates, and in very cold climates, populations can persist through the winter only on the lower part of the stem where they can be protected by snow cover (Greenbank 1970). To feed, BWA insert their stylets intercellularly and feed in the parenchyma. When the stylet is inserted, there is also an injection of saliva that forms a sheath around the stylet and may occasionally flow into adjoining intercellular spaces. During the feeding, the adelgids repeatedly withdraw and reinsert their stylets into a new area (Balch 1952). Their feeding causes several symptoms in susceptible species. There is often gouting, or abnormal swelling of the stem tissue, at the nodes and around the buds, the twigs and smaller branches may show swelling or twisting, and many times apical dominance is lost, with the top of the crown becoming flattened (Brower 1947; Balch 1952). Changes are seen in the cells surrounding the point of insertion of the stylet; the adjacent cells grow in number and in size, have thickened walls and large or multiple nuclei, and a secondary periderm is formed beneath this abnormal tissue (Balch et al. 1964). In addition, the adelgid's feeding causes the formation of xylem tissue that resembles compression wood, and this abnormal tissue is often referred to as "rotholz", meaning red wood in German because of its red color (Balch et al. 1964; Timell 1986). This abnormal xylem tissue consists of cells with thickened cell walls, shorter tracheids that are round rather than rectangular, a larger number of rays, and a greater fibril angle (Doerksen

and Mitchell 1965). These characteristics of the rotholz make the wood dense, brittle, and unfit as lumber (Balch 1952). Heartwood is also formed prematurely (Puritch 1977; Timell 1986). The presence of rotholz is associated with reduced conductance in the sapwood (Mitchell 1967; Hollingsworth and Hain 1991). This suggests that adelgid infestation leads to water stress in the crown, and water stress reduces both photosynthesis and respiration (Puritch 1973), which eventually can cause mortality. Mortality in Fraser fir trees is usually seen after two to five years of infestation (Amman and Speers 1965).

Observations on balsam fir and silver fir have reported that a thickened outer bark is often seen as a result of BWA infestation, and that this seems to aid in the recovery of the tree (Brower 1947; Balch 1952; Kloft 1957). Measurements of moist outer bark, dry outer bark, and total outer bark thicknesses for Fraser fir were found to be greater in those trees with moderate to heavy BWA infestations, which supports the observations that thicker outer bark is the result of BWA infestation in Fraser fir as well (Hollingsworth and Hain 1992). Hollingsworth and Hain (1992) also compared the bark of Fraser fir to that of silver fir, *Abies alba* Miller, from Germany, a species considered tolerant of BWA. The outer bark, which includes all tissues exterior to the necrophylactic periderm, was generally thicker in the silver fir trees than in the Fraser fir trees (Hollingsworth and Hain 1992).

Gene Expression and Plant Defense

Microarrays are a high-throughput technique used to study patterns of gene expression in thousands of genes at one time. This technique has been utilized in studies of a

wide variety of species, and discoveries have been made using microarrays, as well as other methods to analyze levels of gene expression, in the study of plant responses to insects and disease. Many genes involved in defenses against insect attack, pathogens, and wounding have been identified in plants. A small number of signaling pathways have been shown to regulate these defensive genes, with the main signaling pathways being dependent upon jasmonic acid (JA), salicylic acid (SA), or ethylene (Ecker 1995; Howe et al. 1996; Durner et al. 1997; Vijayan et al. 1998). JA has been shown to be crucial in insect defense, such as in tomato (*Lycopersicon esculentum* cv Castlemart) against the tobacco hornworm (*Manduca sexta* L.) (Howe et al. 1996), as well as for pathogen defense in *Arabidopsis* (Vijayan et al. 1998). Insect attacks by leaf feeders such as Lepidopterans have generally been found to induce JA associated genes but not SA associated genes (Hermsmeier et al. 2001; Heidel and Baldwin 2004). Significant differences have been seen between JA levels in *Manduca sexta* L. feeding and the control, but JA levels did not increase due to SA or the SA-analog benzothiadiazole (BTH) treatment of the *Nicotiana attenuata* Torr. plants (Heidel and Baldwin 2004). The JA pathway has also been shown to have different levels of contribution to gene expression for different plant-insect interactions. The use of *Arabidopsis* mutants showed that 67 to 84% of the genes induced by the feeding of the specialist chewing insect, *Pieris rapae* L., are under the control of the jasmonate pathway (Reymond et al. 2004). A constant increase in the levels of jasmonate was seen in leaves damaged by *P. rapae* feeding on *Arabidopsis thaliana* (L.) Heynh. (Reymond et al. 2004). Feeding by the greenbug aphid (*Schizaphis graminum* Rondani), which feeds on the phloem, has been shown to strongly

induce SA regulated defense genes in sorghum (*Sorghum bicolor* (L.) Moench) (Zhu-Salzman et al. 2004).

Studies have shown that there does not appear to be a strict separation into two sets of signaling responses due to attack by insects and pathogens (Fidantsef et al. 1999). Instead, a study on tomato plants found that each different treatment by insects, pathogens, or chemicals had specific patterns of gene expression (Fidantsef et al. 1999). There have also been shown to be differences in gene expression between mechanical wounding and wounding due to insect feeding, as well as for different types of insect feeders. When wounding of leaves was compared to leaf damage due to feeding of *P. rapae* on Arabidopsis, it was found that many genes were induced by both treatments but were generally induced to higher levels in the leaves that were mechanically wounded (Reymond et al. 2000). In addition, there are some cases where genes that were induced by mechanical wounding were not induced by insect feeding, which is likely to be of benefit to the insect (Reymond et al. 2000).

Studies on gene expression responses in Arabidopsis to green peach aphids found that PR (pathogenesis-related) genes associated with an SA-dependent response pathway were induced within 24 to 48 hours after infestation (Moran and Thompson 2001). Aphid feeding did not activate JA-related signaling pathways as strongly as SA pathways in Arabidopsis (Moran and Thompson 2001). Two of the genes that showed greater expression in infested versus uninfested Arabidopsis plants from aphid feeding, PR-1 and BGL2, have also been known to show an increase in expression as a result of Arabidopsis infection by

Pseudomonas syringae van Hall bacteria (Uknes et al. 1992). Artificial wounding with a pin was not found to induce expression of the PR-1 or BGL2 genes in Arabidopsis (Moran and Thompson 2001). It has been hypothesized that aphid feeding is similar to fungal hyphae invasion, which could explain the similarities between responses to pathogens and aphid feeding (Fidantsef et al. 1999). But unlike pathogen induced responses, the increased expression of these genes by aphids did not occur in uninfested leaves, therefore it was not systemic (Moran and Thompson 2001). An increase in PDF1.2 expression was also seen, which shows that the JA and ethylene-dependent pathogen signaling pathway was also induced by the aphid feeding, but it was not induced in uninfested leaves of infested plants (Moran and Thompson 2001). Treatment with the SA-analog benzothiadiazole (BTH) did lead to an increase in PR gene expression, but did not affect PDF1.2 expression (Moran and Thompson 2001).

PINII mRNA expression has been found to generally be associated with wounding and feeding by insect larvae, and P4 has generally been associated with fungal and bacterial infections (Fidantsef et al. 1999). But there were cases with overlap in these responses; aphid feeding induced higher levels of P4 but not PINII expression, which is another example of the similarity in responses to aphids and pathogens (Fidantsef et al. 1999).

Proteinase inhibitor (PI) genes are commonly associated with wound responses and are expressed due to feeding by the aphid *Myzus nicotianae* Blackman in tobacco plants (Voelckel et al. 2004). Feeding from various types of insects, tissue-feeding lepidopterans, mesophyll-sucking insects, and phloem-feeding aphids, all caused an increase in expression

of a common set of genes as well as a decrease in ubiquitin carrier protein transcripts (Voelckel et al. 2004). But compared to the other types of insects, the aphids caused fewer transcriptional responses in tobacco; fewer genes showed changes in expression and those fold changes were smaller (Voelckel et al. 2004). From the aphid feeding, an increase was seen in glutamate synthase, suggesting that the aphids cause an increase in glutamate production, which is a nitrogen transport molecule that supplies nitrogen for amino acid synthesis (Voelckel et al. 2004). A decrease in germin, a H_20_2 generating enzyme, was also observed with aphid feeding (Voelckel et al. 2004). In addition, the aphids did show a preference for feeding on younger leaves, which could be due to either differential gene expression or nutrient content (Voelckel et al. 2004).

Feeding by different types of insect feeders had varying effects on transcription in *Nicotiana attenuata*, but there were many common elements in the transcriptional effects as well (Voelckel and Baldwin 2004b). The transcriptional response to an attack also depended upon the order of attack by different insect species (Voelckel and Baldwin 2004b). Transcriptional changes were seen 24 hours after insect attack, but after 5 days of continual attack, the species-specific changes had disappeared (Voelckel and Baldwin 2004b). The treatments of *Manduca sexta* (chewing feeder) and MeJA treatments showed the most similar effects on localized gene expression, while systemic tissue changes were most similar for the *Tupiocoris notatus* Distant (mesophyll cell feeder), *Spodoptera littoralis* Boisduval (chewing feeder), and *M. sexta* treatments (Heidel and Baldwin 2004). Systemic responses to *Myzus nicotianae* feeding, an aphid phloem-feeder, were very different from the responses to leaf

chewing insect and mesophyll-feeding insect (Heidel and Baldwin 2004). Three nitrogen-uptake and metabolism genes were up-regulated by the aphid feeding and not by the other types of insect feeders (Heidel and Baldwin 2004).

Differences in gene expression have also been seen when comparing generalist and specialist insects of the same feeding guild. When the transcriptional changes due to feeding from three lepidopteran larvae on *N. attenuata* were compared, with two being generalized feeders and one a specialized feeder, the plant's responses to the two generalized feeders were more similar than the responses to the specialized feeder (Voelckel and Baldwin 2004a). Responses to feeding from generalized and specialized chewing insects have also been compared in *A. thaliana*. In this case, similar patterns were seen between the generalized and specialized feeders, with just two genes being induced only by the specialist (Reymond et al. 2004).

A time series microarray study of Arabidopsis wound responses showed that by only 15 minutes after mechanical wounding of leaves, the expression of 20 genes had already been induced (Reymond et al. 2000). These genes included PR genes, touch genes, and genes encoding mitogen-activated kinases (Reymond et al. 2000). The number of genes that were more highly expressed increased to 39 at 90 minutes after wounding, but then decreased; the number of more highly expressed genes was only 13 at 9 hours after wounding and seven by 24 hours after wounding (Reymond et al. 2000). JA levels were also measured through the time series experiment. JA levels reached a maximum peak at two hours after wounding, which corresponded with the peak expression levels of a group of

genes that included some believed to be responsible for JA synthesis (*LOX2* and *AOS*) as well as a gene known to be induced by jasmonate (*JR3*) (Reymond et al. 2000). The levels of a JA precursor, oxophytodienoic acid (OPDA) rose more slowly and reached a maximum peak at six hours after wounding (Reymond et al. 2000). The wounding effects on wild-type Arabidopsis were similar to those of an ethylene insensitive mutant (*ein2-1*), indicating that none of the wound-inducible genes identified here require ethylene (Reymond et al. 2000).

Water stress also appeared to increase expression levels for 31 of the wound inducible genes, and decrease expression of one wound induced gene in Arabidopsis (Reymond et al. 2000). Another study showed that greenbug infestation induced a drought, salt, and low temperature responsive gene and a gene involved in abscisic acid biosynthesis in sorghum (Zhu-Salzman et al. 2004). Photosynthesis-related genes were also found to be suppressed by MeJA treatment of sorghum, possibly for energy reallocation to defense (Zhu-Salzman et al. 2004). Feeding by *M. sexta* has also been found to decrease the expression of photosynthesis-related genes in *N. attenuata* (Hermsmeier et al. 2001).

Much of the gene research on plant defense-related gene expression in tree species has focused on the genus *Populus*. Ralph et al. (2006a) performed one of the first large-scale studies of gene expression in *Populus* related to herbivore feeding, by using microarrays to study *P. trichocarpa* x *P. deltoides* leaves after feeding by forest tent caterpillar (*Malacosoma disstria* Hübner). Over one thousand genes were upregulated after 24 hours of feeding by forest tent caterpillar, while fewer than half that number were downregulated (Ralph et al. 2006a). Genes induced by herbivory included genes known to be involved

octadecanoid and ethylene signaling, transport, general metabolism, secondary metabolism, and transcriptional regulation (Ralph et al. 2006a). Genes associated with photosynthesis were generally down-regulated with forest tent caterpillar feeding (Ralph et al. 2006a).

The effects of gypsy moth (*Lymantria dispar* L.) feeding as well as exogenous jasmonic acid application on gene expression have also been studied in *Populus nigra* L. (Babst et al. 2009). The expression of over 800 genes was affected by one of these two treatments, with only a 14% overlap in the responses to these two treatments (Babst et al. 2009). In particular, gypsy moth feeding affected gene expression in multiple signaling pathways, including jasmonic acid and abscisic acid biosynthetic pathways, as well as primary and secondary metabolism (Babst et al. 2009). Gene expression was also studied systemically, in leaves not being fed on by gypsy moth, and the system effects of feeding were weaker than the local effects, with fewer genes differentially expressed (Babst et al. 2009).

A microarray study of gene expression changes in Sitka spruce (*Picea sitchensis* (Bong) Carrière) with wounding or feeding by spruce budworms (*Choristoneura occidentalis* Freeman) or white pine weevils (*Pissodes strobi* Peck) found many changes in gene expression one or two days after treatment (Ralph et al. 2006b). There was a great amount of overlap in the gene expression changes caused by these three treatments (Ralph et al. 2006b). Certain genes involved in the octadecanoid and ethylene signaling pathways were identified as upregulated, as well as terpenoid and phenolic secondary metabolism genes (Ralph et al. 2006b).

Proteome changes in Sitka spruce due to feeding by the white pine weevil have also been studied (Lippert et al. 2007). The production of ten proteins was found to be induced by white pine weevil feeding, 13 due to wounding, and 32 produced in response to both feeding and wounding (Lippert et al. 2007). Proteins induced by insect feeding include small heat shock proteins, proteins involved in secondary metabolism, and oxidoreductases (Lippert et al. 2007).

Further work is necessary in these tree species to understand transcriptome changes related to defense against insects. The completed genome sequencing of *Populus trichocarpa* (Torr. & Gray) allows for the integration of DNA mapping and transcriptome data (Tuskan et al. 2006). The sequencing of the genome of species in the Pinaceae family will aid in future genetic work with Fraser fir.

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Chapter 2

GENE EXPRESSION CHANGES IN FRASER FIR AFTER APPLICATION OF CHEMICALS INVOLVED IN PLANT DEFENSE SIGNALING

ABSTRACT

Jasmonic acid, salicylic acid, sodium nitroprusside, and quercetin were applied to Fraser fir foliage and gene expression changes were studied 24 hours after application, one week after a single application, and 24 hours after a week of daily chemical applications. Fewer differentially expressed genes were found 24 hours after a single application compared to the other two timepoints studied, one week after a single application and after seven daily applications. Cellular processes, metabolic processes, and response to stimulus were the functional categories with the greatest numbers of differentially regulated genes. The KEGG pathways most highly represented in the differentially regulated genes were metabolic pathways and the biosynthesis of secondary metabolites. Many of the differentially expressed genes seem to be involved in general stress responses, with many having been found to be differentially regulated in *Arabidopsis thaliana* (L.) Heynh. in response to drought stress, abscisic acid treatment, or increased levels of ozone.

INTRODUCTION

Fraser fir (*Abies fraseri* (Pursh) Poir.) is found naturally in several populations at high elevations of the southern Appalachians. In addition, it is grown over a much larger area as a Christmas tree species. It is the main species of the Christmas tree industry in North

Carolina, an industry whose wholesale value exceeds $100 million per year (NC Cooperative Extension 2011). Due to both the ecological and economic importance of Fraser fir, further study of its defense responses to insects is of interest, particularly related to the the balsam woolly adelgid (*Adelges piceae* (Ratzeburg)) (BWA). BWA is an exotic pest that has caused severe mortality to natural stands of Fraser fir, and it is unknown what defense pathways are involved in the tree's response to BWA and other pests.

A small number of signaling pathways have been shown to regulate plant defensive genes, with the main signaling pathways being dependent upon jasmonic acid (JA), salicylic acid (SA), or ethylene (Ecker 1995; Howe et al. 1996; Durner et al. 1997; Vijayan et al. 1998). In addition, nitric oxide (NO) has been shown to be involved in the induction of the hypersensitive response of cell death in plants to pathogens (Delledonne et al. 1998; Delledonne et al. 2001), and the chemical sodium nitroprusside (SN) acts as a donor of NO. SN has been shown to increase the expression of genes related to disease resistance and general defense responses when applied to the roots of *Arabidopsis thaliana* (L.) Heynh. (Parani et al. 2004). When SN is injected into tobacco (*Nicotiana tabacum* L.) leaves, it increases levels of ethylene and SA (Mur et al. 2008).

JA has been shown to be crucial in plant defense against insects, such as in tomato (*Lycopersicon esculentum* cv Castlemart) against the tobacco hornworm (*Manduca sexta* L.) (Howe et al. 1996), as well as for pathogen defense in *A. thaliana* (Vijayan et al. 1998). A number of studies have shown that exogenous application of JA, SA, or ethylene can increase resistance in plants to insects or disease, presumably by activating these signaling pathways.

In sorghum (*Sorghum bicolor* (L.) Moench.), many of the same gene expression changes occur with infestation by the phloem-feeding greenbug aphid (*Schizaphis graminum* (Rondani)) as with the application of SA and methyl jasmonate (Zhu-Salzman et al. 2004). In addition, greenbug infestation was less frequent on sorghum plants that had been treated with methyl jasmonate (Zhu-Salzman et al. 2004). These effects have also been seen in conifer species, including increased resistance in Norway spruce (*Picea abies* (L.) Karst.) to *Ceratocystis polonica* (Siem.) C. Moreau infection and *Ips typographus* (L.) infestation after treatment with JA (Erbilgin et al. 2006; Krokene et al. 2008).

Studies on conifers have shown that JA and ethylene application cause increases in phenolics and formation of traumatic resin ducts, both important parts of induced tree defenses (Franceschi et al. 2002; Hudgins et al. 2004; Hudgins and Franceschi 2004; Erbilgin et al. 2006; Krokene et al. 2008). In Douglas fir (*Pseudotsuga menziesii* Mirbel Franco), application of JA induced ethylene production as well as formation of traumatic resin ducts (Hudgins and Franceschi 2004). The application of an ethylene antagonist on Douglas fir reduced the normal response to the application of JA, where fewer traumatic resin ducts were formed (Hudgins and Franceschi 2004). This indicates that ethylene is downstream from JA and is the ultimate cause of these induced defense responses (Hudgins and Franceschi 2004). Additionally, JA causes increases in terpenoid concentrations in both the wood and the bark, as well as an increase in both concentration and emission of terpenoids from the foliage (Martin et al. 2002; Martin et al. 2003). Both constitutive and inducible terpenoids are known to be a part of the plant defenses of many conifers (Trapp and Croteau 2001). In

Norway spruce, terpene synthase activity increased in the wood and needles of JA-treated trees, indicating that the JA treatment induces *de novo* synthesis of terpenoids (Martin et al. 2002; Martin et al. 2003). In addition to increasing levels of terpenes, in some cases JA treatment also changes the terpene composition (Martin et al. 2003).

Treatment of Norway spruce with JA causes increased levels of SA, indicating a link between the JA and SA pathways in conifers (Kozlowski et al. 1999; Martin et al. 2003). Application of SA to Norway spruce has also been shown to reduce *I. typographus* populations (Urbanek Krajnc et al. 2011). This application of SA to Norway spruce resulted in increased levels of thiols, indicating a relationship between the SA and glutathione pathways (Urbanek Krajnc et al. 2011). Additionally, the level of phenolics decreased more slowly in the SA-treated trees as compared to the untreated trees, which could aid in defense responses (Urbanek Krajnc et al. 2011). Although SA clearly has a role in defense in conifers, it has not been shown to induce the formation of traumatic resin ducts (Hudgins and Franceschi 2004).

In addition to mortality, BWA infestation on Fraser fir also causes the loss of apical dominance, which is an important trait for Christmas tree production. Therefore, along with defense signaling, further study of auxin regulation in Fraser fir is of interest. Quercetin, a flavonoid, is involved in both defense and auxin transport within plants. Flavonoids are inhibitors of auxin transport in *Arabidopsis*, with mutants that do not produce flavonoids showing reduced apical dominance (Brown et al. 2001). The application of quercetin on wheat (*Triticum aestivum* vc Sonora) embryos has been shown to cause an increase in

multiple shoot meristems (Fischer et al. 1997). In addition, quercetin is involved in plant defense, with higher levels of quercetin in groundnut (*Arachis hypogaea* L.) foliage being associated with higher mortality of an insect pest, the tobacco armyworm (*Spodoptera litura* (Fab.)) (Mallikarjuna et al. 2004).

Using microarrays, the expression of thousands of genes can be studied at one time. Although genome sequence information for Fraser fir is limited, more extensive sequence information is known about other members of the Pinaceae family, in particular, loblolly pine (*Pinus taeda* L.). Therefore, the probes for the arrays used in this experiment were based on loblolly pine EST sequences similar to *A. thaliana* genes known to be involved in plant defense. Previous studies have shown success in using heterologous arrays of this type, such as Scots pine (*P. sylvestris* L.) and Norway spruce targets to loblolly pine cDNA probes (van Zyl et al. 2002).

In this study, the chemicals JA, SA, SN, and quercetin were applied to the foliage of Fraser fir seedlings in the greenhouse. Two thirds of the treated seedlings received one chemical application, while one third received chemical applications daily for seven days. Microarrays were used to study gene expression changes in the foliage 24 hours after the single application, seven days after the single application, and 24 hours after the last of seven daily applications in order to identify genes affected by these plant defense signaling compounds.

METHODS

Seventy-five Fraser fir seedlings that had been grown in a greenhouse at North Carolina State University were used in this study. The seedlings were sprayed with chemical or control solutions in July of 2007, with fifteen seedlings per chemical treatment. Five of the fifteen seedlings were treated once and foliage collected 24 hours after treatment. Five seedlings were treated once and foliage collected one week after treatment. Five seedlings were treated daily for seven days and foliage collected 24 hours after the last treatment.

The chemical solutions included were methyl jasmonate (JA), salicylic acid (SA), sodium nitroprusside dehydrate (SN), or quercitin dehydrate (Q). The chemicals were dissolved in ethanol, then de-ionized water and Tween-20 were added to each solution for a final concentration of 9.5% ethanol and 0.1% Tween-20. The solutions were 1.5 mM methyl jasmonate (Cooper and Goggin 2005), 4 mM salicylic acid (Schenk et al. 2000), 150 µM sodium nitroprusside dihydrate (Parani et al. 2004), and 50 µM quercetin dihydrate. A control solution was made with the same concentrations of ethanol and Tween-20. The chemical solutions were sprayed to run-off on the foliage. The quercetin solution was made new each day, and the other solutions were stored at -20°C (Fries et al. 1997).

Sample Preparation

At the end of each treatment period, several branch tips were collected from each seedling and immediately placed into liquid nitrogen and stored at -80°C later that day. The samples were later ground in liquid nitrogen using a mortar and pestle. RNA was extracted

from the ground tissue using the CTAB buffer as described by Chang et al. (1993). The CTAB buffer was added to approximately 100 mg of the ground foliage in a 1.5 µl tube and incubated for 15 minutes at 65°C with shaking. 500 µl of chloroform:isoamyl alcohol (CIA) was added and mixed. The mixture was centrifuged at 4°C for 10 minutes at 10,000 rpm. One-quarter volume of 10M lithium chloride was added to the supernatant and mixed by inverting. The RNA was precipitated overnight at -20°C. The solution was then centrifuged at 4°C for 1 hour at 10,000 rpm. The pellet was re-suspended in 500 µl of SSTE buffer. 500 µl of CIA was added and mixed. This was centrifuged at 4°C for 10 minutes at 14,000 rpm (maximum speed). The supernatant was kept and 1 ml of 100% cold ethanol was added and mixed by inverting. This was kept overnight at -80°C, then centrifuged at 4°C for 1 hour at 14,000 rpm. The supernatant was discarded and 200 µl of 70% cold ethanol was added. This was centrifuged at 4°C for 10 minutes at 14,000 rpm. The supernatant was discarded. The pellet was re-dissolved in 50 µl of water.

A DNase digest was performed to eliminate DNA present in the RNA sample. The starting material was 3 µg of RNA in 6 µl of water, and samples were concentrated in a speed-vac if necessary. Three units of the Promega™ DNase enzyme were used, incubating for 30 minutes at 37°C. Stop solution was added and the sample was incubated at 65°C for 10 minutes for inactivation. The product was purified using a Qiagen® RNeasy® mini spin column and buffers. The RNA product was eluted with 40 µl of water and concentrated down to 10 µl in a speed-vac at 35°C.

A reverse transcriptase reaction was performed to obtain cDNA. 1µl each of oligo dT and dNTP mix (10 mM each) were added and the mixture was heated at 65 °C for 5 minutes. The samples were quickly chilled on ice and spun down. 4µl of 5x first strand buffer and 2 µl of 0.1 M DTT were added and incubated at 42°C for 2 minutes. 1 µl of Superscript® RT II (Invitrogen™) was added and the mixture was incubated at 42°C for 2 hours. The reaction was inactivated by heating at 70°C for 15 minutes. Then 1 µl of RNase H was added and the mixture was incubated at 37°C for 20 minutes.

The cDNA product was cleaned up using Qiaquick® columns (QIAGEN Inc). 200 µl of Qiagen® binding buffer was added to the sample and then was transferred to the column. The column was centrifuged for 1 minute at 13,000 rpm. The column was washed twice with 750 µl of Qiagen® wash buffer. The column was dried by centrifuging for 1 minute, and cDNA was eluted twice with 20 µl of water, incubating for one minute before centrifugation.

Dye incorporation of the cDNA samples was performed using the LabelIT µarray® Cy™3/Cy™5 kits (Mirus Bio Inc). An incubation time of 1 hour was used for the Cy 3 samples and 2.5 hours for the Cy 5 samples. The labeled products were cleaned up again using Qiaquick® columns (QIAGEN Inc), but using a total of 50 µl of water for elution.

Array Design

Combimatrix CustomArray™ 4x2K arrays were used. Probe sequences were designed from EST sequences from other species in the Pinaceae family similar to plant genes known to be involved in plant defense (Chapter 3). The experimental design for the

pairs of samples to be hybridized together is shown in Figure 1. The head and tail of each arrow indicates whether the Cy5 or Cy3 dye, respectively, was used.

Hybridization

Hybridizations were performed using 35 µl of the labeled product. Pre-hybridization and hybridization solutions, as well as wash solutions, were prepared according to the CustomArray™ 4X2K microarray hybridization and imaging protocol (PTL005). Hybridizations were performed in a 45°C rotisserie hybridization oven overnight and washed according to the above protocols. The arrays were scanned in a ScanArray® Lite (PerkinElmer Life Sciences Inc) scanner. The arrays were subsequently stripped following the Combimatrix protocols for stripping and preparation for re-hybridization (PTL001 and PTL002). The pairs of samples were randomly assigned to the order in which they were hybridized. Gridding and data extraction was performed using the CombiMatrix microarray imager software.

Statistical Analysis

Exported data were analyzed in JMP® Genomics (version 4, SAS Institute, Inc.). Intensity values were averaged for any probe that was present on the array more than once. A Loess normalization was performed on the data in JMP® Genomics, using a smoothing parameter of 0.2 and 1 Loess iteration. A mixed model analysis was performed with chemical and dye as fixed effects. Tree(chemical), array, use(array), and chamber(use*array)

were random effects. The degrees of freedom were calculated with the Satterthwaite method. The pFDR multiple testing correction method was used, with α=0.05 (Storey 2002). LS means were calculated to compare each chemical with the control. Mixed model analyses were performed for each collection time separately.

The Blast2GO suite of programs (Conesa et al. 2005) was used to identify the gene ontology of the EST sequences used, based on similarity to annotated genes in public databases. Assignment of the putative genes to biochemical pathways as described in the Kyoto Encyclopedia of Genes and Genomes (KEGG) was also carried out using Blast2GO. The Fisher's exact test in Blast2GO was used to determine significant differences between annotation assignments of significant genes for different chemical treatments or timepoints of collection. The top matches to *A. thaliana* genes were determined using a blastx search in blast+ against the Arabidopsis protein database for each of the EST sequences used to design the probes on the array (Altschul et al. 1990, Altschul et al. 1997). These *A. thaliana* genes were used to compare results here to previous microarray experiments. The *A. thaliana* gene matches for differentially regulated probes were used to search the ArrayExpress Gene Expression Atlas for previous experimental results for these genes; microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the indicated accession numbers (European Bioinformatics Institute 2011).

RESULTS AND DISCUSSION

A total of 538 probes were found to show differential hybridization intensities for the chemical treatments and time periods; these are summarized in Table 1, and a complete list is provided in Table 2. The fewest probes (n=13) showed differential intensities in hybridizations with RNA samples from the tissues collected just 24 hours after the first treatment. In this time period for JA application, six genes were found to be upregulated as compared to the control and two genes were downregulated. The SN treatment caused two genes to be upregulated and three downregulated. The SA treatment had just one gene that was downregulated, while the Q treatment had no significant changes in gene expression as compared to the control. There was one significant gene in common between JA and SN and one gene in common between SA and SN.

For all chemical treatments except for SA, many more probes showed differential intensities between control and treated samples in hybridizations using tissues collected seven days after a single treatment application, compared to the sampling done 24 hours after a single application. A total of 223 different probes were significant for this time period, with only one of these in common for two chemicals. Probe 9692_TC68960, which matches to *A. thaliana* gene AT4G29000, was upregulated one week after a single application of both JA and SN.

For two of the chemical treatments, SN and Q, the greatest number of differentially-hybridizing probes were found in the tissues that had been chemically treated every day for seven days prior to collection. It is not surprising that these multiple chemical treatments

would have a greater effect on gene expression, since they would include genes affected at various time periods after chemical exposure. It is not known why this same pattern was not seen for JA application as well. Ten genes were found to be significant for two different chemicals at this time period (Table 2).

Overall, the JA and SN treatments caused the greatest number of differential hybridization signals compared to the control, with 268 and 303 probes showing differential hybridization expressed over all time periods for the two treatments, respectively. Searching the GO annotation terms from Blast2GO for the 2000 sequences on the array found that 153 contained either the terms jasmonate or jasmonic acid, indicating that these genes are known to be related to the JA signaling pathway, JA biosynthesis, or the response to JA in other plant species. Of these 153 genes, 32 correspond to probes that showed differential hybridization between control samples and samples treated with JA application. Forty-five of these genes were significant in the current experiment for any chemical application.

The SA and Q treatments had only two and 11 differentially expressed genes, respectively. The application of a higher concentration of quercetin solution may have elicited a greater response; previous studies used a much higher concentration, but it was necessary to greatly dilute the quercetin here for it to remain in solution with ethanol and water (Fischer et al. 1997). Interestingly, 99 of the 2000 sequences included on the array contained either salicylic or salicylate as part of the GO terms in the annotation performed in Blast2GO, and 70 of the 99 are known to be involved in the response to salicylic acid stimulus. It is not known why only two probes were found to be differentially regulated due

to SA application here, but the concentration of SA applied in the treatment may not have been high enough; the concentration used was based on a previous study done with *A. thaliana*, which has foliage that is very different than that of Fraser fir. Fraser fir foliage is thick with a waxy cuticle, which may make it harder for chemicals to penetrate. Previous studies applying SA to *A. thaliana* have used concentrations varying from 0.30 mM to 4 mM, while a SA concentration of 100 mM has been applied to the bark of Norway spruce trees and a 10 mM solution was used as a soil drench for slash pine (*Pinus elliottii* Engelm.) seedlings (Schenk et al. 2000; Davis et al. 2002; Kliebenstein et al. 2006; van Leeuwen et al. 2007; Urbanek Krajnc et al. 2011). Therefore, it is not known what the proper concentration of SA solution would be for application to Fraser fir foliage. Additionally, a shorter period of time between treatment and collection may have produced greater numbers of differentially regulated genes; a study with SA application on *A. thaliana* found greater numbers of differentially expressed genes just four hours after the chemical application, as compared to 28 and 52 hours after treatment (van Leeuwen et al. 2007).

Figure 2 shows the gene ontology biological categories of the significant probes at all tissue-collection timepoints for the JA and SN chemical treatments as assigned by Blast2GO as a percentage of the total number of probes in that category found on the entire array. The cell proliferation category was only found to be differentially regulated by the SN treatment and not by JA treatment. Cell wall organization or biogenesis genes were only found to be downregulated by JA and SN treatment and not upregulated. A greater percentage of rhythmic processes appeared to be upregulated by JA treatment than SN, while a larger

percentage of rhythmic processes were downregulated by SN treatment. A larger percentage of probes involved in signaling appeared to be downregulated than upregulated by SN treatment. Figure 3 shows the gene ontology for the probes found to be significant for all chemicals in the collection one week after a single chemical application and the collection made after 7 chemical applications. These are also given as a percentage of the total number of probes in that category found on the entire array. The cell proliferation category was only represented in the chemical treatment each day for seven days; there were not any genes significant in this category one week after a single chemical application. Genes in the cell wall and biogenesis category were only found to be downregulated, there were no upregulated genes in this category. A greater percentage of rhythmic processes appeared to be upregulated one week after a single chemical application than after seven daily applications. No significant annotation differences were found between any of the chemical treatments or time periods when the lists of significant probes were compared using the Fisher's Exact Test in Blast2GO.

The KEGG pathways associated with differentially regulated genes are shown in Table 3. None of the genes found to be differentially regulated by SN or Q treatments had KEGG enzyme code assignments, so these treatments are not included in the table. Metabolic pathways and biosynthesis of secondary metabolites are the pathways most highly represented for all treatments. One gene involved in monoterpenoid synthesis and another gene involved in terpenoid backbone synthesis were upregulated by JA treatment.

A BLAST search was performed to determine the top *A. thaliana* gene match for the EST sequences used to design the probes on the array. These *A. thaliana* genes were used to search the Gene Expression Atlas for previous microarray results (European Bioinformatics Institute 2011). Thirty-one of the genes differentially regulated here have previously been shown to be affected by drought stress in *A. thaliana* (ArrayExpress expts E-MEXP-2435, E-MEXP-2377, E-MEXP-1863, E-ATMX-32). Six of these genes that were affected by drought stress in prior experiments (AT1G67310, AT3G12500, AT3G47090, AT2G27660, AT2G40010, AT1G30900), as well as two additional genes of significance (AT3G48050, AT5G61850), have also been shown to be affected by abscisic acid treatment in *A. thaliana* (ArrayExpress expts E-MEXP-475, E-GEOD-3454). It is expected that similar genes may be expressed under drought stress and abscisic acid treatment since these two are related; abscisic acid levels are often higher in plants that are under drought stress.

Sixteen of the differentially regulated genes have also been found to be affected by increased levels of ozone in previous microarray studies of *A. thaliana* (ArrayExpress expt E-MEXP-1863). Most were up-regulated with ozone levels, regardless of whether they were found to be up- or down-regulated in the current experiment. A gene found to be upregulated by SN application (AT1G64200) in this experiment has also been shown to be upregulated by jasmonate and methyl jasmonate application in *A. thaliana*, as well as by increased ozone levels (ArrayExpress expts E-MEXP-883, E-GEOD-4733, E-MEXP-1863). It is VHA-E3, a vacuolar H^+-ATPase. In addition, it was downregulated with ethylene or drought stress

(ArrayExpress expts E-GEOD-537, E-MEXP-1863). These all indicate that many general stress response genes are being affected by these chemical applications.

Four genes (AT3G47090, AT5G58280, AT5G57420, AT2G27660) have also been shown to be differentially regulated by treatment of *A. thaliana* cell cultures or plants with SA (ArrayExpress expts E-GEOD-3709, E-GEOD-3984). In our experiment, expression levels of three of these genes were found to be affected by SN and one by JA.

A microarray study was done previously by Babst et al. (2009) where JA was applied to *Populus nigra* L. foliage, and gene expression changes one day after JA application were examined. Results from this study were compared with our results by using the *A. thaliana* sequence matches. One gene found to be significantly upregulated by JA application in *P. nigra* was also found to be significantly upregulated in Fraser fir one day after application of JA or SN (AT3G15352). Fifteen other genes found to be upregulated by Babst et al. (2009) were also found to be differentially up- or down-regulated at the one week collection timepoint in the current study by JA, SN, or Q. One gene that was found to be downregulated by JA in *P. nigra* (Babst et al. 2009) was found to be upregulated by JA in Fraser fir one day after application (AT4G22670). This indicates that there may be differences between tree species in gene expression responses. The fact that the same gene might be upregulated one day after JA application in *P. nigra* and downregulated one week after application in Fraser fir could also indicate changes in gene expression over time. There were also ten additional genes downregulated in *P. nigra* that were found to be differentially up- or down-regulated in Fraser fir one week after application of JA or SN.

Gene expression changes after SN application were previously studied in *A. thaliana* by Parani et al. (2004). Eight genes significant here with seven applications of SN were also found to be differentially regulated with SN treatment in *A. thaliana* (Parani et al. 2004). In some cases, the genes were oppositely regulated in the two experiments; this may be because in the current experiment the treatment was seven applications of SN, while in the experiment by Parani et al. (2004) SN was applied only once and plant tissues were collected one hour after the chemical treatment. None of the genes that were significantly differentially regulated here after a single treatment were found to be significant by Parani et al. (2004). This may be because tissues here were never collected less than 24 hours after a chemical treatment; we might have identified other differentially expressed genes if collections had been made sooner after treatment.

One gene found to be significantly downregulated by JA one week after application is AT1G32640, or MYC2, is known to be upregulated by JA in *A. thaliana* for six hours after application, and, along with other factors, is involved in the activation of the JA and ABA pathways (Lorenzo et al. 2004). It is unexpected that this gene would be downregulated in the current experiment, but it is possible that it was upregulated sooner after treatment, but was not sampled soon enough after treatment in the current study.

The gene AT3G01420 is the top BLAST match for two of the probes in the current study. One of these probes was found to be downregulated by JA one week after a single treatment, while the other was upregulated after seven applications of JA. This *A. thaliana*

gene is also known as α-DOX1, and expression has been reported to be induced by bacterial inoculation, as well as by treatment with SA or SN (De León et al. 2002).

Two probes found to be significantly down-regulated in the current study after seven applications of JA and SA correspond to the *A. thaliana* PAD4 gene (AT3G52430). Expression of the PAD4 gene has been shown to be up-regulated in *A. thaliana* from three to 48 hours after infestation by the green peach aphid, and it is believed to be involved in defense against the aphid (Pegadaraju et al. 2005; Pegadaraju et al. 2007). Since this gene involved in insect resistance is down-regulated after seven chemical applications of JA or SA, it indicates that repeated applications of these chemicals may hinder insect resistance, the opposite of the desired effect. Other genes known to be involved in plant defense were also found to be downregulated after seven days of chemical applications. These include AT1G33520, AT3G48090, AT5G06320, and AT2G37040 which were downregulated after seven applications of SN. AT1G33520, or MOS2, is thought to be downstream from PAD4 and required for a disease resistance gene pathway (Zhang et al. 2005). AT3G48090 (EDS1) is a gene that is also required for plant disease resistance R gene function in *A. thaliana* (Falk et al. 1999), and AT5G06320 (NHL10) is known to be upregulated in *A. thaliana* in response to the cucumber mosaic virus (Zheng et al. 2004). PAL1 (AT2G37040) is a phenylalanine-ammonia lyase responsible for the first step in phenlypropanoid biosynthesis. It is known to be upregulated in bean (*Phaseolus vulgaris* L.) by wounding or fungal infection (Liang et al. 1989).

Overall, many of the genes affected by chemical application in this experiment seem to be general stress response genes, because they have been shown to be affected by other environmental stress conditions. Genes involved in many biological pathways were identified as being differentially regulated by JA and SN applications. Future research may include further study of genes found to be differentially regulated here, in order to better determine gene functions in Fraser fir. Eventually, this research on the genome of Fraser fir and its relation to defense and insect infestation could aid in the breeding of Fraser fir trees that are resistant to BWA infestation.

Table 1. The number of up-/down-regulated probes for each treatment.

Chemical	1 application/24 hours	1 application/1 week	7 applications
Methyl Jasmonate	6/2	107/94	30/29
Salicylic acid	0/1	1/0	0/0
Sodium nitroprusside	2/3	8/11	115/164
Quercetin	0/0	3/0	3/5

Table 2. List of probes (n=538) found to be significantly differentially regulated, the chemical treatment(s) and timepoint(s) of significance, whether it was up or down regulated, fold change between the treatment(s) and the control, and the closest match to *A. thaliana*.

Probe Name	Chemical Treatment, Timepoint, Up/Down Regulated	Fold Change		*A. thaliana* Name
10002_TC64971	JA a1t7 up	1.12		AT4G37770
10054_TC74170	JA a1t7 down	0.79		AT3G25600
10066_CF663440	SN a7t7 up and JA a1t7up	1.08	1.22	AT3G13000
10086_TC67945	SN a1t7 up	1.12		AT2G02380
10173_TC73332	SN a7t7 down	0.93		AT4G02890
10187_TC79991	JA a1t7 down	0.93		AT2G30620
10217_TC61402	SN a7t7 up	1.08		AT3G59770
10222_DR100618	JA a1t7 down	0.90		AT4G30250
10241_TC66428	SN a7t7 down	0.94		AT2G42690
10246_TC67072	JA a7t7 up	1.16		AT3G50360
10260_CF401150	JA a7t7 up	1.11		AT4G17500
10287_TC57781	JA a1t7 up	1.60		AT1G15520
10310_TC66681	SN a7t7 down	0.73		AT2G37340
10324_TC61899	SN a7t7 down and JA a1t7 down	0.73	0.86	AT3G16110
10363_TC66903	SN a1t7 down	0.81		AT4G25515
10372_TC60892	SN a7t7 down	0.87		AT1G80870
10410_TC72591	JA a1t7 down	0.83		AT4G06634
10414_TC76384	SN a7t7 down	0.96		AT1G33520
10430_TC62567	JA a1t7 up	1.23		AT4G29270
10434_TC66802	SN a7t7 down and JA a1t7 up	0.81	1.13	AT2G18980
10459_AW011180	JA a1t7 down	0.65		AT4G26370
10468_TC68978	JA a1t7 up	1.25		AT2G36740
10476_TC67561	SN a7t7 down	0.94		AT5G05280
10504_TC58120	JA a7t7 down	0.92		AT3G22400

Table 2 (Continued)

10518_DR694260	SN a7t7 down	0.95		AT4G14465
10525_TC65868	SN a7t7 down	0.96		AT2G06530
10573_TC58744	SN a7t7 up	1.06		AT2G45640
10574_TC77485	SN a7t7 down	0.93		AT1G52150
10590_TC70855	JA a1t7 up	1.22		AT1G23800
10603_DN609729	JA a1t7 down	0.71		AT3G13960
10624_TC73559	JA a1t7 down	0.76		AT5G09810
10654_AW042784	JA a7t7 down	0.71		AT1G07890
10670_TC66131	SN a7t7 up	1.07		AT4G38970
10684_TC61621	JA a1t7 down	0.83		AT2G18790
10697_TC59453	JA a1t7 down	0.86		AT5G40020
10737_TC63428	SN a7t7 down	0.90		AT5G36930
10758_TC58709	JA a1t7 down	0.71		AT2G24270
10778_DR691330	JA a1t7 up	1.25		AT3G06510
1078_TC59650	JA a7t7 up and SN a7t7 up	1.05	1.30	AT1G35720
10836_TC68511	JA a1t7 up	1.12		AT3G48050
10837_TC65800	SN a1t7 down	0.65		AT1G53540
10885_TC69655	SN a1t1 up and JA a1t1 up	1.21	1.28	AT3G15352
10905_TC57805	JA a1t7 down	0.82		AT3G12500
10914_TC76067	JA a1t7 up	1.01		AT3G24590
10929_DR688643	SN a7t7 down	0.89		AT4G38860
10946_TC76050	SN a7t7 up	1.08		AT5G01410
1097_TC67540	JA a7t7 up	1.17		AT4G03520
110_TC73810	SN a7t7 up	1.10		AT2G41840
11022_TC71760	SN a7t7 down and JA a1t7 up	0.97	1.14	AT2G18160
11072_TC74759	JA a1t7 down	0.73		AT5G36970
11117_TC63886	Q a7t7	0.96		AT3G06720
11127_TC65847	SN a7t7 down	0.90		AT1G02305
11130_BE187188	JA a1t7 down	0.86		AT2G28500
11154_TC66901	SN a1t7 down	0.92		AT5G59970
11208_TC72978	JA a7t7 down	0.91		AT4G11650
11240_TC75389	Q a7t7	0.90		AT1G06430
1127_TC64709	JA a1t7 up	1.08		AT5G06150
1128_TC72973	JA a1t7 up	1.06		AT5G41380
11294_DR164092	JA a1t7 down	0.80		AT1G08560
11317_TC69421	JA a1t7 up	1.22		AT4G02570
11322_TC72002	SN a7t7 up	1.04		AT2G38120
11360_TC72973	JA a1t7 down and SN a7t7 up	0.81	1.02	AT5G41380
11413_TC62058	SN a7t7 up	1.09		AT1G49180

Table 2 (Continued)

11440_TC68482	JA a1t7 up	1.24		AT5G06950
11454_DR055152	SN a7t7 up	1.41		AT3G20770
11488_TC57462	SN a7t7 down	0.94		AT3G52430
11549_TC59910	SN a7t7 down	0.87		AT3G58570
11550_new00735	JA a1t7 down	0.96		AT5G19780
11555_TC72348	JA a1t7 up and SN a7t7 up	1.15	1.22	AT1G59660
11578_DR692273	SN a7t7 down	0.90		AT4G24400
11597_TC70508	SN a7t7 up	1.05		AT1G51170
11612_TC59061	JA a1t7 up	1.14		AT3G20740
11621_new16776	SN a7t7 down	0.84		AT3G19820
11637_TC72431	JA a1t7 up	1.18		AT3G15510
1164_TC58167	SN a7t7 down	0.88		AT3G17390
11674_TC62649	SN a7t7 down	1.00		AT2G46690
11691_TC58325	JA a1t7 up	1.45		AT2G36830
11810_TC63456	JA a1t7 up	1.08		AT1G69550
11823_TC74016	SN a7t7 up	1.20		AT4G34700
11824_TC60978	SN a7t7 down	0.94		AT5G12480
11831_TC75093	JA a1t7 up	1.14		ATCG00170
11862_DR168055	JA a1t7 up	1.27		AT2G45660
11900_CO361093	JA a1t7 up	1.34		AT2G02850
11945_TC61592	JA a1t7 down	0.84		AT1G59218
11954_TC78575	JA a7t7 down and SN a7t7 down	0.81	0.88	AT5G08520
12021_TC71485	SN a7t7 down	0.97		AT5G61850
12085_TC63026	SN a7t7 up	1.34		AT1G60620
12094_TC74306	SN a7t7 up	1.39		AT5G51260
12117_TC76493	JA a1t7 down	0.81		AT5G21482
12139_TC63554	JA a1t7 up	1.29		AT3G06590
12143_BE582317	SN a7t7 down and Q a7t7 up	0.95	1.16	AT5G43730
12146_TC59240	SN a7t7 up	1.23		AT1G14920
12147_CV031438	JA a1t1 up	1.16		AT4G23900
12230_TC76161	SN a7t7 down	0.86		AT1G71090
12233_TC60889	SN a7t7 down	0.98		AT5G23420
12258_TC60964	JA a1t7 up	1.06		AT5G65380
12262_TC71025	SN a7t7 down	0.86		AT2G17820
12289_TC75865	SN a7t7 down	0.82		AT4G27270
12319_TC68097	JA a1t7 up	1.27		AT5G28640
12324_TC70095	SN a7t7 down	0.84		AT5G52640
12325_TC74159	SN a7t7 down	0.92		AT5G67510
1234_TC58763	JA a1t7 down	0.81		AT3G23990

Table 2 (Continued)

12347_AA556306	SN a7t7 down	0.87		AT5G15850
12353_TC71976	JA a7t7 up	1.09		AT2G23700
12375_AW870226	SN a7t7 up	1.33		AT5G15450
12389_CX651381	JA a1t7 down	0.91		AT3G01420
12398_TC59832	SN a7t7 up	1.27		AT5G13960
12405_TC65372	SN a7t7 down	0.74		AT3G01910
12413_TC75836	JA a1t7 down	0.81		AT5G05280
1246_new08544	SN a7t7 down	0.83		AT5G49190
12469_DR685815	SN a7t7 up	1.03		AT5G37490
125_TC67241	JA a7t7 down	0.91		AT1G61770
12531_new00588	SN a1t7 down and SN a7t7 down and JA a7t7 down	0.68 0.93	0.69	AT1G30270
12533_TC58009	JA a1t7 down	0.77		AT5G63110
1286_TC79504	JA a1t7 up and SN a7t7 up	1.32	1.42	AT1G17880
1322_TC71384	JA a7t7 up	1.10		AT5G44210
1345_DR017655	JA a1t7 up	1.42		AT1G06760
1391_DR116620	SN a7t7 up	1.02		AT1G56440
143_TC59286	Q a1t7 up	1.00		AT2G04410
151_TC68643	SN a7t7 up	1.16		AT3G51420
1537_TC79995	SN a7t7 down	0.76		AT4G26740
1554_TC57349	JA a1t7 up	1.16		AT2G36530
1583_AW042955	SN a7t7 up	1.03		AT5G52640
1588_TC59039	JA a1t7 up	1.10		AT2G15220
1632_TC74059	JA a1t7 down	0.84		AT3G14290
1662_TC66623	JA a7t7 up	1.08		AT5G08260
1686_BQ700903	JA a7t7 up	1.33		AT3G22400
1689_TC65504	JA a1t7 up	1.06		AT5G60860
172_TC73446	SN a7t7 down	0.89		AT3G48090
1738_TC59532	JA a1t7 up	1.06		AT1G10760
1769_TC70618	JA a1t7 down	0.80		AT4G37800
1781_TC57267	SN a7t7 down	0.85		AT5G06320
1802_DR686575	JA a1t7 up	1.29		AT3G25700
181_DR692879	JA a1t7 up	1.17		AT4G37170
1825_TC75902	SN a7t7 up	1.11		AT1G20990
1907_TC77760	JA a1t7 up	1.19		AT1G68130
1912_TC66635	JA a7t7 down	0.92		AT5G40580
1918_TC78119	JA a1t7 down	0.77		AT1G09250
1928_TC59317	JA a1t7 down	0.74		AT5G62000
1929_TC77760	JA a1t7 up	1.04		AT1G68130

Table 2 (Continued)

1985_TC61849	JA a1t7 down	0.71		AT5G42650
2012_TC64361	SN a7t7 up	1.32		AT5G23240
2058_TC65923	JA a1t7 up	1.11		AT5G15800
2065_TC66815	SN a7t7 up	1.06		AT4G26910
2066_BX681405	JA a7t7 down	0.94		AT3G18140
2100_TC59250	SN a1t7 up	1.12		AT3G19500
2111_TC59673	JA a1t7 up	1.31		AT1G46264
2166_CX651381	JA a7t7 up	1.26		AT3G01420
2189_TC58563	JA a1t7 up	1.22		AT2G45660
2192_TC60849	JA a1t7 up	1.21		AT4G02050
2245_TC77392	JA a1t7 down	0.90		AT5G46050
2249_TC77804	SN a7t7 up and JA a1t7 up	1.17	1.21	AT3G46550
2255_TC76932	JA a7t7 down	0.82		AT2G07560
2267_TC77547	SN a7t7 down	0.84		AT3G61970
2290_AW010840	JA a7t7 up and SN a7t7 up	1.10	1.25	AT4G09960
2292_TC72349	SN a7t7 down	0.81		AT3G09200
230_TC73077	JA a7t7 down	0.88		AT1G59870
233_DR168611	SN a7t7 down and Q a7t7 down	0.61	0.91	AT3G15020
2336_CN852406	JA a7t7 down and Q a1t7 up	0.95	1.28	AT3G15510
2345_TC61190	SN a7t7 up	1.04		AT2G33880
235_TC68944	JA a1t1 down	0.81		AT3G24120
2363_TC77305	JA a1t7 down	0.80		AT1G32640
2370_TC70871	SN a1t7 up	1.31		AT5G47750
2376_TC74830	SN a7t7 down and JA a1t7 up	0.77	1.13	AT2G45570
2379_TC58712	SN a7t7 down	0.92		AT5G23540
2401_DN613155	JA a1t7 up	1.13		AT1G25280
2410_TC68627	SN a7t7 down	0.84		AT1G68920
2426_TC75567	SN a7t7 down	0.77		AT1G35710
2501_TC69847	JA a1t7 down	0.94		AT1G29860
2509_TC74866	SN a7t7 down and JA a1t7 up	0.81	1.16	AT2G02240
2514_TC57650	JA a1t7 down	0.82		AT4G16260
2525_TC67337	SN a7t7 up	1.76		AT1G68810
2569_TC59085	JA a1t7 up and SN a7t7 up	1.15	1.38	AT5G53000
2595_TC68958	SN a7t7 up	1.15		AT5G64950
2627_TC78353	SN a7t7 down	0.84		AT1G76690
2630_DR684850	SN a7t7 down	0.76		AT1G18450
2634_TC75040	SN a1t1 down	0.83		AT3G25860
2637_DR385644	JA a1t7 down	0.85		AT1G66340
2663_TC61121	SN a7t7 up	1.05		AT2G26350

Table 2 (Continued)

2691_TC74228	JA a1t7 up	1.20		AT4G13260
2702_new16341	JA a7t7 up	1.19		AT2G30860
2709_DR079681	SN a7t7 down	0.96		AT3G24490
2710_TC66863	JA a7t7 up	1.10		AT5G20630
2714_TC65372	JA a1t7 up	1.12		AT3G01910
2720_TC65032	JA a1t7 down	0.92		AT1G74520
2725_TC68736	JA a7t7 up	1.04		AT5G18640
2782_TC76351	JA a7t7 up	1.18		AT1G67310
2863_DR110419	JA a1t7 down	0.84		AT3G54260
2871_DR059007	SN a7t7 up and JA a1t7 up	1.11	1.22	AT1G70250
2886_new00576	JA a1t7 up	1.39		AT5G24270
2891_TC73605	SN a7t7 up	1.15		AT5G59320
2939_DR682973	JA a1t7 up	1.18		AT2G40470
2995_BX680984	JA a1t7 down	0.86		AT2G41630
3033_TC73643	JA a7t7 up	1.46		AT1G60710
3042_TC57390	SN a7t7 down	0.90		AT3G53980
3061_TC67395	SN a7t7 down	0.83		AT2G37040
3100_BE582317	Q a1t7 up	1.13		AT5G43730
3143_TC69325	SN a7t7 up	1.03		AT2G35490
3151_CX645455	SN a7t7 down	0.84		AT1G27940
3155_TC75038	SN a7t7 up	1.27		AT2G20560
3162_TC80318	SN a7t7 down	0.95		AT5G48670
3163_TC59556	SN a1t1 down	0.80		AT1G16220
320_TC66527	JA a1t7 up	1.03		AT1G78370
3246_TC62401	SN a7t7 up	1.02		AT1G14980
3325_TC58878	SN a7t7 down	0.85		AT2G46600
3331_TC59202	JA a7t7 down and SN a1t7 up	0.91	1.41	AT1G60690
3353_TC58474	SN a7t7 down	0.99		AT4G36220
3355_TC75500	SN a7t7 down	0.78		AT1G69550
3368_DR095292	SN a7t7 up	1.12		AT5G12040
3403_DR011185	JA a1t7 up	1.79		AT1G80460
341_BX255756	SN a7t7 down	0.79		AT2G44940
3436_new00179	SN a7t7 up and JA a7t7 up	1.00	1.05	AT2G02120
3446_TC74137	SN a7t7 down	0.79		AT4G10250
3447_TC57939	JA a7t7 down	0.84		AT1G20440
3458_CF385349	JA a1t7 up	1.12		AT3G44480
3464_DR694479	SN a7t7 down	0.91		AT4G24250
3485_TC63642	SN a7t7 up	1.03		AT1G66880
3496_TC73583	JA a7t7 up	1.07		AT1G55300

Table 2 (Continued)

3532_TC57905	JA a1t7 down	0.94		AT3G03980
355_TC58996	SN a7t7 up	1.01		AT5G16990
3565_TC61588	JA a1t7 up	1.18		AT5G19690
3568_TC60423	JA a1t7 down and SN a7t7 up	0.80	1.03	AT3G50070
3589_AW755073	SN a7t7 down	0.98		AT2G44120
3598_TC67188	SN a7t7 down	0.84		AT5G57420
360_TC73351	SN a7t7 down	0.95		AT3G15010
3602_TC74059	JA a1t7 down	0.81		AT3G14290
3631_BE187150	SN a7t7 up	1.07		AT5G49630
3652_BX682339	SN a7t7 down	0.77		AT5G45110
3735_TC59106	SN a7t7 up	1.08		AT2G30110
3755_TC69120	JA a1t7 down and SA a1t1 down	0.77	0.80	AT4G27280
3755_TC69120	SN a1t1 down	0.96		AT4G27280
3760_TC67968	JA a1t7 down	0.83		AT3G20570
3773_TC69128	SN a7t7 down	0.93		AT1G07540
3790_CO363752	JA a1t7 down	0.84		AT1G05910
3816_TC74997	SN a7t7 up	1.17		AT2G32300
3840_DR111775	SN a7t7 down	0.75		AT5G20720
3848_TC57723	SN a7t7 up	1.09		AT3G51680
3878_TC66792	JA a1t7 up	1.16		AT1G70080
3914_TC62039	JA a1t7 up	1.33		AT5G48880
3948_TC76766	SN a7t7 up	1.24		AT1G51340
3950_CF667698	Q a7t7 up and JA a1t7 up	1.04	1.29	AT3G28360
3954_CX715832	JA a1t7 up	1.30		AT4G26530
3966_DR388612	SN a1t1 up	1.22		AT5G09810
3982_TC59240	SN a1t7 down	0.84		AT1G14920
4011_TC67480	SN a7t7 down	0.87		AT1G55510
4029_TC73955	JA a1t7 down	0.83		AT4G30210
411_TC59773	SN a7t7 down	0.93		AT1G72680
4111_TC75155	SN a7t7 down	0.96		AT2G46600
4122_BX679843	JA a1t7 up	1.26		AT5G17880
4162_TC74665	SN a7t7 up	1.23		AT3G57290
4243_TC74231	SN a7t7 down	0.86		AT5G61510
4313_TC65260	JA a1t7 up	1.28		AT1G11530
4340_DR069063	SN a7t7 up	1.04		AT3G62260
4355_TC77745	SN a7t7 up	1.17		AT2G45430
4368_TC75154	JA a1t7 up	1.15		AT2G39220
440_TC72200	SN a7t7 down	0.93		AT2G42990
4423_TC59760	SN a7t7 down	0.84		AT1G32230

Table 2 (Continued)

443_TC58886	JA a1t7 down	0.89		AT2G39770
4440_TC60742	JA a1t7 down	0.92		AT4G32551
4443_TC68501	JA a1t7 down	0.98		AT1G08230
4448_CF476660	SN a7t7 up	1.29		AT1G69550
4453_TC62639	SN a7t7 down	0.87		AT2G27660
4472_TC70180	SN a7t7 down	0.90		AT2G35530
4490_TC66207	SN a7t7 down	0.99		AT2G40010
4505_TC75865	SN a7t7 down	0.95		AT4G27270
4552_TC67395	JA a1t7 down	0.83		AT2G37040
4557_TC59660	SN a7t7 down	0.80		AT3G19760
4595_BX000681	SN a7t7 up	1.06		AT1G05850
4602_TC68634	SN a7t7 up	1.04		AT2G13650
4612_TC63147	JA a1t7 down	0.85		AT4G15475
4635_CF475551	SN a7t7 down	0.85		AT3G44750
466_TC57724	JA a1t7 down	0.85		AT2G01250
4679_DR024442	SN a7t7 down and JA a1t7 up	0.87	1.17	AT4G26200
4728_TC60511	JA a1t7 down	0.84		AT2G18160
4731_TC62620	JA a7t7 up	1.02		AT2G23380
4765_TC59361	JA a1t7 down	0.82		AT3G03590
478_TC67914	JA a7t7 down and JA a1t7 down	0.85	0.90	AT3G10030
478_TC67914	SN a7t7 up	1.09		AT3G10030
4782_TC59851	SN a7t7 down and JA a1t7 up	0.84	1.33	AT5G57170
4792_TC77201	JA a1t7 up	1.16		AT4G24210
4808_BF517387	JA a1t7 up	1.25		AT2G01830
4842_TC67076	SN a7t7 up	1.05		AT1G32860
4858_DR060757	JA a1t7 down	0.79		AT1G22380
4864_TC57724	SN a7t7 down	0.87		AT2G01250
4880_DR088528	SN a7t7 up	1.03		AT1G03010
4909_TC59650	SN a7t7 down	0.90		AT1G35720
491_DN609855	SN a7t7 up	1.09		AT3G54940
4929_DR016307	SN a7t7 down	0.86		AT1G79000
4943_TC69363	SN a7t7 down	0.69		AT1G71870
4996_TC66796	SA a1t7 up	1.11		AT5G62940
5012_TC75845	SN a1t7 up	1.05		AT4G03960
5014_TC70485	SN a7t7 down	0.97		AT3G57430
505_TC57015	JA a1t7 up	1.07		AT4G11650
506_DR179068	JA a1t7 down	0.72		AT2G19810
5062_TC75511	SN a7t7 down	0.87		AT5G05580
5077_TC62756	SN a7t7 down	0.91		AT5G48150

Table 2 (Continued)

517_TC57839	SN a7t7 up	1.15		AT2G03200
5186_TC58210	SN a7t7 up	1.07		AT4G01850
5214_TC78723	SN a7t7 down	0.98		AT3G16510
5219_TC72918	SN a7t7 down	0.89		AT1G47128
5223_DR682530	SN a7t7 up	1.49		AT1G32360
5296_TC65864	SN a7t7 down	0.92		AT2G29500
5342_TC73522	JA a1t7 up	1.15		AT5G09810
5370_AW758552	JA a1t7 down	0.90		AT1G20510
5378_BX678239	SN a7t7 down	0.73		AT1G73690
5385_TC68085	JA a1t7 up	1.24		AT2G26430
5432_BF610347	JA a1t7 down	0.77		AT1G36160
5438_TC67978	JA a1t7 down	0.66		AT4G02570
5454_TC76065	SN a7t7 down	0.95		AT3G17070
5503_TC68483	JA a1t7 down	0.70		AT4G22260
5521_TC61437	SN a7t7 down	0.85		AT2G02040
5544_new01285	JA a1t7 down	0.77		AT5G04630
5547_TC70204	JA a1t7 down	0.91		AT3G12120
5560_TC69752	JA a1t7 up	1.17		AT3G63500
5597_NP542707	SN a7t7 up	1.49		AT1G76180
5603_TC77926	JA a7t7 down and SN a7t7 up	0.90	1.14	AT4G31880
5610_TC68442	JA a1t7 up	1.29		AT3G04930
5646_DR682504	SN a7t7 up	1.10		AT3G03660
5651_TC68833	SN a7t7 down	0.98		AT1G62300
5698_new17289	JA a1t7 down	0.89		AT1G77490
5700_TC68582	JA a7t7 up	1.06		AT4G33360
5775_TC74314	SN a7t7 up	1.20		AT5G65270
5788_CO170920	JA a1t7 up	1.15		AT5G01920
5790_TC74066	JA a1t7 down	0.84		AT4G25130
587_TC68766	SN a7t7 up and JA a7t7 up and JA a1t1 up	1.29	1.63	AT1G49950
5885_TC57748	JA a7t7 up	1.16		AT1G73050
5901_TC74120	SN a7t7 up	1.11		AT1G64200
5903_TC68372	JA a1t7 up	1.20		AT1G02065
5930_CO363342	JA a1t7 down and SN a7t7 down	0.90	0.97	AT5G07990
5932_CO169484	JA a1t7 down	0.86		AT1G22380
5945_TC65751	SN a7t7 up	1.18		AT4G33300
5959_CO201375	SN a7t7 up	1.08		AT3G12580
5980_TC67834	SN a7t7 up	1.24		AT3G51780
5990_TC63122	SN a7t7 down	0.83		AT4G18910
5998_TC74502	SN a7t7 down	0.74		AT4G24210

Table 2 (Continued)

6023_CO175144	JA a7t7 up	1.09		AT5G20250
6040_TC78246	JA a1t1 up	1.05		AT3G62550
6055_CO169087	SN a7t7 up	1.25		AT5G01240
6096_TC69428	SN a7t7 up	1.06		AT4G18550
6129_TC70547	SN a7t7 up	1.05		AT2G40950
6153_TC73890	JA a1t7 down	0.78		AT2G34420
6156_DR081791	SN a7t7 down	0.96		AT2G29500
6159_TC62512	SN a7t7 down	0.86		AT1G20000
6172_TC69998	JA a1t7 down	0.94		AT4G33720
6180_TC64083	SN a7t7 down	0.82		AT5G54250
6189_DR060450	SN a7t7 up	1.25		AT2G43330
6192_TC69212	SN a7t7 up	1.21		AT3G25810
6232_TC74066	JA a1t7 up	1.37		AT4G25130
6252_TC66342	SN a7t7 up	1.12		AT2G16770
6284_TC76626	SN a7t7 up and JA a1t7 up	1.23	1.38	AT1G75510
63_DR012434	JA a1t7 down	0.77		AT3G04880
6342_BQ635260	SN a7t7 up	1.18		AT4G02570
6359_TC59106	JA a1t7 down	0.95		AT2G30110
6372_TC74733	JA a1t7 down	0.92		AT4G29040
641_DR692647	SN a7t7 down	0.90		AT4G00370
6415_TC76696	JA a7t7 down	0.99		AT2G02090
6443_TC74636	SN a1t7 down	0.93		AT1G10630
6461_CO364351	SN a1t7 down	0.94		AT1G67030
6472_TC68769	Q a7t7	0.75		AT4G02680
6486_TC68722	JA a1t7 down	0.91		AT1G80820
6499_AI812599	JA a1t7 up	1.63		AT1G76900
6568_TC76937	JA a1t7 down	0.77		AT1G34420
6646_TC58856	SN a7t7 down	0.95		AT2G38120
6665_TC75148	SN a7t7 down	0.63		AT4G29230
671_TC74998	JA a7t7 up	1.20		AT5G48150
6723_TC59834	SN a7t7 down	0.80		AT5G06710
674_new08724	JA a1t7 down	0.79		AT1G13440
6752_TC69440	SN a7t7 down	0.85		AT1G12910
6756_TC67636	SN a7t7 down	0.80		AT3G17460
6785_TC59518	SN a7t7 down	0.85		AT1G30900
6800_BM157643	SN a7t7 down	0.63		AT1G58170
6818_CD020615	JA a1t7 up	1.18		AT3G27000
6845_TC71081	SN a7t7 down	0.98		AT5G11260
6894_TC59620	JA a1t7 up	1.20		AT2G36780

Table 2 (Continued)

6907_CX714820	SN a7t7 down	0.81		AT2G35110
6956_TC75121	SN a7t7 down and JA a1t7 up	0.76	1.27	AT5G66320
6971_TC76780	JA a7t7 down	0.91		AT1G35710
7014_TC74347	SN a7t7 up	1.49		AT5G02810
7016_TC74618	SN a7t7 down	0.98		AT1G15520
7036_TC58492	SN a7t7 down	0.92		AT5G42800
7053_TC68826	JA a7t7 up	1.16		AT5G16370
7087_TC58841	SN a7t7 down	0.88		AT5G20510
7104_TC70320	SN a7t7 down	0.81		AT3G30530
7136_TC65923	SN a7t7 up	1.04		AT5G15800
7170_TC57358	SN a7t7 down	0.95		AT3G48090
7189_TC78724	SN a7t7 up	1.07		AT4G38510
7214_DR691051	JA a1t7 up and SN a7t7 up	1.09	1.12	AT4G36400
7259_DR178847	SN a7t7 down	0.78		AT1G24360
7260_TC62009	SN a7t7 down	0.78		ATCG00190
7339_TC59631	JA a7t7 up	1.14		AT3G16100
7370_TC74516	JA a7t7 down	0.99		AT3G08710
7378_TC58691	JA a1t7 up	1.06		AT3G59380
7382_TC75461	JA a1t7 up	1.17		AT5G05350
7395_TC58456	SN a7t7 down	0.64		AT4G05320
7472_TC58189	SN a7t7 up	1.04		AT4G38130
7487_TC70513	SN a7t7 down	0.82		AT2G37060
7500_TC62646	JA a1t7 down	0.70		AT2G26580
7512_new16993	SN a7t7 down and JA a1t7 up	0.83	1.35	AT1G60710
7516_TC78164	SN a7t7 down	0.97		AT5G66750
7520_TC68482	JA a1t7 down	0.73		AT5G06950
753_BF610347	SN a7t7 down	0.85		AT1G36160
7537_CO366247	JA a1t7 down	0.89		AT4G26850
7541_TC74997	JA a1t7 up	1.26		AT2G32300
7545_BI077229	SN a7t7 up	1.19		AT1G69040
7546_TC57084	JA a1t7 up	1.26		AT5G26990
7549_TC74434	JA a1t7 up	1.20		AT4G10250
756_TC73554	SN a7t7 up	1.08		AT3G23810
7569_TC61464	JA a7t7 up	1.14		AT1G09070
7585_CF395157	JA a1t7 down	0.80		AT3G47570
76_TC58520	SN a7t7 down	0.96		AT5G66390
7601_new18180	SN a7t7 up and JA a1t7 up	1.06	1.29	AT4G21150
7635_CO369472	JA a7t7 up	1.07		AT4G34150
7648_TC60787	SN a7t7 up	1.35		AT3G45080

Table 2 (Continued)

7661_TC76504	JA a1t1 down	0.70		AT1G80350
7662_CV033156	JA a7t7 down	0.98		AT4G36220
7679_TC61109	JA a7t7 down	0.96		AT1G24120
7709_DR689687	SN a7t7 down	0.92		AT1G17840
7726_TC69116	SN a7t7 up	1.01		AT5G55760
7758_TC75345	SN a7t7 up	1.24		AT2G46690
7767_TC61884	SN a7t7 down and JA a7t7 down	0.73	0.95	AT4G22690
7781_DR164264	SN a7t7 down	0.87		AT3G04740
7792_DR049490	JA a7t7 down	0.90		AT2G40300
7801_TC61916	SN a7t7 down	0.76		AT5G62700
7806_NP1369328	SN a7t7 down	0.89		AT4G11650
7853_DR694006	SN a7t7 up and SN a1t7 up	1.17	1.22	AT5G06150
7861_CF664620	SN a7t7 down	0.70		AT3G44110
7867_TC66235	SN a7t7 up	1.12		AT4G01700
7906_new00004	SN a7t7 down	0.94		AT2G41430
7915_TC59085	SN a7t7 down	0.91		AT5G53000
7933_TC67648	JA a1t7 up	1.17		AT5G63980
7938_TC76639	SN a7t7 up	1.08		AT5G58280
7965_TC72716	SN a7t7 down	0.87		AT3G46230
800_CN852393	SN a7t7 down	0.89		AT3G18130
8000_DR162242	JA a1t7 up	1.13		AT1G37130
8022_TC59481	SN a7t7 down	0.87		AT2G37810
8049_new00152	JA a1t7 up	1.09		AT5G48540
8061_DR049490	SN a7t7 up	1.13		AT2G40300
8070_TC57612	SN a7t7 down	0.71		AT2G01250
8093_AL750356	SN a7t7 up	1.10		AT5G59845
8107_TC58238	Q a7t7	0.90		AT4G27670
8110_TC74501	SN a7t7 down	0.74		AT4G24210
8142_TC57176	JA a7t7 down	0.87		AT5G08670
8164_TC61641	JA a7t7 down	0.91		AT5G02120
8196_new01039	JA a1t7 up	1.22		AT5G10930
8202_DR386396	JA a1t7 up	1.24		AT5G14520
8215_TC69292	SN a7t7 down	0.81		AT5G44790
8246_TC71799	JA a7t7 down	0.88		AT3G53020
8261_TC73531	JA a1t7 down	0.86		AT3G05950
8296_TC66394	SN a7t7 down	0.84		AT2G30050
8350_TC59924	JA a7t7 down	0.84		AT5G51970
837_TC73315	SN a7t7 down	0.72		AT3G52590
8373_DR051728	SN a7t7 down	0.66		AT4G27270

Table 2 (Continued)

8389_DR684486	JA a7t7 down	0.93		AT1G80830
8427_TC70763	SN a7t7 down	0.98		AT2G40830
8523_new08357	JA a1t7 up	1.41		AT1G79550
8543_TC59906	SN a7t7 up	1.13		AT5G16650
8544_TC60049	SN a7t7 down	0.90		AT5G35735
8548_new09433	JA a1t7 down	0.82		AT5G66140
8563_TC60235	SN a7t7 up	1.25		AT2G33800
8588_TC66554	SN a1t7 up	1.12		AT3G55530
8602_TC63004	JA a1t7 up	1.26		AT1G51940
8617_DR055425	JA a7t7 up	1.07		AT1G20640
8618_DR689687	Q a7t7 up	1.20		AT1G17840
8622_TC64070	SN a1t7 down	0.79		AT1G02335
8626_TC71788	SN a7t7 up	1.09		AT4G23280
8637_TC76580	SN a7t7 down	0.78		AT4G30080
8652_TC79637	SN a7t7 down and JA a1t7 down	0.84	0.86	AT1G32360
8679_TC59762	JA a1t7 down	0.86		AT3G24140
8681_TC61121	SN a7t7 down	0.90		AT2G26350
8693_TC66325	JA a1t7 up	1.17		AT4G16260
8700_DR060135	SN a7t7 up	1.17		AT1G55730
8710_CX650473	JA a7t7 down	0.95		AT5G44720
8711_TC73238	JA a1t7 down	0.77		AT1G49760
8720_TC70427	SN a7t7 up	1.19		AT4G12240
8725_TC71892	SN a7t7 up	1.31		AT1G65730
8743_AL749632	SN a7t7 up	1.21		AT5G13650
8761_TC60892	JA a1t7 down	0.92		AT1G80870
8775_TC58960	SN a7t7 down	0.95		AT5G61210
8777_TC65948	JA a1t7 up	1.08		AT5G13930
8819_TC68059	SN a7t7 up	1.01		AT4G28450
8837_TC63216	SN a7t7 down	0.96		AT3G06480
8844_TC79555	JA a1t7 down	0.96		AT1G75390
8847_TC72163	SN a7t7 down	0.77		AT4G37250
8858_TC67978	SN a7t7 down	0.92		AT4G02570
8892_new08210	SN a7t7 down	0.97		AT1G61800
8897_TC61899	SN a7t7 down	0.99		AT3G16110
8908_TC76975	JA a1t7 down	0.86		AT1G70060
8979_TC73661	SN a7t7 up	1.07		AT1G60710
9009_CF395070	SN a7t7 down and JA a1t7 up	0.91	1.10	AT3G04220
9022_TC68643	JA a1t7 up	1.90		AT3G51420
9028_TC62340	SN a7t7 down	0.91		AT3G10330

Table 2 (Continued)

9031_TC75027	JA a1t7 up	1.29		AT1G59940
9040_TC70658	SN a7t7 down	0.76		AT1G02040
9048_TC70254	JA a1t7 down and SN a7t7 up	0.71	1.20	AT3G47090
9058_TC67831	JA a7t7 up	1.11		AT1G29340
9083_TC75737	JA a1t7 up	1.06		AT4G17260
9173_TC61367	SN a7t7 up	1.38		AT2G34680
9211_TC70851	SN a7t7 up	1.15		AT3G04220
9260_TC76271	JA a1t7 down	0.90		AT2G47460
9286_TC59918	JA a1t7 down	0.81		AT1G08830
9295_TC76584	SN a7t7 down	0.87		AT4G39470
9319_BM158807	JA a1t7 down	0.80		AT4G32640
9321_TC65653	JA a1t7 up	1.20		AT3G43810
9325_TC60269	SN a7t7 down	0.88		AT5G27380
9358_TC64083	SN a7t7 down	0.90		AT5G54250
9397_TC76764	JA a1t7 up	1.18		AT1G80830
9448_TC68939	JA a1t7 up	1.02		AT1G20610
9478_DR388612	SN a1t7 down	0.97		AT5G09810
9491_TC58752	JA a1t7 up	1.10		AT1G12520
9507_TC74288	JA a1t1 up	1.42		AT4G22670
9518_TC67241	SN a7t7 up	1.09		AT1G61770
9560_TC64722	JA a1t7 down	0.85		AT2G45570
9575_TC59832	JA a1t7 up	1.27		AT5G13960
9617_TC57371	JA a7t7 down	0.92		AT3G52430
9632_TC60964	SN a7t7 up	1.41		AT5G65380
9634_TC77150	JA a1t7 down	0.88		AT5G51230
9692_TC68960	JA a1t7 up and SN a1t7 up	1.42	1.44	AT4G29000
9704_DR100215	SN a7t7 up	1.11		AT1G19715
9707_TC60933	SN a7t7 down	0.90		AT2G03430
9708_TC62067	JA a1t7 down	0.62		AT1G45249
972_TC60622	JA a1t1 up	1.18		AT3G19380
9723_CF665627	SN a7t7 down	0.96		AT4G19970
9756_TC76228	SN a1t7 down	0.94		AT5G18830
9764_TC66454	JA a1t7 up	1.28		AT3G48990
9768_TC61276	SN a7t7 down	0.95		AT3G24660
9786_TC65929	JA a1t7 down	0.72		AT5G13930
9818_TC69403	SN a7t7 down	0.86		AT1G09320
9824_TC60529	SN a7t7 down and JA a1t7 up	0.86	1.11	AT4G32640
9838_DN632937	SN a7t7 up	1.07		AT1G73660
9853_BX682614	SN a7t7 up	1.01		AT1G75250

Table 2 (Continued)

9894_TC70878	SN a7t7 up	1.31		AT5G06700
9898_TC66471	SN a7t7 up	1.24		AT3G10920
9915_TC74504	JA a7t7 up	1.05		AT4G24210
9941_TC77775	SN a7t7 up	1.11		AT3G27740
9957_TC69136	JA a1t7 down	0.84		AT5G62440
9989_TC76051	JA a1t7 down and SN a7t7 down	0.79	0.89	AT5G01410
9992_TC61956	SN a1t7 down	0.86		AT4G02570
9994_DR016465	SN a7t7 down	0.96		AT1G12570

Table 3. KEGG pathways associated with probes differentially regulated by JA and SN treatment, at all collection timepoints.

Pathway	upregulated by JA	downregulated by JA	upregulated by SN	downregulated by SN
Alanine, aspartate and glutamate metabolism	1	0	1	0
alpha-Linolenic acid metabolism	3	2	0	2
Amino sugar and nucleotide sugar metabolism	1	3	2	0
Arachidonic acid metabolism	1	0	0	0
Arginine and proline metabolism	0	1	0	0
Ascorbate and aldarate metabolism	0	2	0	0
Benzoate degradation	1	0	0	0
Biosynthesis of secondary metabolites	16	11	6	15
Biosynthesis of unsaturated fatty acids	1	0	0	0
Caprolactam degradation	0	0	0	1
Carbon fixation in photosynthetic organisms	1	0	1	0
Carotenoid biosynthesis	0	0	1	0
Chloroalkane and chloroalkene degradation	0	1	0	0
Citrate cycle (TCA cycle)	0	0	0	1
Cyanoamino acid metabolism	2	1	0	1
Cysteine and methionine metabolism	2	0	1	2
Drug metabolism - cytochrome P450	2	2	1	0
Drug metabolism - other enzymes	0	1	0	2
Ethylbenzene degradation	1	0	0	0
Fatty acid biosynthesis	0	1	0	0
Fatty acid elongation	1	0	0	0
Fatty acid metabolism	1	1	0	0
Flavone and flavonol biosynthesis	2	2	0	4
Flavonoid biosynthesis	2	3	0	5
Fructose and mannose metabolism	2	2	1	0
Geraniol degradation	1	0	0	0

Table 3 (Continued)

Glucosinolate biosynthesis	0	0	1	0
Glutathione metabolism	2	5	1	1
Glycerolipid metabolism	2	0	0	1
Glycerophospholipid metabolism	0	0	0	1
Glycine, serine and threonine metabolism	0	2	0	0
Glycolysis / Gluconeogenesis	2	3	1	1
Glyoxylate and dicarboxylate metabolism	1	1	0	0
Histidine metabolism	0	1	0	0
Isoquinoline alkaloid biosynthesis	0	1	0	0
Linoleic acid metabolism	2	3	0	1
Metabolic pathways	24	17	8	20
Metabolism of xenobiotics by cytochrome P450	2	1	1	0
Methane metabolism	4	2	4	3
Microbial metabolism in diverse environments	8	4	2	6
Monoterpenoid biosynthesis	1	0	0	0
Naphthalene degradation	0	1	0	0
Nitrogen metabolism	2	0	0	1
Oxidative phosphorylation	0	1	2	0
Pentose phosphate pathway	1	0	1	0
Phenylalanine metabolism	2	2	1	4
Phenylalanine, tyrosine and tryptophan biosynthesis	1	0	0	2
Phenylpropanoid biosynthesis	6	3	1	4
Photosynthesis	0	1	2	0
Porphyrin and chlorophyll metabolism	0	0	0	1
Purine metabolism	2	0	1	1
Pyrimidine metabolism	2	0	2	1
Pyruvate metabolism	0	0	0	1
Retinol metabolism	0	1	0	0
Starch and sucrose metabolism	2	1	1	1
Sulfur metabolism	4	0	0	2
T cell receptor signaling pathway	1	0	2	0
Terpenoid backbone biosynthesis	1	0	0	0
Toluene degradation	0	0	0	1
Tryptophan metabolism	0	1	0	0
Tyrosine metabolism	0	2	0	1
Ubiquinone and other	0	1	0	0

Table 3 (Continued)

terpenoid-quinone biosynthesis				
Valine, leucine and isoleucine degradation	1	0	0	1
Xylene degradation	0	0	0	1
Zeatin biosynthesis	1	3	0	0

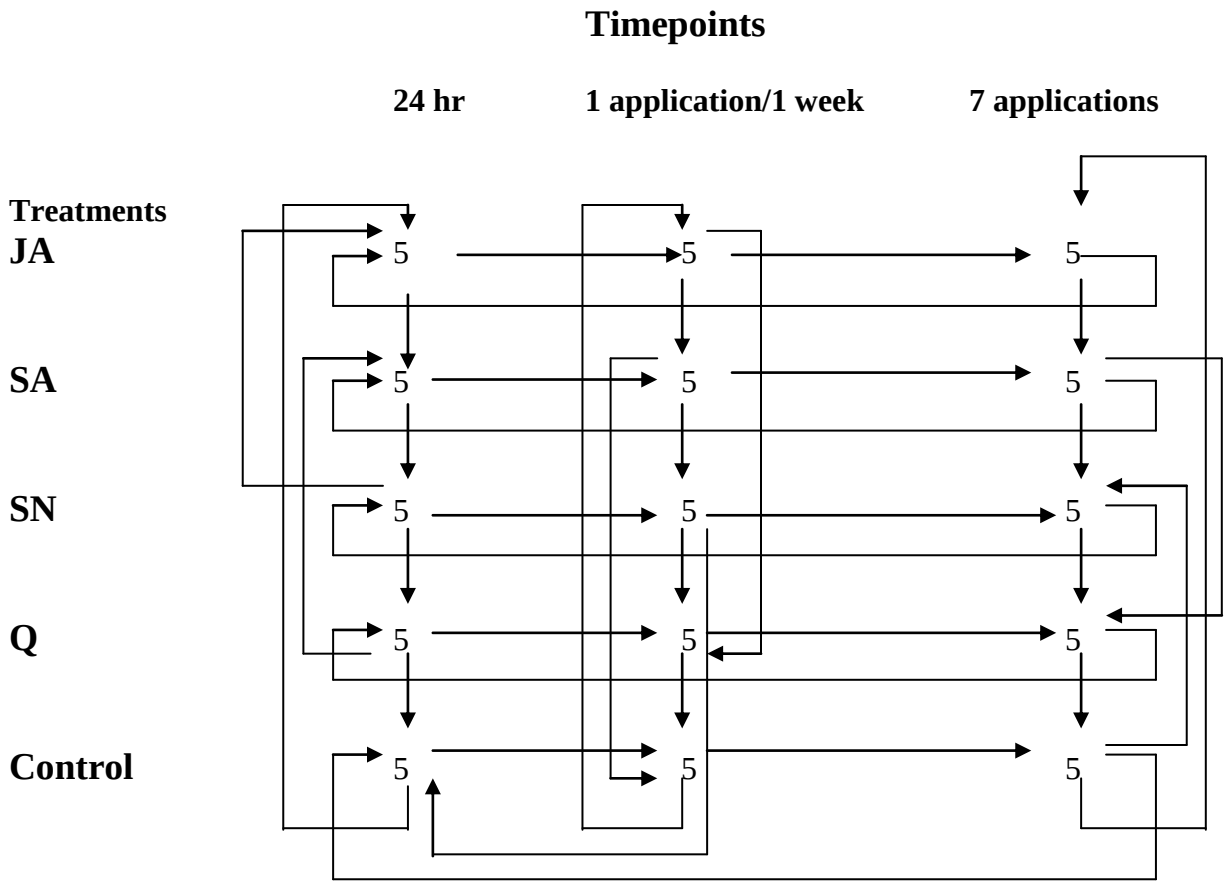

Figure 1. Experimental design for each pair of samples to be hybridized together on the same array. The head and tail of each arrow indicates whether Cy5 or Cy3 dye, respectively, was used for the labeling.

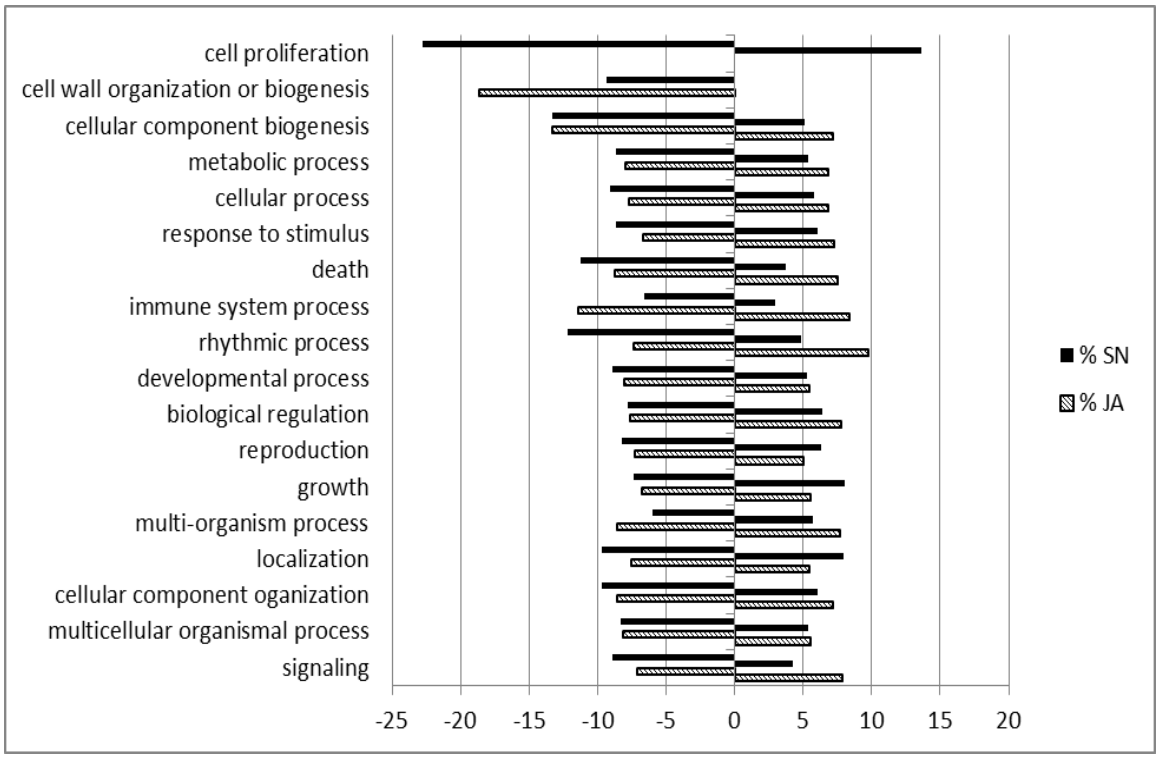

Figure 2. Biological process level 2 categories from Blast2GO for the up-/down-regulated probes for the JA and SN chemical treatments, given as a percentage of the total number of probes on the array in each category. The upregulated genes are shown to the right of the center axis, and the downregulated genes are to the left.

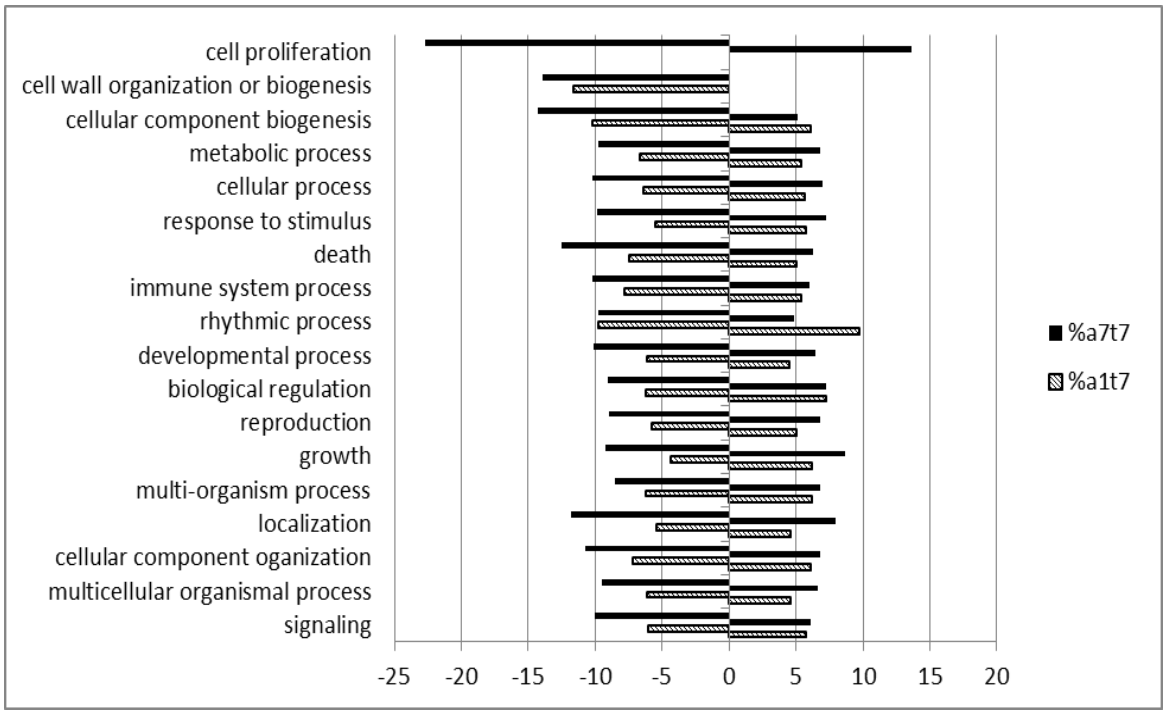

Figure 3. Biological process level 2 categories from Blast2GO for the up-/down-regulated probes for the collections made after one week, comparing those that were treated once (a1t7) and those that were treated daily for seven days (a7t7). These are given as a percentage of the total number of probes on the array in each category. The upregulated genes are shown to the right of the center axis, and the downregulated genes are to the left.

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Chapter 3

GENE EXPRESSION CHANGES IN FRASER FIR (*ABIES FRASERI*) RELATED TO INFESTATION BY THE BALSAM WOOLLY ADELGID (*ADELGES PICEAE*) IN THE FIELD

ABSTRACT

Microarrays were used to study gene expression in Fraser fir (*Abies fraseri* (Pursh.) Poir.) infested by the balsam woolly adelgid (*Adelges piceae* (Ratzeburg)) (BWA). Fraser fir trees were naturally infested by BWA at varying levels. The trees also showed varying intensities of their response to the BWA infestation, such as their level of apical dominance loss and bark reaction. The microarray probes were designed from pine EST sequences similar to *Arabidopsis thaliana* (L.) Heynh. genes known to be involved in plant defense. One hundred and thirty different ESTs were found to be significant for one or more of the phenotypic traits studied in the first year of sampling. In the second year of sampling, 338 ESTs were found to be significant for at least one of the phenotypic traits. *A. thaliana* genes similar to the significant ESTs included several known to be affected by abscisic acid treatment, as well as one gene thought to be involved in the regulation of jasmonic acid and ethylene signaling. Also found to be significant were AT3G25810, which is a terpene synthase, and PIN3, which is involved in plant tropism and may be related to the loss of apical dominance caused by BWA infestation.

INTRODUCTION

Fraser fir (*Abies fraseri* (Pursh.) Poir.) is native to the southeastern United States and is found naturally at elevations of approximately 1300 meters to over 2000 meters in North Carolina, Virginia, and Tennessee (Dull et al. 1988). Fraser fir is also a premier Christmas tree species that is grown in plantations throughout the southern Appalachians. It has been found to be very susceptible to the balsam woolly adelgid, *Adelges piceae* (Ratzeburg), an exotic insect accidentally introduced to the United States from Europe (Mitchell 1966). The balsam woolly adelgid (BWA) was first found in the United States in Maine in 1908, and Fraser fir trees in the southern Appalachians were first found to be infested with BWA by 1957 (Kotinsky 1916; Speers 1958). Since then, BWA has spread to all natural stands of Fraser fir. Fraser fir is highly susceptible to the adelgid, with as much as 95 to 98% mortality seen in some natural stands (Witter and Ragenovich 1986). Christmas tree plantations must regularly be scouted for BWA infestation; if an infestation goes untreated it can cause significant economic losses, through quality degradation and eventual tree mortality.

On mature Fraser fir, BWA is generally found on the bole of the tree, where it feeds on parenchyma cells in the outer bark. Their feeding causes several symptoms in susceptible species. There is often gouting, an abnormal tissue growth that results in swelling at the nodes and around the buds, the twigs and smaller branches may show swelling or twisting, and many times apical dominance is lost, with the top of the crown becoming flattened (Brower 1947; Balch 1952). There can be a generalized bark reaction in the area of the infestation, where the bark becomes thicker and harder (Hollingsworth and Hain 1992,

Appendix A). The adelgid's feeding also causes the formation of xylem tissue that resembles compression wood (Balch et al. 1964; Timell 1986). This abnormal xylem tissue, referred to as rotholz, is dense, brittle, and is associated with reduced conductance in the sapwood (Balch 1952; Mitchell 1967; Hollingsworth and Hain 1991). This suggests that adelgid infestation leads to water stress in the crown, and water stress reduces both photosynthesis and respiration (Puritch 1973), which eventually can cause mortality. Mortality in Fraser fir trees is usually seen after two to five years of infestation (Amman and Speers 1965).

Microarray technology allows for the study of the expression levels of thousands of genes simultaneously, and microarrays have been used in many studies of plant-insect interactions, particularly for plant species such as *Arabidopsis thaliana* (L.) Heynh. and *Nicotiana attenuata* Torr. (Reymond et al. 2000; Hermsmeier et al. 2001; Moran and Thompson 2001; Moran et al. 2002; Heidel and Baldwin 2004; Voelckel and Baldwin 2004). Their use has been somewhat limited thus far in woody plant-insect interactions, but examples include the study of gene expression changes in hybrid poplar (*Populus trichocarpa* Torr.&Gray × *P. deltoides* Bartr.(Salicaceae)) leaves due to forest tent caterpillar (*Malacosoma disstria* Hübner) feeding (Ralph et al. 2006a), as well as changes in Sitka spruce (*Picea sitchensis* (Bongard) Carrière) due to wounding or feeding by spruce budworm (*Choristoneura occidentalis* Freeman) or white pine weevil (*Pissodes strobi* Peck.) (Ralph et al. 2006b; Ralph et al. 2006c).

To date, genome sequence information for conifers is limited, but loblolly pine (*Pinus taeda* L.) is a species in the Pinaceae family for which extensive EST DNA sequence

information is available. Therefore, the probes for the arrays used in this experiment were designed using EST sequences from other species in the Pinaceae family, mainly loblolly pine. Previous studies have shown success using heterologous arrays, specifically with hybridization of Scots pine (*P. sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) targets to loblolly pine cDNA probes (van Zyl et al. 2002). It is expected that there would also be a high level of gene sequence conservation between loblolly pine and Fraser fir.

The objective of this study was to identify genes involved in the defense mechanisms or reactions to BWA infestation. Phloem tissues from field grown Fraser fir trees of varying levels of infestation, apical dominance loss, and bark reaction were collected to compare gene expression between trees with high levels of BWA infestation and symptoms to those with low or no infestations.

MATERIALS AND METHODS

Tissue Collection

Phloem tissue samples were collected from field grown Fraser fir trees in two counties in western North Carolina during the month of June in 2006 and 2007. In 2006, one sample was collected from each of sixteen trees at a site near Sparta, NC, in Alleghany County, and each of twelve trees at a site in Banner Elk, NC, in Avery County. At each site, the trees were ranked from 0 to 5 for level of balsam woolly adelgid infestation, with 0 being uninfested and 5 being heavily infested. The bark reaction level was ranked from 0 to 5, with 0 being thin, smooth bark, and 5 being very thick, rough, and hardened bark. The level of

apical dominance loss was also ranked from 0 to 5, with 0 being a leader that has had no loss of apical dominance, and 5 being a leader that is bent at a 90 degree angle. A piece of bark was removed from each tree at approximately breast height and the phloem tissue was peeled off using a knife. The tissue was immediately frozen in liquid nitrogen.

In 2007, the second year of sampling, another collection was made at the same site that had been sampled in 2006 near Sparta, NC, as well as from a site near Minneapolis, NC, in Avery County. Six trees were sampled from each site; none were trees from which samples had been collected in the previous year. Two to three samples per tree were collected at heights varying from 0.6 m to 3.2 m on each of the trees in order to obtain areas of bark containing differing levels of adelgid infestation. Each sample was again ranked from 0 to 5 for infestation level and the level of bark reaction. Each tree was ranked from 0 to 5 for the level of apical dominance loss. After collection, phloem samples were immediately placed in a solution of RNA*later*® to protect RNA integrity until extraction.

RNA Extraction

RNA was extracted by grinding the phloem tissue in liquid nitrogen with a mortar and pestle. Approximately 100 mg of ground tissue was placed in a tube with 700 µl of lysis buffer containing 4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.3, 25 mM EDTA, 2.5% PVP-10, and 1% beta-mercaptoethanol, which was added just before use. Seven µl of 20% sarkosyl was added, and the tube was vortexed and incubated at 65°C for 10 minutes, with vortexing every few minutes. The tube was centrifuged at 14,000 rpm

(maximum speed) for one minute and the supernatant was transferred to a shredder column from an RNeasy® kit (QIAGEN Inc). The column was spun and an equal volume of 100% ethanol was added to the flowthrough. This mixture was transferred to an RNeasy® spin column; the column was spun and the flowthrough discarded. The column was first washed with 600 µl of Qiagen® RW1 buffer and then twice with 500 µl of Qiagen® RPE buffer. Finally, the column was washed with 500 µl of 100% ethanol. The column was then centrifuged for two minutes at 14,000 rpm to dry it. To elute the RNA, 60 µl of RNase-free water was added, incubated for 5 minutes, and centrifuged at 14,000 rpm for one minute.

Target Preparation

A reverse transcriptase reaction was performed to obtain cDNA. The reaction was started with 5 µg of total RNA at a volume of 10 µl. 1µl each of oligo dT and dNTP mix (10 mM each) were added and the mixture was heated at 65 °C for 5 minutes. The samples were quickly chilled on ice and spun down. 4µl of 5x first strand buffer and 2 µl of 0.1 M DTT were added and incubated at 42°C for 2 minutes. 1 µl of Superscript® RT II (Invitrogen™) was added and the mixture was incubated at 42°C for 2 hours. The reaction was inactivated by heating at 70°C for 15 minutes. Then 1 µl of RNase H was added and the mixture was incubated at 37°C for 20 minutes.

The cDNA product was cleaned up using Qiaquick® columns (QIAGEN Inc). 200 µl of Qiagen® binding buffer was added to the sample and then was transferred to the column. The column was centrifuged for 1 minute at 13,000 rpm. The column was washed twice with

750 µl of Qiagen® wash buffer. The column was dried by centrifuging for 1 minute, and cDNA was eluted twice with 20 µl of water, incubating for one minute before centrifugation.

Dye incorporation of the cDNA samples was performed using the LabelIT µarray® Cy™3/Cy™5 kits (Mirus Bio Inc). An incubation time of 1 hour was used for the Cy 3 samples and 2.5 hours for the Cy 5 samples.

Array Design

For the 2006 samples, Combimatrix CustomArray™ 12K arrays were designed. Probe sequences were designed from EST sequences from other species in the Pinaceae family known to be involved in plant defense or stress responses, and were chosen through a gene ontology search (R. Whetten, personal communication).

For the 2007 samples, the number of probes was decreased to 2,000, and a Combimatrix CustomArray™ 4x2K array was used. This subset of probes was determined based on the results from the 2006 sampling. The 2,000 probes used included those that were significant for any of the phenotypic traits in the 2006 experiments, as well as those that showed high levels of expression.

Hybridization

Pre-hybridization and hybridization solutions, as well as wash solutions, were prepared according to the CustomArray™ 12K microarray hybridization and imaging protocol (PTL006) for the 2006 samples and the CustomArray™ 4X2K microarray

hybridization and imaging protocol (PTL005) for the 2007 samples. Hybridizations were performed in a 45°C rotisserie hybridization oven overnight and washed according to the above protocols. The arrays were scanned in a ScanArray® Lite (PerkinElmer Life Sciences Inc) scanner. The arrays were subsequently stripped following the Combimatrix protocols for stripping and preparation for re-hybridization (PTL001 and PTL002). Gridding and data extraction was performed using the CombiMatrix microarray imager software.

Statistical Analysis

Exported data were analyzed in JMP® Genomics (version 4, SAS Institute, Inc.). Intensity values were averaged for any EST with multiple probes present on the array. A Loess normalization was performed on the data, using a smoothing parameter of 0.2 and 1 Loess iteration. A mixed model analysis was performed with dye and either infestation level, level of apical dominance, or bark reaction level included as fixed variables. For the 2006 sampling, the array pairing was used as a random effect, which gave each pair of samples that was hybridized together on an array the same value. For the 2007 sampling, the array use number, array chamber, tree number, and the sample height were used as random variables. Analyses were performed with infestation, apical dominance, and bark reaction treated as linear effects. Since there was only one measure of leader loss per tree, the low and high height samples were analyzed separately for this trait. The mixed model analyses were first performed on the data for both sites combined, with the site main effect and site*infestation, site*apical dominance, or site*bark reaction interactions as fixed effects.

Any probes found to have a significant site interaction were also tested for significance in each site alone, using the same mixed model with the site and site interaction effects removed. The degrees of freedom were calculated with the Satterthwaite method. LS means were not calculated to determine up- and down-regulation of genes since there were up to six measurement values for each phenotypic trait.

Blast2GO was used to identify the gene ontology and biological categories of the EST sequences used (Conesa et al. 2005). KEGG pathway assignments for these sequences were also found using Blast2GO (Conesa et al. 2005). The top matches to *A. thaliana* genes were determined using a blastx search in blast+ against the Arabidopsis protein database for each of the EST sequences used to design the probes on the array (Altschul et al. 1990, Altschul et al. 1997). These *A. thaliana* genes were used to compare results here to previous microarray experiments.

RESULTS AND DISCUSSION

2006 Sampling

Thirty-two different probes were found to show significant differences in signal intensity as a function of infestation level across the two sites combined, and there was one additional probe significant for one site alone. Seventy-two probes showed significant differences in intensity as a function of overall level of bark reaction for the two sites combined, with an additional six probes significant for one of the two sites. There were 77 probes significant for loss of apical dominance for both sites and one additional probe

significant for one of the two sites. Forty-one of the probes were found to be significant for more than one of these phenotypic traits. Figure 1 is a Venn diagram indicating the numbers of significant probes in each category and the overlap between categories.

A different array design with 12,000 probes was used for the 2006 samples than for any of the other microarray experiments reported in this thesis, so the names of EST sequences from which probes were designed, rather than the names of the probes themselves, were used to compare the results of this experiment with the others performed. Four ESTs found to be differentially regulated due to BWA infestation in the field were also significant due to seedling infestation in the lab (Chapter 4). Two of the ESTs here were upregulated in Fraser fir with BWA infestation in the lab, AI812599 and TC74170. Two others were downregulated with BWA infestation in the lab, one in Fraser fir (TC75093) and one in noble fir (TC76351). None of the top *A. thaliana* gene matches for these ESTs (AT1G76900, AT3G25600, ATCG00170, AT1G67310) are known to be involved in plant defense reactions. Fifty-four different ESTs that were found to be significant in the 2006 field sampling were also differentially regulated with the application of jasmonic acid (JA), salicylic acid (SA), sodium nitroprusside (SN), or quercetin to Fraser fir seedlings (Chapter 2).

One probe (TC75513_1116_1150) that was found to be differentially regulated by infestation, bark reaction, and apical dominance loss matches to the *A. thaliana* gene PIN3 (AT1G70940). PIN3 regulates auxin efflux, and has been shown to be involved in regulating

plant tropism (Friml et al. 2002). This gene could be involved in the loss of apical dominance seen with BWA infestation.

Production of terpenes is an important part of general tree defense responses, and the *A. thaliana* gene match for probe TC64624_217_251 is AT3G25810, which is known to be involved in production of multiple terpenes (Chen et al. 2003). This probe was found to be differentially regulated by the general bark reaction and leader loss traits. This gene is a terpene synthase thought to catalyze the formation of several monoterpenes (Chen et al. 2003).

The jasmonic acid signaling pathway is known to play a major role in plant stress response, and has been shown to be important in plant defense against insects, such as in tomato (*Lycopersicon esculentum* Mill., cv Castlemart) against the tobacco hornworm (*Manduca sexta* L.) (Howe et al. 1996), as well as for pathogen defense in *Arabidopsis* (Vijayan et al. 1998). Probe TC73191 was significant for apical dominance loss in this field experiment and its *A. thaliana* gene match, AT4G26850, has been shown to be induced by JA treatment in *A. thaliana* (Sasaki-Sekimoto et al. 2005). In addition, AtHD1 (AT4G38130) is known to regulate many plant processes (Fong et al. 2006), and may regulate the expression of genes involved in jasmonic acid and ethylene signaling in response to pathogens (Zhou et al. 2005). The probe that matches to this gene (CO370277) was found to be differentially regulated by the general bark reaction trait in Fraser fir.

Several of the *A. thaliana* gene matches for significant probes are related to drought stress or abscisic acid signaling. These include NCED3 (AT3G14440), which has been

found to be more highly expressed in *A. thaliana* plants under drought stress and is believed to be involved in the biosynthesis of abscisic acid in the plant (Iuchi et al. 2001). An over-expression of NCED3 increased the drought tolerance of *A. thaliana* plants, possibly due to closure of the stomata and therefore reduced transpiration rates, and caused higher abscisic acid levels (Iuchi et al. 2001). The response of Fraser fir to infestation by the balsam woolly adelgid involves the development of rotholz in the xylem, which prevents water transport and creates drought stress conditions (Mitchell 1967; Puritch 1973; Hollingsworth and Hain 1991). Increased expression of the NCED3 gene in *A. fraseri* with increased levels of bark reaction to the adelgid may be due to these drought stress conditions and might be helping the tree to tolerate the infestation for a longer period of time.

In addition, probe TC59317_920_954 was significantly differentially expressed for the apical dominance loss trait in the field, and was also found to be downregulated in Fraser fir both by BWA infestation in the lab and one week after treatment with JA (Chapter 2, Chapter 4). This probe corresponds to ARF2 (AT5G62000), which is an auxin response transcription factor known to be induced by ABA application in *A. thaliana*, indicating that it is involved in ABA responses (Wang et al. 2011). Accumulation of the ARF2 protein has also been found to be decreased by ethylene (Li et al. 2004). It has been shown to be related to several abnormal growth patterns in *A. thaliana* due to auxin regulation (Li et al. 2004; Okushima et al. 2005). The differential expression of ARF2 seen in Fraser fir may be either related to the loss of apical dominance or simply due to the drought stress conditions in the field from BWA infestation, since it is also related to ABA signalling.

The *A. thaliana* gene matches were used to compare the differentially regulated genes discovered here with a previous study of gene expression changes in *P. nigra* following feeding by gypsy moth (*Lymantria dispar* (L.)). Four genes found to be significant in the field sampling here were also found to be differentially regulated in *P. nigra* when fed on by gypsy moth (Babst et al. 2009). One was differentially regulated due to apical dominance loss (AT3G12800), one for the general bark reaction (AT5G07990), one due to both infestation and bark reaction (AT1G75280), and one for both apical dominance loss and bark reaction (AT4G38900).

2007 Sampling

Sixteen probes were found to be significantly differentially regulated for infestation for the two sites combined, and an additional four probes that were significant for the site*infestation interaction were also significant for one of the two sites alone. The average intensity values at varying levels of infestation are shown in Figure 4 for four of these significant probes. 169 probes were significant for apical dominance loss for the lower sample height on both sites, and an additional three were found to be significant when the two sites were analyzed separately. For the higher sample height, 168 probes were significant for apical dominance loss for the two sites combined, and an additional 12 probes were significant for one site alone. Only 36 probes were significant for both the high and low sample height. Ten probes were significant for the overall bark reaction trait for both sites, with an additional five significant for one of the two sites. Only three probes were

found to be significant for more than one of the traits; one probe was found to be significant for both infestation and bark reaction. There were also two probes significant for leader loss that were significant for other traits; one was significant for infestation and the other significant for the bark reaction. The number of probes significant for each trait is shown in Figure 2. It is not clear why there were so many more probes found to be significant for the apical dominance loss trait in 2007 than in 2006. None of the trees from which samples were collected in 2007 had the highest levels of leader loss that were seen in the 2006 sampling, so there was a smaller range of values for the apical dominance trait for the 2007 samples. This may have contributed to finding a greater number of probes significant in 2007.

Forty-seven ESTs found to be significant in the 2006 sampling were also significant in 2007. Thirty-six of these were significant for the leader loss trait in the 2007 sampling, while they were significant for various traits in the 2006 sampling. Just one probe was found to be significant for BWA infestation level in both years of sampling. This EST is TC78119, and the best *A. thaliana* gene match for this is AT1G09250, which is a transcription factor. This gene may be of interest for future studies.

The results here were also compared with a previous study of gene expression changes in *P. nigra* with feeding by gypsy moth, by looking at the *A. thaliana* gene matches for the significant probes. Fifteen genes found to be differentially regulated for the leader loss trait were also found to be upregulated by gypsy moth feeding on *P. nigra* (Babst et al. 2009).

When looking at the probes found to be significant for infestation, four were also found to be significant in the chemical application study (Chapter 2). Probe 2192_TC60849 was upregulated by JA one week after treatment, while 1918_TC78119 was downregulated by JA one week after treatment. Probe 7758_TC75345 was upregulated after seven daily chemical treatments of sodium nitroprusside (SN). SN acts as a donor of nitric oxide, which has been shown to be involved in the induction of the hypersensitive response of cell death in plants to pathogens (Delledonne et al. 1998; Delledonne et al. 2001). While probe 10574_TC77485 was downregulated with SN treatment, it showed generally greater expression levels with higher levels of BWA infestation in the field, with an infestation level of 4 having the highest level of gene expression. The best match in *A. thaliana* for this probe is AT1G52150 (ATHB15 or CNA) which is known to be required for vascular development (Kim et al. 2005). Overexpression of this gene in *A. thaliana* does affect xylem growth and differentiation, causing greater amounts of lignification and greater numbers of vascular bundles (Kim et al. 2005). This may be of interest because BWA infestation in Fraser fir causes the development of unusual xylem tissue, rotholz, which is the eventual cause of tree death (Balch et al. 1964; Timell 1986). Only one probe significant here for infestation was also found to be upregulated in Fraser fir seedlings after infestation by BWA for four to six days (Chapter 4). The closest *A. thaliana* match for this EST is AT3G18380, which is thought to be a transcription factor.

Of the *A. thaliana* gene matches for those probes found to be significant for the general bark reaction, NHL10 (AT2G35980) is known to be upregulated in response to a

virus (Zheng et al. 2004). It has been hypothesized that aphid feeding is similar to fungal hyphae invasion, which could explain the similarities between responses to pathogens and aphid feeding (Fidantsef et al. 1999). It is expected that the response to the adelgid could be similar, since the adelgid, like the aphid, has a close interaction with the plant it feeds on. Another gene significant for the bark reaction is known to be involved in abscisic acid response; AT2G33380, or RD20, has been shown to be induced by drought stress and by treatment with ABA (Takahashi et al. 2000), as well as after leaf wounding (Aubert et al. 2010).

When looking at the total number of probes significant for any trait in this study, 35 additional probes match to *A. thaliana* genes known to be associated with ABA or drought stress responses. These include ARF2 (AT5G62000), which was also significant for the 2006 sampling, PDR12 (AT1G15520), and HD2C (AT5G03740). PDR12 is a plasma membrane abscisic acid transporter present in plant guard cells, which has been shown to be involved in stomatal closure in response to drought stress (Kang et al. 2010). This was also found to be downregulated in Fraser fir with BWA infestation in the laboratory (Chapter 4). HD2C was significant for the loss of apical dominance in the field sampling, and was also found to be downregulated in Fraser fir with BWA infestation in the laboratory (Chapter 4). HD2C expression is affected by ABA and it is thought to be involved in the regulation of drought stress responses in *A. thaliana* (Sridha and Wu 2006).

The biological processes for the probes found to be significant for each phenotypic trait are shown in Figure 3. The biological processes most highly represented are cellular

processes and response to stimulus. The significant probes were also assigned to KEGG pathways in Blast2GO. The pathways that were most highly represented by the probes significant for leader loss were glutathione metabolism (8 probes), phenylpropanoid biosynthesis (6 probes), methane metabolism (5 probes), and amino sugar and nucleotide sugar metabolism (5 probes). The glutathione and phenylpropanoid pathways are known to be involved in stress and defense response, and one probe significant for infestation was also involved in phenylpropanoid biosynthesis. The *A. thaliana* genes associated with probes assigned to the phenylpropanoid biosynthesis pathways include AT3G48000, AT3G48170, AT2G36780, AT1G35720, AT5G06720. Two of these genes involved in the phenylpropanoid pathway are also known to be associated with ABA treatment. Both AT3G48170, an aldehyde dehydrogenase (ALDH10AD9), and AT1G35720 (AnnAt1) have been shown to be induced by ABA treatment (Lee et al. 2004; Cantero et al. 2006; Missihoun et al. 2011).

CONCLUSIONS

Genes from a number of different resistance or stress response pathways have been identified here as differentially expressed in Fraser fir infested by BWA, including pathways associated with ABA and JA signaling, as well as terpenoid production. It appears that many of the genes identified here, particularly those that have been shown to be related to abscisic acid treatment, are likely a response to the drought stress in the tree due to the rotholz production, rather than a direct response to the insect infestation itself. Of particular interest

for further research may be the gene ATHB15, which is related to vascular development, and PIN3, which is involved in plant tropism and may be related to the loss of apical dominance seen with BWA infestation. Further work is necessary to determine the functions of these genes in Fraser fir, as well as their relationship to BWA infestation responses, and this information can be used in future breeding of firs resistant to BWA.

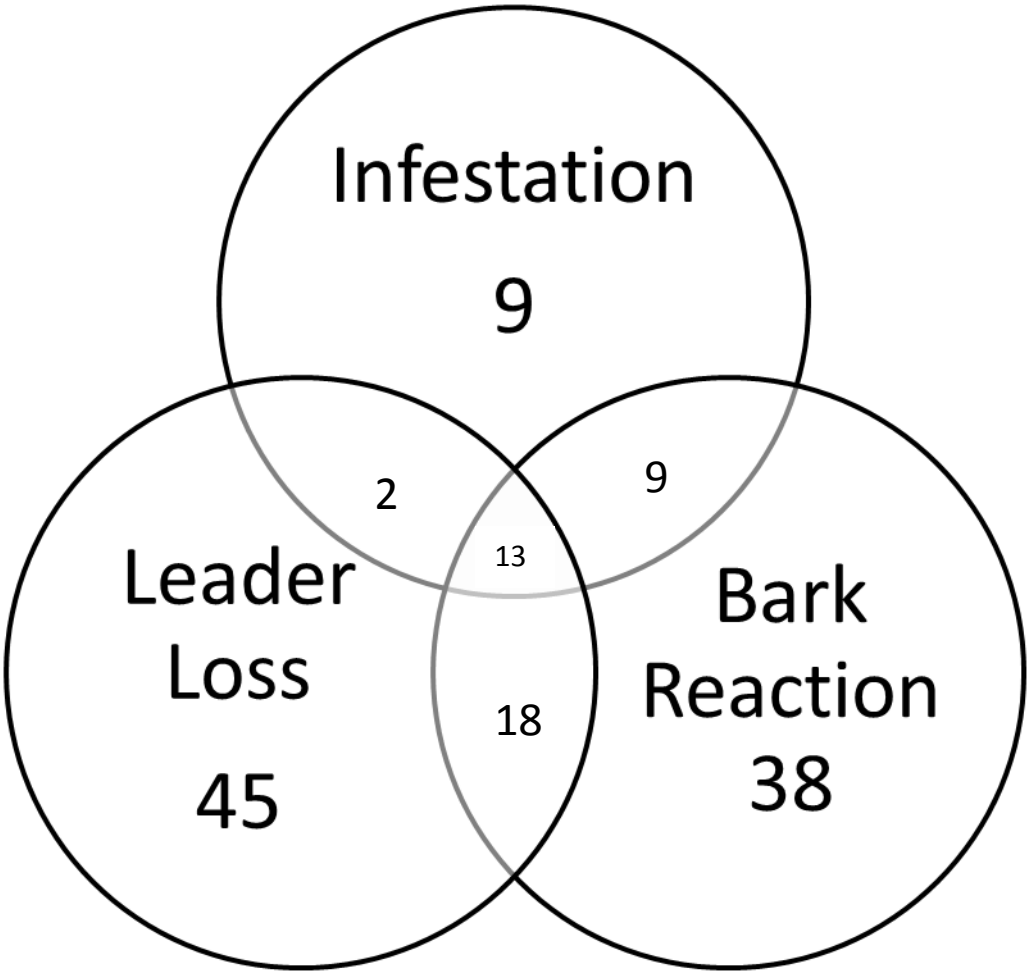

Figure 1. The number of probes significant for each of the phenotypic traits and the overlap between traits for the 2006 sampling.

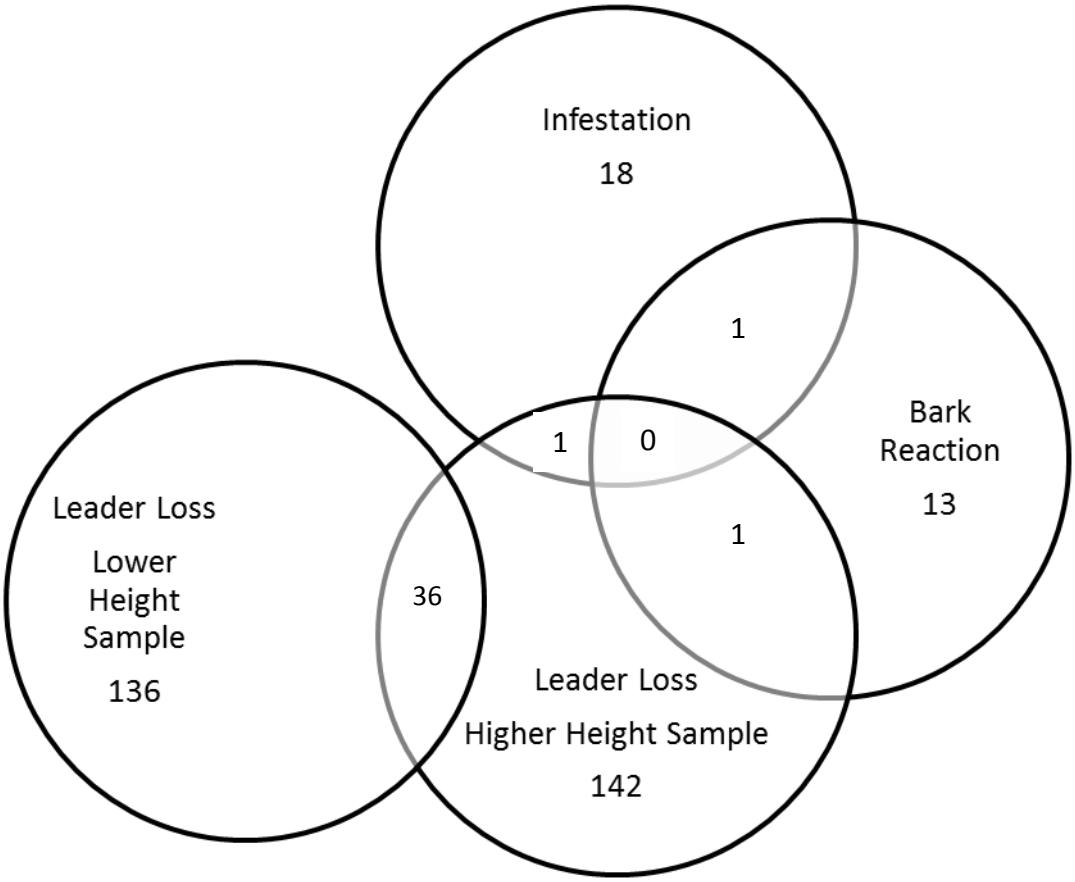

Figure 2. The number of probes significant for each of the phenotypic traits for at least one site and the overlap between traits for the 2007 sampling.

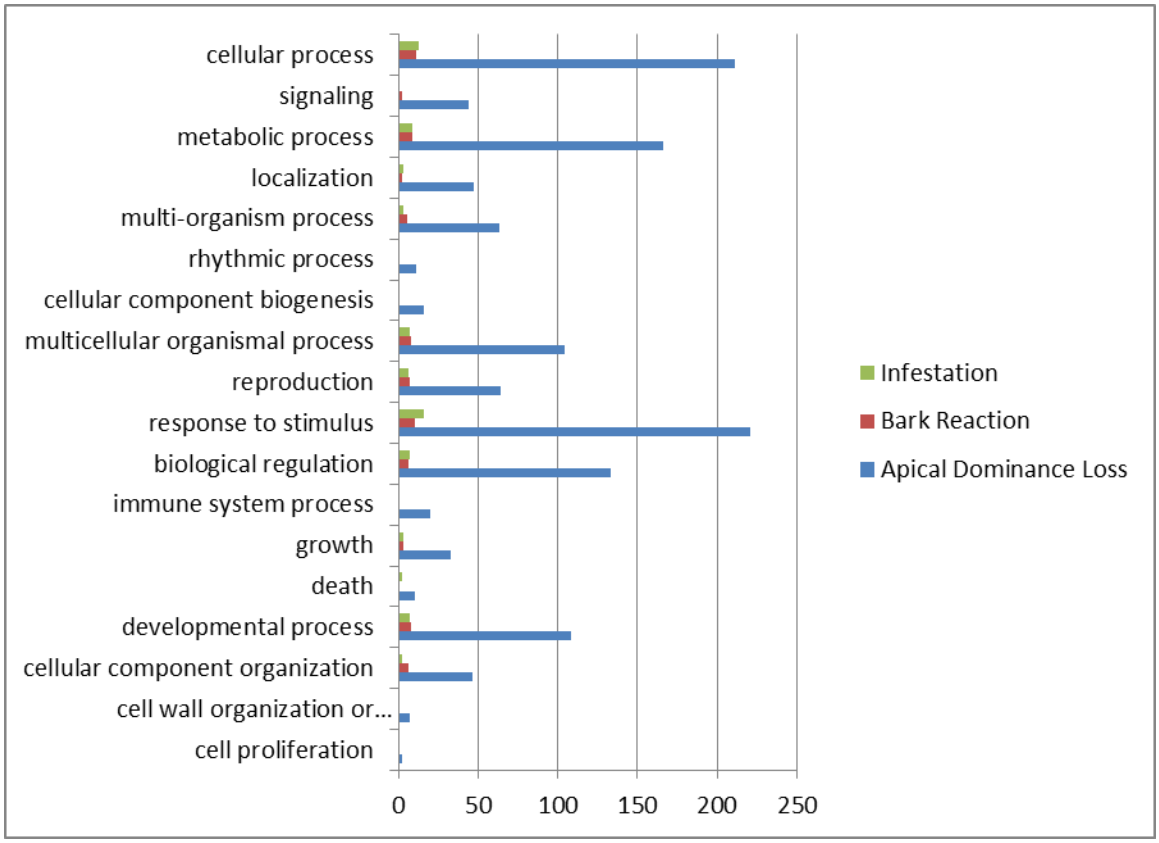

Figure 3. Biological process level 2 categories from Blast2GO for the probes found to be significant for each phenotypic trait in the 2007 sampling.

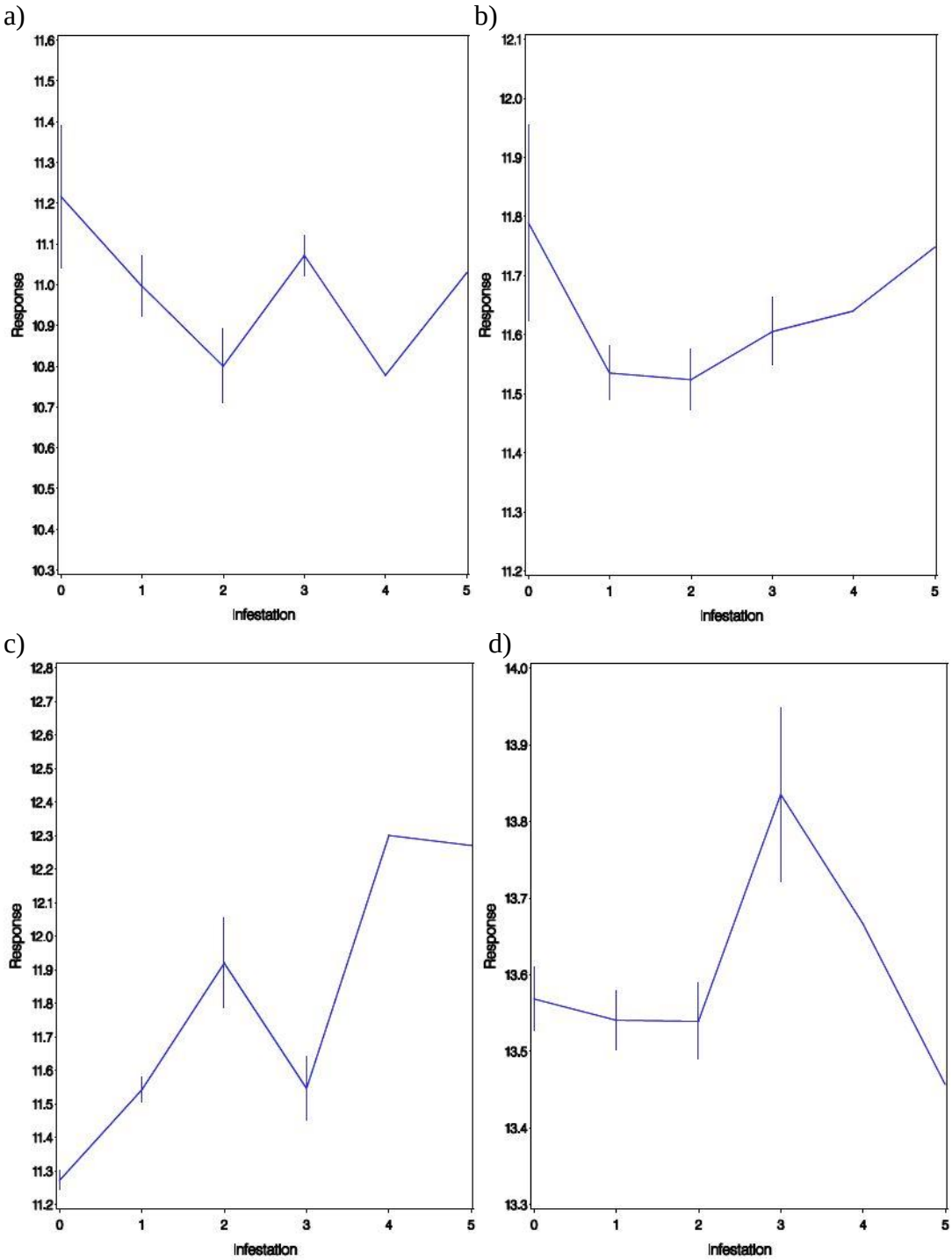

Figure 4. Average intensity (response), with standard error bars, as a function of level of infestation for four probes differentially regulated with infestation level, a) 298-TC57397, b) 3734-TC65793, c) 5092-TC58985, and d) 781-TC65534.

Table 1. List of probes significant in the 2006 field sampling, the phenotypic trait(s) for which they were significant in the 2006 sampling, the closest *A. thaliana* gene match for the EST, and which species or chemical the EST was significant for in the laboratory infestation or chemical application microarray experiments (Chapter 2, Chapter 4). Up- or down-regulation of genes not indicated.

Probe Name	**Significant Trait**	***A. Thaliana* gene match**	**Significant in laboratory infestation**	**Significant in chemical application study**
AI812599_227_261	bark reaction	AT1G76900	Fraser	JA
AI812599_227_261	infestation	AT1G76900	Fraser	JA
AI812599_227_261	leader	AT1G76900	Fraser	JA
AI812599_289_323	infestation	AT1G76900	Fraser	JA
AW010637_428_462	leader	AT3G07260		
AW011616_409_443	bark reaction	AT5G07920		
AW011616_409_443	infestation	AT5G07920		
AW011616_409_443	leader	AT5G07920		
AW981610_13_48	bark reaction	AT5G50260		
BE582317_21_58	infestation	AT5G43730		SN, Q
BI397836_192_227	bark reaction	AT1G08970		
BI643993_364_399	bark reaction	AT3G02570		
BQ696140_12_47	leader	AT3G05660		
CD020615_135_169	leader	AT3G27000	Fraser	JA
CF385835_65_99	bark reaction	AT1G75280		
CF385835_65_99	infestation	AT1G75280		
CF479472_265_299	bark reaction	AT3G59030		
CF479472_265_299	leader	AT3G59030		
CF667698_346_381	bark reaction	AT3G28360		JA, Q
CF667698_346_381	leader	AT3G28360		JA, Q
CO363342_155_193	bark reaction	AT5G07990		JA, SN
CO366886_13_47	infestation	AT3G48340		
CO370277_106_140	bark reaction	AT4G38130		
CV031438_321_355	infestation	AT4G23900		JA
CX650473_801_835	bark reaction	AT5G44720		JA
CX650473_801_835	infestation	AT5G44720		JA
CX650473_801_835	leader	AT5G44720		JA
DR013334_589_623	bark reaction	AT1G59750		
DR016307_281_315	infestation	AT1G79000		SN
DR022514_5_39	bark reaction			

Table 1 (Continued)

DR052835_619_653	leader	AT4G12780		
DR079461_552_586	leader	AT4G36220		
DR167573_187_221	leader	AT3G22400		
DR681969_647_681	bark reaction	AT3G24590		
DR681969_647_681	infestation	AT3G24590		
DR681969_647_681	leader	AT3G24590		
DR682504_389_426	bark reaction	AT3G03660		SN
DR682504_389_426	infestation	AT3G03660		SN
DR686575_509_543	bark reaction	AT3G25700	Fraser	JA
DR690242_361_395	bark reaction	AT3G25700		
DR690242_361_395	leader	AT3G25700		
new00995_379_413	bark reaction	AT3G48000		
new01039_12_46	infestation	AT5G10930		JA
new01039_291_325	bark reaction	AT5G10930		JA
new08724_1068_1102	leader	AT1G13440		JA
new16326_991_1028	leader	AT1G21450		
TC57176_1008_1045	bark reaction	AT5G08670		JA
TC57637_944_978	bark reaction	AT2G46280		
TC57637_944_978	infestation	AT2G46280		
TC57724_726_762	infestation	AT2G01250		JA
TC57748_577_611	bark reaction	AT1G73050		JA
TC57748_577_611	leader	AT1G73050		JA
TC58134_594_629	leader			
TC58396_2153_2187	leader	AT5G67360		
TC59250_1769_1803	leader	AT3G19500		SN
TC59296_650_686	leader	AT3G12800		
TC59317_920_954	leader	AT5G62000	Fraser	JA
TC59453_528_562	bark reaction	AT5G40020		JA
TC59453_528_562	leader	AT5G40020		JA
TC59673_1080_1114	bark reaction	AT1G46264	Fraser	JA
TC60280_583_617	bark reaction	AT4G38900		
TC60280_583_617	leader	AT4G38900		
TC60511_1447_1481	leader	AT2G18160		JA
TC60529_1929_1965	bark reaction	AT4G32640		JA, SN
TC60548_547_581	bark reaction	AT2G40600		
TC60548_547_581	infestation	AT2G40600		
TC60548_547_581	leader	AT2G40600		
TC60641_123_157	leader	AT1G24620		
TC60742_859_893	bark reaction	AT4G32551		JA

Table 1 (Continued)

TC60933_443_477	leader	AT2G03430	Fraser	SN
TC61204_398_433	bark reaction	AT2G36800		
TC61863_598_632	bark reaction	AT2G42520		
TC62756_810_844	leader	AT5G48150	Fraser	SN
TC62925_808_842	bark reaction	AT2G01630	Fraser	
TC63554_257_291	leader	AT3G06590	Fraser	JA
TC63898_769_803	infestation	AT3G10920		
TC63903_177_211	bark reaction	AT4G16110		
TC64402_518_552	leader	AT4G13940		
TC64624_217_251	bark reaction	AT3G25810		
TC64624_217_251	leader	AT3G25810		
TC64988_1154_1188	bark reaction	AT3G11660		
TC64988_1154_1188	leader	AT3G11660		
TC65152_140_175	infestation	AT3G05890		
TC65383_412_446	leader			
TC65780_589_623	bark reaction	AT2G21250		
TC65780_589_623	infestation	AT2G21250		
TC65780_589_623	leader	AT2G21250		
TC65790_274_308	bark reaction	AT5G60910	Fraser	
TC65790_274_308	leader	AT5G60910	Fraser	
TC65853_1894_1928	leader	AT4G33720		
TC66131_1191_1225	bark reaction	AT4G38970		SN
TC66207_710_744	bark reaction	AT2G40010		SN
TC66317_1178_1212	leader	AT5G43940		
TC66428_855_889	bark reaction	AT2G42690		SN
TC66428_855_889	infestation	AT2G42690		SN
TC66428_855_889	leader	AT2G42690		SN
TC66752_214_248	bark reaction	AT3G14440		
TC66868_997_1031	bark reaction	AT5G03740	Fraser	
TC66868_997_1031	leader	AT5G03740	Fraser	
TC66983_743_777	leader	AT3G51440		
TC67332_332_366	leader	AT5G18230		
TC67480_690_724	bark reaction	AT1G55510		SN
TC67480_690_724	leader	AT1G55510		SN
TC67636_573_607	bark reaction	AT3G17460		SN
TC67636_573_607	infestation	AT3G17460		SN
TC67636_573_607	leader	AT3G17460		SN
TC67914_353_387	leader	AT3G10030		JA
TC68319_1727_1761	bark reaction	AT5G47750		

Table 1 (Continued)

TC68319_1727_1761	infestation	AT5G47750		
TC68319_1727_1761	leader	AT5G47750		
TC68497_1021_1055	bark reaction	AT1G30080		
TC68643_1242_1276	bark reaction	AT3G51420		SN
TC68643_1242_1276	leader	AT3G51420		SN
TC68722_601_635	leader	AT1G80820		JA
TC68736_1208_1242	leader	AT5G18640		JA
TC68930_1346_1381	bark reaction	AT2G24260		
TC68958_1117_1156	bark reaction	AT5G64950		SN
TC69116_1346_1380	bark reaction	AT5G55760		SN
TC69440_629_663	bark reaction	AT1G12910		SN
TC70225_857_891	leader	AT3G47620		
TC70508_85_119	bark reaction	AT1G51170		SN
TC71386_829_863	bark reaction	AT2G23740		
TC71624_149_184	bark reaction	AT4G14370		
TC71624_149_184	leader	AT4G14370		
TC71629_113_147	bark reaction	AT1G19210		
TC71629_113_147	infestation	AT1G19210		
TC71629_113_147	leader	AT1G19210		
TC72200_865_899	bark reaction	AT2G42990		SN
TC72200_865_899	leader	AT2G42990		SN
TC72606_161_195	bark reaction	AT5G46180	Fraser	
TC72606_161_195	leader	AT5G46180	Fraser	
TC72869_96_130	bark reaction	AT1G62500		
TC72952_1754_1788	leader	AT2G19810		
TC72963_1002_1036	bark reaction	AT2G30860		
TC72973_928_962	leader	AT5G41380		JA
TC73044_1211_1245	leader	AT4G35450		
TC73191_1965_1999	leader	AT4G26850		
TC73505_547_581	bark reaction	AT4G36040		
TC73605_8_42	bark reaction	AT5G59320	Fraser	SN
TC73643_523_557	leader	AT1G60710		JA
TC74059_398_432	bark reaction	AT3G14290	Fraser	JA
TC74155_801_835	bark reaction	AT5G25610		
TC74170_389_423	bark reaction	AT3G25600	Fraser	JA
TC74170_389_423	infestation	AT3G25600	Fraser	JA
TC74170_389_423	leader	AT3G25600	Fraser	JA
TC74228_740_774	leader	AT4G13260		JA
TC74418_1558_1592	bark reaction	AT1G48030		

Table 1 (Continued)

TC74418_1558_1592	leader	AT1G48030		
TC74500_852_887	leader	AT4G24210	Fraser	
TC74850_2164_2198	leader	AT2G34750		
TC74866_443_478	bark reaction	AT2G02240		JA, SN
TC74866_443_478	infestation	AT2G02240		JA, SN
TC74866_506_540	bark reaction	AT2G02240		JA, SN
TC74866_506_540	infestation	AT2G02240		JA, SN
TC74866_506_540	leader	AT2G02240		JA, SN
TC75093_4348_4387	bark reaction	ATCG00170	Fraser	JA
TC75093_4348_4387	infestation	ATCG00170	Fraser	JA
TC75367_593_627	infestation	AT1G55910		
TC75367_593_627	leader	AT1G55910		
TC75513_1116_1150	bark reaction	AT1G70940		
TC75513_1116_1150	infestation	AT1G70940		
TC75513_1116_1150	leader	AT1G70940		
TC75612_851_885	leader	AT2G29380		
TC75865_340_374	leader	AT4G27270		SN
TC76051_121_155	leader	AT5G01410		JA, SN
TC76351_655_690	infestation	AT1G67310	Noble	JA
TC76351_655_690	leader	AT1G67310	Noble	JA
TC76351_696_730	bark reaction	AT1G67310	Noble	JA
TC76444_785_819	bark reaction	AT1G69080		
TC76444_785_819	infestation	AT1G69080		
TC76575_652_686	bark reaction	AT3G17100		
TC76575_652_686	infestation	AT3G17100		
TC76830_736_771	leader	AT1G01720		
TC76851_1405_1439	bark reaction	AT2G33610		
TC76915_692_726	leader	AT5G35410		
TC76925_870_904	bark reaction	AT4G35290		
TC77089_770_807	bark reaction	AT1G10370		
TC77089_770_807	leader	AT1G10370		
TC77150_1006_1040	leader	AT5G51230	Fraser	JA
TC77547_568_602	leader	AT3G61970		SN
TC78090_76_110	bark reaction	AT4G11600		
TC78090_76_110	infestation	AT4G11600		
TC78119_745_779	bark reaction	AT1G09250		JA
TC78119_745_779	infestation	AT1G09250		JA
TC79052_241_275	bark reaction	AT5G19690	Fraser	
TC79052_241_275	leader	AT5G19690	Fraser	

Table 1 (Continued)

TC79302_1425_1460	bark reaction	AT4G30250	
TC80034_321_355	leader	AT5G06490	
TC80318_803_837	leader	AT5G48670	SN

Table 2. List of probes significant in the 2007 field sampling that were also significant for either the laboratory infestation or chemical application microarray experiments (Chapter 2, Chapter 4). Also included are the phenotypic trait(s) for which they were significant in the 2007 sampling, the closest *A. thaliana* gene match for the EST, and which species or chemical the EST was significant for in the laboratory infestation or chemical application microarray experiments (Chapter 2, Chapter 4). Up- or down-regulation of genes not indicated.

Probe Name	**Significant Trait**	***A. Thaliana* gene match**	**Significant in laboratory infestation**	**Significant in chemical application study**
11022_TC71760	bark reaction	AT2G18160		JA, SN
2663_TC61121	bark reaction	AT2G26350		SN
7260_TC62009	bark reaction	ATCG00190		SN
10574_TC77485	infestation	AT1G52150		SN
1918_TC78119	infestation	AT1G09250		JA
2192_TC60849	infestation	AT4G02050		JA
6173_TC80513	infestation	AT3G18380	Fraser	
7758_TC75345	infestation	AT2G46690		SN
8049_new00152	infestation	AT5G48540		JA
10002_TC64971	leader	AT4G37770		JA
10053_TC57877	leader	AT2G37060		
10054_TC74170	leader	AT3G25600		JA
10217_TC61402	leader	AT3G59770		SN
10287_TC57781	leader	AT1G15520		JA
10640_new00444	leader	AT4G00330		
10658_TC77889	leader	AT1G22920		
1078_TC59650	leader	AT1G35720		JA, SN
10889_TC66486	leader	AT5G28540	Fraser	
10905_TC57805	leader	AT3G12500		JA
10914_TC76067	leader	AT3G24590		JA
10969_TC76584	leader	AT4G39470	Fraser	
11112_TC69673	leader	AT2G47800		
11287_TC75713	leader	AT4G00830	Fraser	
11294_DR164092	leader	AT1G08560		JA
11317_TC69421	leader	AT4G02570		JA
11459_TC68061	leader	AT4G35985	Fraser	

Table 2 (Continued)

11493_CF667983	leader	AT1G55870	Noble	
11550_new00735	leader	AT5G19780		JA
11578_DR692273	leader	AT4G24400		SN
11597_TC70508	leader	AT1G51170		SN
11837_TC66334	leader	AT5G13930		
11862_DR168055	leader	AT2G45660		JA
12021_TC71485	leader	AT5G61850		SN
12070_TC70549	leader	AT5G15800	Fraser	
12085_TC63026	leader	AT1G60620		SN
12251_TC67475	leader	AT1G65910	Fraser	
12275_TC76703	leader	AT3G30530	Fraser	
12347_AA556306	leader	AT5G15850	Fraser	SN
12353_TC71976	leader	AT2G23700		JA
12379_TC73934	leader	AT1G77490	Fraser	
12389_CX651381	leader	AT3G01420		JA
12398_TC59832	leader	AT5G13960		SN
12531_new00588	leader	AT1G30270	Fraser	JA, SN
1300_TC74957	leader	AT1G19715		
1306_DR695339	leader	AT1G02040	Fraser	
1391_DR116620	leader	AT1G56440		SN
1501_TC77150	leader	AT5G51230	Fraser	
151_TC68643	leader	AT3G51420		SN
1588_TC59039	leader	AT2G15220		JA
1760_TC65612	leader	AT3G51030		
1781_TC57267	leader	AT5G06320		SN
1802_DR686575	leader	AT3G25700		JA
1928_TC59317	leader	AT5G62000	Fraser	JA
2037_TC76351	leader	AT1G67310	Noble	
2166_CX651381	leader	AT3G01420		JA
2190_TC74636	leader	AT1G10630	Fraser	
2192_TC60849	leader	AT4G02050		JA
2283_TC76095	leader	AT4G21450	Fraser	
2315_TC59673	leader	AT1G46264	Fraser	
233_DR168611	leader	AT3G15020		SN, Q
2336_CN852406	leader	AT3G15510		JA, Q
2449_TC62968	leader	AT2G27710	Fraser	
2544_TC61139	leader	AT4G35850	Fraser	
2627_TC78353	leader	AT1G76690		SN
2698_TC70895	leader	AT1G61720		

Table 2 (Continued)

2702_new16341	leader	AT2G30860		JA
2727_TC68055	leader	AT1G07980	Fraser	
2863_DR110419	leader	AT3G54260		JA
2891_TC73605	leader	AT5G59320	Fraser	SN
3100_BE582317	leader	AT5G43730		Q
3331_TC59202	leader	AT1G60690		JA, SN
3458_CF385349	leader	AT3G44480	Fraser	JA
3568_TC60423	leader	AT3G50070		JA, SN
3760_TC67968	leader	AT3G20570	Fraser	JA
3848_TC57723	leader	AT3G51680		SN
3966_DR388612	leader	AT5G09810		SN
456_AL750347	leader	AT4G30210		
4602_TC68634	leader	AT2G13650		SN
4966_TC77060	leader	AT1G50320	Fraser	
504_BX252812	leader	AT1G63750	Fraser	
5299_TC74950	leader	AT5G54580	Fraser	
5454_TC76065	leader	AT3G17070		SN
5521_TC61437	leader	AT2G02040		SN
5651_TC68833	leader	AT1G62300	Noble	SN
570_TC60470	leader	AT3G26935	Fraser	
5853_TC60485	leader	AT1G74030		
5980_TC67834	leader	AT3G51780		SN
5990_TC63122	leader	AT4G18910		SN
6159_TC62512	leader	AT1G20000		SN
6180_TC64083	leader	AT5G54250		SN
6232_TC74066	leader	AT4G25130		JA
6532_TC76503	leader	AT4G13640	Fraser	
6553_TC69356	leader	AT3G53570	Fraser	
6804_TC61367	leader	AT2G34680		
6845_TC71081	leader	AT5G11260		SN
7014_TC74347	leader	AT5G02810		SN
7016_TC74618	leader	AT1G15520	Fraser	SN
7063_TC68528	leader	AT3G15510	Fraser	
7546_TC57084	leader	AT5G26990		JA
7647_TC72895	leader	AT3G54420		
7801_TC61916	leader	AT5G62700		SN
7867_TC66235	leader	AT4G01700		SN
7965_TC72716	leader	AT3G46230		SN
8000_DR162242	leader	AT1G37130		JA

Table 2 (Continued)

8049_new00152	leader	AT5G48540		JA
8093_AL750356	leader	AT5G59845		SN
8225_TC62400	leader	AT3G48000	Fraser	
8243_TC66868	leader	AT5G03740	Fraser	
8261_TC73531	leader	AT3G05950		JA
8568_DN613481	leader	AT3G07650	Fraser	
8618_DR689687	leader	AT1G17840		Q
8652_TC79637	leader	AT1G32360		JA, SN
8725_TC71892	leader	AT1G65730		SN
8775_TC58960	leader	AT5G61210		SN
8777_TC65948	leader	AT5G13930		JA
8858_TC67978	leader	AT4G02570		SN
9040_TC70658	leader	AT1G02040		SN
9083_TC75737	leader	AT4G17260	Fraser	JA
9156_TC66681	leader	AT2G37340	Fraser	
9321_TC65653	leader	AT3G43810	Fraser	JA
9491_TC58752	leader	AT1G12520		JA
9560_TC64722	leader	AT2G45570		JA
9625_DR387851	leader	AT3G57330	Fraser	
972_TC60622	leader	AT3G19380		JA
9786_TC65929	leader	AT5G13930		JA
9894_TC70878	leader	AT5G06700	Fraser	SN
9992_TC61956	leader	AT4G02570		SN

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Chapter 4

GENE EXPRESSION CHANGES IN FRASER FIR AND NOBLE FIR DUE TO BALSAM WOOLLY ADELGID INFESTATION

ABSTRACT

Seedlings of two fir species, Fraser fir (*Abies fraseri* (Pursh) Poir.) and noble fir (*Abies procera* Rehder), were infested with BWA under controlled environmental conditions and differences in gene expression between the infested seedlings and uninfested controls were studied using microarrays. Fraser fir is highly susceptible to BWA, with high levels of mortality seen in natural stands. Noble fir is less susceptible, with low mortality seen in natural stands. A much greater number of genes were found to be differentially regulated in Fraser fir than in noble fir. Cellular processes, metabolic processes, and response to stimulus were the annotation categories most highly represented by the significant genes. Multiple genes involved in jasmonic acid (JA) signaling pathways were found to be upregulated in infested Fraser fir. Two significant probes were found to have high sequence similarity to the *Arabidopsis thaliana* (L.) Heynh. PAL1 gene; one was upregulated in infested noble fir, while the other was downregulated in infested Fraser fir. Additional phenylpropanoid biosynthesis genes showed significant changes in transcript levels; five were downregulated in infested Fraser fir while only one was upregulated.

INTRODUCTION

The balsam woolly adelgid, *Adelges piceae* (Ratzeburg), is a pest of fir (*Abies*) species native to central Europe and was introduced into the United States in 1908 (Kotinsky 1916; Balch 1952). Different species of fir show varying levels of susceptibility to infestation by the balsam woolly adelgid (BWA), with many of the Asian and European fir species being completely tolerant or resistant to BWA infestation (Mitchell 1966). Fraser fir (*Abies fraseri* (Pursh) Poir.), which is found naturally only in a few isolated stands in the mountains of Virginia, North Carolina, and Tennessee, has been found to be highly susceptible and has been subject to mortality rates as high as 95% in some areas of the southern Appalachians (Mitchell 1966; Witter and Ragenovich 1986). Noble fir (*Abies procera* Rehder), native to the Pacific Northwest, is generally less susceptible to damage from BWA than Fraser fir; mortality from BWA has been seen in low elevation plantings, but noble fir has been shown to have resistance in stands at elevations above 300 meters in the Pacific Northwest (Mitchell 1966; Mitchell and Buffam 2001).

In susceptible species, such as Fraser fir, several symptoms of adelgid infestation are seen. There is often gouting, which is a swelling of the tissue at the nodes and around the buds, the twigs and smaller branches may show swelling or twisting, and many times apical dominance is lost, with the top of the crown becoming flattened (Brower 1947; Balch 1952). In addition, the adelgid's feeding causes formation of xylem tissue that resembles compression wood, and this abnormal tissue is referred to as "rotholz" because of its red color (Balch et al. 1964; Timell 1986). This damaged xylem tissue does not conduct water

well, causing drought stress, reduced photosynthesis and respiration, and eventual tree death (Mitchell 1967; Puritch 1973; Hollingsworth and Hain 1991). Mortality in Fraser fir trees is usually seen after two to five years of infestation (Amman and Speers 1965). It is unknown what genes and defense pathways are involved in the reaction to this insect by either susceptible or resistant species.

Microarrays can be used to measure levels of gene expression across thousands of genes at one time. There are several examples of microarray technology being utilized to study gene expression responses in forest trees to insect feeding, such as forest tent caterpillar (*Malacosoma disstria* Hübner) on hybrid poplar (*Populus trichocarpa* x *deltoides*) (Ralph et al. 2006a), spruce budworm (*Choristoneura occidentalis* Freeman) or white pine weevils (*Pissodes strobi* Peck) on Sitka spruce (*Picea sitchensis* (Bong.) Carrière) (Ralph et al. 2006b; Ralph et al. 2006c), or gypsy moth (*Lymantria dispar* L.) on black poplar (*Populus nigra* L.) (Babst et al. 2009).

Probes for microarrays used here were designed from EST sequences from other species in the Pinaceae family, mainly loblolly pine (*Pinus taeda* L.), that were similar to *Arabidopsis thaliana* (L.) Heynh. genes known to be involved in plant defense or stress responses. Previous experiments have shown that results can be obtained using arrays designed from sequence information of another species in the same taxonomic family (van Zyl et al. 2002). In this study, both Fraser fir and noble fir seedlings were infested with BWA under controlled environmental conditions. Gene expression differences between

infested and control seedlings were studied to determine genes and defense pathways involved in the reactions to the adelgid infestation.

METHODS

Three-year-old seedlings of Fraser fir, noble fir, and Veitch's fir (*Abies veitchii* Lindley) were grown in a controlled environment in Anderson bands (Anderson Die & Manufacturing, Portland, OR) in a finely crushed bark mulch at North Carolina State University. They were kept at 18°C with approximately 50% relative humidity, under fluorescent lighting on a 16 hour:8 hour light:dark cycle. Seedlings were randomly assigned to either the infested or uninfested treatment. The two treatments were kept on opposite sides of the same room, both enclosed in a mesh fabric to keep the adelgids from moving to the uninfested controls. Small bark discs from Fraser fir trees containing at least one BWA egg mass were placed on the seedling branches to infest the seedlings with the adelgid. The seedlings were examined at least every other day for any crawlers found on the branches. The crawlers settled on or near the buds, so the branch tips and buds were collected and immediately frozen in liquid nitrogen. Branch tips were collected for two categories of infestation length, the short infestation length was one to two days after infestation, and the longer infestation length was four to six days after an adelgid was found settled on the branch tip. Either one or two samples were used from each tree; in cases where two samples were used from the same tree, both were collected on the same date. All but one seedling also had

another branch tip with BWA that had been feeding for a longer period, from one to five weeks, which were not included in the gene expression analysis.

Infested branch tips were only able to be collected from three Veitch's fir seedlings, so this species was not included in the gene expression study. Additionally, a dead crawler that had inserted its stylet was found on one of the Veitch's fir seedlings. It is likely that a large number of infested branch tips from this species were not able to be obtained because Veitch's fir is highly resistant to BWA infestation (Mitchell 1966).

Sample Preparation

For RNA extraction, the buds were placed in a 2 ml screw-cap tube between two ceramic beads. The tube was shaken for 30 seconds in a FastPrep® instrument to grind the tissue. RNA was extracted using Qiagen® RNeasy® spin columns, with a modified protocol (Dharmawardhana, unpublished method).

Five micrograms of RNA were used as the starting material for a reverse transcriptase reaction to obtain cDNA. 1µl each of oligo dT and dNTP mix (10 mM each) were added and the mixture was heated at 65 °C for 5 minutes. The samples were quickly chilled on ice and spun down. 4µl of 5x first strand buffer and 2 µl of 0.1 M DTT were added and incubated at 42°C for 2 minutes. 1 µl of Superscript® RT II (Invitrogen™) was added and the mixture was incubated at 42°C overnight. The reaction was inactivated by heating at 70°C for 15 minutes, then 1 µl of RNase H was added and the mixture was incubated at 37°C for 20 minutes.

The cDNA product was purified using Qiaquick® columns (QIAGEN Inc). 200 µl of Qiagen® binding buffer was added to the sample and then was transferred to the column. The column was centrifuged for 1 minute at 13,000 rpm then washed twice with 750 µl of Qiagen® wash buffer. The column was dried by centrifuging for 1 minute, and cDNA was eluted twice with 20 µl of water, incubating for one minute before centrifugation.

The Ulysis® AlexaFluor® 647 kit was used for the fragmenting and labeling of the cDNA samples. To fragment the cDNA, a digest was performed with a 10,000-fold dilution of DNase I, with incubation for 10 minutes at 37°C. The labeling reaction was performed with 7.5 ul of labeling dye as described in the product information.

Hybridization

Combimatrix CustomArray™ 4x2K arrays were used. The probes on the array were designed from *Pinus taeda* sequences similar to *A. thaliana* genes known to be involved in plant defense or stress responses, as described in Chapter 3.

The volume of the labeled product was reduced down to 15.75µl in a speed-vac. Pre-hybridization and hybridization solutions, as well as wash solutions, were prepared according to the CustomArray™ 4X2K microarray hybridization and imaging protocol (PTL005). Hybridizations were performed in a 45°C rotisserie hybridization oven overnight and washed according to the above protocols. The arrays were scanned in a ScanArray® Lite (PerkinElmer Life Sciences Inc) scanner. The arrays were subsequently stripped following the Combimatrix protocols for stripping and preparation for re-hybridization (PTL001 and

PTL002); two arrays were used for three hybridizations each. The samples were randomly assigned to the order in which they were hybridized. Gridding and data extraction was performed using the CombiMatrix microarray imager software.

Statistical Analysis

Exported data were analyzed in JMP® Genomics (version 4, SAS Institute, Inc.). Intensity values were averaged for any probe that was present on the array more than once. A Loess normalization was performed on the data, using a smoothing parameter of 0.2 and 1 Loess iteration. Samples infested for one to two days were categorized as a short infestation length, and samples infested for four to six days were categorized as long infestation length. Since samples were collected over a period of several weeks, the number of days between the start of the experiment and the day a sample was collected was calculated. A mixed model was performed for each species separately, with an infestation value of 0 or 1 as a fixed effect. Tree number, the number of days in the experiment, and the use number of the array were considered random variables. Tree, infestation, and use number were treated as class variables. The category of length of infestation was used as a design level by-variable, meaning analyses were performed separately for each infestation length. The degrees of freedom were calculated with the Satterthwaite method. The pFDR multiple testing correction method was used, with α=0.05 (Storey 2002). LS means were calculated to compare infested samples with the control.

Blast2GO was used to determine annotations for the EST sequences from which the probes for the array were designed (Conesa et al. 2005). A Fisher's exact test was performed in Blast2GO to determine significant differences between annotation assignments for upregulated and downregulated genes. KEGG pathway assignments for these sequences were also found using Blast2GO. The top matches to *A. thaliana* genes were determined using a blastx search in blast+ against the Arabidopsis protein database for each of the EST sequences used to design the probes on the array (Altschul et al. 1990, Altschul et al. 1997). These *A. thaliana* genes were used to compare results here to previous microarray experiments.

RESULTS AND DISCUSSION

Two hundred and thirty-seven probes were found to be significantly differentially regulated due to BWA infestation of fir seedlings in this experiment. Only 11 were found to be differentially regulated in noble fir infested seedlings compared to the controls, and the remaining probes were differentially regulated in Fraser fir. No probes were found to be differentially regulated in both species. Within noble fir, nine of the 11 probes were differentially regulated in the shorter infestation length of one to two days. Within Fraser fir, more probes (90% of the total) were differentially regulated after a longer infestation length of four to six days than after the shorter infestation length. The numbers of up- and down-regulated probes for each species and infestation period is shown in Table 1, and all the significant probes are listed in Table 2. Although the total number of differentially regulated

genes for noble fir is quite low, this gives some indication that this species, that is more resistant to BWA, may have a greater defense response right after BWA infestation, while the susceptible species, Fraser fir, continues to respond to BWA for several days after the infestation begins. The quicker response by noble fir may be indicative of a normal wound response, while the gene expression changes in Fraser fir may be part of its longer term response that includes rotholz production in the wood. Three probes (10875_TC59101, 8450_TC72606, 8609_TC71676) were found to be differentially regulated in Fraser fir after both the shorter and longer infestation period, and in all three of these cases, the probes were regulated in the opposite directions for the two infestation lengths. Two of the three probes were upregulated after the shorter period of infestation and then downregulated after the longer period of infestation.

Although some of the changes in gene expression seen here could be due to systemic responses from the other branch tip on the seedling not used in the study that had been infested for one to five weeks, it is expected that most of the differentially expressed genes are localized and due to the adelgid feeding on the branch tip sampled. In most cases, branch tips for both infestation lengths were collected on the same date from one seedling, so it may be expected that systemic gene expression changes from branch tips not included in the study would be similar in both of the categories of infestation length. Since there is little overlap in gene expression changes seen for the two infestation lengths, it suggests that the local gene expression changes are greater than the systemic effects. Also, previous work by Babst et al. (2009) looking at gene expression changes in *Populus nigra* L. with feeding by gypsy moth

(*Lymantria dispar* L.) larvae found that only a small number of differentially regulated genes were affected systemically, when compared to the number of localized differentially expressed genes.

Several annotation categories were significantly over or under represented when comparing the upregulated genes to those that were downregulated when the single test p-values with a significance level of 0.05 were used, whereas no categories were significant if the pFDR correction for multiple testing was included. In general, metabolic and biosynthetic processes tended to be more highly represented in the upregulated genes, whereas complex assembly and subunit organization annotations were more highly represented in the downregulated genes.

In comparing the results here to previous microarray experiments with field collected samples from infested Fraser fir, one EST significant here for infestation in Fraser fir was also significant in the samples collected from the field in 2007 (TC80513) (Chapter 3). Additionally, one EST significant in the 2006 field experiment was significant here for infestation in noble fir (TC76351) (Chapter 3). The corresponding genes in *A. thaliana* are both known to be regulators of transcription (AT1G67310, AT3G18380). Also, over 40 probes significant for apical dominance loss in the field sampling of infested Fraser fir were found to be differentially expressed here. This indicates that many of the genes affected early after BWA infestation continue to be differentially expressed for a long period after infestation, since the trees sampled in the field had been infested for much longer periods of time, years rather than weeks.

The *A. thaliana* gene matches for the ESTs used to design the arrays here were used to compare the results of this experiment with a previous study by Babst et. al. (2009). Ten of the significant genes in our study were also found to be differentially regulated in *P. nigra* when fed on by gypsy moth larvae (Babst et al. 2009). Interestingly, for half of these ten genes, BWA infestation had an effect opposite that of gypsy moth (Babst et al. 2009). The differences in gene responses between these two studies could be due to the fact that Fraser fir and poplar are tree species that are not closely related, Fraser fir is a conifer and poplar is a deciduous species and they may respond to insect feeding in very different ways. In addition, gypsy moth and BWA have different feeding mechanisms; gypsy moth chews on the foliage, while BWA uses a piercing-sucking mouthpart to insert a stylet into the bark of the tree. Also, the *P. nigra* samples collected by Babst et al. (2009) were collected 22 hours after the gypsy moth larvae were placed in the leaf, and in the current study, the tissue samples were collected after a slightly longer period of infestation, either one to two days or four to six days after infestation.

MYC2 (AT1G32640) was found to be upregulated by both BWA and gypsy moth infestation, and was downregulated in Fraser fir one week after treatment with JA (Chapter 2; Babst et al. 2009). MYC2 is involved in the activation of the JA and ABA pathways, as well as activating JA-dependent wound and insect responses in *A. thaliana* (Lorenzo et al. 2004; Dombrecht et al. 2007).

PAL1 (AT2G37040) was the best *A. thaliana* match for two probes that were differentially regulated with BWA infestation; one probe was upregulated in noble fir and the

other was downregulated in Fraser fir. PAL1 was also found to be downregulated in Fraser fir one week after treatment with JA (Chapter 2). PAL1 is a phenylalanine-ammonia lyase, which is known to be upregulated in bean (*Phaseolus vulgaris* L.) by wounding or fungal infection (Liang et al. 1989). Phenylalanine-ammonia lyase is responsible for the first step in phenlypropanoid biosynthesis. Only one additional phenylpropanoid biosynthesis gene was upregulated in infested Fraser fir (AT3G06050) in this study while five were downregulated in Fraser fir (AT3G48000, AT4G16260, AT4G01070, AT4G16270, AT1G71695). AT3G06050 (PRXIIF) has been shown to be upregulated in mitochondria due to oxidative stress, and is thought to be important in antioxidant defense (Sweetlove et al. 2002). Some of the downregulated phenylpropanoid genes are also related to stress or defense responses; AT4G16260 is a cell wall related protein in the glycosyl hydrolase family, which has been shown to be upregulated with NaCl treatment (Jiang et al. 2007), as well as with *Alternaria brassicicola* (Schweinitz, Wiltshire) infection (Mukherjee et al. 2010). AT1G71695 is a putative peroxidase found in the vacuole (Carter et al. 2004). It is interesting that many of these genes related to the phenylpropanoid pathway, and related to stress responses, were found to be downregulated in Fraser fir. This indicates a possibility that chemicals in the adelgid's saliva are negatively affecting Fraser fir's normal defense responses. This does not seem to be the case in the less susceptible species, noble fir, where PAL1 was upregulated after infestation.

Two additional genes known to be involved in stress responses were upregulated in noble fir with BWA infestation, but not in Fraser fir. The first is APG6 (AT5G15450),

which is known to be upregulated with heat stress, but thus far has not been related to other types of plant stressors (Myouga et al. 2006). The second is KIN10 (AT3G01090), which is a protein kinase that regulates the activation of many genes. A large number of these genes have been shown to be activated by KIN10 under stress conditions (Baena-Gonzalez et al. 2007). KIN10 has also been identified as related to abscisic acid signaling (Jossier et al. 2009).

A total of seven genes found to be upregulated in Fraser fir with BWA infestation were also upregulated with the foliar application of JA to Fraser fir foliage (Chapter 2). One of these is AT3G44480, which was upregulated 1 week after treatment with JA (Chapter 2). This is also known as RPP1, which is involved in resistance of *A. thaliana* to the fungal parasite *Hyaloperonospora parasitica* (Pers.) Constant. (Botella et al. 1998).

The gene AT5G09810, ACT7, was found to be downregulated in Fraser fir both by BWA infestation and one week after treatment with sodium nitroprusside (SN), which acts as a donor of nitric oxide (Chapter 2). This is an actin gene that has previously been shown to be downregulated with ABA application and upregulated with auxin treatment in *A. thaliana* roots (McDowell et al. 1996; Kandasamy et al. 2001). Although ACT7 expression has been studied in roots and is involved in root callus formation, it is unknown how this compares to its function in other plant tissues.

The gene PDR12 was found to be downregulated in Fraser fir both by BWA infestation and one week after treatment with SN (Chapter 2). PDR12 is in the pleiotropic drug resistance (PDR) subfamily of ATP-binding cassette (ABC) transporters (Sanchez-

Fernandez et al. 2001). PDR12 is a plasma membrane abscisic acid transporter present in plant guard cells, which has been shown to be involved in stomatal closure in response to drought stress (Kang et al. 2010).

MYB6 (AT4G09460) was upregulated in Fraser fir by BWA infestation. MYB6 is part of the MYB transcription factor family, and has been shown to be upregulated by stress and by treatment with chemicals including abscisic acid, ethylene, jasmonic acid, and salicylic acid (Yanhui et al. 2006). The gene JAR1 (AT2G46370) was also upregulated in Fraser fir with BWA infestation. JAR1 is a JA-amino synthetase that activates JA (Staswick and Tiryaki 2004). This suggests that BWA infestation activates the JA-signaling pathway in Fraser fir.

Additional evidence to suggest that the JA-signaling pathway is activated by BWA infestation in Fraser fir is the fact that the gene ARF6 (AT1G30330) was upregulated in Fraser fir after BWA infestation. ARF6 is an auxin response transcription factor that is required for auxin response in flowers, as well as for JA production in flowers (Nagpal et al. 2005). It is possible that ARF6 may regulate genes involved in JA production (Nagpal et al. 2005). It was also shown to be downregulated in *P. nigra* when fed on by gypsy moth larvae (Babst et al. 2009). The opposite results for BWA infestation compared with gypsy moth infestation may be due to the fact that different types of tissues were used in the two experiments, leaf tissue was collected from *P. nigra* (Babst et al. 2009).

Production of terpenes is a major part of the general defense system of conifers in the Pinaceae family. Surprisingly, only one terpenoid synthase gene is upregulated with

infestation, and it was upregulated in Fraser fir after the longer length of infestation by the adelgid. The gene is TPS02 (AT4G16730), which is a (E)-β-Ocimene and (E,E)-α-Farnesene synthase gene (Huang et al. 2010). TPS02 has also been shown to be induced in *A. thaliana* by treatment of coronalon, a jasmonate mimic, as well as by feeding by *Plutella xylostella* L. (Huang et al. 2010). (E)-β-Ocimene and (E,E)-α-Farnesene are plant volatile compounds that are known to be emitted due to insect feeding on a number of different plant species and are believed to possibly be used to attract natural enemies of insect herbivores (Loughrin et al. 1994; Rose et al. 1996; Pare and Tumlinson 1997; Kessler and Baldwin 2001).

CONCLUSIONS

Several genes known to be involved in plant defense responses have been identified here as part of the defense response to BWA. Noble fir had a greater number of differentially regulated genes after the shorter period of infestation, while most of the differentially regulated genes in Fraser fir were seen after the longer infestation period. This indicates that noble fir may be showing a normal wound response to the adelgid's stylet that is over quickly. The gene expression changes in Fraser fir may be part of the many symptoms seen after infestation, including rotholz production, loss of apical dominance, gouting, and eventually tree death.

Metabolic and biosynthetic processes were found to be more highly represented in the upregulated genes. Genes involved in JA and abscisic acid signaling were also found to be upregulated with BWA infestation. Further investigation of these genes is necessary to

differentiate the responses of susceptible versus resistant species as well as the responses in susceptible species leading to rotholz production. In particular, PAL1 genes that were upregulated in noble fir but downregulated in Fraser fir may be of interest, including whether BWA saliva is affecting the expression of these genes.

Table 1. The number of up-/down-regulated probes for each species and category of infestation length.

	Short Infestation	Long Infestation
Fraser fir	12/11	84/122
Noble fir	4/5	1/1

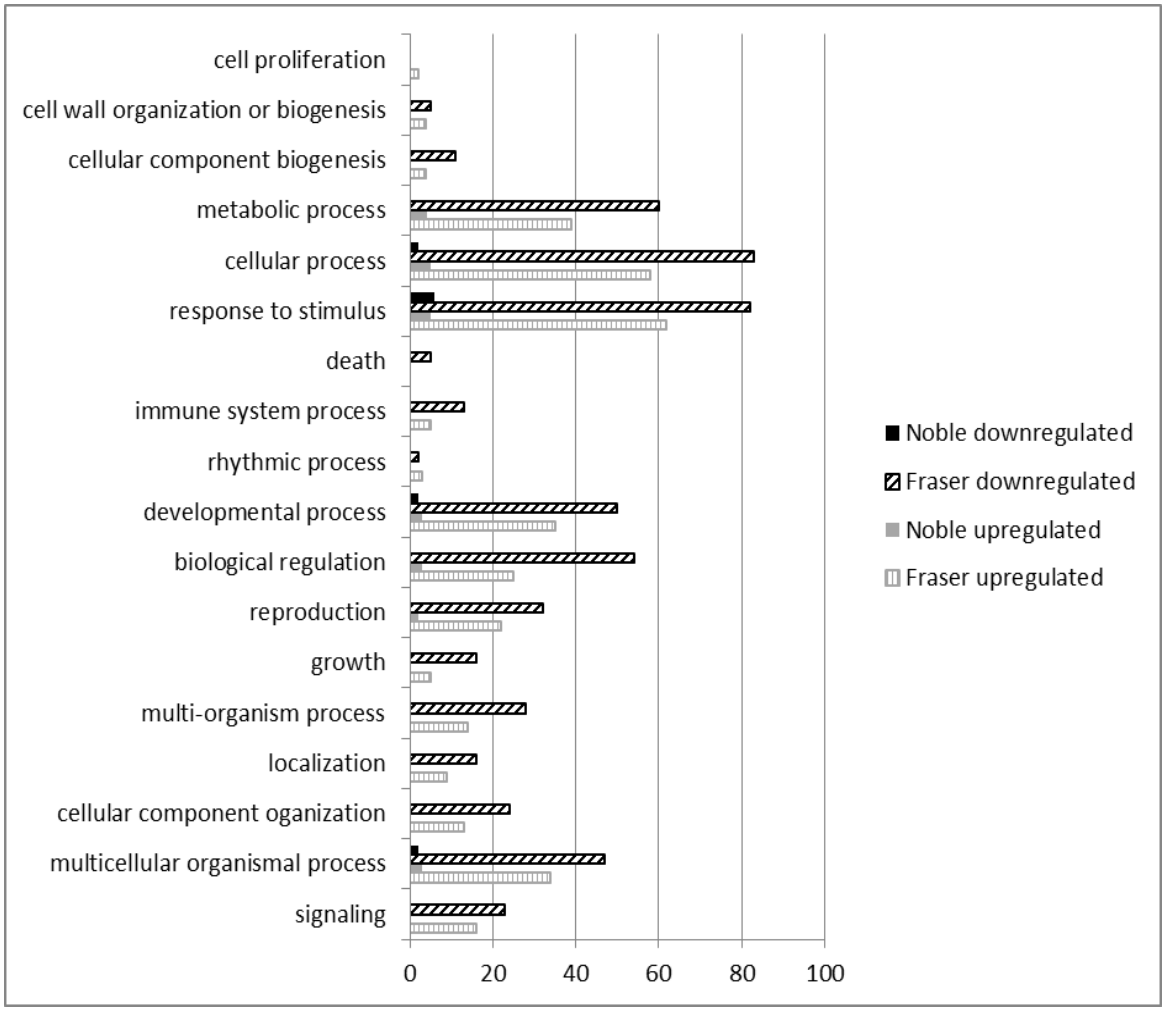

Figure 1. Biological process level 2 categories from Blast2GO for the probes found to be up- or down-regulated for each species in this experiment.

Table 2. List of probes found to be significantly differentially regulated for each species and infestation length, with fold change between the infested and the control, whether it was up or down regulated, and the closest match to *A. thaliana*.

Probe Name	**Up/Down Regulated**	**Fold Change**	**Species**	**Infestation Length**	**A. thaliana match**
10053_TC57877	up	1.27	Fraser	Long	AT2G37060
10086_TC67945	up	1.34	Fraser	Long	AT2G02380
10205_TC65318	down	0.71	Fraser	Long	AT4G30270
1023_TC61029	up	1.28	Fraser	Long	AT4G24210
10244_TC68523	down	0.78	Fraser	Long	AT5G25810
10247_AA739937	down	0.73	Fraser	Short	AT3G28917
10430_TC62567	down	0.88	Fraser	Long	AT4G29270
10504_TC58120	down	0.77	Fraser	Long	AT3G22400
1060_TC69663	down	0.84	Fraser	Long	AT2G21100
10603_DN609729	up	1.18	Fraser	Long	AT3G13960
10751_TC68645	up	1.18	Fraser	Long	AT3G02260
1080_TC61916	down	0.76	Fraser	Long	AT5G62700
10827_TC65298	up	1.38	Fraser	Long	AT4G30270
10854_AW870226	up	1.19	Noble	Short	AT5G15450
10875_TC59101	down	0.86	Fraser	Long	AT4G08150
10875_TC59101	up	1.14	Fraser	Short	AT4G08150
10878_new00644	up	1.15	Fraser	Long	AT4G09320
10889_TC66486	down	0.91	Fraser	Short	AT5G28540
10913_TC57093	up	1.22	Fraser	Long	AT4G10750
10924_TC78174	down	0.85	Fraser	Long	AT1G44110
10929_DR688643	down	0.88	Fraser	Long	AT4G38860
10969_TC76584	down	0.70	Fraser	Long	AT4G39470
11035_TC72911	down	0.73	Fraser	Long	AT2G17390
11205_TC70547	down	0.88	Fraser	Long	AT2G40950
11206_TC64376	down	0.81	Fraser	Long	AT1G51310
11287_TC75713	up	1.59	Fraser	Long	AT4G00830
11459_TC68061	up	1.24	Fraser	Short	AT4G35985
11472_CO175551	down	0.64	Fraser	Long	AT1G16670
11493_CF667983	down	0.94	Noble	Long	AT1G55870
11575_TC65790	up	1.26	Fraser	Long	AT5G60910
11586_TC67109	down	0.71	Fraser	Long	AT2G42810
116_TC73913	up	1.16	Fraser	Long	AT4G16730
11674_TC62649	down	0.68	Fraser	Long	AT2G46690
11704_TC76243	up	1.54	Fraser	Short	AT3G53000
11863_TC76792	up	1.25	Fraser	Long	AT3G05970
11866_TC68077	down	0.72	Fraser	Long	AT4G01070
11895_new40640	down	0.74	Fraser	Short	AT4G35300
11945_TC61592	down	0.76	Fraser	Long	AT1G59218
11964_TC70798	down	0.87	Fraser	Long	AT1G11080
120_DR119369	up	1.30	Fraser	Long	AT5G19160

Table 2 (Continued)

12070_TC70549	down	0.89	Fraser	Long	AT5G15800
12133_TC58202	up	1.19	Fraser	Long	AT1G75540
12139_TC63554	up	1.22	Fraser	Long	AT3G06590
12194_TC74503	down	0.80	Fraser	Long	AT4G24210
12233_TC60889	down	0.86	Fraser	Long	AT5G23420
12251_TC67475	down	0.66	Fraser	Long	AT1G65910
12258_TC60964	down	0.92	Fraser	Long	AT5G65380
12275_TC76703	down	0.90	Fraser	Long	AT3G30530
12279_TC57958	down	0.92	Fraser	Long	AT5G07990
12307_TC67885	up	1.24	Fraser	Long	AT2G18030
12319_TC68097	down	0.67	Fraser	Short	AT5G28640
12347_AA556306	up	1.27	Fraser	Long	AT5G15850
12379_TC73934	down	0.71	Fraser	Long	AT1G77490
1243_TC61394	down	0.83	Fraser	Long	AT2G27920
12501_DN611590	down	0.63	Fraser	Long	AT1G22690
12531_new00588	down	0.95	Fraser	Long	AT1G30270
1255_TC78293	up	1.09	Fraser	Long	AT1G76880
1306_DR695339	up	1.19	Fraser	Long	AT1G02040
1501_TC77150	up	1.10	Fraser	Long	AT5G51230
1518_TC68719	down	0.81	Fraser	Long	AT4G37580
1521_DR691856	up	1.50	Fraser	Long	AT5G59320
1540_TC73940	up	1.14	Fraser	Long	AT1G67750
1546_new09433	up	1.21	Fraser	Long	AT5G66140
1583_AW042955	up	1.11	Fraser	Long	AT5G52640
1632_TC74059	down	0.84	Fraser	Long	AT3G14290
172_TC73446	up	1.32	Fraser	Long	AT3G48090
1819_TC61519	up	1.28	Fraser	Long	AT3G54220
1823_DR177302	down	0.92	Fraser	Long	AT1G27170
1825_TC75902	down	0.76	Fraser	Long	AT1G20990
1832_CX650407	up	1.03	Noble	Long	AT3G01090
1839_TC79138	down	0.77	Fraser	Short	AT4G16270
1842_new01459	up	1.23	Fraser	Long	AT3G55360
1846_AI812437	down	0.77	Fraser	Long	AT5G01650
1915_DR686575	up	1.35	Fraser	Long	AT3G25700
1928_TC59317	down	0.83	Fraser	Long	AT5G62000
1931_DR693059	down	0.73	Fraser	Long	AT2G01830
2011_TC77256	down	0.75	Fraser	Long	AT5G24930
2031_TC57602	up	1.27	Fraser	Long	AT3G12500
2037_TC76351	down	0.89	Noble	Short	AT1G67310
2114_DR387292	up	1.29	Fraser	Long	AT2G26300
2124_DR049213	down	0.82	Fraser	Long	AT4G30410
2190_TC74636	up	1.09	Fraser	Long	AT1G10630
2213_TC76243	down	0.83	Fraser	Long	AT3G53000
2232_BM492972	down	0.84	Fraser	Long	AT5G39340
2283_TC76095	up	1.23	Fraser	Long	AT4G21450
2315_TC59673	down	0.77	Fraser	Long	AT1G46264

Table 2 (Continued)

235_TC68944	up	1.17	Fraser	Long	AT3G24120
2363_TC77305	up	1.34	Fraser	Short	AT1G32640
2449_TC62968	down	0.73	Fraser	Long	AT2G27710
2461_TC74726	down	0.85	Fraser	Long	AT1G70370
2514_TC57650	down	0.82	Fraser	Long	AT4G16260
2539_TC62687	down	0.82	Fraser	Long	AT1G61290
2544_TC61139	up	1.23	Fraser	Long	AT4G35850
2611_BX250346	down	0.93	Fraser	Long	AT3G20810
2692_TC75207	down	0.73	Fraser	Long	AT5G62000
2710_TC66863	up	1.56	Fraser	Long	AT5G20630
2727_TC68055	down	0.74	Fraser	Long	AT1G07980
2772_TC75493	down	0.82	Fraser	Long	AT2G30105
2891_TC73605	down	0.64	Fraser	Long	AT5G59320
3065_TC73281	up	1.24	Fraser	Long	AT5G20620
3071_TC72351	down	0.77	Fraser	Long	AT1G48030
3149_TC72566	up	1.35	Fraser	Long	AT1G44100
3228_TC75393	down	0.80	Fraser	Short	AT2G35940
3365_TC57307	up	1.29	Noble	Short	AT2G44745
3458_CF385349	up	1.28	Fraser	Long	AT3G44480
3496_TC73583	up	1.30	Fraser	Long	AT1G55300
358_TC73511	up	1.25	Fraser	Long	AT2G46370
3588_TC69340	down	0.65	Fraser	Long	AT3G47450
3601_TC60933	down	0.86	Fraser	Long	AT2G03430
3760_TC67968	down	0.82	Fraser	Long	AT3G20570
3763_TC62925	down	0.86	Fraser	Long	AT2G01630
3897_TC66145	down	0.71	Fraser	Long	AT1G71695
3914_TC62039	up	1.25	Fraser	Long	AT5G48880
3998_CO167117	up	1.27	Fraser	Long	AT4G14890
406_BX678446	up	1.36	Fraser	Long	AT2G32720
4123_TC76249	up	1.18	Fraser	Long	AT4G39890
4294_DR386472	down	0.82	Fraser	Long	AT5G14520
4343_TC79153	down	0.63	Fraser	Long	AT1G27170
4359_TC65317	up	1.47	Fraser	Short	AT3G06050
4362_TC59627	down	0.71	Fraser	Long	AT3G23990
4451_TC67318	up	1.16	Fraser	Long	AT2G33320
4552_TC67395	up	1.07	Noble	Short	AT2G37040
4653_DR691856	down	0.86	Fraser	Long	AT5G59320
4796_TC76828	down	0.79	Fraser	Long	AT2G43040
4824_TC66235	down	0.75	Fraser	Long	AT4G01700
491_DN609855	down	0.74	Fraser	Long	AT3G54940
4914_TC58109	down	0.84	Noble	Short	AT1G67100
4966_TC77060	down	0.84	Fraser	Long	AT1G50320
504_BX252812	down	0.76	Fraser	Long	AT1G63750
5077_TC62756	up	1.18	Fraser	Long	AT5G48150
509_TC61522	up	1.07	Fraser	Long	AT3G55560
5165_TC68635	up	1.76	Fraser	Long	AT4G22810

Table 2 (Continued)

5182_TC70623	up	1.31	Fraser	Long	AT4G15440
5286_TC74724	up	1.26	Fraser	Long	AT3G51880
5299_TC74950	down	0.91	Fraser	Long	AT5G54580
5359_TC72716	down	0.52	Fraser	Long	AT3G46230
5378_BX678239	up	1.42	Fraser	Long	AT1G73690
5490_TC75609	up	1.23	Fraser	Long	AT1G30330
5503_TC68483	down	0.81	Fraser	Long	AT4G22260
5503_TC68483	up	1.27	Fraser	Short	AT4G22260
5515_TC78127	down	0.85	Fraser	Long	AT1G06840
5560_TC69752	down	0.88	Fraser	Short	AT3G63500
5596_DR693476	up	1.19	Fraser	Long	AT1G71870
5633_TC79621	up	1.42	Fraser	Long	AT4G21070
5651_TC68833	down	0.94	Noble	Short	AT1G62300
570_TC60470	up	1.29	Fraser	Long	AT3G26935
5760_AW010304	up	1.36	Fraser	Long	AT4G35640
5901_TC74120	up	1.22	Fraser	Short	AT1G64200
5936_BX675149	down	0.74	Fraser	Long	AT3G55090
5987_DR683700	down	0.87	Fraser	Long	AT5G55990
6013_TC62223	down	0.78	Fraser	Long	AT3G18690
6094_TC69748	up	1.13	Fraser	Long	AT1G13450
6122_AJ309092	up	2.08	Fraser	Short	AT5G42650
6145_TC74716	up	1.46	Fraser	Long	AT4G36750
6173_TC80513	up	1.26	Fraser	Long	AT3G18380
6186_TC80511	down	0.81	Fraser	Long	AT1G80780
6189_DR060450	down	0.66	Fraser	Long	AT2G43330
6295_TC64594	down	0.76	Fraser	Short	AT5G49980
6342_BQ635260	down	0.78	Fraser	Long	AT4G02570
6402_TC75002	down	0.78	Fraser	Long	AT5G55260
6449_AI812599	up	1.23	Fraser	Long	AT1G76900
6532_TC76503	down	0.73	Fraser	Long	AT4G13640
6553_TC69356	down	0.77	Fraser	Long	AT3G53570
6676_TC65978	down	0.76	Fraser	Long	AT5G13930
6777_TC74213	up	1.28	Fraser	Long	AT1G75290
6818_CD020615	down	0.77	Fraser	Long	AT3G27000
6847_TC61848	down	0.95	Fraser	Long	AT2G39940
6981_TC66819	down	0.82	Fraser	Long	AT5G55990
7016_TC74618	down	0.86	Fraser	Long	AT1G15520
7058_TC75093	down	0.61	Fraser	Short	ATCG00170
7063_TC68528	down	0.80	Fraser	Long	AT3G15510
7082_TC68729	down	0.80	Fraser	Long	AT1G13960
7095_TC71845	up	1.19	Fraser	Long	AT4G09460
7124_TC71889	down	0.87	Fraser	Long	AT4G35020
7208_TC65052	down	0.74	Fraser	Long	AT1G61800
7255_BF609586	down	0.84	Fraser	Long	AT2G43710
7271_TC71703	up	1.16	Noble	Short	AT5G45980
7382_TC75461	down	0.95	Fraser	Long	AT5G05350

Table 2 (Continued)

7411_CO175144	down	0.70	Fraser	Long	AT5G20250
7444_TC79052	up	1.10	Fraser	Long	AT5G19690
7485_CO369520	up	1.18	Fraser	Long	AT2G41835
7492_TC68959	down	0.92	Fraser	Long	AT3G24010
7506_TC72421	up	1.20	Fraser	Long	AT1G77670
7517_TC62193	down	0.83	Fraser	Long	AT2G24610
7526_DR010812	down	0.90	Noble	Short	AT3G09640
7539_CF395824	down	0.85	Fraser	Long	AT4G37050
7576_TC74170	up	1.08	Fraser	Long	AT3G25600
7588_BM902657	down	0.95	Fraser	Long	AT1G42970
7661_TC76504	up	1.25	Fraser	Short	AT1G80350
7861_CF664620	up	1.25	Fraser	Long	AT3G44110
7924_BF610198	down	0.79	Fraser	Long	AT5G11590
793_TC61269	down	0.81	Fraser	Long	AT3G22840
7959_TC74626	down	0.87	Fraser	Long	AT4G36760
7974_TC57668	down	0.79	Fraser	Long	AT4G38410
8058_TC75957	down	0.76	Fraser	Long	AT5G19910
8107_TC58238	down	0.74	Fraser	Long	AT4G27670
8127_TC67395	down	0.77	Fraser	Long	AT2G37040
8150_TC74500	up	1.28	Fraser	Long	AT4G24210
8225_TC62400	down	0.89	Fraser	Long	AT3G48000
8243_TC66868	down	0.73	Fraser	Long	AT5G03740
826_TC77875	down	0.76	Fraser	Long	AT2G25140
8296_TC66394	up	1.30	Fraser	Long	AT2G30050
8297_TC77768	down	0.67	Fraser	Long	AT3G60490
8350_TC59924	up	1.18	Fraser	Long	AT5G51970
8450_TC72606	down	0.89	Fraser	Long	AT5G46180
8450_TC72606	up	1.07	Fraser	Short	AT5G46180
8486_new21895	down	0.88	Fraser	Short	AT1G17880
8522_TC77201	up	1.18	Fraser	Long	AT4G24210
8564_TC71890	down	0.64	Fraser	Long	AT2G41710
8568_DN613481	down	0.82	Fraser	Long	AT3G07650
8609_TC71676	up	1.44	Fraser	Long	AT4G16970
8609_TC71676	down	0.69	Fraser	Short	AT4G16970
8659_TC69454	down	0.73	Fraser	Long	AT3G55200
8674_TC75721	down	0.76	Fraser	Long	AT5G05850
8682_CX646810	up	1.26	Fraser	Long	AT3G53630
8709_BX681729	up	1.13	Fraser	Long	AT3G62250
8776_TC68703	down	0.74	Fraser	Long	AT1G06760
8844_TC79555	up	1.18	Fraser	Long	AT1G75390
8846_TC68087	down	0.78	Fraser	Long	AT3G20640
8847_TC72163	up	1.29	Fraser	Long	AT4G37250
9031_TC75027	up	1.46	Fraser	Long	AT1G59940
9083_TC75737	up	1.37	Fraser	Long	AT4G17260
910_DR068879	down	0.84	Fraser	Long	AT2G33500
9156_TC66681	down	0.78	Fraser	Long	AT2G37340

Table 2 (Continued)

9171_TC74097	down	0.63	Fraser	Long	AT3G13470
9192_DR055340	up	1.14	Fraser	Long	AT3G19080
9295_TC76584	up	1.19	Fraser	Long	AT4G39470
9319_BM158807	down	0.78	Fraser	Long	AT4G32640
9321_TC65653	down	0.66	Fraser	Long	AT3G43810
9352_DR693345	up	1.13	Fraser	Long	AT5G59340
9416_TC62434	up	1.08	Fraser	Long	AT5G07990
9448_TC68939	down	0.83	Fraser	Long	AT1G20610
9478_DR388612	down	0.78	Fraser	Long	AT5G09810
9486_DR685191	down	0.48	Fraser	Long	AT2G21970
9528_TC79390	up	1.20	Fraser	Short	AT2G03500
9572_BQ654844	up	1.25	Fraser	Long	AT4G36250
9625_DR387851	up	1.27	Fraser	Short	AT3G57330
9881_CF672009	down	0.86	Noble	Short	AT1G78900
9894_TC70878	up	1.10	Fraser	Long	AT5G06700
9995_TC65807	down	0.76	Fraser	Long	AT5G60910

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DISSERTATION CONCLUSION

Prior to these studies, there had been limited research performed on gene expression and gene function in tree species, and even less on fir species in particular. The studies of gene expression here have identified a number of genes as being involved in plant defense responses in Fraser fir (*Abies fraseri* (Pursh.) Poir.) and noble fir (*A. procera* Rehder). The research utilizing the application of defense signaling chemicals on Fraser fir seedlings identified a number of genes with unknown functions as being affected by foliar application of one or more of the chemicals. Many of the genes differentially regulated by chemical application are also known to be involved in drought stress in other plant species. Although in some plant species foliar application of chemicals involved in plant defense signaling can increase plant resistance, there is some evidence here that repeated application of jasmonic acid and salicylic acid actually has the opposite effect in Fraser fir. Five probes corresponding to genes involved in plant defense in *Arabidopsis thaliana* (L.) Heynh., including the PAD4 gene, were found to be downregulated in Fraser fir after seven daily applications of jasmonic acid or salicylic acid.

The gene expression studies of fir infested with balsam woolly adelgid (*Adelges piceae* (Ratzeburg)) (BWA) have identified many genes differentially regulated by adelgid feeding, as well as genes involved in the long-term reaction by the tree to an infestation that has been ongoing for many years. Many of the genes differentially regulated for various levels of infestation in the field were known to be associated with drought stress. This is not surprising, since the adelgid infestation causes Fraser fir to produce a type of wood referred

to as rotholz, which does not conduct water very well, and the eventual death of the tree from the infestation is due to water stress in the crown. Also identified in the field study as being differentially regulated by multiple traits is a gene corresponding to *A. thaliana* PIN3, which is involved in plant tropism and may be related to the loss of apical dominance seen with BWA infestation.

The fir seedlings infested in the laboratory allowed for identification of genes that respond just days after infestation by BWA. Most of these genes are likely due to the plant's response to the adelgid itself, rather than the symptoms such as drought stress that are seen after a longer period of infestation. Of particular interest may be a gene corresponding to the *A. thaliana* PAL1 gene, which is related to plant defense and is part of the phenylpropanoid pathway. This gene was upregulated with BWA infestation in noble fir, a species that shows some resistance to the adelgid, while it was downregulated in Fraser fir with BWA infestation. Four additional phenylpropanoid biosynthesis genes were found to be downregulated in Fraser fir after infestation, so future work investigating whether the saliva of BWA is affecting the normal insect defense responses in Fraser fir is important.

For the Fraser fir samples collected in the field, the environmental variability from one site to another, as well as variation within one site, may have added to differences in gene expression among the trees. Some of this was overcome in the second year of sampling, when multiple samples of varying levels of infestation were collected from each tree. This may be part of the reason why there were far fewer significantly differentially regulated probes in the first year of field sampling as compared to the second year, 134

versus 348, respectively. In each year of the field sampling, 10 to 20 percent of the genes found to be differentially regulated by one or more traits in the field were also differentially regulated by BWA infestation in the lab. In future research of gene expression related to insect infestation, the use of plants in controlled environmental conditions would be preferable.

The results obtained from this series of gene expression studies can serve as a list of genes that would warrant further study in relation to plant defense. In the future, qRT-PCR could be used for additional studies of gene expression of genes of interest that have been identified here. Further studies to understand the functions of these genes in fir species are important. In addition, a better understanding of the effects of adelgid saliva on the normal defense response in Fraser fir would greatly aid in understanding the interaction between host and insect. A study of differentially regulated genes in BWA infested trees as compared to trees wounded in a manner similar to that of the feeding of the adelgid would help to separate the gene expression changes caused by the aldgid's saliva alone. Part of the difficulty in performing infestation studies with BWA is the small size of the insect and the fact that it is hard to determine the exact point in time at which the adelgid inserts its stylet into the tree. If there was a better way to pinpoint the exact time of insertion, a more controlled time course of gene expression changes after infestation could be undertaken.

If genes required for resistance to BWA infestation can one day be determined, this could aid in breeding resistant fir trees. Conversely, if the genes responsible for the tree's

production of rotholz wood, which is the eventual cause of tree death, can be discovered, it might be possible to use RNA silencing technology to prevent the expression of these genes.

When this research began several years ago, microarrays were the best method for studying gene expression of a large number of genes at once for candidate gene identification. But in recent years, sequencing technologies have improved greatly, and at the same time, costs associated with this technology have decreased greatly. Therefore, if this research was being undertaken today, transcriptome sequencing would be a better method to use, providing expression data on a greater number of genes.

Appendix A

INFESTATION STATUS AND BARK PHENOTYPES IN OPEN-POLLINATED FRASER FIR FAMILIES

ABSTRACT

Level of balsam woolly adelgid (*Adelges piceae* Ratzeburg) infestation, loss of apical dominance, gouting, bark appearance, and bark thickness were studied on Fraser fir (*Abies fraseri* (Pursh) Poir.) trees remaining on an abandoned open-pollinated progeny trial site. Highly significant correlations were seen between bark thickness, color, roughness, and hardness ($p<0.0001$). A significant negative correlation was also seen between the number of balsam woolly adelgid second instar larvae present and the bark hardness and thickness ($p<0.01$). The family within seed source variable was significant ($p=0.0098$) for bark thickness, and bark thickness had a heritability value of 0.58.

INTRODUCTION

Fraser fir (*Abies fraseri* (Pursh) Poir.) is native to the southeastern United States, where it is found only at a few populations at high elevations in North Carolina, Tennessee, and Virginia. It is grown over a much larger area as a Christmas tree species; it is grown in Christmas tree plantations on over 25,000 acres in North Carolina alone (NC Christmas Tree Association 2012). In natural stands, it has suffered high levels of mortality due to the balsam woolly adelgid (*Adelges piceae* Ratzeburg) (BWA), which was introduced from Europe.

Progeny tests have previously been performed on Fraser fir families to select those exhibiting traits preferred for Christmas tree growth. One such progeny trial was performed at a site in western North Carolina, Purchase Knob, planted in 1984 and containing open-pollinated families from five seed sources (Li et al. 1988; Jett et al. 1993). In 2002, the trees remaining in this progeny trial were surveyed for BWA infestation and bark appearance. It has been observed that Fraser fir bark tends to get rougher and thicker due to BWA infestation (Hollingsworth and Hain 1992). The relationship between levels of infestation and bark reaction, as well as the heritability of these traits were studied here.

METHODS

All trees remaining of the Purchase Knob progeny trial were surveyed for level of BWA infestation, height, level of apical dominance loss, and the presence or absence of branch gouting. The infestation level was categorized as high, medium, low, or not infested. The level of apical dominance loss was rated on a scale of 0 to 5, with 0 being no apical dominance loss and 5 having an apical branch bent at a 90° angle. The overall status of alive, dead, or that it was a stump sprout was noted. The overall appearance and health of the tree was rated from 0 to 2, with 0 being poor and 2 being extremely good. A piece of bark was removed from each tree at approximately breast height and brought back to the lab for further measurements. The bark pieces that were removed were approximately 2” x 2”. The thickness in four locations around the edge of the piece of bark was measured with calipers. The color was ranked on a scale of 1 to 4, with 1 being light colored and 4 being nearly black

in color. The roughness was measured as a percentage of the total area that contained bumps, and the overall bark reaction was rated from 1 to 4, with 1 being smooth, light colored, soft bark and 4 being rough, dark, and hard. The number of woolly masses and the number of second instars were counted inside a subset of each bark piece. The number of woolly masses falling within two or three 10 mm in diameter circles, depending on the size of the bark piece, were counted, and the number of second instars falling within two or three 8 mm in diameter circles were counted.

To measure the hardness of the bark pieces collected, a resistograph was used, which provided a measure of density (Isik and Li 2003). These resistograph measurements are given as varying levels of resistance, or density, as it moves through the piece of bark, and the maximum resistance level was used in further calculations. Resistograph measurements were taken for 277 of the bark samples.

Data analysis was performed using SAS software using the general linear model (GLM) procedure (SAS Institute, Inc. 2003). Trees that were stump sprouts were removed from the analysis, as well as any families not present in at least two reps. Rep was treated as a fixed variable, while source, family(source), rep*source, and rep*family(source) were random variables. Individual tree within population heritabilities for the bark thickness and hardness traits were calculated using equation (1)

$$h^2_{i(p)}=4\sigma^2_{f(s)}/(\sigma^2_{f(s)}+\sigma^2_{f(s)*rep}+\sigma^2_e) \qquad (1)$$

where $\sigma^2_{f(s)}$ is the variance due to family(source) differences, $\sigma^2_{f(s)*rep}$ is the variance due to family(source) x rep interaction, and σ^2_e is the error variance (Zobel and Talbert 1991). The

variance components of random effects were estimated by the restricted maximum likelihood method in the varcomp procedure in SAS (SAS Institute, Inc. 2003). Correlations were also calculated for the bark traits measured using the correlations procedure in SAS (Proc CORR) (SAS Institute, Inc. 2003).

Additionally, bark samples were taken from 6 other trees outside the progeny trial at the Purchase Knob site. Samples were collected at five different heights on the tree and measurements were collected for the following bark characteristics: roughness, color, thickness, overall bark reaction, number of BWA adults, and the number of BWA second instars.

RESULTS AND DISCUSSION

Highly significant correlations exist between the bark traits of thickness, color, roughness, overall bark reaction, and hardness (Table 1). With a level of significance of $\alpha=0.05$, there is also a significant negative correlation between the number of second instars present on the bark and both the hardness and thickness of the bark. Additionally, correlations between the number of adults and second instars with other bark traits are significant to a level of $\alpha=0.10$. This suggests that harder, thicker bark may not provide suitable feeding sites for BWA. The growth of harder, thicker bark may be a possible host resistance mechanism for Fraser fir.

The Type III sums of squares F values for the analysis of bark traits for the remaining progeny trial trees are shown in Table 2. Trees that had been cut and were stump sprouts

were removed from the analysis, as well as any families not present in at least two reps. The bark thickness trait is significant (p=0.0098) for family(source). This indicates that there are family differences in bark thickness, which could provide certain families with better resistance or tolerance to BWA infestation. The variance components and heritability values for the bark thickness and hardness traits are shown in Table 3, and the heritability of the bark thickness trait is 0.58. All the raw data collected is given in Table 4.

Figure 1 shows the average level of bark reaction, color, roughness, and thickness, as well as numbers of BWA adults and second instars for bark collected at varying heights from six trees. The level of bark roughness is highest lower on the bole of the trees and this decreases moving up the tree. The patterns are similar, though not as pronounced, for the other bark traits measured. The levels of BWA are low at the bottom of the tree, likely due to greater level of the bark reaction here. The BWA populations peak on the bark collected at the second highest height in the tree, where the overall bark reaction is lower and the adelgids are likely able to find the most suitable feeding sites.

Table 1. Correlations for bark traits measured, all trees included.

	# adults	# second instars	Color	Roughness	Overall bark reaction	Hardness
Thickness	-0.05669 0.3151	**-0.14567** **0.0095**	**0.47924** **<0.0001**	**0.65778** **<0.0001**	**0.59304** **<0.0001**	**0.54483** **<0.0001**
# adults		**0.35818** **<0.0001**	-0.09878 0.0796	-0.07316 0.1946	-0.06993 0.2151	-0.11627 0.0537
# second instars			-0.10482 0.0627	-0.10729 0.0568	-0.10265 0.0684	**-0.17705** **0.0032**
Color				**0.63323** **<0.0001**	**0.68440** **<0.0001**	**0.37487** **<0.0001**
Roughness					**0.86889** **<0.0001**	**0.49129** **<0.0001**
Overall bark reaction						**0.47547** **<0.0001**

Table 2. Type III sums of squares for all bark traits measured. **Significant to p<0.01.

	Rep	Source	Family(Source)	Rep*Source	Rep*Family(Source)
Degrees of Freedom	5	4	57	20	95
Thickness	2.05	1.24	1.70**	1.35	0.80
# adults	1.84	0.68	0.92	1.12	0.92
#second instars	1.45	1.38	1.27	1.39	2.65
Color	1.29	1.54	1.22	0.77	1.00
Roughness	0.58	0.96	1.07	1.52	1.15
Stage of reaction	1.10	1.75	1.03	1.26	0.97
Degrees of Freedom	5	4	57	20	72
Hardness	1.58	0.91	1.19	1.24	0.90

Table 3. Variance and heritability values for bark traits.

	Bark Thickness	**Bark Hardness**
Var(Rep)	0.07275	0.04081
Var(Rep*Source)	0.0001141	0.06665
Var(Family(Source))	0.21493	0.1757
Var(Rep*Family(Source))	0	0
Var(Error)	1.27011	1.60865
Heritability	0.57892043	0.393868916

Table 4. All bark and field measurements collected for remaining progeny trial trees.

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
1	1	15001-2	7.075	0.67	0.00	2	80	3	N	4.17	3.47	l	2	1	0
1	1	15005-1	5.4125	3.67	9.00	1	70	3	N						
1	1	15005-2	5.3	1.67	0.33	3	100	3	N	2.97	1.82	m	3	1	0
1	1	15502-2	5.5125	1.67	5.67	3	100	4	N	3.80	4.23	l	2	1	0
1	1	15507-1	6.6625	0.33	0.00	2	100	4	N	4.03	3.45	m	1	1	0
1	1	15507-2	6.05	0.00	0.00	2	80	3	N	4.97	5.02		3	1	0
1	1	15507-3	6.725	1.33	0.00	4	100	4	N	2.37	4.32	m	3	1	0
1	1	16001-4	7.025	1.33	1.33	3	70	3	N	4.30	3.95	h	1	1	0
1	1	16002-3	4.0375	0.00	7.33	2	40	2	N	3.77	5.42	h	5	0	1
1	1	16002-4	5.075	0.67	1.33	3	70	3	N		4.39	m	5	1	0
1	1	16003-5	6.7375	0.00	0.50	2	40	2	N	4.30	4.55	l	3	1	0
1	1	16004-3	7.225	0.00	0.00	4	90	4	N	2.63	4.31	m	3	1	0
1	1	16009-1	4.4875	0.00	0.00	3	50	2	N		3.42	h	3	1	0
1	1	16010-2	4.525	0.00	0.00	3	40	2	N	3.37	2.89	l	5	1	0
1	1	16010-3	4.525	0.33	1.67	1	10	1	N	3.23	3.27	h	2	1	0
2	1	25501-1	3.7	0.33	6.33	1	20	1	N	1.60	2.47	h	3	0	0
2	1	25502-4	5.075	3.33	7.67	3	80	3	Y	2.73	2.54	h	2	1	0
2	1	25503-1	6.9625	0.67	0.67	3	100	4	N	4.43	5.87	l	5	1	1
2	1	25503-4	6.7375	3.00	0.33	3	90	3	N	4.43	4.79	l	3	1	2
2	1	25507-1	3.625	0.33	9.67	2	50	3	Y	1.87	3.57	h	5	1	1
2	1	25507-3	7.85	1.67	0.00	4	90	4	N	3.77	4.29	h	4	1	0
2	1	26010-3	8.2375	1.33	0.00	2	80	3	N	4.17	3.8	h	2	1	0
2	1	26010-4	7.2625	0.33	0.67	2	80	3	N	3.37	3.94	h	3	1	0
3	1	35501-1	6.5875	0.00	0.00	2	60	2	N	3.77	3.86	m	3	1	0
3	1	35502-2	3.35	0.67	0.33	2	30	2	N	1.97	3.22	h	3	1	0
3	1	35504-5	5.4125	0.00	0.00	2	40	2	N	3.40	4.05	l	3	1	0
3	1	35507-3	3.9375	0.33	4.33	3	60	2	N	2.50	1.3	m		1	0
3	1	36003-3	6.35	3.00	3.00	3	50	2	N	3.90	4.1	h	2	1	0
3	1	36004-1	5.8	0.33	1.33	3	30	2	N		3.64	m	4	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
3	1	36004-4	3.6125	0.00	0.00	2	20	2	N	2.83	1.73	l	3	1	0
3	1	36006-4	5.475	0.00	7.67	3	60	3	N		4.29	m	2	1	0
3	1	36007-2	5.6875	0.00	0.00	2	90	3	N	5.50	3.31	m	3	1	0
3	1	36008-2	6.8625	2.00	1.33	3	80	3	N	3.43	4.4	h	3	1	0
4	1	46003-4	4.8125	0.00	0.00	3	90	3	N		4.81	l	3	1	0
4	1	46004-3	5.075	0.00	0.33	4	90	4	N		3.4	l	4	1	0
4	1	46005-5	5.9125	1.67	1.67	3	80	3	N		4.71	h	5	1	0
4	1	46006-4	7.1	2.67	1.00	3	80	3	N	3.37	4.32	h	1	1	0
4	1	46008-5	2.8	10.67	18.67	2	60	2	N	2.00	1.94	h	1	1	0
4	1	46009-5	7	0.33	0.00	2	100	3	N	6.57	3.4	l	2	1	0
4	1	46503-3	5.6375	0.33	4.67	4	100	4	N	4.47	2.78	l	2	1	0
4	1	46507-1	8.1875	0.00	0.00	4	100	4	N	6.57	3.94	h	1	1	0
4	1	46508-2	3.625	7.00	2.00	2	70	3	N	3.23	1.52	h	1	1	0
4	1	46510-5	3.825	0.00	0.00	2	10	2	N	1.87	2.27	l	1	1	0
5	1	55001-2	2.45	0.00	0.00	2	10	1	Y		2.15	m	1	1	0
5	1	55003-1	5.1625	0.00	0.00	3	90	4	N	5.23	3.66	h	4	1	0
5	1	55003-2	1.1875	0.00	0.00	1	5	1	N		1.3	l	1	1	0
5	1	55005-3	7.175	1.00	7.00	4	80	3	N	3.23	3.67	h	2	1	0
5	1	55006-2	5.6375	0.67	6.00	3	90	3	N	4.43	3.79	m	3	1	0
5	1	55008-3	5.9875	0.33	5.33	3	80	3	N	3.77	3.45	h	2	1	0
5	1	55010-5	1.9125	0.00	0.00	1	5	1	N	1.97	1.69	l	3	1	0
1	2	15003-3	5.85	3.33	0.33	3	100	4	Y	5.23	1.58	h	2	1	0
1	2	15004-2	6.1625	0.00	0.00	4	100	4	N		4.31	h	1	1	0
1	2	15007-1	9.1125	0.00	0.00	4	100	4	N	6.17	3.4	m	3	1	0
1	2	15007-2	6.075	0.00	0.00	3	100	4	N	4.70	3.89	h	1	1	0
1	2	15007-4	7.7625	1.33	0.00	4	90	4	N	4.97	3.31	h	2	1	0
1	2	15008-2	6.8625	0.00	0.00	4	90	4	N		3.01	m	3	1	0
1	2	15502-3	6.5375	1.00	1.33	4	100	4	N	7.30	3.31	h	1	1	0
1	2	15504-4	8.2375	0.33	0.00	4	100	4	N	5.37	4.03	h	2	1	0
1	2	15506-5	4	7.00	8.50	2	60	3	Y	1.33	1.8	h	2	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
1	2	16001-3	7.1	5.67	0.33	3	100	3	Y	4.57	2.27	h	2	1	0
1	2	16002-2	4.525	0.00	6.50	3	60	3	Y	3.50	2.73	h	2	1	0
1	2	16002-4	6.2	0.67	0.00	3	70	3	N	3.77	4.47	l	4	1	0
1	2	16005-4	6.8625	1.33	0.33	4	90	4	N	4.17	4.89	h	2	1	0
1	2	16007-4	8	1.50	0.00	3	95	3	N	6.17	3.48	m	2	1	0
2	2	25501-1	8.275	0.00	0.00	3	90	3	N	4.03	4.89	h	3	1	1
2	2	25508-2	6.15	0.00	0.67	3	80	3	Y	3.15	1.35	1	3	1	0
2	2	25508-5	5.8625	0.67	1.67	4	90	3	N	4.03	3.44	h	3	1	0
2	2	26001-1	6.3625	3.67	0.00	2	50	2	N	2.23	4.19	h	3	0	0
3	2	35503-1	6.0375	0.00	2.67	3	80	3	N		4.91	h	4	1	0
3	2	35503-4	4.45	1.00	0.00	3	90	3	N	2.90	3.67	h	3	1	0
3	2	35508-2	7.1375	3.67	2.33	3	50	3	N	4.30	4.45	h	3	1	1
3	2	35509-3	4.85	1.33	0.00	3	70	3	Y	2.13	1.5	h	1	1	0
3	2	35510-1	7.025	1.33	0.00	3	100	4	N	5.23	4.34	l	3	1	0
3	2	35510-5	4.875	0.50	0.00	3	90	3	N	4.43					
3	2	36002-3	5.3875	2.00	3.00	3	40	2	N	3.90	2.9	h	2	1	0
3	2	36008-4	6.0625	0.33	0.00	4	100	4	N	12.67	1.71	h	0	1	0
3	2	36008-5	8.3	0.00	0.00	4	100	4	N		3.44	m	3	1	0
3	2	36009-3	6.325	2.67	1.67	3	60	3	N	2.87	3.45	h	2	1	0
4	2	46004-3	5.175	0.00	0.00	3	100	4	N	5.77	2.15	l	2	1	0
4	2	46004-5	6.225	0.67	0.67	3	100	3	N	4.03	3.44	h	1	1	0
4	2	46005-5	7.25	0.00	0.00	4	100	4	N	4.30	3.49	h	2	1	0
4	2	46006-2	5.625	0.33	5.67	3	80	3	N		2.4	h	4	1	0
4	2	46006-3	7.6125	1.67	1.67	2	100	4	N	3.13	3.24	m	3	1	0
4	2	46007-4	8.2625	1.33	9.00	3	80	3	N	4.97	4.15	h	2	1	0
4	2	46009-3	6.6375	1.00	7.50	2	60	3	N	4.83	3.05	h	1	1	0
4	2	46010-1	4.35	0.00	0.00	3	60	3	N		3.77	h	2	1	0
4	2	46507-3	5.65	0.33	0.00	2	100	4	N	4.30	3.27	l	3	1	0
5	2	55003-4	1.8375	0.67	2.33	1	20	2	N	0.67	1.55	h	2	0	0
5	2	55005-2	8.1375	2.33	1.33	4	70	3	N	3.77	3.19	h	1	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
5	2	55006-1	8.325	0.00	0.00	4	100	4	N		3.43	l	2	1	0
5	2	55008-2	7.8875	0.00	0.67	2	80	3	N	6.77	3.27	m	2	1	0
5	2	55001-1	6	7.00	2.50	3	70	3	N	2.23	1.69	h	3	1	0
5	2	55009-5	3.35	0.00	0.00	1	10	1	N	1.07	5.94	h	5	0	1
1	3	15001-4	5.675	4.33	5.00	3	50	3	N	1.70	3.63	h	4	1	0
1	3	15002-1	5.8625	6.00	10.67	3	40	3	N	3.10	3.72	h	3	1	0
1	3	15002-3	4.3125	1.00	2.00	3	50	2	N		3.33	h	1	1	0
1	3	15003-5	5.8125	0.00	0.00	4	100	4	N		3.53	l	3	1	0
1	3	15007-4	7.325	0.67	9.33	3	90	3	N		4.37	m	1	1	0
1	3	15008-5	3.7875	0.00	5.67	2	60	3	Y	2.50	3.35	h	5	0	1
1	3	15009-3	3.2375	0.00	0.00	3	60	2	Y	0.80	4.24	h	3	0	1
1	3	16001-2	7.5	0.00	0.00	4	90	3	N	4.70	2081	h	3	1	0
1	3	16001-4	7.3	0.33	0.00	4	100	4	N		3.36	h	3	1	0
1	3	16003-3	2.15	0.00	0.00	1	5	1	Y	0.53	2.01	h	3	0	0
1	3	16008-1	4.2875	0.00	0.00	3	50	3	N	3.30	3.34	l	2	1	0
1	3	16008-4	5.4	0.67	1.67	2	70	3	Y	1.17	2.49	h	1	1	0
1	3	16008-5	4.7	2.33	5.33	2	40	2	N	3.63	2.21	m	1	0	0
1	3	16009-2	5.15	0.67	2.33	3	60	3	N		2.81	h	3	1	0
1	3	16009-3	6.55	3.00	0.00	2	50	2	N	4.57	3.46	m	2	1	0
2	3	25503-1	7.5	1.00	6.33	3	80	3	N	4.03	4.37	m	3	1	0
2	3	25506-3	3.9875	0.00	1.33	3	60	3	Y	3.37	1.88	h	1	1	0
2	3	25507-2	4.8575	1.50	0.00	3	100	4	N	3.80	3.51	l	1	1	0
3	3	35501-3	6.275	1.00	3.67	3	70	3	N		2.74	l	1	1	0
3	3	35502-5	5.425	5.00	18.33	3	90	3	Y	2.63	2.18	h	1	1	0
3	2	35504-5	4.95	0.00	0.00	2	40	2	N		3.21	up	3	1	0
3	3	35505-3	4.0125	0.00	3.33	2	30	2	Y	3.37	2.71	h	2	1	0
3	3	35507-4	3.175	0.67	6.00	2	20	2	N		2.22	h	1	1	0
3	3	35508-4	6.825	0.00	0.00	3	60	3	N		2.97	h	3	1	0
3	3	35509-3	3.2875	1.50	1.50	3	40	2	N	5.90	1.54	m	1	1	0
3	3	36003-5	6.025	0.33	0.00	3	80	3	N	2.10	2.87	l	2	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
3	3	36004-3	5.9125	4.00	2.00	3	60	2	N	2.37	1.99	h	2	1	0
3	3	36006-1	4.225	0.00	3.33	2	40	2	N	2.87	2.12	h	2	1	0
3	3	36006-3	5.65	5.33	5.67	3	80	3	N	3.77	3.9	h	2	1	0
3	3	36008-3	1.8625	0.00	0.00	1	5	1	N	0.40	1.71	m	2	0	0
3	3	36010-1	5.4375	0.00	0.00	4	80	3	N	5.23	3.38	l	3	1	0
4	3	46005-5	6.325	2.33	17.33	2	50	2	N	4.83	4.23	h	2	1	0
4	3	46006-3	5.7	0.00	0.00	4	100	4	N						
4	3	46008-1	4.725	2.67	0.00	3	65	3	Y	2.77	2062	m	3	1	0
4	3	46507-1	5.933333	0.00	0.00	2	70	3	N		2.67	l	1	1	0
4	3	46510-2	6.3875	0.00	8.33	3	80	3	N		2.47	h	3	1	0
5	3	55004-1	4.8875	0.00	5.00	4	80	3	N	3.23	1.68	h	1	1	0
5	3	55005-1	4.75	9.67	42.00	3	50	3	N	3.23	4.51	h	3	0	0
5	3	55005-3	4.025	1.33	1.33	2	30	2	Y	2.33	4.06	h	4	1	0
5	3	55006-4	5.4	2.33	16.00	3	70	3	N	2.83	2.79	h	1	1	0
5	3	55008-4	3.8	0.33	6.33	3	70	3	N		1.3	m	2	1	0
5	3	55007-1	8.675	0.33	0.00	4	100	4	N	7.43	4.16	h	3	1	0
1	4	15004-4	7.2375	0.67	2.00	4	100	4	N	4.57	3.51	h	2	1	0
1	4	15004-5	5.675	2.33	0.00	3	90	3	N	3.90	3.56	h	3	1	0
1	4	15005-5	4.875	0.00	6.00	2	50	3	N	4.57	2.59	m	2	1	0
1	4	15006-4	6.625	1.33	0.00	3	80	3	N	5.23	2.39	m	2	1	0
1	4	15007-1	6.4	1.67	0.33	3	90	3	N	3.90	2.82	m	1	1	0
1	4	15009-1	6	2.00	12.67	4	70	3	N	3.77	4.79	h	1	1	0
1	4	15501-3	4.225	3.67	5.67	3	80	3	Y	2.40	3.55	h	1	1	0
1	4	15502-1	4.9	0.67	3.00	1	40	1	N	3.40	4.7	h	4	1	0
1	4	16010-5	3.325	1.00	2.00	2	30	2	Y	3.23	1.43	h	1	1	0
1	4	16001-3	5	2.67	0.33	3	40	2	N	5.23	4.27	h	2	1	0
1	4	16002-3	4.45	2.33	42.33	3	60	3	N	2.83	2.28	h	2	1	0
1	4	16004-1	5.5	0.67	0.00	2	80	3	N	4.30	2.93	l	1	1	0
1	4	16005-5	4.9625	0.33	3.67	3	40	3	N	3.10	3.5	l	2	1	0
1	4	16007-3	5.7375	1.00	0.00	3	70	3	N	2.77	2.31	l	3	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
1	4	16008-2	2.175	0.33	7.00	1	2	1	Y	0.13	1.91	m	2	0	0
2	4	25501-3	2.3375	0.33	13.67	3	40	3	Y	2.73	3.83	h	3	1	0
2	4	25503-2	5.05	2.00	42.33	2	80	3	Y	2.00	1.69	h	3	1	0
2	4	25503-5	7.5375	1.67	1.33	3	70	3	N	6.93	5.31	h	3	1	0
2	4	25507-1	6.5875	0.00	20.50	3	50	3	N	4.17	4.38	h	3	1	0
2	4	26001-1	3.1125	0.00	0.00	1	5	1	N	1.07	4.5	l	5	0	1
3	4	35501-4	3.8625	0.33	3.00	4	20	2	N		1.54	h	1	1	0
3	4	35503-5	7.075	1.00	0.00	2	90	4	N	7.47	4.33	l	5	1	0
3	4	35504-4	5.6	0.00	0.67	2	90	3	N	2.87	1.57	l	3	1	0
3	4	35506-1	5.1875	0.00	0.00	2	40	2	N	3.63	2.68	l	2	1	0
3	4	35508-3	5.8375	1.00	10.67	2	50	2	N	3.50	2.66	h	3	1	0
3	4	35510-2	6.7625	2.00	1.33	2	100	4	N	6.53	3.19	m	3	1	0
3	4	36003-1	6.5	5.00	1.33	2	50	2	N	2.83	3.41	h	1	1	0
3	4	36006-4	5.5	4.00	0.33	3	60	3	N	4.17	4.18	m	1	1	0
3	4	36010-2	3.575	0.50	3.50	2	40	2	N	2.13	1.6	h	1	1	0
3	4	35503-1	4.525	0.67	1.33	4	80	3	N		3.48	m	2	1	0
4	4	46003-3	5.175	4.33	2.00	4	80	3	N	2.70	10.7	h	1	1	0
4	4	46004-1	4.75	4.00	0.00	3	70	2	N	2.60	3.65	h	2	1	0
4	4	46005-2	4.275	0.00	0.00	3	70	3	N		2.8	h	1	1	0
4	4	46009-3	9.1375	0.67	0.00	3	90	4	N	6.43	3.28	l	2	1	0
4	4	46502-2	3.7	0.33	0.67	2	40	2	N	2.83	1.91	m	5	2	0
4	4	46507-1	7.6125	2.00	0.00	3	80	3	N	6.53	3.24	m	1	1	0
4	4	46510-1	4.65	4.67	0.67	3	80	3	N	4.57	3.73	h	3	1	0
4	4	46510-4	6.25	2.67	4.00	2	65	2	N	2.83	3.1	h	1	1	0
5	4	55003-4	5.225	3.67	0.67	3	90	3	N	1.83					
5	4	55003-4	6.05	1.00	0.33	3	90	3	N	5.50					
1	5	15001-5	5.75	0.00	0.00	2	60	3	N	0.80	4.24	h	3	1	0
1	5	15002-3	6.325	0.33	0.00	4	100	4	N	0.93	5.52	h	4	1	1
1	5	15003-1	4.575	0.33	0.00	3	90	3	Y	2.00	2.78	h	4	1	0
1	5	15006-3	4.1875	2.00	6.33	2	30	2	Y	4.30	3.04	h	1	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
1	5	15007-4	10.2375	1.00	0.00	4	100	4	N		4.61	h	3	1	0
1	5	15009-2	7.625	0.00	0.50	3	80	3	N		2.77	m	1	1	0
1	5	15504-1	7.3375	0.67	9.67	4	80	3	N	3.37	5.56	h	2	1	1
1	5	15507-5	5.325	0.67	0.00	3	70	3	N	3.40	4.36	h	2	1	0
1	5	16001-4	7.4875	0.00	0.00	4	100	4	N	4.17	3.63	m	2	1	0
1	5	16003-5	7.925	0.00	0.00	4	100	4	N	3.50	4.15	h	3	1	0
1	5	16006-3	4.7625	0.00	2.33	3	50	3	N		4.7	h	2	1	0
1	5	16007-3	5.8125	1.00	13.67	3	80	3	N	3.23	3.97	h	2	1	0
1	5	16009-4	6.625	1.67	1.67	4	90	3	N	5.10	4.3	l	4	1	0
2	5	25501-3	7.1875	0.33	1.00	4	90	4	N	4.03	6.27	h	4	1	1
2	5	25503-5	2.9375	1.33	0.00	2	10	2	N	1.97	3.78	l	4	0	0
2	5	25506-4	8.1375	0.00	3.00	4	100	4	N	7.60	5.12	h	5	1	0
2	5	25506-5	3.3625	0.33	0.67	2	20	2	N		5.47	h	2	0	1
2	5	25507-1	6.4	0.00	0.33	3	80	3	N	4.83	6.19	m	5	1	1
2	5	25508-2	4.925	0.00	4.67	2	50	2	N	2.60	4.9	h	1	1	0
2	5	25509-5	9.25	3.67	5.33	2	90	3	N	7.43	5.03	h	1	1	0
2	5	26001-4	6.2375	0.00	0.00	4	100	4	N	3.80	4	m	2	1	0
3	5	35502-5	4.8	0.00	0.00	3	95	3	N	3.37	3.99	m	3	1	0
3	5	35503-5	3.075	0.00	1.67	2	10	1	N	1.70	4.12	m	3	1	0
3	5	35504-3	5.4	2.33	0.00	4	100	4	N	1.83	4.44	h	3	1	0
3	5	35504-4	5.5375	0.00	6.67	2	50	2	N	2.87	4.2	h	3	1	0
3	5	35505-1	8.1125	1.00	1.67	4	100	4	N	3.37	5.27	h	1	1	0
3	5	35505-3	6.95	2.33	2.33	4	100	4	N	5.10	3.68	m	2	1	0
3	5	35506-4	4.9375	0.00	0.00	2	60	3	N	2.87	1.5	m	3	1	0
3	5	35508-5	7.75	0.33	0.00	4	100	4	N	5.10	4.12	m	1	1	0
3	5	35509-3	5.3375	4.67	3.67	4	90	4	N	4.30	3.9	m	3	1	0
3	5	35509-5	5.925	3.00	0.00	4	100	4	N	7.17	3.74	l		2	1
3	5	35510-4	5.6	1.00	1.50	2	90	3	N	5.50	2.77	l	4	1	0
3	5	36002-2	7.3875	0.33	0.00	4	100	4	N	4.17	4.11	h	3	1	0
3	5	36004-4	6.0625	1.33	11.33	4	80	3	N	4.03	4.07	h	3	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
3	5	36005-1	5.9375	1.00	0.00	3	70	3	N	4.43	4.41	h	3	1	0
3	5	36008-2	4.9625	2.00	2.50	4	80	3	N	4.07	4.18	m	1	1	0
3	5	36008-4	3.6125	13.00	39.33	2	40	2	N	2.83	2.66	h	1	1	0
3	5	36009-1	7.075	1.00	2.67	4	100	4	N	2.77	4.96	h	3	1	0
4	5	46003-5	4.8125	0.50	1.50	3	80	3	N	4.03	1.72	h	1	1	0
4	5	46008-3	4.0375	0.00	9.50	3	70	3	N	3.00	5.66	h	4	1	1
4	5	46009-3	2.4375	0.33	2.33	2	5	2	N	2.37	3.09	l	4	1	0
4	5	46010-1	5.5125	0.33	0.00	3	70	3	N	2.97	4.89	h	1	1	0
4	5	46010-3	4.1875	2.50	4.00	2	60	3	N	2.23	1.49	l	1	0	0
4	5	46503-1	5.5625	2.00	0.00	4	90	4	N	4.03	4.4	h	4	1	0
4	5	46503-2	4.35	0.00	0.00	2	20	2	N	1.43	3.86	l	3	1	0
4	5	46503-5	4.8125	0.00	0.00	4	90	4	N	4.07	1.68	m	2	1	0
4	5	46505-3	5.8875	0.00	0.00	4	60	3	N	3.27	2.72	l	2	1	0
4	5	46505-4	6.5375	0.67	0.00	4	90	3	N	4.57	5.48	h	2	1	0
4	5	46507-2	5.9	0.00	2.00	3	80	3	N		4.24	h	5	1	1
4	5	46510-1	5.3875	1.33	1.00	3	70	3	N	1.87	3.24	l	1	1	0
5	5	55001-3	7.475	1.67	0.00	4	100	4	N	3.53	3.85	h	1	1	0
5	5	55003-3	7	3.00	1.00	4	100	4	N	4.30	4.87	h	1	1	0
5	5	55005-2	6.375	0.33	2.00	4	70	3	N	3.90	3.62	h	1	1	0
5	5	55005-5	5.35	2.33	3.00	2	60	2	N	2.63	3.96	m	5	1	1
5	5	55006-4	6.5875	1.67	28.67	4	80	3	N	3.80	4.57	h	1	1	0
5	5	55008-3	4.875	0.33	0.00	3	100	3	N	4.70	1.63	h	1	1	0
5	5	55009-2	4.9875	0.00	0.33	3	60	3	N	2.50	6.45	m	5	0	1
1	6	15001-2	5.7375	0.00	0.00	3	70	3	N	3.80	3.66	h	1	1	0
1	6	15002-1	6.3125	2.33	5.67	2	60	2	N	4.83	4.94	h	1	1	0
1	6	15002-2	6.5	3.33	10.33	2	40	2	N	3.00	5.17	h	2	1	1
1	6	15003-3	5.775	1.00	5.33	3	70	3	N	3.77	5.15	h	2	1	1
1	6	15003-4	7.7125	0.00	4.00	3	80	3	N	3.37	5.28	h	1	1	0
1	6	15005-3	4.5	1.50	0.50	3	20	2	N	3.90	3.09	h	2	1	0
1	6	15006-3	7.3875	2.67	0.00	3	100	4	N	3.77	3.85	h	4	1	0

Table 4 (Continued)

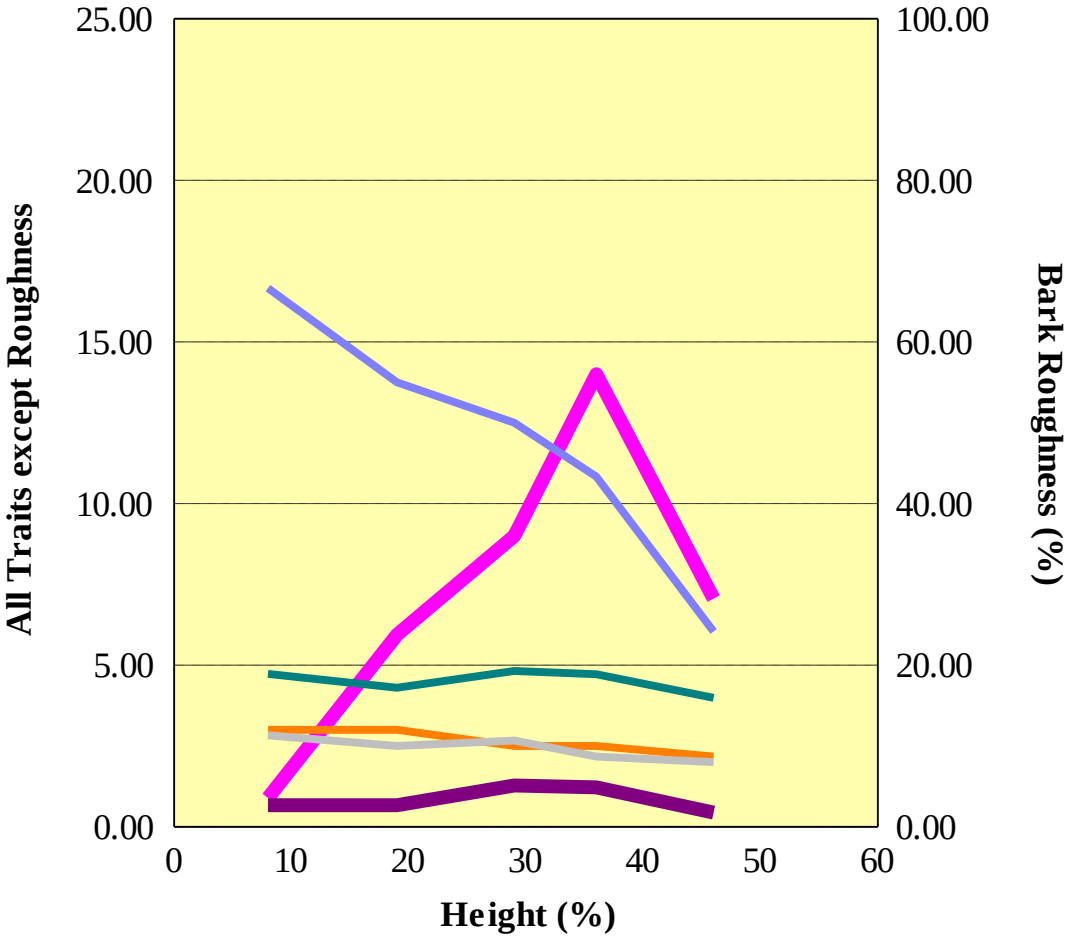
Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
1	6	15008-3	5.3625	1.00	16.00	3	50	3	N	4.17	4.73	h	1	1	0
1	6	15506-5	6.3875	0.33	1.33	2	90	3	N	3.90	3.01	h	3	1	0
1	6	15507-3	5	3.33	2.33	3	75	3	N	3.63	6.44	h	5	1	1
1	6	15507-4	5.825	1.00	2.50	3	90	4	N	6.00	4.98	h	3	1	0
1	6	16001-3	5.625	0.00	0.00	4	90	4	N	6.00	5.53	h	3	1	0
1	6	16001-4	8.1375	0.67	0.00	4	100	4	N	6.30	4.51	m	3	1	0
1	6	16001-5	4.9125	0.00	0.00	4	90	3	N	6.17	3.92	h	2	1	0
1	6	16002-1	4.575	1.33	2.33	3	70	3	N	3.77	4.85	h	3	1	0
1	6	16002-4	6.5625	0.00	0.33	4	90	3	N	3.00	5.58	h	1	1	0
1	6	16002-5	7.7	0.00	0.00	4	90	4	N	5.23	4.99	h	3	1	0
1	6	16004-3	6.4	0.67	0.00	3	100	4	N	6.27	3.31	l	2	1	0
1	6	16005-2	5.85	0.67	0.00	4	100	4	N	4.03	4.94	h	2	1	0
1	6	16005-5	4.05	0.33	2.00	3	75	3	N	3.10	4.31	h	2	1	0
1	6	16006-3	5.8875	0.00	0.00	3	30	2	N	4.43	1.52	l	1	0	0
1	6	16008-1	5.775	6.33	0.33	3	60	3	N	2.73	3.82	h	1	1	0
1	6	16008-3	4.8375	0.33	0.67	4	80	3	N	3.37	2.49	l	1	1	0
1	6	16008-4	4.875	0.67	0.00	3	60	3	N	3.77	5.07	h	2	1	0
1	6	16008-5	6.475	1.00	1.33	4	100	4	N	6.57	3.04	m	2	1	0
1	6	16009-3	3.4625	1.33	3.00	2	20	2	N	2.73	2.62	m	3	1	0
2	6	25503-1	6.575	0.00	0.00	4	80	3	N						
2	6	25503-1	6.575	0.00	0.00	4	80	3	N	5.10					
2	6	25506-3	4.65	0.33	49.33	3	70	3	N	3.10	7.21	h	5	1	1
2	6	25507-1	5.5875	0.33	1.00	4	90	3	N	4.43	5.25	h	3	1	0
2	6	25507-4	5.675	1.33	6.00	3	90	3	N	6.27	4.52	h	3	1	0
2	6	25508-1	5.7625	0.67	4.00	3	80	3	N	3.03	4.37	h	2	1	0
2	6	25508-5	5.675	7.67	6.00	3	50	3	N	2.83	2.68	m	2	1	0
2	6	26010-3	4.4375	2.67	12.00	2	30	2	N	3.10	5.22	h	1	1	0
2	6	26010-4	5.75	2.33	0.67	4	90	4	N	4.17	5.27	h	1	1	1
3	6	35502-4	3.975	0.00	0.67	3	90	3	N	3.37	2.68	h	1	1	0
3	6	35505-3	5.4	1.33	1.67	4	80	3	N	3.63	5.29	m	4	1	0

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
3	6	35506-1	5.775	1.67	5.67	3	70	3	N	3.40	4.05	h	1	1	0
3	6	35507-1	6.875	0.00	0.00	3	90	3	N	5.37	3.81	h	2	1	0
3	6	36002-2	4.925	2.50	0.00	3	80	3	N	5.07	3	l	1	1	0
3	6	36002-5	5.05	0.00	0.00	3	30	2	N	3.00	3.73	m	1	1	0
3	6	36003-5	5.2	2.00	0.33	3	40	3	N	4.57	5.13	l	4	1	0
3	6	36004-5	5.5125	5.00	3.33	3	80	3	N	3.10	2.53	m	1	1	0
3	6	36005-1	6.025	5.00	0.00	3	30	2	N	4.43	3.52	h	1	1	0
3	5	36006-1	6.6125	3.33	18.33	4	90	3	N	3.90	4.48	h	3	1	0
3	6	36006-2	3.5125	0.67	3.67	3	20	2	N	3.63	5.74	h	4	1	1
3	6	36006-3	5.475	0.00	0.00	3	60	3	N	2.77	4.99	h	4	1	0
3	6	36007-4	3.425	7.00	10.00	2	20	2	N	3.63	2.94	h	3	1	0
3	6	36008-1	6.2875	1.33	1.00	4	100	4	N	5.90	5.13	h	3	1	0
3	6	36009-1	6.05	0.67	1.00	2	70	3	N	3.93	3.27	m	1	1	0
3	6	36009-3	5.825	2.00	9.00	3	80	3	N	3.53	4.88	h	3	1	0
3	6	36009-4	6.225	4.33	3.67	3	50	3	N	2.37	5.28	h	4	1	0
3	6	36010-1	4.8125	0.67	0.00	4	50	3	N	2.90	5.29	h	3	1	0
4	6	46003-3	6.875	2.00	0.00	3	30	2	N	6.13	4.77	h	1	1	0
4	6	46004-1	3.375	6.00	4.67	2	50	3	N	3.50	6.35	h	4	1	1
4	6	46004-4	3.4375	2.00	4.33	3	60	3	N	2.83	4.13	h	3	1	0
4	6	46005-2	6.125	2.00	3.67	3	50	2	N	3.77	4.49	h	2	1	0
4	6	46005-4	2.5	1.33	0.00	3	10	2	N	2.50	1.96	l	2	1	0
4	6	46005-5	6.3125	2.50	0.00	3	70	3	N	3.90	3.08	l	1	1	0
4	6	46006-2	5.2125	1.33	1.00	2	40	2	N	1.47	4.57	h	3	1	0
4	6	46007-1	3.575	2.50	4.50	3	60	3	N	3.67	2.4	h	1	1	0
4	6	46007-4	5.6625	5.50	3.50	3	50	3	N	4.43	4.43	h	2	1	0
4	6	46008-5	6.0625	0.00	0.00	4	60	2	N	4.83	4.7	h	3	1	0
4	6	46502-3	6.1625	2.67	1.33	2	50	2	N	3.27	3.49	h	2	1	0
4	6	46502-5	5.95	1.00	2.33	4	90	3	N	1.60	5.1	h	1	1	0
4	6	46504-4	5.275	1.67	9.33	3	80	3	N	3.10	4.82	h	3	1	0
4	6	46505-2	6.925	0.67	21.67	3	80	3	N	3.40	5.86	3	3	1	1

Table 4 (Continued)

Source	Rep	Family-Tree Number	Average Thickness (mm)	# adults	# second instars	color	roughness	Overall bark reaction	stump sprout	Hardness	Height (m)	Infestation Level	Apical Dominance Loss	Gouting	Overall Looks
4	6	46507-2	4.55	1.50	27.00	3	80	3	N	1.73	3.84	h	1	1	0
4	6	46507-3	6.6375	1.33	0.00	2	40	2	N	6.67	4.62	h	3	1	1
5	6	55001-5	3.325	0.33	10.33	3	70	3	N	3.50	2.23	h	1	1	0
5	6	55004-1	5.575	0.00	0.33	4	90	4	N	4.70	6.44	h	2	1	0
5	6	55004-2	7.025	0.33	0.00	4	100	4	N	6.93	4.42	h	1	1	0
5	6	55005-5	5.675	0.00	0.00	4	80	3	N	5.87	5.1	h	2	1	0
5	6	55009-2	5.65	0.67	5.33	3	60	3	N	2.20	4.2	h	2	1	0
1	1	15004-1	5.825	1.00	0.00	4	80	3	N	3.90	4.43	m	2	1	0
1	6	15009-5	5.2875	0.33	1.67	4	80	3	N		5.78	h	4	1	0
5	2	55007-5	6.15	0.67	0.00	4	100	4	N	4.97	2.7	l	3	1	0
1	2	15005-5	5	0.33	0.00	3	10	2	N	2.20	3.71	h	2	1	0
3	6	36006-1	6.275	0.00	0.00	4	100	4	N	8.63	5.37	h	1	1	0
1	4	15007-2	7.6875	1.33	1.00	4	70	3	N	3.63	5.25	h	2	1	0
3	3	35504-5	5.9125	0.00	0.00	4	100	4	N	4.03	1.81	l	1	1	0
1	6	16006-2	6.1	0.00	2.67	3	75	3	N	4.17	5.03	h	3	1	0
1	3	16006-4	6.125	1.00	1.00	4	100	4	N	6.03	3.18	h	3	1	0
1	4	15003-1	7.875	0.33	0.00	4	100	4	N	5.90	4.33	h	1	1	0



Reaction	2.83	2.50	2.67	2.17	2.00
Rough	66.67	55.00	50.00	43.33	24.17
Color	3.00	3.00	2.50	2.50	2.17
Thickness	4.73	4.30	4.82	4.72	3.99
Adults	0.67	0.67	1.28	1.22	0.44
2nd Instars	0.89	5.94	9.00	14.00	7.06

Figure 1. Average measurements for bark traits at five height levels on 6 trees.

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