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Title: Identifying Candidate Genes Associated with Cold Adaptation in Douglas-fir using DNA Microarrays.

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Glenn T. Howe Amy M. Brunner

In forest trees from temperate and boreal regions, cold acclimation is an important adaptive trait that involves changes in gene expression and physiology. Genecological, quantitative genetic, and QTL studies have been used to study the genetics of cold acclimation in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), but the genes responsible for variation among individuals and populations remain unknown. Douglas-fir seedlings from a low elevation, coastal Oregon population and a high elevation, inland Washington population were grown in a common garden in Corvallis, Oregon, and artificial freeze tests were used to compare their cold hardiness from September, 2004 to May, 2005. Cold hardiness was associated with the timing of bud set and bud flush, and there were large population differences in cold acclimation, deacclimation, and bud phenology. In late October, for example, population differences in percent cold damage (predicted at -7°C) were as large as 58% for needles (OR=62% and WA=3%), 48% for stems (OR=53% and WA=5%), and 43% for buds (OR=43% and WA=0%). A custom oligonucleotide microarray

was used to analyze gene expression differences between cold susceptible and cold hardy seedlings from the two populations during acclimation. The microarray was developed from >18,000 ESTs from four Douglas-fir cDNA libraries, including three new libraries specifically targeting the stages of cold acclimation, maximum hardiness, and cold deacclimation. Oligonucleotides designed from loblolly pine and white spruce ESTs were included on the microarray to test whether heterologous hybridizations could be used to study Douglas-fir cold acclimation and other processes. Three hundred forty-three differentially expressed Douglas-fir genes were identified (FDR adjusted p-value ≤ 0.01). Many of these genes had similarities to genes encoding dehydrins, heat shock proteins, pathogenesis-related proteins, and genes associated with ABA, GA, and dormancy. Preliminary results suggest that loblolly pine and white spruce ESTs are useful for identifying genes that are differentially expressed during cold acclimation. These differentially expressed genes are considered cold hardiness candidate genes and will be used in genetic association studies of adaptive traits in Douglas-fir from Oregon and Washington.

Identifying Candidate Genes Associated with Cold Adaptation in Douglas-fir using DNA Microarrays

by
Dana Kathryn Howe

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APPROVED:

Co-Major Professor, representing Forest Science

Co-Major Professor, representing Forest Science

Head of the Department of Forest Science

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Dana Kathryn Howe, Author

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In memory of my mother, Jeanne Elaine Salo Larsen, whose spirit and smile will always be with me.

Identifying Candidate Genes Associated with Cold Adaptation in Douglas-fir using DNA Microarrays.

INTRODUCTION

In forest trees from temperate and boreal regions, cold acclimation is an important adaptive trait that involves changes in physiology and gene expression. In Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), genecological, quantitative genetic, and QTL studies have shown that cold acclimation has geographic patterns of genetic variation and is under moderately strong genetic control (reviewed in Aitken and Hannerz 2001; Howe et al 2003). A genetic association study is underway to identify which genes and alleles are associated with genetic variation in cold adaptation in populations of Douglas-fir from Oregon and Washington. I used two populations of Douglas-fir seedlings that differed in cold hardiness to screen thousands of genes on a DNA microarray to identify genes that have patterns of gene expression that are associated with cold adaptation. These "expression candidate genes" will be used in the association study of Douglas-fir.

Cold adaptation

Actively growing tissues are the most vulnerable to cold damage; therefore trees in temperate and boreal regions have annual growing cycles that limit cold damage by restricting their growth to the summer. The transition from a stage of active growth to one of cold tolerance and dormancy is a complex process involving changes in physiology and gene expression that occur in response to environmental stimuli and

endogenous signals (Weiser 1970; Hughes and Dunn 1996; Thomashow 1998; Welling and Palva 2006).

In trees, the process of cold adaptation involves: (1) growth cessation and bud set, (2) cold acclimation, (3) initiation of endodormancy (dormancy regulated by internal physiological factors; Lang et al 1987), (4) development of maximum cold hardiness, (5) release of endodormancy, (6) cold deacclimation, and (7) growth initiation and bud flush. These processes are not discrete, but represent a continuum.

The cessation of elongation and bud set can occur months before diameter growth ceases and long before there is a danger of fall frosts (Weiser 1970; Perry 1971). Day length, low temperatures, nutrient availability, and moisture all influence the timing of growth cessation and bud set (van den Driessche 1969; Campbell and Sugano 1979; Bigras et al 2001).

Cold acclimation is the gradual acquisition of freezing tolerance, usually in response to environmental signals. It occurs slowly as trees are exposed to decreasing temperatures and short days, and leads to maximum cold hardiness by mid-winter. Timmis and Worrall (1975) described three stages of cold acclimation in Douglas-fir. In the first stage, visible growth ceases as a result of short photoperiods. In the second stage, cold hardiness increases upon exposure to cooler night temperatures. The final stage of maximum hardiness is signaled by short cold days and freezing nights. During this period trees are endodormant.

The release of endodormancy occurs once chilling requirements are met. Exposure to temperatures slightly above freezing are needed to fulfill chilling requirements (Howe et al 1999). Growth initiation and spring bud flush are stimulated

by lengthening photoperiods and increasing temperatures, once chilling requirements have been met (Lavender et al 1968).

Cold damage and acclimation

Most of the cellular damage caused by cold results from dehydration associated with freezing (Thomashow 1998). When exposed to low temperatures, intercellular water freezes and creates a water potential across the cellular membrane. This potential can cause intracellular water to move out of the cell, thus causing dehydration and injury. Cellular dehydration results in damage to membranes, denaturation of proteins, and accumulation of salts.

Cold acclimation involves the stabilization of membranes against damage from freezing (Thomashow 1998). This membrane stabilization involves changes in biochemistry and gene expression. Cold exposure induces genes that encode hydrophilic polypeptides that change the lipid composition of cellular membranes to reduce this injury from frost.

Many different classes of proteins are regulated by low temperatures and are associated with dehydration response and protection during cold acclimation (reviewed in Renaut et al 2006). These include anti-freeze proteins (AFPs), dehydrins and late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs) and chaperonins, and pathogenesis-related (PR) proteins. In addition, transcription factors and genes involved in translation and signaling pathways are responsive to low temperatures. Numerous studies in annual and woody plants have begun to identify genes encoding proteins involved in to cold acclimation (reviewed in Rowland and

Arora 1997; Welling and Palva 2006; Clapham et al 2001; Thomashow 1999; Hughes and Dunn 1996).

Cold acclimation gene expression

Genetic evidence for cold acclimation has existed for some time (Weiser 1970; Perry 1971), and Guy et al (1985) demonstrated that changes in gene expression occur during cold acclimation. In *Arabidopsis*, microarrays have been used to measure changes in gene expression in response to cold. Provart et al (2003) used chilling-sensitive mutants to identify 634 chilling responsive genes involved in acclimation, including genes related to metabolism, photosynthesis, and free radical detoxification. Seki et al (2002) identified 53 cold responsive genes, and found similarities among genes that responded to cold, drought, and high salinity stress. The differential expression of up- and down-regulated genes were divided into two major categories (1) functional proteins involved in stress tolerance, and (2) regulatory proteins involved in signal transduction and gene expression. The expression of many *Arabidopsis* cold acclimation genes are regulated by the CBF/DREB (C-repeat/ dehydration-responsive element-binding factor) transcription factor (Seki et al 2001, 2002; Fowler and Thomashow 2002; Provart et al 2003; Van Buskirk and Thomashow 2006). Although more than a thousand genes have been identified in short (< 12 hours) and long (> 48 hours) term cold exposure (Hannah et al 2005), *Arabidopsis* is an annual plant that does not reach the same levels of cold tolerance as trees. Therefore, studying cold acclimation in a woody perennial species may reveal additional pathways and genes that could not be explored in *Arabidopsis*.

Genetics of Douglas-fir cold hardiness

There is significant genetic variation in cold adaptation among Douglas-fir populations. The timing of vegetative bud set and bud flush, cold hardiness, and frost damage are strongly associated with a population's latitude, longitude (distance from the ocean), and elevation (Campbell and Sorenson 1978; Griffin and Ching 1977; Loopstra and Adams 1989; Rehfeldt 1989; St. Clair et al 2005; St. Clair 2006).

Latitudinal variation in bud phenology and cold hardiness is found in comparisons between Oregon and Washington seed sources. In common garden studies, southern Oregon seed sources generally set bud later and experienced more damage from fall frost than did sources from northern Oregon and Washington (Campbell and Sorenson 1973; Campbell and Sugano 1979). Latitudinal variation in cold hardiness is stronger among coastal populations of Douglas-fir than among populations from further inland (Griffin and Ching 1977; St. Clair 2006; Campbell and Sugano 1979). The buds of northern coastal sources generally responded more slowly to warm temperatures and flushed later than did southern coastal sources.

Differences are also seen in Douglas-fir cold hardiness related to a population's proximity to the ocean, as the risk of fall frost damage is generally lower in the coastal maritime climates (Timmis et al 1994). In the fall, coastal seedlings set bud later and experienced more frost damage than inland populations (Griffin and Ching 1977; Loopstra and Adams 1989; Schuch et al 1989a; St. Clair 2006; O'Neill et al 2000). The date of bud set is strongly and positively correlated with frost damage ($r = 0.93$ to 0.95; Griffin and Ching 1977; Loopstra and Adams 1989). Cold hardiness patterns reverse in the spring, however, and seedlings from the milder coastal sources flush

later and have less cold damage than inland sources (Balduman et al 1999; Schuch et al 1989a, 1989b; O'Neill et al 2000; Aitken and Adams 1997).

High and low elevation populations are strongly differentiated for cold damage, and variation in bud set and growth is related to elevation (Campbell 1979; St. Clair et al 2005; Hermann and Lavender 1967). Populations from higher elevations tend to set bud earlier and experience less frost damage than lower elevation populations (Griffin and Ching 1977; Loopstra and Adams 1989; Rehfeldt 1989). These high elevation sources also tend to deacclimate earlier in the spring (van den Driessche 1970; Schuch et al 1989b). As elevation increases, temperatures decrease. Fall cold damage is more strongly associated with cold temperatures than with latitude or longitude, with populations from cold climates setting bud earlier and suffering less damage (Balduman et al 1999; St. Clair et al 2005; St. Clair 2006).

Although cold hardiness can differ among needles, stems, and buds (Schuch et al 1989a; Aitken and Adams 1996; Aitken et al 1996), quantitative genetic studies demonstrate that cold adaptation traits are under genetic control and heritable. Correlations in cold damage between tissues are stronger within a season than across seasons (Anekonda et al 2000b; St. Clair 2006; O'Neill et al 2000; Aitken and Adams 1995; Jermstad et al 2001b). This suggests that cold hardiness in spring and fall are largely controlled by different suites of genes and should be handled as separate traits.

Fall cold damage is moderately to strongly associated with bud set in seedlings ($r = 0.57$ to 0.74; St. Clair 2006; Campbell and Sorenson 1973), but the timing of bud set is not a good predictor of fall cold hardiness in saplings ($r = -0.29$, Aitken and Adams 1995). Genetic correlations between spring cold hardiness and bud flush are

high in saplings (r = -0.86 to -0.90; Aitken and Adams 1995; Aitken and Adams 1997; Li and Adams 1993). Therefore, the timing of bud flush is often used as a surrogate for spring cold hardiness.

Fall and spring cold hardiness also have large amounts of genetic variation (Aitken and Adams 1996; O'Neill et al 2000, 2001). Heritabilities for cold hardiness traits vary with season—they are higher in spring than in fall, and lowest in winter (Anekonda et al 2000b; O'Neill et al 2001; Aitken and Adams 1995; Aitken and Adams 1996).

The cold adaptation traits of bud flush, spring cold hardiness, and fall cold hardiness have been mapped in Douglas-fir as quantitative traits in family pedigree studies (Jermstad et al 2001a, 2001b, 2003; Wheeler et al 2005). Only one linkage group contains QTL for both spring and fall cold hardiness (Jermstad et al 2001b). Several QTL were associated with spring cold hardiness in all three shoot tissues, suggesting the same set of genes control spring cold hardiness in all three tissues. Nearly all of the bud flush QTL were detected at each test location, although the test sites differed in elevation, latitude, and climate (Jermstad et al 2003). This is another indication that bud flush is a highly heritable trait. In addition to the mapped QTL, 29 cold hardiness candidate genes were placed on the linkage map, seventeen of which mapped within 95% confidence intervals of 6 QTL regions for needle cold hardiness in the spring (Wheeler et al 2005).

Measuring cold hardiness

Cold hardiness is typically assessed by measuring the amount of damage to a tissue or whole plant after exposure to cold temperatures. It can be measured under natural conditions or after subjecting tissues to artificial freezing tests (AFTs) in the laboratory. Using programmable freezers, artificial freeze tests simulate natural cold night temperatures by gradually decreasing the temperature a few degrees per hour (Weiser 1970; Anekonda et al. 2000a). High correlations between cold hardiness measured after a natural frost event and artificial freezing test demonstrate that AFTs can be used to reliably predict cold hardiness ($r = 0.72$ to 0.78; O'Neill et al 2001).

Common methods of measuring damage include visual scoring of tissues or whole plants, leakage of electrolytes from tissues, and chlorophyll fluorescence. Freeze testing using intact plants or cuttings allows for the evaluation of multiple tissues and the interactions among tissues and organs (Anekonda et al 2000b). Although it requires a larger sample size, more time for symptom development, and consistent scoring, it provides the best estimate of "real" cold hardiness (Burr et al 2001). Electrolyte leakage and chlorophyll fluorescence require only a small amount of tissue and provide results relatively quickly. Electrolyte leakage may not accurately measure cold hardiness differences among species, families, and treatments because it can be affected by cuticle properties, can not be used to determine a precise lethal temperature, and produces injury curves that vary with season (reviewed in Burr et al 2001). Chlorophyll fluorescence is limited because it must be used on green tissues. Genetic correlations between cold injury to shoot tissues (i.e., needles, stems, and buds) can vary considerably, and are sometimes weak (Aitken and Adams 1996).

Therefore it may be necessary to evaluate more than one tissue to assess overall hardiness. Therefore, I used cuttings in my freeze tests to evaluate cold hardiness, and I measured damage separately on needles, stems, and buds.

Candidate gene identification

The range of Douglas-fir spans a wide diversity of geographic areas, and there are significant phenotypic differences in cold adaptation traits among populations (described above). These differences may be the result of natural selection on cold adaptation traits, as temperature greatly affects the adaptation of Douglas-fir to Pacific Northwest environments (Howe et al 2003; St. Clair et al 2005). Although allozymes have been used to assess ecological variation among Douglas-fir populations (Neale 1985; El-Kassaby and Ritland 1996; Adams et al 1998), their association with variation in quantitative traits is weak. Future research aims to understand how genetic variation is associated among unrelated individuals in a population (Howe et al 2003). This is done through association studies of cold adaptation traits.

Association studies identify correlations between alleles and phenotypic differences in a population of unrelated individuals. This can be done using genome-wide scans to find associations between specific DNA markers (e.g., candidate gene alleles) and phenotypes (Howe et al 2003; Neale and Savolainen 2004). A candidate gene is any gene presumed to have an important functional role in a trait based on indirect evidence. An association study of adaptive traits in Douglas-fir is currently underway using the candidate gene approach. The candidate genes being used are (1) functional candidates, i.e., those with direct physiological evidence or strong sequence

homology to genes with known functions in other species; (2) positional candidates, i.e., those with map positions near a QTL of interest; and (3) expression candidates, i.e., those with differential messenger RNA (mRNA) abundance associated with an adaptive trait. The main goal of my study was to identify expression-based candidate genes.

Thesis project

For my thesis, I tested the hypotheses that population differences in cold acclimation in Douglas-fir seedlings are associated with (1) the expression of different genes in the different populations, or (2) differences in the timing of gene expression. Three expressed sequence tag (EST) libraries were created to discover genes that are actively transcribed into mRNA during fall cold acclimation, vegetative bud set, winter dormancy, spring deacclimation, and vegetative bud flush. These EST libraries were used to create a custom oligonucleotide microarray for gene expression profiling. Additional oligonucleotides were designed from the large EST resources available in loblolly pine and white spruce.

I tested the difference in cold hardiness between two Douglas-fir seedling populations—one from a southern, low-elevation, coastal seed source and one from a northern, high-elevation, inland seed source. Seedlings were used in my study because they are more vulnerable to cold damage than older trees. Compared to older trees, seedlings (1) grow later into the fall, (2) are within the zone of cold air that pools above the forest floor, (3) are often in open areas that are exposed to radiation frosts, and (4) tend to flush earlier in the spring (O'Neill et al 2001; Howe et al 2003). Cold

susceptible and cold hardy buds and stems from these populations were compared using gene expression profiling and oligonucleotide microarrays. The genes identified with differential expression patterns of cold acclimation over time or between populations will be incorporated into the larger association study of adaptation traits in coastal Douglas-fir. In addition, heterologous hybridization of loblolly pine and white spruce will provide valuable information about how well cold hardiness differences in Douglas-fir populations can be studied using the current EST resources available from other conifers.

MATERIALS AND METHODS

Plant material

I used Douglas-fir seedlings to construct three EST libraries. These seedlings were grown from seed collected from two open pollinated seed orchards, one in Lincoln County, and the other in eastern Lane County, Oregon. The trees in both orchards originated from scions collected from Douglas-fir trees growing in a low elevation population near Toledo, Oregon (TL seed source). TL seeds were sown in February, 2003, in type-8 styroblocks in greenhouses in Cottage Grove, Oregon. After seven months of growth, the seedlings were moved to Corvallis, Oregon (44° 30' N, 123° 17' W), maintained in styroblocks outdoors under natural conditions, and sampled monthly from September to April. On each harvest date, bud, needle, and stem tissues were collected from 20 seedlings and used to create three EST libraries (described below).

I used two seed sources for cold hardiness testing and gene expression analysis. The Coos Bay (CB) seedlings were derived from an open pollinated seed orchard in Curry County, Oregon that contains parents from a low elevation (<300 m) coastal population near Coos Bay, Oregon. The Yakima (YK) seedlings were derived from parents growing in a high elevation (>1500 m) inland population near Yakima, Washington. The CB and YK seeds were sown in February, 2004 in type-8 styroblocks in greenhouses in Cottage Grove, Oregon. In September, the CB and YK seedlings were moved to Corvallis, Oregon, maintained in styroblocks outdoors under natural conditions from September to May. The seedlings were grown in a

randomized block design. Each of the seven blocks contained one styroblock (plot) of CB seedlings and one styroblock of YK seedlings, except for one block, which had only a single styroblock of YK seedlings. Twenty-four seedlings from each population were randomly sampled across all blocks on nine dates for RNA extraction and cold hardiness testing (Table 1).

Phenotypic measurements

Terminal bud development of the CB and YK seedlings was monitored weekly in the fall of 2004 and used to choose sample dates for measuring fall cold hardiness. The terminal bud was scored on a scale of 0 (no bud present) to 6 (fully developed bud). The heights of the seedlings selected for measuring fall cold hardiness were recorded at the time of the freezing tests. In 2005, spring bud flush was periodically monitored and used to choose sample dates for measuring spring cold hardiness. The buds were scored about twice a week from mid-March through mid-April on a scale from 0 (firm bud) to 6 (needles emerged from the bud scales). The development of the lateral buds was also scored in the spring as 0 (not flushed) or 1 (flushed).

Artificial freeze tests

In 2004-2005, artificial freeze tests were conducted on seedlings from the CB and YK populations on nine dates from September to May (Table 1). Needle electrolyte leakage and visual assessment of needles, stems, and buds were used to measure damage at three test temperatures (Table 1). On each sample date, 24 CB and 24 YK seedlings were removed from the styroblocks and brought back to the laboratory. The

Table 1. Seedling collection dates and temperatures for cold hardiness testing of Douglas-fir seedlings.

Sample date	Set[†]	Temperature (°C)[‡]		Test temperature (°C)[§]		
		Max	Min	Max	Mid	Min
EST libraries						
3 Sep 2003	CA	30	10			
7 Oct 2003	CA	21	9			
7 Nov 2003	CA	8	-1			
18 Dec 2003	MH	9	3			
21 Jan 2004	MH	11	4			
19 Feb 2004	CD	12	2			
19 Mar 2004	CD	18	3			
24 Mar 2004	CD	18	3			
5 Apr 2004	CD	18	2			
Cold hardiness tests						
19 Sep 2004	F	22	8	-4	-6	-8
26 Oct 2004	F	14	6	-4	-6	-8
4 Nov 2004	F	13	4	-4	-6	-8
24 Nov 2004	F	9	1	-6 / -8	-8 / -10	-10 / -12
14 Jan 2005	W	6	-1	-33	-38	-43
8 Mar 2005	S	15	2	-6	-8	-10
29 Mar 2005	S	11	5	-4	-6	-8
15 Apr 2005	S	13	1	-4	-6	-8
17 May 2005	S	19	9	-4	-6	-8

[†] EST library sets include cold acclimating (CA), maximum hardiness (MH), and cold deacclimating (CD); cold hardiness test sets include fall (F), winter (W), and spring (S).

[‡] Mean daily maximum (Max) and minimum (Min) temperatures for the week prior to sampling.

[§] In contrast to the other dates, I used a different series of test temperatures for the Coos Bay (CB) and Yakima (YK) populations on 24 Nov 2004.

seedlings were kept in bags with moist towels at 2-4°C before processing. The top 6 cm was removed from each seedling and frozen for future RNA isolation, then the seedlings were cut at the root collar, and the roots and soil were discarded. Twenty undamaged needles from directly below the top cut were subsequently removed for measuring electrolyte leakage (Glerum 1985). Both ends of the needles were cut with a razor blade, resulting in 1-cm segments. One needle segment from each seedling was placed into a glass scintillation vial with a small amount of silver iodide. Four vials were used for each of 3 test temperatures (Table 1) and 2 control temperatures (2ºC and -2ºC), for a total of 20 vials per sampling date. All vials were placed at 2ºC overnight, and then at -2ºC for two hours before running the test.

The remainder of each seedling was used for visual assessments of cold hardiness, essentially as described by Anekonda et al (2000a). Eight seedlings from each population were placed on moistened cheesecloth on top of aluminum foil. The foil was then folded over and taped shut to create an envelope. The ends of the envelopes were left open and were placed at -2ºC overnight. Six envelopes were used per sample date, one per population for each of the three test temperatures. The freezing tests were conducted in a Forma Scientific Model 8270/859M ultra-low freezer with a West M3750 temperature controller (West Instruments, East Greenwich, RI).

Although three test temperatures were used for each sample date, these temperatures differed depending on the expected cold hardiness of the seedlings (Table 1). The freezer was programmed to decrease the temperature at a rate of 5ºC per hour, and then hold a constant test temperature for one hour. The samples were then removed from the freezer. The ends of the aluminum foil envelopes were folded

over to decrease desiccation and prevent light exposure, and the electrolyte leakage vials were placed in a black bag to exclude light. All samples were then placed at 4ºC overnight to thaw.

Damage assessments

After thawing overnight, the electrolyte leakage vials were placed at room temperature for 20-24 hours, and 13 mL of deionized water were subsequently added to each vial. After 24 hours of incubation at room temperature, each vial was shaken and the electrical conductivity (EC_1) of the water solution was measured using a conductivity meter (VWR Model 2052, West Chester, PA). The vials were then placed in an oven at 90ºC for one hour to kill the needle segments. The vials were removed from the oven and placed at room temperature for 24 hours. The electrical conductivity (EC_2) of the solution was measured for the killed needles, and then used to calculate the relative conductivity (RC) of each sample:

[1] $$RC(\%) = (EC_1 - B_1) \times 100 / (EC_2 - B_2)$$

where EC_1 and EC_2 are as described above, B_1 is the electrical conductivity of the frozen blank (i.e., vials with no needles), and B_2 is the electrical conductivity of the heat-killed blank (i.e., heated vials with no needles; Burr et al 2001).

After thawing overnight, the foil envelopes were placed at room temperature for 8-9 days. Damage assessment was based on guidelines from Anekonda et al (2000a). The damage to needles, stems, and buds was compared to healthy unfrozen tissues and measured on a scale of 0 (no damage) to 100% (completely damaged), in increments of 5%. Needle damage was scored based on discoloration and the ease with which the

needles could be detached from the stem. Stem damage was based on the discoloration of the cambial and phloem tissues on longitudinally cut stems (i.e., after removing the outer bark). Buds were cut lengthwise and the damage assessment was based the discoloration of the inner tissues.

Statistical analyses of cold hardiness data

Statistical analyses were performed using SAS Version 9.1 (Statistical Analysis System; Cary, NC). For each population, SAS PROC CORR was used to calculate correlations between different measures of cold damage in the fall (n=12; 4 dates x 3 temperatures), winter (n=3; 1 date x 3 temperatures), and spring (n=12; 4 dates x 3 temperatures). Correlations were also performed across all test dates (n=27; 9 dates x 3 temperatures).

Differences in cold hardiness are typically assessed by estimating (1) the *temperature* that elicits a particular response (i.e., minimum temperature that causes no damage or temperature at which 50% of the population is killed) or (2) the amount of *damage or mortality* that results from exposure to a specific test temperature. The AFT temperatures selected maximized population differences, but produced damage ranges with too little overlap between YK and CB to effectively estimate a temperature that would elicit a set response in both populations (Figures A1 to A4). Therefore, I estimated cold damage using models that predict the amount of *damage* expected from exposure to a temperature of either -7°C (fall and spring) or -35°C (winter).

For each date, simple linear regression was used to study the influence of test temperature on tissue damage (i.e., RC or visual score), using the general equation:

[2] $$Y_{ij} = \beta_0 + \beta_1 P_i + \beta_2 T_j + \beta_3 P_i T_j + \varepsilon_{ij}$$

where Y_{ij} is the tissue damage for the *i*th population and *j*th test temperature, P_i is the effect of the *i*th population, T_j is the effect of the *j*th test temperature, P_iT_j is the interaction between the *i*th population and the *j*th test temperature, and ε_{ij} is the residual error. At each sample date, the slopes of the regression line for each population were compared using the general linear models (GLM) procedure in SAS. I predicted damage for each population and date using separate regression equations (Table A1), because the slopes for the CB and YK populations were significantly different for fifteen of the thirty-six slopes (p-value ≤ 0.05; Table A2). I used ESTIMATE statements to estimate the amount of damage when seedlings where exposed to -7°C in the fall and spring (CD_{-7}) and -35°C in the winter (CD_{-35}), and to test the difference in slopes between the regression lines for the two populations.

RNA samples

For the EST libraries, ten TL seedlings were pooled into two 5-tree groups on each sample date (Table 1). Each pooled sample was divided into needles, stems, and buds, and RNA was extracted separately from each tissue. Total RNA was isolated according to Chang et al (1993), except that the resulting RNA pellets were purified on RNeasy® mini-columns according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). Each RNA sample was eluted in 40 µl of RNase-free water. The

RNA concentration was measured on a Beckman Coulter DU640 spectrophotometer (Fullerton, CA), and RNA integrity was verified using gel electrophoresis.

To examine population differences in cold hardiness, RNA was isolated from 12 CB and 12 YK seedlings from each sample date (Table 1). The twelve trees from each population were pooled into two 6-tree samples; each pooled sample was divided into needles, stems, and buds; and total RNA was extracted separately from each tissue. For the spring collections, the pooled bud tissue represented all developmental stages (i.e., firm buds to elongating new growth). The RNA concentration was measured on a NanoDrop ND-1000 spectrophotometer (Wilmington, DE), and RNA integrity was verified on an Agilent Bioanalyzer 2100 (Palo Alto, CA).

EST libraries

I constructed three expressed sequence tag (EST) libraries to represent different stages of cold hardiness. Seedlings used in the cold acclimating (CA) library were collected during vegetative bud set in September, October, and in November, after the seedlings had experienced five consecutive nights of below-freezing temperatures. Seedlings used for the maximum hardiness (MH) library were collected in December and January. The cold deacclimating (CD) library was made from seedlings collected in February and March during bud swell and bud flush, and in April during new shoot extension. The mean daily maximum and minimum temperatures for the week prior to each sampling date are shown in Table 1. RNA from each tissue was diluted to 1 $\mu g\ \mu l^{-1}$, and equal quantities of the diluted RNA from each tissue and sample date were pooled for each of the three libraries. The pooled total RNA was used by

Evrogen JCS (Moscow, Russia) for double-stranded cDNA synthesis, using the SMART approach (Zhu et al 2001), and then normalized using the DSN normalization method (Zhulidov et al 2004). The resulting cDNA was directionally inserted into the pAL17.1 vector and transformed into *E. coli* with the BioRad Micropulser (Hercules, CA). SymBio Corporation (Menlo Park, CA) amplified the cDNA clones using rolling circle amplification, and then sequenced about 4,000 cDNA clones per library using a MegaBASE 4000 sequencer (GE Healthcare, Little Chalfont, UK).

Bioinformatics

The raw EST sequences were analyzed using the EST Analysis Pipeline (ESTAP; Mao et al 2003). End sequences were trimmed to remove poly-A or poly-T tracts, vector, and low quality sequences (Phred quality score below 20; Ewing et al 1998). Sequences resulting from chimeras or contamination with *E. coli* DNA were removed. BLASTX homology searches were performed against the NCBI non-redundant protein (nr), Swiss-Prot, PIR, and PRF databases (default settings; Altschul et al 1997). All EST sequences ≥150 quality bases were submitted to GenBank.

The EST sequences from the three cold hardiness libraries were assembled together with 6,760 sequences from a Douglas-fir EST library prepared from actively growing seedlings in their first growing season (Krutovsky et al 2004). CAP3 was used to form consensus sequences (unigenes) that were then used for designing microarray oligonucleotides (Huang and Madan 1999). The CAP3 default parameters were used with a range of percent identity cutoffs (90-99%), and the resulting contig sets were compared (Figure A5). The sequence orientations of the ESTs were

deduced from the presence of poly-A or poly-T tracts at the ends of the sequences, known orientations of similar proteins in other species (BLASTX e-value $\leq e^{-6}$), and cloning orientations for the three directionally-cloned cold hardiness libraries. The orientations of the contigs were inferred from the orientations of their component ESTs. The unigenes whose sequence orientations could be determined with confidence were placed into a Douglas-fir "correct orientation" group (D_c, step1; Table 2). If the sequence orientation could not be determined, the unigenes were placed in a "non-oriented" group, and two reverse complementary sequences were used to design probes for the oligonucleotide microarrays (discussed below). These non-oriented unigenes were later placed into one of three groups, D_c (correct orientation), D_i (incorrect orientation), or D_u (unknown orientation; Table 2), based on microarray analyses (discussed below).

Unigene sets from *Pinus taeda* (loblolly pine; Pratt et al 2005) and *Picea glauca* (white spruce; Pavy et al 2005) were also used to design probes for the oligonucleotide microarrays. The loblolly pine EST database included >180,000 sequences in >31,000 contigs (downloaded 9 Nov 2005 from MAGIC DB http://fungen.org/Projects/Pine-/Pine.htm). The spruce database contained >48,000 sequences in >16,000 contigs (downloaded 9 Feb 2006 from SpruceDB http://ccgb.umn.edu:8309/Pub_SpruceDB/).

The unigenes from pine and spruce were divided into three groups (Table 2). The first (correctly oriented) group consisted of sequences with significant similarity (e-value $\leq e^{-10}$) to the D_c (step1) unigenes. The second (non-oriented) group consisted

Table 2. Orientation of unigene sequences from Douglas-fir, loblolly pine, and white spruce used as oligonucleotide probes on microarrays.

Unigene group[†]	Orientation[‡]	Criteria[§]
Unigenes used in differential gene expression analyses		
D_c	Correct	BLASTX (e-value $\leq e^{-10}$), poly-A tail, cloning orientation (step 1) or High RE compared to its reverse complement (step 2)
P_c, S_c	Correct	High similarity to D_c (BLASTN e-value $\leq e^{-10}$) (step 1) or High RE compared to its reverse complement (step 2)
P_{c*}, S_{c*}	Assumed correct	No similarity to Douglas-fir unigenes (step 1)
Unigenes used to determine expression threshold, but not used in differential gene expression analyses		
D_i	Incorrect	Low RE compared to its reverse complement (step 2)
Unigenes not used in differential gene expression analyses		
P_i, S_i	Incorrect	Low RE compared to its reverse complement (step 2)
D_u, P_u, S_u	Unknown	RE not significantly different than its reverse complement (step 2)

[†] D = Douglas-fir; P = loblolly pine; and S = white spruce.

[‡] Correct = strong support for the unigene sequence being in the correct orientation; Incorrect = strong support for the unigene sequence being in the incorrect orientation; Assumed correct = the orientation was inferred form the cloning orientation, and assumed to be correct.

[§] In step 1, bioinformatic analyses were used to infer the orientation of the unigene and to design probes for the microarrays. For sequences that could not be confidently oriented in step 1, RMA $\log_2$ expression (RE) values from microarray analyses were used in step 2 to infer the correct sequence orientation.

of sequences with weak similarity (e-value > e^{-10}) to the D_c (step1) unigenes, or similarity to the non-orientated Douglas-fir unigenes. The third group consisted of sequences with no significant similarity (e-value ≥ e^{-2}) to our Douglas-fir unigenes. Functional annotations and categories were assigned to the Douglas-fir unigenes based on BLASTX comparisons (downloaded 29 Aug 2006) and the Munich Information Center for Protein Sequences (MIPS; Ruepp et al 2004) set of *Arabidopsis* proteins (http://mips.gsf.de/proj/thal/db/) at an e-value ≤ e^{-6}.

Microarray design and hybridization

NimbleGen Systems, Inc. (Madison, WI) designed ten 60-mer probes for each unigene of each species. Each 60-mer probe is a composite of 37 overlapping 24-mer probes which NimbleGen ranked based on the frequency of each 24-mer within the genome of interest, and the minimum number of mismatches between the probe and any other 24-mer probe in the target genome (see Appendix 1). I selected as many as six 60-mer probes per sequence based on the (1) NimbleGen ranks and (2) BLASTN relationships of the probes to the other unigene sequences of the same species. Only probes with < 97% sequence similarity to other unigenes within a species were used. Probe selection was done in a stepwise process, giving preference to probes with high NimbleGen ranks and low percent identity BLASTN matches. If less than six probes were selected, I added additional probes by sequentially increasing the percent identity match criteria from 94% to 97%, in increments of 1%. For each species, a probe set was designed for the oriented unigenes and each of the two reverse complementary non-oriented unigenes. For the pine and spruce unigenes that had no significant

Douglas-fir matches (group 3), the probe set was designed using the most probable sequence orientation (derived from cloning orientation). Due to space limitations on the microarrays, probes from the unigenes of the three loblolly pine groups and the unknown spruce group were randomly selected for inclusion on the array.

Using our selected probes, NimbleGen constructed two microarray platforms using their maskless array synthesis technology. Both arrays contained 86,880 Douglas-fir probes, representing 10,757 unigenes. One array platform incorporated 105,573 loblolly pine probes, representing 18,341 unigenes. The other array platform incorporated 105,573 white spruce probes, representing 18,076 unigenes. Each array (17.4 mm x 13 mm) contained 390,000 (16 μm x 16 μm) probe features. Probes for Ambion ArrayControl™ RNA spikes (Austin, TX) were included on both microarrays. Each set of probes was replicated randomly on the microarray, but the probes for the Douglas-fir unigenes and Ambion spikes are in the same location on both array platforms (pine and spruce).

Stem and bud RNA samples isolated from the CB and YK seedlings on Sept.19, Oct. 26, and Nov. 4 were labeled with biotinylated-11-UTP using the Ambion MessageAmp™ II aRNA amplification kit according to the manufacturer's protocol. Labeling reactions were prepared for two 6-tree samples per population, for a total of 24 reactions (2 6-tree samples x 2 populations x 2 tissues x 3 dates). The Ambion RNA spikes were mixed according to the manufacture's recommendations to provide a range of RNA concentrations, and then added to each RNA sample before labeling. Total RNA (1500 ng) from each sample was labeled using a 14-hour in vitro

transcription reaction. The concentration of the amplified RNA was measured on a NanoDrop ND-1000 spectrophotometer, and the integrity was verified on an Agilent Bioanalyzer 2100. For each of the 24 samples, 22 μg μl^{-1} was sent to the NimbleGen facility in Iceland for fragmentation and hybridization using their proprietary system. Twelve samples (2 populations x 2 tissues x 3 dates) were hybridized to each array platform, and the two array platforms served as biological replicates for statistical analyses.

Statistical analyses of gene expression data

NimbleGen used GenePix® (Molecular Devices Corporation; Chicago, IL) to create TIF files of the arrays. NimbleScan™ was used to extract and quantify the feature intensities.

Robust Mulitchip Average (RMA; Irizarry et al 2003; Bolstad et al 2003) expression values were computed for all twenty-four Douglas-fir arrays using NimbleScan. The following model was used to detect differences between the average RMA $\log_2$ expression (RE) values of each gene in the reverse complement group (i.e., for the unigenes in the non-oriented group).

[3] $$Y_{ij} = \mu + A_i + G_j + \varepsilon_{ij}$$

where Y_{ij} is the RE value for the *i*th array and *j*th group, A_i is the effect of the *i*th array, G_j is the effect of the *j*th group, and ε_{ij} is the residual error. Using the SAS ANOVA procedure, genes with a significant group effect (p-value < 0.1) were oriented using the difference between the mean RE value of the reverse complements. The reverse complement with the lower mean RE value was assumed to be in the incorrect

orientation, assigned to group D_i, and removed from the gene expression data set. The reverse complement with the higher mean RE value was assumed to be in the correct orientation and was added to the group of correctly oriented sequences to form the final group of correctly oriented sequences D_c (step 2; Table 2). The D_c group was used in further analyses for detecting differential gene expression. The unigenes with a non-significant group effect (p-value ≥ 0.1) were designated as having an unknown orientation (D_u) and were removed from further analyses.

The distributions of RE values for the D_c and D_i groups were compared to determine a signal cutoff for non-specific cross-hybridization (see Results). NimbleScan was used to re-calculate the RE values for the D_c group. D_c unigenes were excluded from further analyzes if their RE values were below the RE cutoff of 7.24 on every array. After the cutoff for cross-hybridization was applied, NimbleScan was used to re-calculate the RE values for the D_c group. Array quality was assessed using the SAS ROBUSTREG procedure. For each array, correlations were calculated for the two replicate probe sets for each unigene in the D_c group.

All statistical tests for differential gene expression were performed with J/MAANOVA using F_s, a modified F statistic using James-Stein shrinkage estimates of the variance (Wu et al 2003; Churchill 2004; Cui et al 2005). P-values were calculated from 1000 permutations of the model residuals. A false discovery rate adjustment of < 0.01 was used to estimate the proportion of false positives within the unigene lists (Storey 2002). The following model was used for analyzing each unigene.

[4] $$Y_i = \mu + T_i + \varepsilon_i$$

where Y_i is the RE value for the *i*th treatment, μ is the population mean, T_i is the effect of the *i*th treatment, and ε_i is the residual error. Six treatments were used in the analysis: (1) CB-September, (2) CB-October, (3) CB-November, (4) YK-September, (5) YK-October, and (6) YK-November. Contrast statements were used to test for significant differences in RE between cold susceptible and cold hardy samples. The less cold hardy CB-October and YK-September treatments were contrasted against the more cold hardy CB-November and YK-October treatments (Figure 1). Contrasts were tested for buds and stems pooled together, as well as for buds and stems individually.

Heterologous hybridizations

Orientation groups were assigned to the pine and spruce reverse complements using the same approach outlined for Douglas-fir, except that the non-oriented pine and spruce unigenes were first assigned to groups P_c, S_c, P_i, and S_i based on similarities to the unigenes in the D_c and D_i groups. SAS ANOVA was used to assign the correct orientation for the remaining unigenes (Table 2). As in Douglas-fir, the unigenes with a non-significant group effect (p-value ≥ 0.1) were designated as unknown orientation (L_u and S_u) and removed from further analyses. The P_i and S_i groups were also removed from the gene expression data sets. The P_c, P_{c*}, S_c, and S_{c*} groups were used in further analyses to detect differential gene expression (Table 2).

The procedures that were applied to the Douglas-fir arrays for computing RE values and assessing array quality were also applied to the twelve pine and twelve

spruce arrays. The distributions of RE values were also compared among the three species. SAS ROBUSTREG was used to calculate correlations between the corresponding unigenes in the D_c and P_c groups across the twelve loblolly array platforms, and between corresponding unigenes in the D_c and S_c groups across the twelve spruce array platforms. Statistical tests for differential gene expression were performed using the same model as outlined for Douglas-fir, except that all contrast were tested for buds and stems pooled together (i.e., the bud and stem samples served as biological replicates for these analyses).

RESULTS

EL and VA are comparable measures of needle cold hardiness.

Correlations between the two measures of needle hardiness (electrolyte leakage, EL, and visual assessment, VA) were comparable for both populations across all test dates (Table 3). Correlations were high in the fall and winter ($r = 0.940$ to 0.998) and moderate in the spring ($r = 0.548$ to 0.696). The VA of needle damage was more difficult to assess in the spring than in the fall and winter because of increased fungal growth within the sealed foil envelopes. This probably accounts for the unexpected decrease in CD_{-7} for the mid-April test (Figure 1) and the decreased correlation between spring needle measures. Therefore, for our experiments, needle EL scores may be more reliable for assessing the cold hardiness of needles in the spring. The needle VA scores are emphasized in my findings, except when noted in my discussions of the spring data.

Population differences in response to cold temperatures

<u>CB and YK seedlings responded differently to fall cold temperatures</u>

Cold acclimation occurred earlier in the YK seed source, and large population differences were apparent for the first three fall test dates ($p \leq 0.05$; Figure 1). For example, in late October, the YK tissues were cold hardy to -8°C, and population differences in CD_{-7} were as large as 58% for needles (CB=62% and YK=3%), 48% for stems (CB=53% and YK=5%), and 43% for buds (CB=43% and YK=0%). By late November, however, the CB tissues were more cold hardy, and the population differences in CD_{-7} were small (~2%).

Table 3. Correlations between alternative measures of cold damage from artificial freezing tests to Douglas-fir seedlings. The fall test dates were 19 Sep, 26 Oct, 4 Nov, and 24 Nov; the winter test date was 14 Jan; and the spring test dates were 8 Mar, 29 Mar, 15 Apr, and 17 May. For each season, the correlations above the diagonal line are for cold damage traits measured on Yakima seedlings whereas the correlations below the diagonal line are for cold damage traits measured on Coos Bay seedlings.

Tissue	Needle EL[†]	Needle[‡]	Stem[‡]	Bud[‡]
Fall (n=12)				
Needle EL		0.986**	0.984**	0.942**
Needle	0.940**		0.996**	0.958**
Stem	0.893**	0.874**		0.952**
Bud	0.825**	0.869**	0.873**	
Winter (n=3)				
Needle EL		0.977	0.883	0.991
Needle	0.998*		0.963	0.939
Stem	0.999*	0.995		0.811
Bud	0.939	0.914	0.950	
Spring (n=12)				
Needle EL		0.696*	0.796**	0.372
Needle	0.548		0.813**	0.231
Stem	0.687*	0.489		0.575
Bud	0.719**	0.028	0.567	
All Dates (n=27)				
Needle EL		0.859**	0.865**	0.585**
Needle	0.822**		0.863**	0.520**
Stem	0.746**	0.721**		0.566**
Bud	0.768**	0.546**	0.520**	

[†] Relative conductivity of electrolyte leakage (EL) from needles.
[‡] Visual assessment (VA) of damage.
* p-value ≤0.05
** p-value ≤0.01

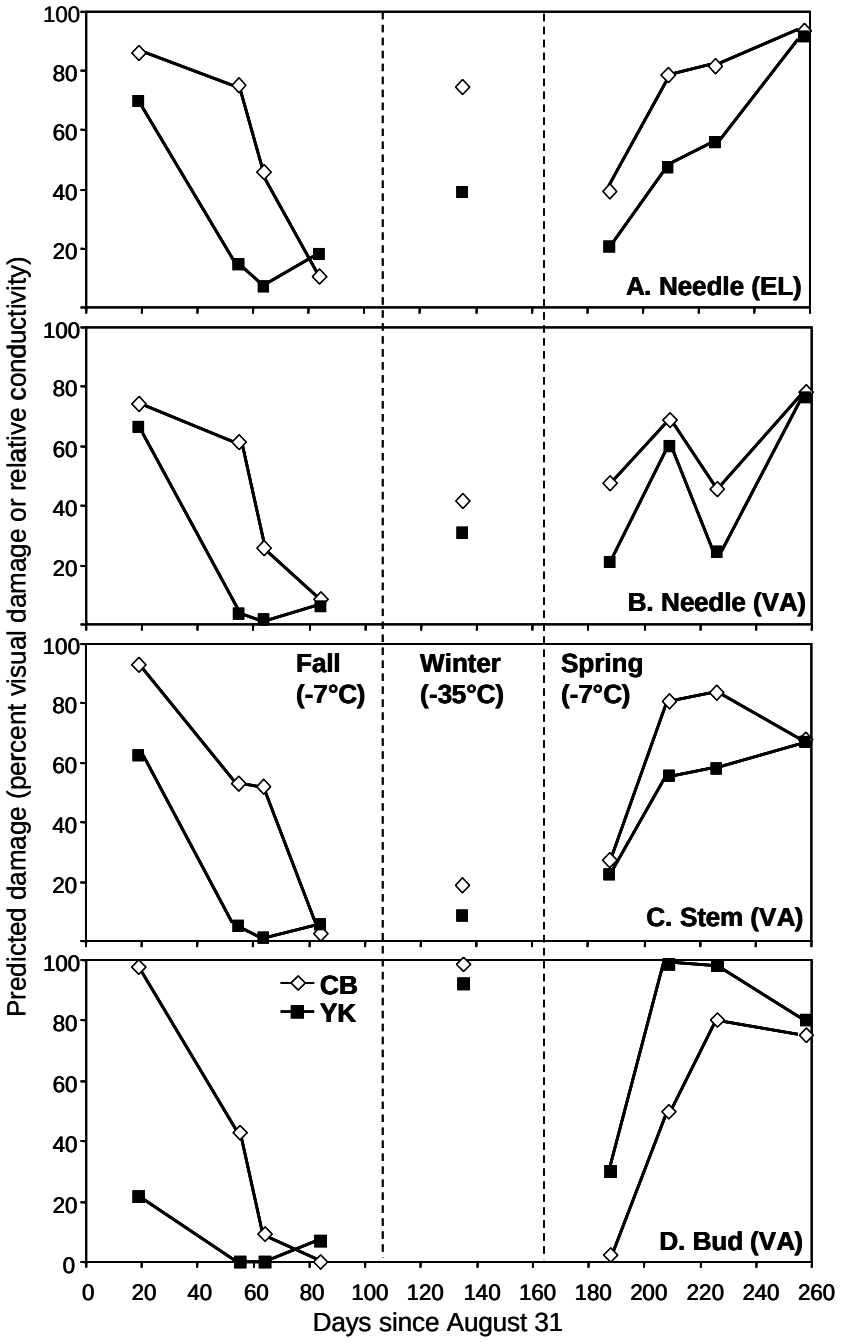

Figure 1. Predicted damage of Douglas-fir Coos Bay (CB; —◇—) and Yakima (YK; —■—) seedlings across different tissues and measurement techniques using artificial freeze tests. Fall and spring damage was predicted at -7°C, whereas winter damage was predicted at -35°C. Electrolyte leakage (EL) was used to predict damage to needles (A), whereas visual assessment (VA) was used to predict damage to needles (B), stems (C), and buds (D). Population differences were significant ($p \leq 0.05$) for all dates, except on 24 Nov and 17 May for needle (EL); 19 Sep, 24 Nov, 14 Jan, and 17 May for needle (VA); 24 Nov, 14 Jan, and 17 May for stems; and 17 May for buds.

Population differences in cold damage were smaller in winter than in fall.

In January, both populations were more cold hardy than in the fall. The mean difference in CD_{-35} between the populations was 11% for needles, 11% for stems, and 6% for buds (Figure 1).

In spring, population differences in cold damage varied by tissue.

In the early spring, the YK seedlings were more cold hardy than the CB seedlings based on stems and needles, but they were less hardy based on buds ($p \leq 0.05$; Figure 1). In late March, for example, the population differences in the CD_{-7} were as large as 30% for needle EL (CB=78% and YK=48%), 26% for stems (CB=81% and YK=55%), and 49% for buds (CB=50% and YK=99%). From early March to mid-April, the buds of the YK seedlings had greater CD_{-7} than the buds of the CB seedlings. By May, both populations had deacclimated and CD_{-7} did not differ for any of the three tissues.

Tissue differences in response to cold temperature

Buds responded differently to cold than did needles and stems. Buds were the first tissue to acclimate in the fall (Figure 1), but they suffered the most cold damage in the winter (95%) compared to needles (36%) and stems (14%). Stems were the least damaged winter tissue, and therefore the most cold hardy. In the spring, the buds were the only tissue for which YK was less cold hardy than CB (discussed above).

Correlation of cold damage among shoot tissues

Cold damage among shoot tissues was highly correlated in the fall and winter, but only moderately correlated in the spring. Strong, significant correlations were found between the cold hardiness of needles, stems, and buds in the fall ($0.869 \leq r \leq 0.996$) and winter ($0.811 \leq r \leq 0.995$; Table 3). In the spring, moderate to strong correlations where found between the tissues ($0.567 \leq r \leq 0.796$), except for the YK buds and needles ($r = 0.372$ for correlation involving needle EL). Across all dates, YK and CB had similar correlations between tissues.

Cold hardiness was associated with the timing of bud set and bud flush

The YK seedlings set bud earlier and had less cold damage than the CB seedlings until early November (Figures 1D and 2A). By mid-October, the populations had large differences in CD_{-7} (discussed above), and bud set was complete for the YK population, but bud set was only 38% for the CB population. All of the YK seedlings had set bud before the first frost on November 5, 2004, but bud set was only ~85% for the CB seedlings on this date. By late November, bud set was complete for the CB population as well.

Compared to the CB seedlings, the YK seedlings flushed earlier in the spring and had greater cold damage to the buds (Figures 1D and 2B). In mid-March, for example, more than 50% of the YK seedlings had flushed compared to less than 5% of the CB seedlings. The lateral buds also flushed earlier in the YK seedlings compared to the CB seedlings. By mid-March there was a 60% difference in lateral bud flush between the two populations (CB=13% and YK=73%; data not shown).

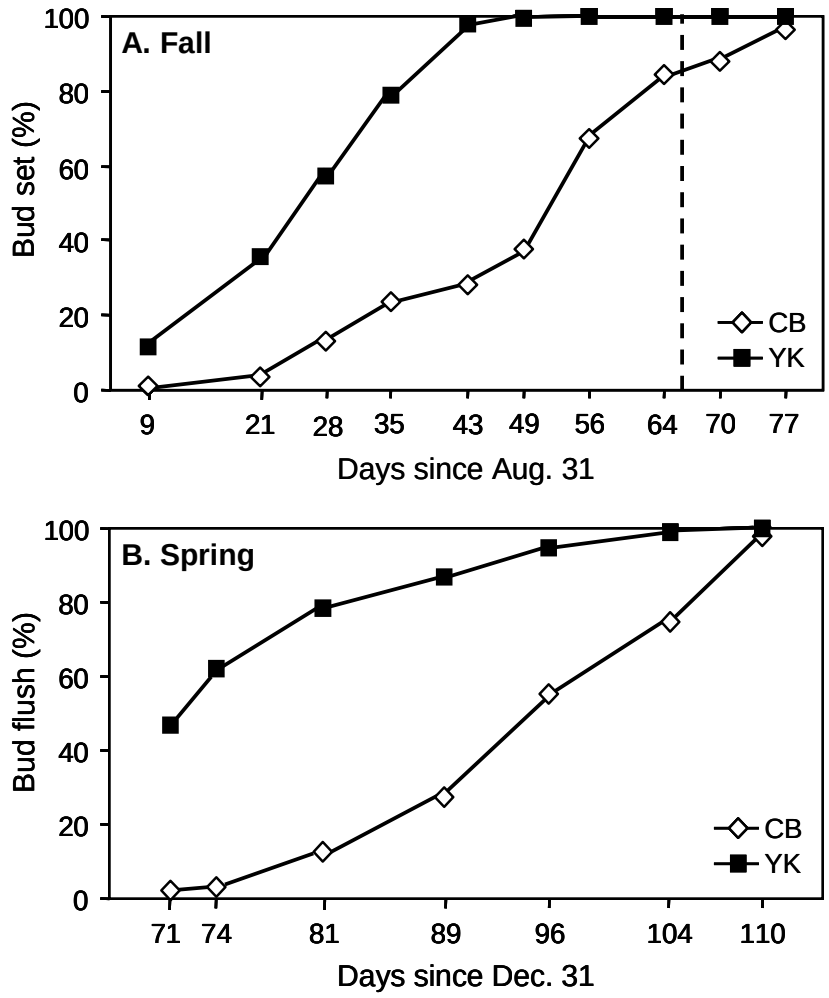

Figure 2. Timing of bud set and bud flush for populations of Douglas-fir seedlings used for cold hardiness testing. The percentages of seedlings which have set bud in the fall of 2004 (A) and flushed in the spring of 2005 (B) are shown. The Coos Bay (CB) seedlings are from a cold susceptible seed source from a low elevation coastal area, whereas the Yakima (YK) seedlings are from a cold hardy seed source from a high elevation inland area. The dashed line indicates the first night of frost in Corvallis, Oregon, in the fall of 2004.

Three new Douglas-fir ESTs libraries were created and used to develop a unigene set.

Three new EST libraries were constructed from Douglas-fir seedlings, specifically focusing on the stages of cold acclimation, maximum hardiness, and cold deacclimation. A total of 12,157 raw EST sequences were generated from these new cold hardiness libraries (Table 4). On average, 93% of the sequence reads resulted in a high quality EST sequence. The average sequence length submitted to GenBank for these libraries was 515 bp. The cold hardiness libraries make up 63% of the Douglas-fir ESTs currently available in GenBank. The libraries were constructed using directional cloning methods, and the resulting clones were sequenced from the 3'end. Therefore, the sequences were presumed to be in the 3'-5' orientation. Based on bioinformatics analyses and microarray results, I estimated that 2-3% of the cDNAs were cloned in the incorrect orientation.

The ESTs from the three cold hardiness libraries were combined with the ESTs from actively growing Douglas-fir seedlings, resulting in 18,094 high quality sequences that were contigged using CAP3. Contig sets were generated using different CAP3 percent identity cutoffs (90-99%), and the number of singleton and contigs were determined for each set (Figure A5). The contig set constructed using a 94% identity cutoff was chosen as the unigene set for the design of the 60-mer oligonucleotides used in microarray construction. This unigene set had a total of 10,757 sequences, consisting of 7,774 singletons and 2,983 multi-sequence contigs (Table 5). The contigs were composed of 2 to 38 sequences, with an average length of 644 bp. The average length of the singletons was 447 bp. The gene discovery rate,

Table 4. DNA sequences derived from expressed sequence tag (EST) libraries constructed from Douglas-fir seedlings. Quality sequences have an average Phred score greater than 20 after vector trimming. Unigene sequences were generated using CAP3 with an overlap identity cutoff of 94%.

	EST libraries[†]				
Description	CA	MH	CD	GR[‡]	Total
Raw sequences					
Number of reads	4128	4029	4000	11770	23927
Average length (bp)	955	1013	1089	762	955
Quality sequences					
Number of reads	3949	3701	3684	6760	18094
Average length (bp)	545	497	504	356	476
Unigene set					
Number of singletons	1335	1711	1777	2951	7774
Number of contigs*	N/A	N/A	N/A	N/A	2983

[†] EST libraries were prepared from first-year seedlings that were cold acclimating (CA), at maximum cold hardiness (MH), cold deacclimating (CD), or actively growing (GR).

[‡] The GR library was described by Krutovsky et al (2004).

* N/A = not applicable because many contigs were derived form ESTs from multiple libraries.

Table 5. Douglas-fir unigenes constructed from four seedling EST libraries using CAP3 with an overlap identity cutoff of 94%. Functional annotations were assigned to sequences based on BLASTX similarities to NCBI non-redundant protein database and MIPS database of *Arabidopsis* proteins.

ESTs per contig	Number of contigs	Average length of contigs	Percent annotated
1	7774	447	37
2	1513	590	55
3	621	660	64
4	328	699	72
5	158	704	80
6	94	741	82
7	67	750	93
8	61	754	85
9	31	753	90
≥10	110	814	91
Total	10757	691	48

calculated by dividing the number of unigenes (10,757) by the total number of high quality sequences (18,094; Lorenz et al 2005), was 59% for the four Douglas-fir libraries.

Contigging revealed that 80% of the unigenes were library specific. The GR library, which contains the largest number of ESTs, had the greatest number of library-specific contigs (Figure 3) and the greatest number of singletons. Inter-library contigs made up the remaining 20% of the unigenes, with 149 contigs made up of ESTs from all four libraries. On average, more inter-library contigs were composed of sequences from chronologically "adjacent" libraries (ranging from 245 to 299) than from non-adjacent libraries (ranging from 219 to 234).

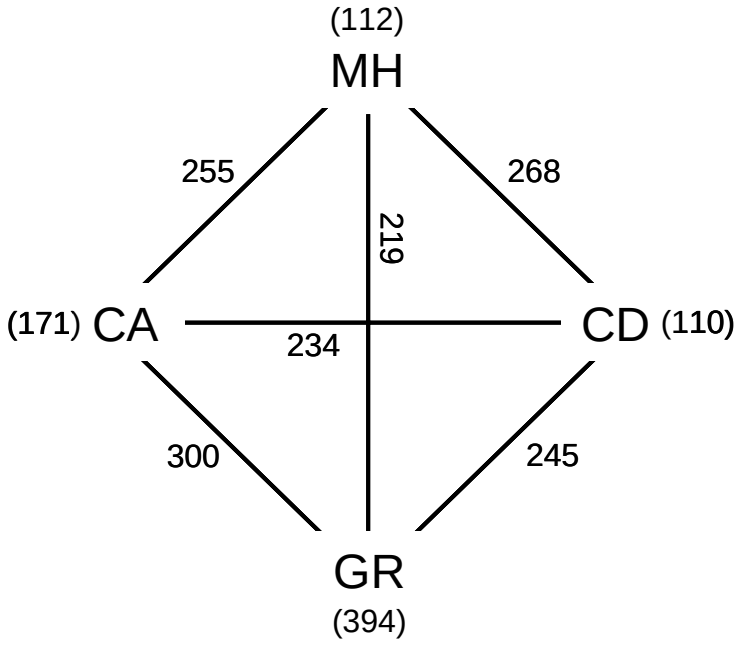

Figure 3. Distribution of multi-sequence contigs from four Douglas-fir EST libraries prepared from first-year seedlings that were cold acclimating (CA), at maximum cold hardiness (MH), cold deacclimating (CD), or actively growing (GR). The numbers of library-specific contigs are shown in parentheses. The numbers of inter-library contigs are shown along each connecting line. In addition, 675 contigs were comprised of ESTs from three or more libraries.

Functional annotation and categorization of the unigenes

Functional annotations were assigned to 48% of the unigenes based on BLASTX similarities to NCBI nr (e-value $\leq e^{-6}$). The functional categorization of the unigenes was based on the broad functional categories of the MIPS database of *Arabidopsis* proteins (Figure 4). The greatest numbers of matches were to the experimentally uncharacterized proteins (35%), metabolism (4%), and information pathways (4%).

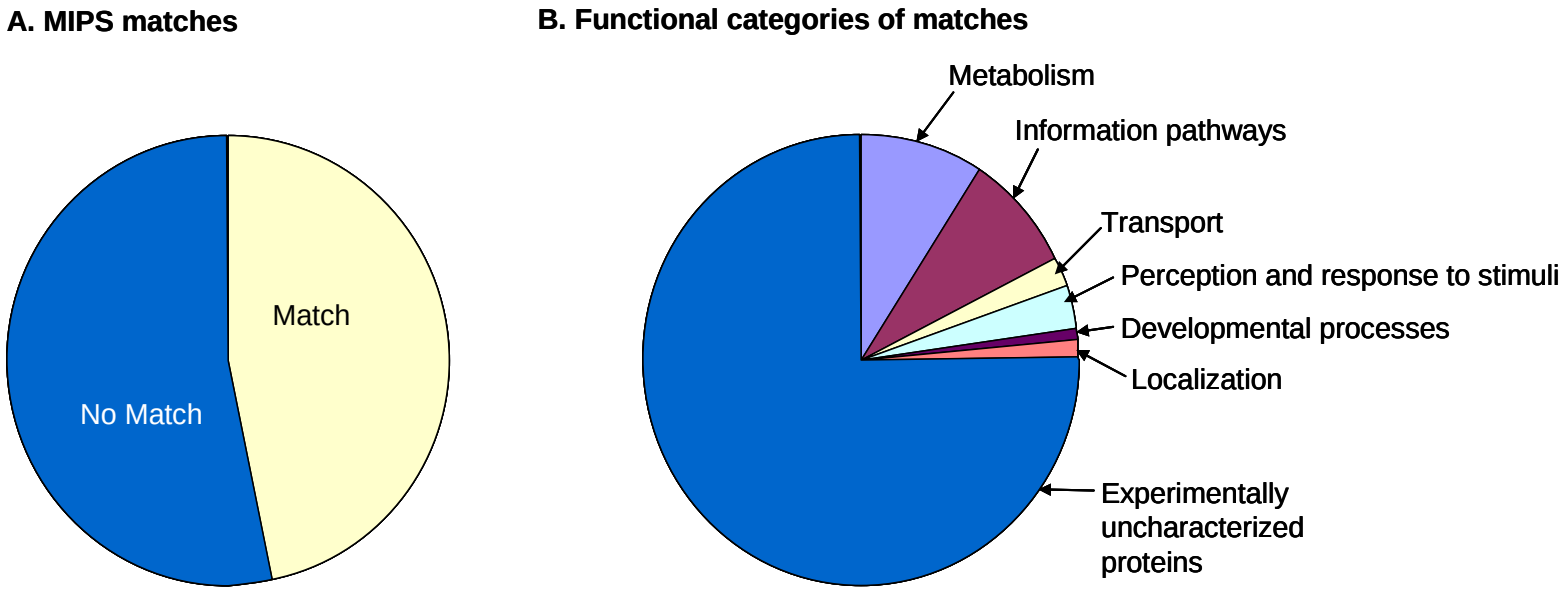

Figure 4. Functional characterization of the correctly oriented Douglas-fir unigenes (D_c) based on BLASTX similarities to the MIPS *Arabidopsis* protein database. **A.** Protein matches (e-value $\leq e^{-6}$) were found for 48% of the D_c unigenes. **B.** The greatest number of significant matches were to the experimentally uncharacterized proteins category.

Cross-hybridization levels were determined from incorrectly oriented sequences.

When the orientation of the unigenes could not be confidently determined using polyA-tail, cDNA cloning orientation, and BLASTX information, probes were designed from the two complementary sequences. This provided an opportunity to examine the amount of signal resulting from non-specific cross-hybridization of RNA samples to oligonucleotide probes that were in the incorrect orientation. The RE values for sequences in the incorrect orientation (D_i) ranged from 5.7 to 11.4, with an average of 6.8 (Figure 5). RE values for sequences in the correct orientation (D_c, step 2) ranged from 6 to 15.7, with an average of 8.1. By comparing the frequency distributions of the RE values from the reverse complement unigenes, a cutoff for "valid" RE values was determined. A RE value of 7.24 was used as a cutoff because <25% of the RE values from incorrectly oriented sequences were greater than 7.24. A total of 8,395 unigenes remained after applying the cutoff. The correlations between the two replicate probe sets for the in D_c group were high, ranging from 0.73 to 0.92 (Table 6), validating the hybridization procedure and probe performances.

Table 6. Range of correlations between the RMA $\log_2$ expression values for the two replicate probe sets for the Douglas-fir, loblolly pine, and white spruce unigenes groups used in differential expression analyses. Correlations were calculated using the SAS ROBUSTREG procedure for 12 arrays from each platform.

	Microarray platforms[‡]	
Unigene set[†]	LP	WS
D_c	0.84-0.92	0.73-0.90
P_c	0.76-0.87	N/A
S_c	N/A	0.59-0.83

[†] Unigene sets as outlined in Table 2.
[‡] LP = Douglas-fir and loblolly pine; and WS = Douglas-fir and white spruce.

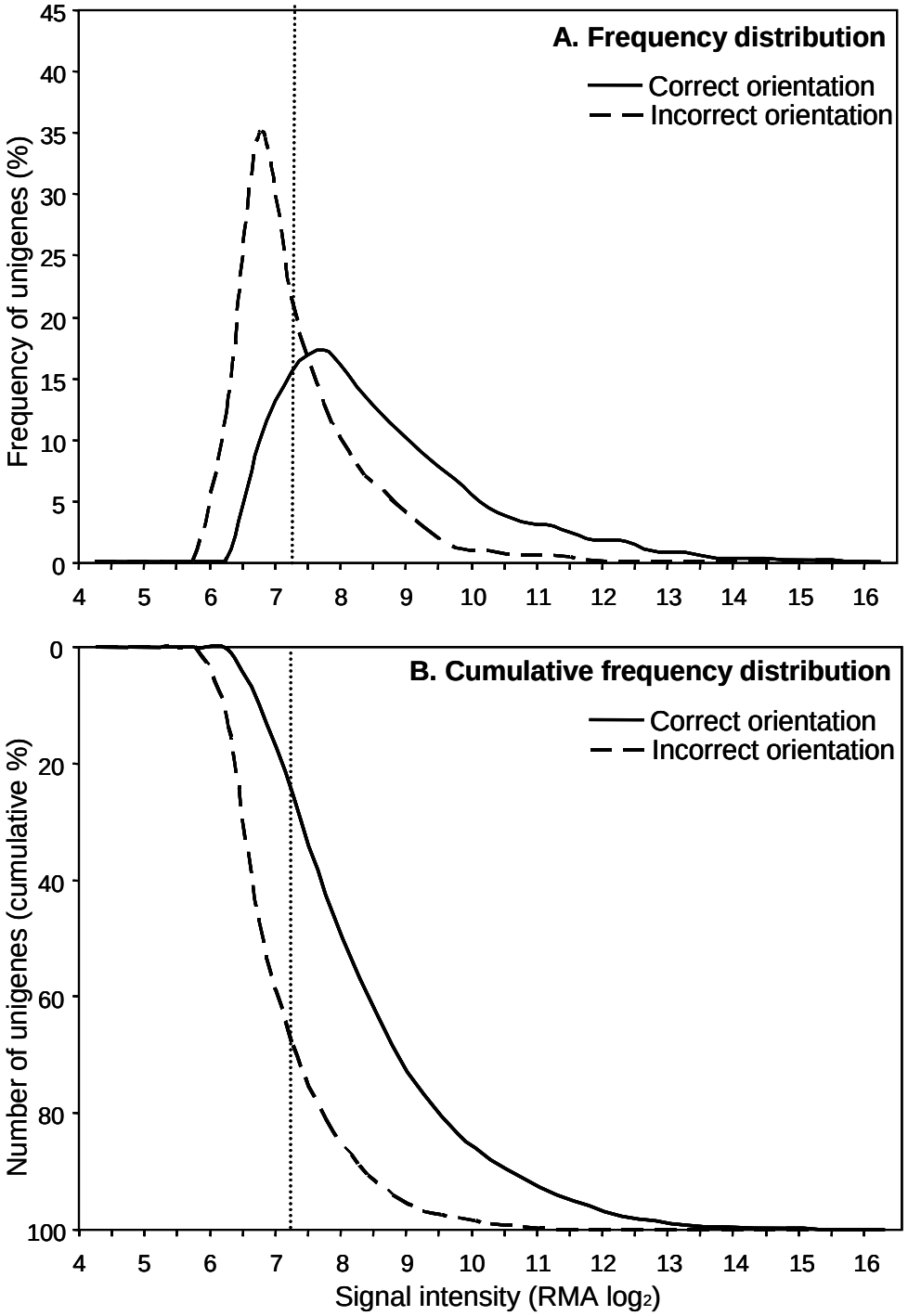

Figure 5. Comparison of RMA $\log_2$ expression (RE) values for Douglas-fir reverse complementary sequences. The distributions of RE values for sequences in the correct and incorrect orientations (A) and the cumulative percentage of sequences at a RE value (B) are shown. The dotted line indicates the cutoff of 7.24 that was used for including unigenes in the analyses of differential gene expression.

Differential gene expression during cold acclimation

I identified a total of 343 differentially expressed unigenes between cold susceptible and cold hardy seedlings (FDR adjusted p-value ≤ 0.01). Because cold acclimation occurred earlier in the YK seed source (Figure 1), I used different months for YK and CB to test for differences in gene expression during cold acclimation. I contrasted the seedlings from CB in October and YK in September (i.e., cold susceptible) against the seedlings from CB in November and YK in October (i.e., more cold hardy). Analyses of the different tissues revealed 26 differentially expressed unigenes in buds, 101 unigenes in stems, and 310 unigenes in a combined analysis of buds and stems (Tables A3 to A5). I created a list of 159 unigenes from the 26 unigenes from buds, the top 100 p-values from stems, and the top 100 p-values from the combined analysis of buds and stems (Table 7). Within this set, 9% of the unigenes were similar to gene sequences for proteins of unknown function, and 40% had no significant similarity to genes encoding known proteins (e-value ≤ e^{-6}). The unigenes that were annotated (51%) were divided into 11 categories, including dehydrins, heat shock proteins, PR and stress proteins, and genes related to ABA and water stress, GA, cold acclimation, dormancy-related, metabolism, translation/ribosomal, transcription factors, and other processes.

The 26 differentially expressed genes in buds were classified into five of the categories: dehydrins, dormancy-related, metabolism, translation/ribosomal, and unknown. All of the dehydrin and dormancy-related genes from buds were

Table 7. Differentially expressed Douglas-fir unigenes between cold susceptible and cold hardy seedlings' buds, stems, and both buds and stems combined (FDR adjusted p-value ≤ 0.01). Function was inferred for each unigene based on BLASTX similarities to the NCBI non-redundant protein database (e-value ≤ e^{-6}).

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Dehydrin					
Pm_UCD_GR_250G04	putative dehydrin	*Picea glauca*		-2.71	
Contig1609	Lea protein	*Pseudotsuga menziesii*	1.94	1.71	1.82
Pm_OSU_CD_010D07	late embryogenesis abundant protein				1.57
Pm_OSU _CA_028E03	late embryogenesis abundant protein		1.96	2.20	2.08
Pm_OSU _MD_037B09	late embryogenesis abundant protein	*Picea mariana*			1.32
Pm_OSU _MD_010C03	putative LEA protein	*Pinus monticola*	1.81	2.01	1.91
Pm_OSU _MD_030F10	embryo-abundant protein	*Picea glauca*			1.21
Pm_OSU _CA_006B08	dehydrin 1	*Picea abies*			1.28
Pm_OSU _CA_007D10	dehydrin 1	*Picea abies*		1.74	1.61
Pm_OSU _CA_012C08	dehydrin 1	*Picea abies*	1.60		1.55
Heat shock					
Contig2	HSP associated protein like	*Arabidopsis thaliana*			1.55
Contig2523	heat shock protein	*Picea glauca*		-1.48	
Pm_OSU _CA_014H07	DegP protease-like	*Oryza sativa*		-1.25	-1.19
PR and stress					
Pm_OSU _CA_042A09	disease resistance associated protein	*Picea abies*			0.94
Pm_UCD _GR_155E12	elicitor inducible beta-1,3-glucanase	*Nicotiana tabacum*		-1.80	
Contig1664	universal stress protein family protein, expressed	*Oryza sativa*		1.28	
Contig1389	putative ethylene response protein	*Capsicum chinense*			0.80
Contig410	stress responsive protein	*Triticum aestivum*		-2.16	
Contig907	putative universal stress protein	*Mirabilis jalapa*		1.03	

Table 7 cont. Differentially expressed Douglas-fir unigenes.

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
ABA					
Pm_UCD_GR_210F02	ASR protein induced by ABA, stress, and ripening	*Ginkgo biloba*		-2.36	-1.87
Contig146	ASR protein induced by ABA, stress, and ripening	*Ginkgo biloba*		-2.51	-2.09
Pm_UCD_GR_163B11	ASR protein induced by ABA, stress, and ripening	*Ginkgo biloba*		1.48	1.59
Pm_UCD_GR_251H10	water deficit inducible LP3-like protein	*Pseudotsuga menziesii*		-2.08	-1.78
Pm_UCD_GR_282A02	abscisic acid water deficit stress and ripening inducible-like protein	*Pseudotsuga menziesii*		1.16	1.05
Contig1065	glyceraldehyde-phosphate dehydrogenase	*Ginkgo biloba*		1.82	
Contig1549	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic			1.74	
Contig2289	putative pyruvate dehydrogenase E1 alpha subunit	*Oryza sativa*			1.36
Contig331	probable aquaporin	*Picea abies*		1.37	
GA					
Pm_UCD_GR_153E03	Gonadotropin, beta chain; Gibberellin regulated protein	*Medicago truncatula*		1.46	1.37
Contig2486	Gibberellin regulated protein	*Medicago truncatula*		-1.67	
Cold acclimation					
Contig1608	Clt1 citrus low temperature inducible	*Poncirus trifoliata*			1.20
Contig2200	COR413-PM2	*Arabidopsis thaliana*		1.71	1.50
Contig2869	cold acclimation protein Picg5a	*Picea glauca*		1.76	1.48
Dormancy					
Contig1409	dormancy-associated protein	*Codonopsis lanceolata*			0.96
Contig340	auxin-repressed protein-like protein ARP1	*Manihot esculenta*	1.60		1.11

Table 7 cont. Differentially expressed Douglas-fir unigenes.

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Metabolism					
Pm_UCD_GR_107G01	FAD binding /aldehydelyase/oxidoreductase	*Arabidopsis thaliana*	-1.46		-1.31
Pm_UCD_GR_122C11	putative serine/threonine-specific protein kinase	*Oryza sativa*			-1.10
Pm_UCD_GR_210D05	cellulase	*Tropaeolum majus*	-1.37		
Pm_UCD_GR_261E02	3-ketoacyl-CoA thiolase; acetyl-CoA acyltransferase	*Cucumis sativus*		1.47	
Pm_UCD_GR_291D09	chalcone synthase	*Abies alba*		1.38	
Contig371	chalcone synthase	*Abies alba*		1.26	
Contig719	chalcone synthase	*Abies alba*		1.80	1.33
Pm_OSU _CA_045B04	chalcone synthase	*Abies alba*		2.10	
Pm_OSU _CA_047D01	chalcone synthase	*Abies alba*		2.40	
Pm_UCD_GR_301H03	nodule autoregulation receptor-like protein kinase precursor	*Glycine max*	-1.23		-0.92
Contig1333	cell wall hydrolase	*Hyacinthus orientalis*			0.91
Contig2134	20S proteasome beta subunit PBG1	*Arabidopsis thaliana*		1.75	
Contig2167	phopholipid hydroperoxide glutathione peroxidase-like protein	*Spinacia oleracea*		1.51	
Contig2299	20S proteasome beta subunit	*Oryza sativa*		1.43	
Contig2318	ubiquinol-cytochrome-c reductase	*Arabidopsis thaliana*		1.79	
Contig2467	galactinol synthase 3, putative, expressed	*Oryza sativa*		2.29	1.86
Contig2733	putative pyruvate kinase	*Arabidopsis thaliana*		1.23	
Contig937	anthocyanidin reductase	*Ginkgo biloba*		1.23	
Pm_UCD_GR_157H09	kinase/ ribokinase	*Arabidopsis thaliana*		1.34	1.29
Contig1675_2	dihydroflavonol 4-reductase	*Malus x domestica*		1.62	
Pm_OSU _CD_014H04	Alpha-glucan phosphorylase, H isozyme (Starch phosphorylase H)			1.95	

Table 7 cont. Differentially expressed Douglas-fir unigenes

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Pm_OSU _MD_038F04	Thioredoxin domain 2	*Medicago truncatula*			1.55
Pm_OSU _CA_035B10	cytochrome b5 isoform Cb5-B	*Vernicia fordii*			0.98
Transcription factors					
Contig1109	MYB-like transcriptional factor MBF1	*Picea mariana*		1.73	
Contig147	putative DNA binding protein	*Atriplex hortensis*		1.70	1.52
Contig1121	putative nucleic acid binding (PHD-finger) protein	*Oryza sativa*			1.10
Contig315	putative AT-hook DNA-binding protein	*Oryza sativa*			-0.39
Translation/ribosomal					
Contig1562	putative translation initiation factor eIF-1A-like	*Solanum tuberosum*			1.00
Contig1569	structural constituent of ribosome	*Arabidopsis thaliana*			1.63
Contig1714	putative translation factor	*Pinus pinaster*	1.71	2.06	1.89
Contig196	40S ribosomal protein S25	*Glycine max*		1.84	1.56
Contig2489	DNA-directed RNA-polymerase II subunit	*Brassica rapa*			1.02
Pm_OSU _CD_052D02	RNA helicase	*Arabidopsis thaliana*			0.95
Other					
Pm_UCD_GR_127F02	putative histidinol dehydrogenase precursor	*Oryza sativa*			-0.66
Pm_UCD_GR_202E04	Cystatin-like protein	*Boechera stricta*		1.06	
Pm_UCD_GR_222B11	histidine-containing phosphotransfer protein	*Catharanthus roseus*		1.34	
Pm_UCD_GR_267H09	Zinc finger, C2H2 type family protein, expressed	*Oryza sativa*			-1.03
Contig1301	leucine-rich repeat protein	*x Citrofortunella mitis*		1.47	1.18
Contig1516	glucose-regulated endoplasmic reticular protein precursor	*Medicago sativa*		1.23	
Contig2279	peptide methionine sulfoxide reductase	*Gossypium barbadense*		1.39	1.35
Contig694	similar to Trp_Asp repeat protein	*Arabidopsis thaliana*			1.36

Table 7 cont. Differentially expressed Douglas-fir unigenes

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Contig827	histone H2A	*Picea abies*		-1.07	
Contig926	Bax inhibitor-1 (BI-1) (OsBI-1)	*Oryza sativa*			1.25
Contig95	eIF4-gamma/eIF5/eIF2-epsilon	*Medicago truncatula*			1.40
Pm_OSU _MD_032E10	nonsymbiotic hemoglobin	*Alnus firma*		1.25	
Pm_OSU _CA_006G07	carbonic anhydrase			-1.32	-1.11
Unknown					
Pm_UCD_GR_110C03	expressed protein	*Oryza sativa*			-0.96
Pm_UCD_GR_118B04	unknown protein	*Oryza sativa*		-1.29	
Pm_UCD_GR_156B08	unknown protein	*Arabidopsis thaliana*			1.47
Contig1082	Protein of unknown function	*Medicago truncatula*		1.39	
Contig1168	putative protein	*Arabidopsis thaliana*	-1.40	-1.58	-1.49
Contig1431	hypothetical protein	*Oryza sativa*			1.30
Contig1868	unknown protein	*Oryza sativa*		1.93	1.73
Contig2632	unnamed protein product	*Solanum tuberosum*		1.17	
Contig1113	unnamed protein product	*Lupinus polyphyllus*			0.98
Contig2889	Conserved hypothetical protein	*Medicago truncatula*			0.66
Pm_OSU _CD_038E01	hypothetical protein	*Arabidopsis thaliana*		1.91	1.68
Pm_OSU _CD_047G11	hypothetical protein	*Thellungiella halophila*		1.67	
Pm_OSU _MD_006B08	unnamed protein product	*Lupinus polyphyllus*			1.43
Pm_OSU _MD_023G06	putative protein	*Arabidopsis thaliana*		1.24	
Pm_UCD_GR_105A02	N/A	N/A	2.23	1.52	1.87
Pm_UCD_GR_112C04	N/A	N/A		-1.10	
Pm_UCD_GR_131H08	N/A	N/A		1.44	
Pm_UCD_GR_136F10	N/A	N/A		1.98	1.52
Pm_UCD_GR_144E04	N/A	N/A	-1.45		

Table 7 cont. Differentially expressed Douglas-fir unigenes

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Pm_UCD_GR_148E01	N/A	N/A	2.31	1.55	1.93
Pm_UCD_GR_163D06	N/A	N/A		1.91	
Pm_UCD_GR_200H10	N/A	N/A	1.82	1.42	1.62
Pm_UCD_GR_202A11	N/A	N/A		-1.33	
Pm_UCD_GR_211E09	N/A	N/A		2.11	
Pm_UCD_GR_216D02	N/A	N/A			-1.33
Pm_UCD_GR_222B07	N/A	N/A	1.84	2.20	2.02
Pm_UCD_GR_267H03	N/A	N/A		-1.63	
Pm_UCD_GR_269F05	N/A	N/A		1.87	
Pm_UCD_GR_279H07	N/A	N/A		1.76	1.45
Contig1019	N/A	N/A		2.12	1.91
Contig1102	N/A	N/A			-0.71
Contig1214	N/A	N/A			1.62
Contig1263	N/A	N/A	1.55		1.36
Contig1266	N/A	N/A		1.58	1.53
Contig1302	N/A	N/A		1.32	
Contig1393	N/A	N/A	2.24	2.08	2.16
Contig1400	N/A	N/A			0.99
Contig1764	N/A	N/A		1.41	
Contig191	N/A	N/A	2.37	2.30	2.33
Contig2086	N/A	N/A		1.87	1.52
Contig265	N/A	N/A		-2.11	
Contig2841	N/A	N/A		-1.26	
Contig2863	N/A	N/A			1.43
Contig312	N/A	N/A	1.90		
Contig385	N/A	N/A		1.89	1.40

Table 7 cont. Differentially expressed Douglas-fir unigenes

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Contig776	N/A	N/A	2.25		
Contig791	N/A	N/A			0.96
Contig90	N/A	N/A		1.65	
Pm_UCD_GR_110F10	N/A	N/A		2.19	
Pm_UCD_GR_123A10	N/A	N/A		2.00	
Pm_UCD_GR_132D07	N/A	N/A		1.79	1.65
Pm_UCD_GR_220G07	N/A	N/A		1.28	
Pm_UCD_GR_224G11	N/A	N/A		1.39	1.27
Pm_UCD_GR_257G09	N/A	N/A	2.38		1.98
Pm_OSU _CD_012B10	N/A	N/A			1.10
Pm_OSU _CD_013E10	N/A	N/A		1.62	
Pm_OSU _CD_035C10	N/A	N/A			0.76
Pm_OSU _CD_036F03	N/A	N/A			1.24
Pm_OSU _CD_042A12	N/A	N/A			-0.82
Pm_OSU _CD_042D11	N/A	N/A		1.64	
Pm_OSU _CD_043E08	N/A	N/A		-1.25	-1.23
Pm_OSU _CD_045G07	N/A	N/A		-1.54	-1.40
Pm_OSU _CD_051G11	N/A	N/A		1.69	
Pm_OSU _MD_016C07	N/A	N/A			1.01
Pm_OSU _MD_020H01	N/A	N/A		1.74	
Pm_OSU _MD_021A12	N/A	N/A	2.43	2.53	2.48
Pm_OSU _MD_023D09	N/A	N/A	1.82	3.02	2.42
Pm_OSU _MD_034A12	N/A	N/A		1.59	
Pm_OSU _MD_042B06	N/A	N/A	1.34		1.12
Pm_OSU _MD_053A06	N/A	N/A	2.38	3.38	2.88
Pm_OSU _MD_054D06	N/A	N/A			1.02

Table 7 cont. Differentially expressed Douglas-fir unigenes

	Functional annotation (BLASTX)		Transcript abundance[†]		
Douglas-fir unigene[‡]	Gene product	Species	Buds	Stems	Both
Pm_OSU _MD_055H07	N/A	N/A			1.49
Pm_OSU _CA_003H11	N/A	N/A			0.41
Pm_OSU _CA_016D03	N/A	N/A			0.88
Pm_OSU _CA_032H12	N/A	N/A			1.40
Pm_OSU _CA_033A11	N/A	N/A			1.44
Pm_OSU _CA_043C12	N/A	N/A		1.85	
Pm_OSU _CA_045A06	N/A	N/A	1.12	1.47	1.29

[†] Difference between RMA $\log_2$ expression values of the cold hardy and cold susceptible seedlings (i.e., cold hardy – cold susceptible).
[‡] Unigene sequences contain contigs and singletons from cold acclimating (CA), at maximum cold hardiness (MH), cold deacclimating (CD), or actively growing (GR) EST libraries.

up-regulated, whereas all of the metabolism genes were down-regulated. There were four differentially expressed unigenes that were only identified in buds.

The 100 unigenes in stems were included in all categories except dormancy-related proteins. The stem genes with the greatest change in abundance within the ABA and water stress group were down-regulated, whereas all the unigenes in the stem metabolism group were up-regulated. There were 55 differentially expressed unigenes that were only identified in stems.

The 100 unigenes from the combined analyses of buds and stems were included in all categories. There were 48 differentially expressed unigenes that were only identified as in the combined analyses of buds and stems. Fifteen differentially expressed unigenes were identified in all three analyses (buds, stems, and combined buds and stems), and three of these were dehydrins.

Heterologous hybridizations

The frequency distributions of the RE values for the P_c unigenes and S_c unigenes were compared to D_c (Figure 6). The RE values for the P_c unigenes ranged from 5.76 to 15.27, with an average of 7.38, whereas the values for the S_c unigenes ranged from 6.35 to 15.34, with an average of 7.32. A total of 4,794 pine unigenes and 6,322 spruce unigenes remained after applying the cutoff. The correlations between the two replicate probe sets for the P_c and S_c unigene groups were high, validating the hybridization procedure and probe performances (Table 6).

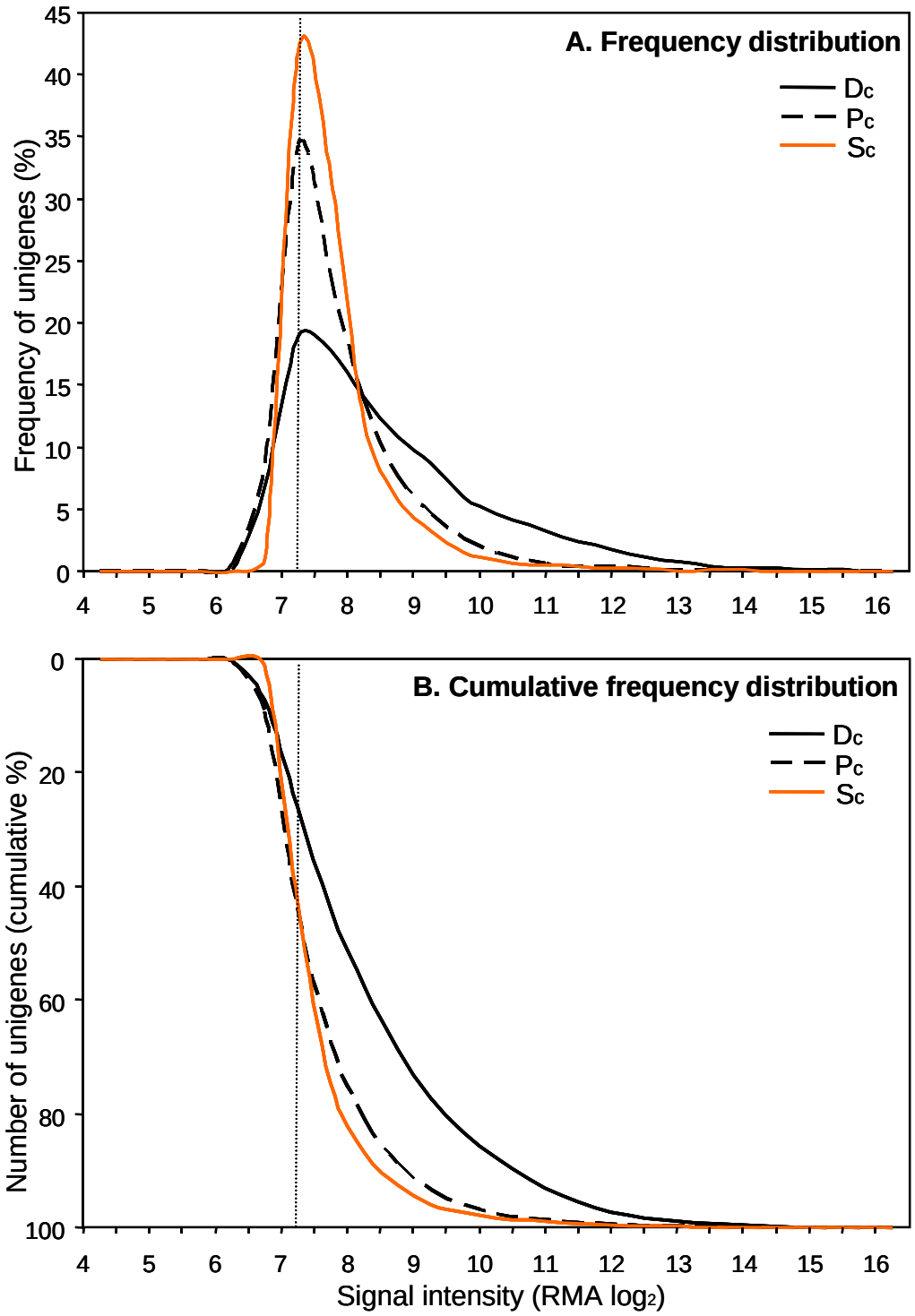

Figure 6. Comparison of RMA $\log_2$ expression (RE) values for Douglas-fir (D_c), loblolly pine (P_c) and white spruce (S_c). The distributions of the RE values for each species (A) and the cumulative percentage of sequences at a RE value (B) are shown. The dotted line indicates the cutoff of 7.24 that was used for including unigenes in the analyses of differential gene expression.

For the P_c and S_c unigenes with significant similarity (e-value $\leq e^{-10}$) to a D_c unigene, correlations between the corresponding unigenes were calculated across the 12 arrays. The average correlation between D_c and P_c was 0.25 (from a total of 5769 correlations). The average correlation between the D_c and S_c groups was 0.27 (from a total of 4087 correlations).

In my heterologous hybridizations of Douglas-fir cold susceptible and cold hardy seedlings to the loblolly array platform, I identified 17 differentially expressed loblolly pine unigenes (FDR adjusted p-value ≤ 0.05; Table 8). Thirteen of these unigenes are similar to Douglas-fir unigenes (e-value $\leq e^{-10}$), but only two of the corresponding D_c unigenes were found to be differently expressed (FDR adjusted p-value ≤ 0.01). Therefore, 15 additional genes were identified using the loblolly pine probes.

In my heterologous hybridizations of Douglas-fir cold susceptible and cold hardy seedlings to the spruce array platform, I identified 65 differentially expressed white spruce unigenes (FDR adjusted p-value ≤ 0.05; Table 8). Fifty-five of these unigenes are similar to Douglas-fir unigenes (e-value $\leq e^{-10}$), but only three of the corresponding D_c unigenes were found to be differently expressed (FDR adjusted p-value ≤ 0.01). Therefore, 62 additional genes were identified using the white spruce probes.

Table 8. Differentially expressed unigenes from heterologous hybridizations of Douglas-fir RNA to loblolly pine and white spruce oligonucleotides (FDR adjusted p-value ≤ 0.05).

Number of unigenes[†]	P_c	S_c
Differentially expressed	17	65
Sequences similar to D_c unigene[‡]	13	55
Differentially expressed in D_c[§]	2	3
Newly identified differentially expressed gene	15	62

[†] Unigene sets as outlined in Table 2.
[‡] e-value ≤ e^{-6}
[§] Differential expression at FDR adjusted p-value ≤ 0.01

DISCUSSION

YK and CB populations responded differently to cold.

Acclimation occurred earlier for the high elevation, inland YK seed source compared to the low elevation, coastal CB seed source. Compared to the CB seedlings, the YK seedlings set bud earlier and had less cold damage in early fall (Figures 1 and 2A). Similar differences in population cold hardiness have been studied in relation to differences in latitude, longitude, and elevation. Campbell and Sorenson (1973) found that a comparable CB seed source also set bud later and experienced more fall cold damage than more northern seed sources. For example, the CB seed source had 78% damage, whereas the most northern seed source from Soleduck, Washington had only 10% damage. In Oregon, longitudinal differences in fall cold hardiness were found between a more hardy inland Cascade seed source compared to a less hardy coastal seed source (O'Neill et al 2000). Elevational differences in cold hardiness were found among seed sources from southern Oregon (Loopstra and Adams 1989). The low-elevation families suffered more cold damage than the high-elevation families. St. Clair (2006) modeled fall cold damage for western Oregon and Washington seed sources as a function of location, including longitude, latitude, and elevation. All three location variables were negatively related to fall cold damage, indicating that seed sources from locations farther north, higher elevations, and more inland (i.e., further east) had less fall cold damage. Because large population differences in fall cold hardiness are associated with environmental

gradients, natural selection seems to be important in determining genetic variation in cold adaptation (Howe et al 2003; St. Clair 2006).

Differences between the YK and CB seedlings' acclimation may also be the result from varietal differences. The two varieties of Douglas-fir, the coastal variety *menziesii* and the interior variety *glauca*, differ in cold adaptation. Interior Douglas-fir sets bud earlier and is more fall cold hardy than the coastal variety (Rehfeldt 1977; Sorenson 1979). St. Clair et al (2005) found that the Washington crest of the Cascade Mountains is a sharp transition zone between var. *menziesii* and var. *glauca*, and the YK seed source comes from the east of the crest. Therefore, the YK seedlings may represent var. *glauca*.

Population differences in cold hardiness were the largest in early fall, but the two populations reached comparable levels of hardiness by late November (Figure 1). In other cold damage studies, family differences were greatest in early fall (September and October) and smaller in late fall and winter (Aitken and Adams 1996). The population differences found in early spring cold hardiness varied by tissue. The YK buds experienced greater cold damage than the CB buds, but the YK needles and stems experienced less damage than the CB needles and stems. By mid-May, when all the seedlings had flushed, CD_{-7} was comparable for the two populations. Earlier deacclimation and greater spring cold damage for inland and high elevation seed sources has been observed in other studies (van den Driessche 1970; Schuch et al 1989b; Aitken and Adams 1997; O'Neill et al 2000, 2001; Aitken and Hannerz 2001; Howe et al 2003).

Large population differences for fall and spring cold damage were also found in previous studies comparing seed sources from the Oregon Cascades versus the coast, and across a wide range of western Oregon and Washington (O'Neill et al 2000; St. Clair 2006). The largest population differences in cold hardiness were in the early fall and spring, supporting the conclusion that differences largely result from different timings or rates of acclimation and deacclimation, rather than differences between the populations' capacity to acclimate and deacclimate (Aitken and Adams 1996; Howe et al 2003).

Compared to needles and stems, buds responded differently to cold (Figure 1). Other studies also found considerable variation in the fall cold hardiness of stems, needles, and buds among other populations of Douglas-fir (Schuch et al 1989a; St. Clair 2006). Buds were the first tissue to acclimate in the fall, and the inland source was hardier than the coastal seed source (Schuch et al 1989a; Aitken and Adams 1996). Buds were the most damaged tissue in the winter. Cold hardiness studies of family variation using AFT also found that Douglas-fir buds were less winter cold hardy than needles and stems (O'Neill et al 2001; Aitken and Adams 1996). Buds only acclimated to -25°C, whereas needles and stems acclimated to -40°C. Stems were the most cold hardy winter tissue in my study, although I did not find significant differences between the populations as previously reported (Schuch et al 1989a). In the spring, buds were the only tissue with greater damage in the YK seedlings. Needles, stems, and buds deacclimate at different rates (Schuch et al 1989b). Previous studies, however, have not reported significant population differences in spring cold hardiness between needles, stems, and buds (O'Neill et al 2000, 2001).

Cold damage among tissues was highly correlated in the fall and winter, but moderately correlated in the spring.

Fall cold hardiness was significantly correlated among all three shoot tissues in the YK and CB seedlings, suggesting that only one tissue is needed to measure population differences in fall cold acclimation (Table 3). Furthermore, a mid-fall (October) date seems to be best for assessing cold hardiness differences among populations. At this time, the seedlings had the largest differences in CD_{-7} and percent bud set (Figures 1 and 2A). However, if these differences result from prior differences in gene expression, multiple dates may be needed to detect the changes in gene activity responsible for differences in cold hardiness. Previous studies also found that fall cold damage is highly correlated among shoot tissues (r = 0.78 to 0.98), suggesting a common set of genes may be involved in cold acclimation of needles, stems, and buds (St. Clair 2006; Anekonda et al 2000b). Studies of saplings, however, found that fall cold hardiness varied considerably among shoot tissues (r = 0.16 to 0.58; Aitken et al 1996; Aitken and Adams 1996), perhaps because bud set occurs much earlier (i.e., June to July) in saplings compared to seedlings.

In the spring, there was low correlation between bud and needle damage in the YK seedlings, suggesting that more than one tissue is needed to effectively evaluate population differences in cold deacclimation of seedling shoots (Table 3). For Douglas-fir seedlings and saplings, stems and buds had the strongest genetic correlations between tissues (seedling r = 0.84; sapling r = 0.52 to 0.59). The corresponding correlations between needles and buds were weaker (seedling r = 0.59; sapling r = 0.42 to 0.31; Aitken et al 1996; O'Neill et al 2000, 2001). In contrast,

other studies found that genetic correlations are high (r = 0.74 to 0.98) between all three sapling shoot tissues in early spring (March and April) for both coastal and Cascade seed sources (Aitken and Adams 1995, 1997).

The similar correlations found between tissues across all dates support the conclusion that the population differences in acclimation and deacclimation result from timing differences and not differences between the populations' ability to acclimate and deacclimate (Aitken and Adams 1996; Howe et al 2003).

Cold hardiness was associated with bud set and bud flush.

Bud set in the YK seedlings was completed more than a month before bud set was completed in the CB seedlings (Figure 2A), suggesting that the two populations respond differently to environmental stimuli such as night temperatures, photoperiod, and nutrient availability (van den Driessche 1969; Campbell and Sugano 1979; Bigras et al 2001). The trend towards earlier bud set and decreased cold damage for seed sources from high elevations, inland locations, and cooler climates is well documented for Douglas-fir populations (Hermann and Lavender 1967; Griffin and Ching 1977; Campbell 1979; Loopstra and Adams 1989; Rehfeldt 1989; St. Clair et al 2005; St. Clair 2006). In a large study of families from western Oregon and Washington, fall cold damage was moderately correlated with later bud set (r = 0.57; St. Clair 2006).

Bud flush is highly correlated with spring cold hardiness (Aitken and Hannerz 2001; Howe et al 2003). In fact, the Pacific Northwest Tree Improvement Research Cooperative recommends that breeders interested in improving spring cold hardiness select for bud flush, rather than measuring cold hardiness per se. The YK buds

flushed earlier than the CB buds, and were the only tissue to have more cold damage in the YK seedlings than in the CB seedlings (Figures 1 and 2B). Later bud flush was associated with greater spring cold hardiness in previous studies of Douglas-fir (Balduman et al 1999; Stevenson et al 1999). In contrast to our populations, other common garden studies found relatively little variation in flushing among seed sources (O'Neill et al 2000, 2001; St. Clair 2006).

Design of the microarray study

The large differences in cold acclimation, deacclimation, and bud phenololgy of the YK and CB seedlings make them excellent candidates for studying population differences in gene expression associated with cold hardiness. I hypothesize that these population differences result from (1) the expression of different genes in the different populations, or (2) differences in the timing of gene expression. I addressed these two hypotheses by analyzing gene expression patterns from YK and CB stems and buds collected in September, October, and November. I emphasized fall cold hardiness in my gene expression studies because it had the largest population differences, and these differences were consistent across all three tissues. The high correlations among seedling shoot tissues in the fall suggest that a common set of genes may be involved; therefore, testing both buds and stems provided additional replication and verification of gene expression, while at the same time allowing me to study multiple tissues.

Douglas-fir EST libraries

I created three new Douglas-fir ESTs libraries from cold acclimating and deacclimating tissues, and then used them to develop a unigene set (Table 4). The

three normalized cDNA libraries were developed from needles, stems, and buds to study cold adaptation in Douglas-fir seedlings. Other EST sequencing projects have recently focused on cold acclimation and dormancy in woody plants, but these studies produced fewer sequences, sampled fewer developmental stages and tissues, and used targeted approaches such as subtractive libraries. cDNA libraries specifically targeting cold acclimated and non-acclimated tissues have been made from *Rhododendron* leaves (Wei et al 2005), blueberry floral buds (Dhanaraj et al 2004), Scots pine buds (Joosen et al 2006), and poplar vascular cambium (Schrader et al 2004). There was limited similarity between the sequences from any two of these libraries (e.g., the overlap ranged from 4 to 8%; Dhanaraj et al 2004; Schrader et al 2004; Wei et al 2005). My results for acclimated and non-acclimated libraries are similar; only 5% of my unigenes were composed of ESTs from the actively growing and maximum hardiness libraries. Overall, 20% of my unigenes were composed of ESTs from more than one library, with greater overlap between chronologically adjacent libraries. This may be explained by the relatively wide range of developmental stages represented within the library seedling population during acclimation and deacclimation.

The three libraries I constructed added 11,334 new Douglas-fir sequences to GenBank, more than doubling the current number of Douglas-fir ESTs. The cold adaptation ESTs were combined with ESTs from actively growing Douglas-fir seedlings to create a unigene set spanning the entire annual growth cycle. All four libraries were constructed from the same three shoot tissues, and from seedlings harvested during their first growing season. Douglas-fir has a large and repetitive

genome that is unlikely to be fully sequenced. Therefore, additional ESTs from roots and reproductive organs, as well as other age classes and developmental stages, would greatly increase the genomic resources for this ecologically and economically important tree. Whereas large EST collections are available for poplar (Sterky et al 2004), spruce (Pavy et al 2005), pine (Lorenz et al 2006), citrus (Forment et al 2005), and apple (Newcomb et al 2006), I found sequence similarities between the Douglas-fir unigenes and pine or spruce unigenes to be only 41 to 48%, respectively. This suggests that sequences from Douglas-fir are needed to better understand and study this species. My results also demonstrate the potential for discovering novel genes because less than half of our current unigenes have significant similarity to identified proteins (e-value $\leq e^{-6}$).

Estimates of gene discovery rates from unigenes are influenced by the contigging criteria. Our unigenes were constructed with a moderate overlap stringency of 94% to provide flexibility to re-evaluate contigs given differential expression results. My estimated 59% gene discovery rate is higher than that reported for other conifers, and our current set of unigenes will be re-evaluated using the differential expression information from the microarrays.

Differential gene expression during cold acclimation

Compared to older trees, seedlings are more likely to continue growing late into the fall. Therefore, the processes of bud set, cold acclimation, and dormancy initiation overlap, and the differentially expressed unigenes I identified may be associated with any of these three key processes (Table 7).

Dehydrins/late embryogenesis abundant (LEA) proteins

LEA proteins are induced by stresses that cause cellular dehydration, including low and freezing temperatures. Dehydrins are a group of LEA proteins (group II or D-11 family) with the conserved K-segment motif EKKGIMDKIKEKLPG and molecular masses ranging from 9 to 200 kDa (Close 1996). The K-segment forms an amphipathic α–helix that has hydrophobic and hydrophilic regions (Dure 1993). Acidic dehydrins accumulate in plants in response to cold temperatures. Previous studies indicate that dehydrins play important roles in membrane stabilization, protein protection, starch degradation, heavy metal detoxification, and free radical sequestration (Allagulova et al 2003; Close 1996, 1997; Wisniewski et al 1999; Rinne et al 1994, 1999; Hara et al 2004; Thomashow 1999).

Two studies in Douglas-fir provide evidence that dehydrins are involved in response to cold. A specific 30-kDa protein accumulated in the terminal buds of samples collected in early November, soon after bud set, reached its maximum level by late November, and then declined in early spring until it was no longer detectable at bud flush in mid-April (Roberts et al 1991). The localization and expression pattern of this protein suggests it plays a role in cold hardiness. Jarvis et al (1996) found three genes that encode dehydrins in Douglas-fir seeds. Although these proteins were identified in seeds, there are common cold response characteristics between seeds and buds (Rohde et al 2000). Accumulation of dehydrin transcripts or proteins has been found in cold acclimation and dormancy development studies in at least 15 other woody plant species (reviewed in Welling and Palva 2006).

Ten of the differentially expressed genes I identified appear to encode dehydrins or LEA proteins. Although one gene was down regulated in the stems, the remaining unigene transcripts increased during acclimation, which is consistent with previous results. Three of the ten unigenes had significant sequence similarity to the same dehydrin protein sequence. This suggests that they are either derived from a single gene or from a closely related gene family.

Heat shock proteins (HSPs)

Heat shock proteins are stress proteins, originally identified in organisms that had been exposed to high temperatures. It is now known that cold temperatures and other environmental stresses that disrupt the osmotic stability of cells also induce the production of HSPs (Sabehat et al 1998). HSPs are involved in translation, translocation, protein refolding, and membrane protection (Vierling 1991). Low temperature induction of HSPs has been reported in peach (Wisniewski et al 1999), poplar (Renaut et al 2005), mulberry (Ukaji et al 1999), and sweet chestnut (Lopez-Matas et al 2004).

I identified three differentially expressed heat shock unigenes—two in stems and two in the combined analyses of stems and buds. Of these three, only one showed an increase in transcript abundance as the tissues became more cold hardy, suggesting that a transient response may have occurred for the other transcripts.

Pathogenesis-related (PR) and stress-related proteins

Pathogen infection, environmental stress, chemical compounds, and wounding can cause PR proteins to be expressed in plants (van Loon 1997). PR proteins are induced

systemically, suggesting they play an important role in the plants' ability to respond to biotic and abiotic stresses (van Loon and van Strien 1999). Fourteen families of PR proteins have been categorized in tobacco, tomato, cucumber, parsley, radish, *Arabidopsis*, and barley. Multiple families of PR proteins are known to accumulate during cold acclimation.

Expression of PR proteins during cold acclimation occurs in western white pine (Ekramoddoullah et al 1995; Yu et al 2000; Liu et al 2003), white spruce (Liu et al 2004), peach (Wisniewski et al 2004) and mulberry (Ukaji et al 2004). In western white pine, accumulation of the PR-10 protein *Pin m* III was significantly correlated with frost hardiness (Ekramoddoullah et al 1995). Accumulation of *Pse m* I, a homolog of *Pin m* III, was described in Douglas-fir needles, but was not associated with frost hardiness (Ekramoddoullah et al 2000).

In stems, and in the combined analyses of buds and stems, I identified two differentially expressed unigenes that seem to encode PR proteins and four unigenes that are associated with stress in other species. These unigenes, however, were not differentially expressed in buds. In stems, two of these unigenes had a decrease in transcript abundance, suggesting that they may not be associated with cold acclimation, as is *Pse m* I.

Abscisic acid (ABA) and water stress

ABA is a plant growth inhibitor that accumulates prior to cold acclimation and may be involved in the induction of dehydrins and LEAs (Welling and Palva 2006).

ABA accumulation is associated with increased chilling and freezing tolerance in woody plants (Rinne et al 1998, 1999; Welling et al 1997, 2002; Li et al 2002).

Rinne et al (1998) found that ABA is important in the photoperiodic control of cold acclimation. In their study, short day treatments resulted in elevated levels of ABA, accumulation of group-2 and group-4 LEAs, and increased freezing tolerance in wild-type birch. An ABA-deficient mutant birch did not have increased ABA levels, accumulate group-2 LEAs, and had delayed and reduced freezing tolerance compared to the wild-type.

I identified nine differentially expressed unigenes that are similar to ABA-related proteins. Although ABA accumulates with cold acclimation, I found up- and down-regulation of unigenes. In *Arabidopsis*, ABA accumulates during cold acclimation, but this accumulation is transient (Hannah et al 2005). ABA decreases in birch buds in the winter (Rinne et al 1999). A greater decrease in transcript abundance was detected in YK tissues compared to CB tissues, suggesting that the earlier acclimation of YK could also result in earlier down regulation of ABA-related proteins. Three genes had significant sequence similarity to the same ABA-related protein sequence, but two were down-regulated and one was up-regulated. These genes may represent a gene family or differences associated with interactions between transcription factors and these genes.

Gibberellic acid (GA)

GA is a plant hormone that enhances cell elongation and accelerates growth. It is often referred to as an antagonist to ABA. GA stimulates the production of α-amylase

in plant cells, which is needed to breakdown starch. In angiosperm trees such as willow, GA is involved in the regulation of growth cessation and cold hardening of stems (Junttila et al 1991; Junttila 1993). Under SD conditions, *Salix* shoot tips had a decrease in cell division and GA_1 levels (Hansen et al 1999). However, cell division was stimulated under SDs when GA_1 was applied. Olsen et al (1995, 1997) found that GA_1 levels decreased by 50% under SD conditions.

I identified two differentially expressed unigenes that are similar to GA-regulated proteins. One unigene decreased in transcript abundance (as would be expected in the fall), but the Gonadotropin beta chain protein increased, suggesting it may have roles in pathways that are not regulated by GA.

Cold acclimation

Analyses of differential expression revealed three Douglas-fir unigenes that are similar to proteins associated with cold acclimation. Two of these unigenes were identified in the analysis of stems and in the analysis of buds and stems combined. One unigene is similar to the Picg5 protein from white spruce (Liu et al 2004). The other unigene is similar to a cold-regulated (COR) gene from *Arabidopsis*. Many COR genes are up-regulated in *Arabidopsis* in response to low temperatures (Thomashow 1999; Hannah et al 2005; Fowler and Thomashow 2002; Seki et al 2001, 2002).

Dormancy-related proteins

Auxin is a plant hormone essential for growth. Although the relationship between auxin and cold acclimation is not clearly understood, auxin is involved in apical

dominance (Rohde et al 2000) and paradormancy (Lang et al 1987). In studies of *Arabidopsis* cold acclimation, auxin-induced genes were the most abundant down-regulated group compared to other hormone-regulated genes (Hannah et al 2005).

I identified two unigenes that seem to encode proteins associated with dormancy and auxin. Both genes are auxin-repressors and increase in abundance during cold acclimation. The greatest change in transcript abundance was found in buds.

Metabolism

Low temperatures induce changes in the expression and activity of metabolic enzymes, including enzymes involved in photosynthesis, detoxification, and the metabolism of carbohydrates, proline, and lignin (Guy 1990; Hurry et al 1995; Renaut et al 2006). Twenty-four differentially expressed unigenes with metabolic functions were identified, four of which decreased in abundance. The down-regulated unigenes encode proteins for FAD-binding, two kinases, and cellulose.

In *Arabidopsis*, genes for secondary metabolism were up-regulated during cold acclimation (Hannah et al 2005). Six up-regulated genes involved in secondary metabolism were included in my metabolism group (chalcone synthase and dihydroflavonol reductase). Five unigenes had significant similarities to the same chalcone synthase sequence. Contigging of these unigenes should be re-evaluated to see if they represent a single gene or a gene family.

Transcription factors

In *Arabidopsis*, transcription factors are among the first genes to increase in expression in response to cold (Fowler and Thomashow 2002; Seki et al 2002). I

found four differentially expressed unigenes associated with transcription, three increased and one decreased in abundance. Among these, one MYB-like transcription factor was up-regulated. In *Arabidopsis*, MYB genes respond to multiple hormone and stress treatments (Yanhui et al 2006). No differentially expressed unigenes were identified as CBF/DREB (C-repeat/dehydration-responsive element-binding) transcription factors.

Translation/ribosomal

Cold acclimation involves many cellular changes and the expression of many genes (Thomashow 1999; Fowler and Thomashow 2002; Seki et al 2001, 2002). Therefore, it is expected that differential expression of genes associated with translation and ribosomes would be detected. Six up-regulated unigenes were identified as ribosomal proteins or as proteins with roles in translation.

Others and unknowns

I identified 13 differentially expressed unigenes with similarity to proteins in other species, but whose association to cold acclimation is not clear. Further analyses of these unigenes are needed to determine if these transcript changes are associated with cold acclimation, bud set, dormancy initiation, or some other process.

I also identified 78 unknown unigenes. Fourteen of these are similar to genes encoding proteins of unknown function in other species, whereas 64 have no significant similarity to genes in other species (e-value $\leq e^{-6}$). This set of unigenes could represent novel cold acclimation genes, or may be "novel" because of lower sequence quality (i.e., length) compared to the other Douglas-fir unigenes. The

average length of the differentially expressed unigenes that had no matches to genes in other species was 403 bp, whereas the average length of the unigenes with matches was 566 bp.

The differentially expressed unigenes I identified during cold acclimation were found by comparing CB and YK cold susceptible tissues against CB and YK cold hardy tissues. Therefore, the results provide support for the hypothesis that the population differences in cold hardiness result from differences in the timing of gene expression. To fully address the hypothesis that populations differences also result from the expression of different genes, more replication is needed.

Heterologous hybridizations

The differentially expressed unigenes identified in the heterologous hybridizations to pine and spruce provide preliminary evidence that Douglas-fir gene expression can be studied using other conifer species (Table 8). There was, however, reduced replication for the pine and spruce comparisons because just 12 samples were hybridized per array platform and there were no biological replicates. Therefore, unigenes were only identified in the combined analyses of buds and stems. In Douglas-fir, the combined analyses of buds and stems identified more unigenes than either tissue alone. This demonstrates the importance of increased replication in microarray analyses. Additional hybridizations are needed to determine the extent that these species can be used to identify additional differentially expressed genes in Douglas-fir during cold acclimation.

Future work

The differentially expressed unigenes I identified will be incorporated into an association study of adaptive traits in Douglas-fir. Association studies are used to identify correlations between alleles and phenotypic differences in populations of unrelated individuals. Douglas-fir is an excellent model to use for association studies because it (1) is evolutionarily old with limited domestication, (2) is distributed in large, natural, out-crossing populations with high gene-flow via pollen (Slavov et al 2005), (3) has relatively high levels of nucleotide diversity ($\pi = 0.00655 \pm 0.00082$; Krutovsky and Neale 2005), (4) has limited linkage disequilibrium ($r^2 = 0.25$ to 0.10 within 2000 bp; Krutovsky and Neale 2005), and (5) has haploid megegametophytes for SNP genotyping.

The Douglas-fir association study is taking a candidate gene approach to studying cold adaptation. Candidate genes are genes identified as having an important functional role in the phenotypic trait of interest based on indirect evidence. The unigenes I identified as being differentially expressed between the cold susceptible and cold hardy seedlings are excellent "expression candidates" for cold acclimation. In addition, the unigenes that have similarity to genes encoding proteins related to cold acclimation provide even stronger evidence of their role in cold hardiness.

My unigenes and microarray information could also be used for mapping. The unigenes I identified as being differentially expressed could be mapped using EST polymorphisms. Wheeler et al (2005) mapped 29 potential cold tolerance ESTs from Douglas-fir, 17 of which were placed within 95% CI of spring cold hardiness QTL. These ESTs were selected using expression and functional evidence from other

species. My expression candidates provide additional ESTs that could be mapped. Many of those highlighted above would be strong candidates because they have significant differential expression in Douglas-fir cold acclimation and sequence similarity to genes associated with cold acclimation in other species. Expression candidate unigenes that map to regions of known cold adaptation QTL would provide even stronger evidence of their role in cold hardiness.

In addition, my microarray results could be used to map expression based quantitative trait loci (eQTL). Primers could be designed from the differentially expressed genes and used in real time reverse transcription PCR reactions with the Douglas-fir mapping population (Jermstad et al 1998). The transcription abundances could then be treated as quantitative traits for mapping (Schadt et al 2003). The mapping of eQTL could provide information about regulatory hotspots (i.e., chromosomal regions with linkages between eQTL and mapped markers) for cold acclimation.

The differentially expressed unigenes could also be used to generate an assay to assess the timing of cold acclimation. In general, trees are most vulnerable to frost damage in the late fall and early spring (Timmis et al 1994), therefore an assay to determine the timing of cold acclimation of seedlings would aid in Douglas-fir tree improvement efforts to deploy the most desirable trees while minimizing the risk of frost damage. The 15 differentially expressed unigenes that were identified in all three analyses are good candidates for developing a screening assay because (1) three of the 15 are dehydrins and known to be involved in cold acclimation, (2) their repeated detection provides increased evidence for their importance in cold acclimation, and (3)

they provide flexibility in the tissue tested. A similar diagnostic assay is being developed in Scots pine (Jossen et al 2006; van Wordragen et al submitted).

CONCLUSIONS

In this thesis I studied cold acclimation differences between two populations of Douglas-fir seedlings in their first year of growth. I developed three EST libraries for Douglas-fir, and combined them with ESTs from an actively growing library to create a unigene set representing the annual growth cycle. I used these unigenes to design oligonucleotides for a custom microarray. With this microarray I tested the hypotheses that population differences in cold hardiness are the result of (1) the expression of different genes in the different populations, or (2) differences in the timing of gene expression.

Cold hardiness was measured using needles, stems, and buds from two Douglas-fir populations—CB seedlings from a southern, low-elevation, coastal seed source and YK seedlings from a northern, high-elevation, inland seed source. The populations and tissues differed in response to cold temperatures. Bud set and cold acclimation occurred earlier in the YK seedlings. In the spring, the YK seedlings flushed earlier, resulting in greater cold damage of the YK buds compared to the CB buds.

In both populations, cold damage among needles, stems, and buds was highly correlated in the fall and winter, but only moderately correlated in the spring. Cold hardiness of the YK and CB seedlings was associated with the timing of bud set and bud flush.

I created three EST libraries to discover genes that are actively transcribed into messenger RNA during fall cold acclimation, vegetative bud set, winter dormancy, spring deacclimation, and vegetative bud flush. I found modest sequence similarities

between my Douglas-fir unigenes and known proteins from other species. This suggests that sequencing from Douglas-fir offers the potential to discover novel genes.

The unigenes developed from the EST libraries were used to create a custom oligonucleotide microarray for gene expression profiling. Additional oligonucleotides, designed from the large EST resources available in loblolly pine and white spruce, were also incorporated on the microarray. I compared the differential expression patterns between cold susceptible and cold hardy buds and stems from my two Douglas-fir populations.

I identified 343 unique differentially expressed unigenes between the cold susceptible and cold hardy seedlings (FDR adjusted p-value ≤ 0.01). These unigenes were similar to genes that encode dehydrins, heat shock proteins, pathogenesis-related proteins, and transcription factors; and genes related to ABA and water stress, GA, cold acclimation, dormancy, metabolism, and translation. The differential expression patterns of these genes were found in both populations, although different time points were used for the cold susceptible and hardy samples. This supports the hypothesis that population differences result from differences in the timing of gene expression. To fully address the hypothesis that population differences result from the expression of different genes, more replication is needed.

The heterologous hybridizations provide preliminary evidence that loblolly pine and white spruce ESTs are useful for identifying genes that are differently expressed in Douglas-fir during cold acclimation. More replication is needed to determine the extent to which these species can identify additional differentially expressed genes in Douglas-fir during cold acclimation.

The differentially expressed cold acclimation genes identified in my study will be incorporated into a larger association study of adaptation traits in coastal Douglas-fir. These studies will increase our understanding of the genetic variation in cold adaptation among populations of Douglas-fir, provide information for conserving genetic variation, and provide breeders with specific alleles to use in marker assisted selection in tree improvement.

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APPENDICES

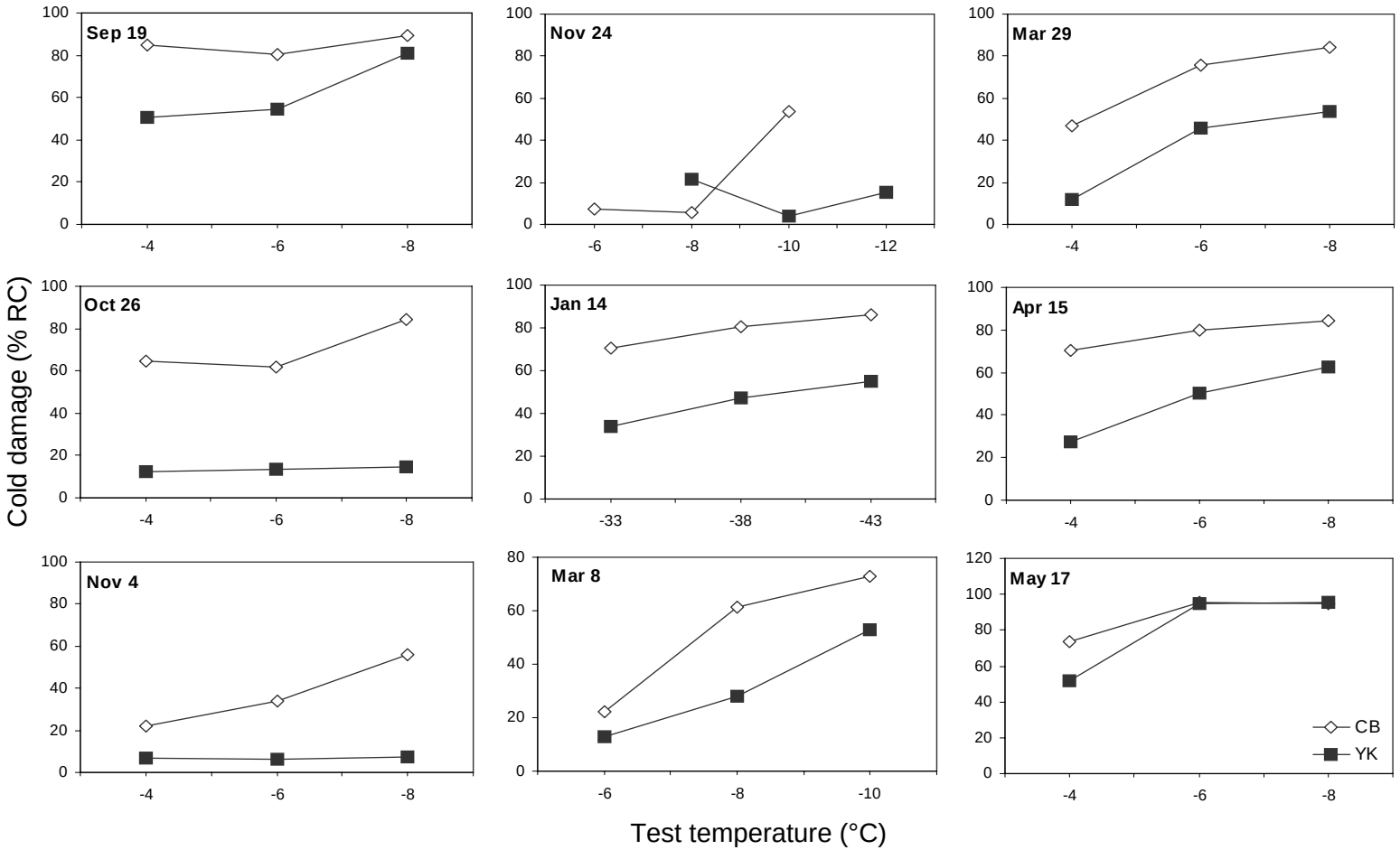

Figure A1. Relative conductivity of electrolyte leakage cold damage to needles, based on artificial freezing tests. Each data point is the average damage score of eight Coos Bay (CB; —◇—) or eight Yakima (YK; —■—) seedlings for each test temperature.

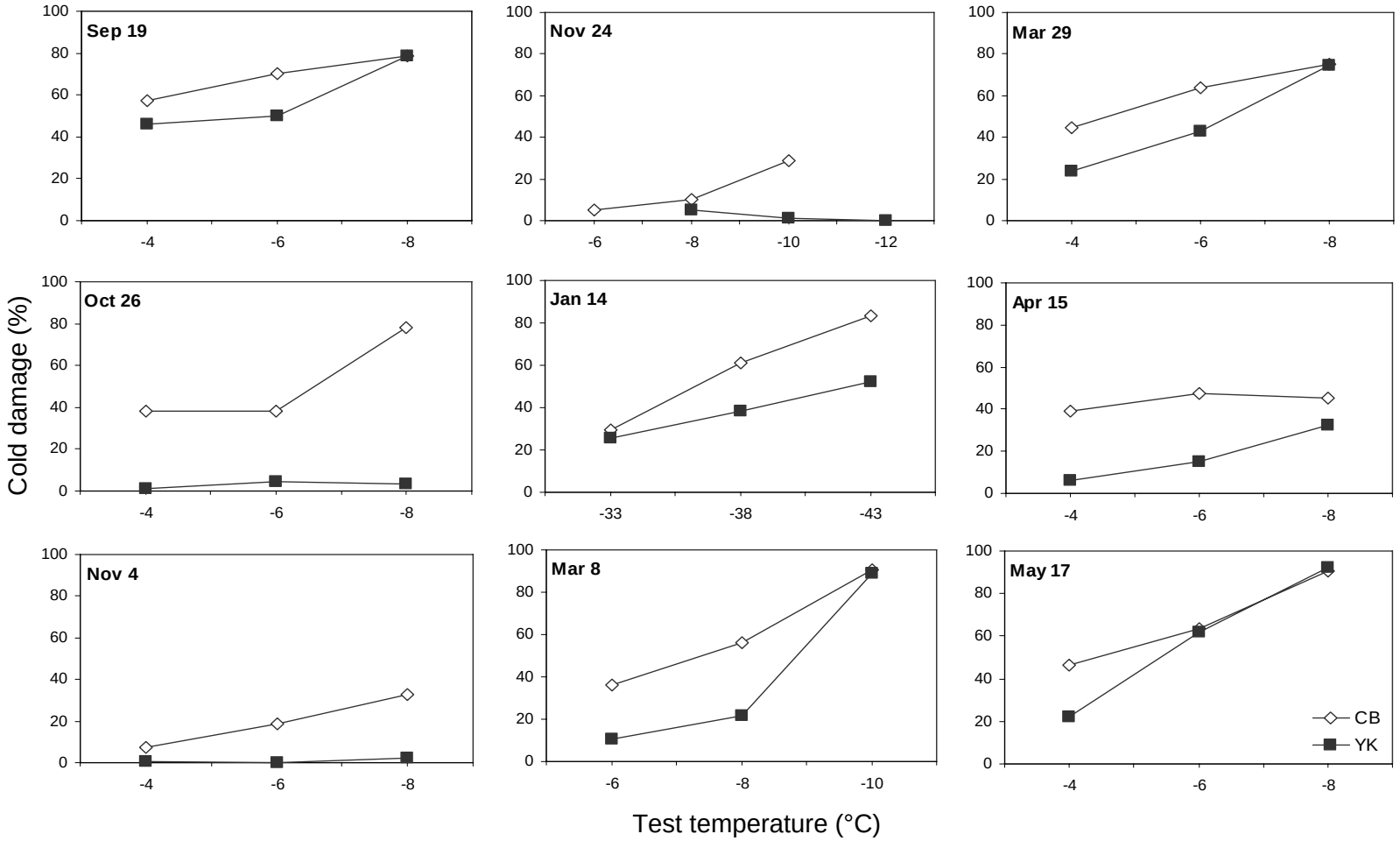

Figure A2. Visual assessment of cold damage to needles, based on artificial freezing tests. Each data point is the average damage score of eight Coos Bay (CB; ◇) or eight Yakima (YK; ■) seedlings for each test temperature.

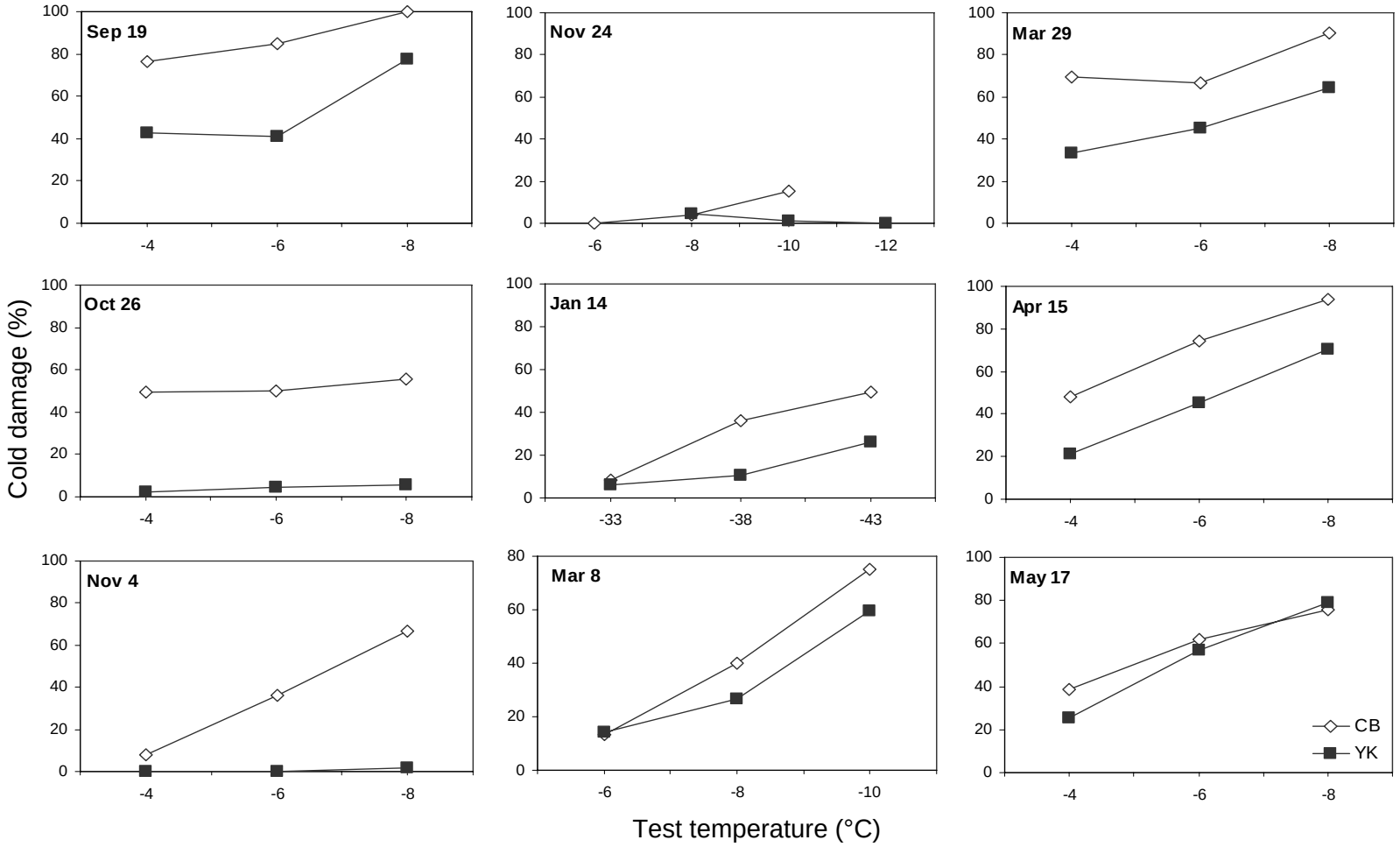

Figure A3. Visual assessment of cold damage to stems, based on artificial freezing tests. Each data point is the average damage score of eight Coos Bay (CB; —◇—) or eight Yakima (YK; —■—) seedlings for each test temperature.

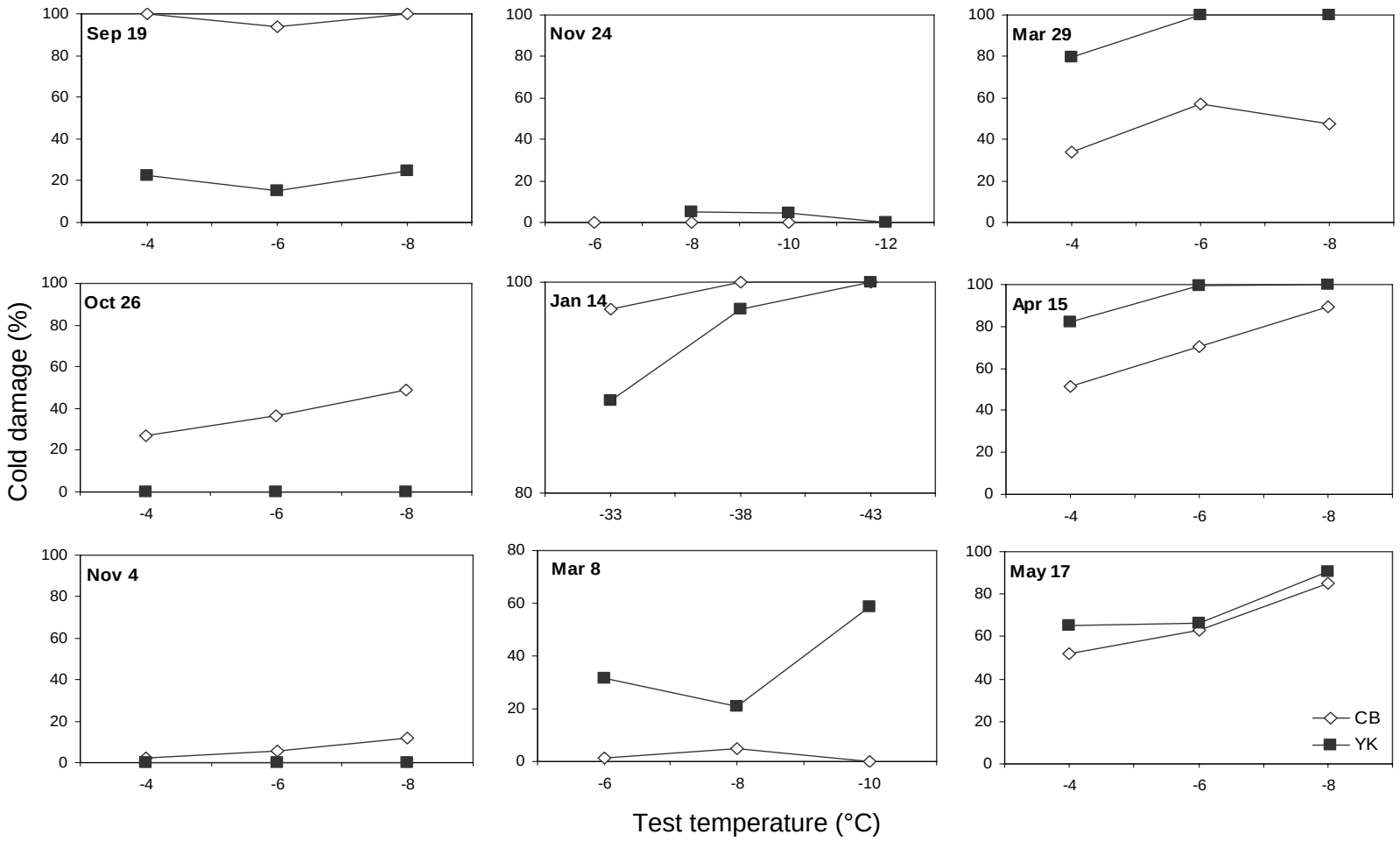

Figure A4. Visual assessment of cold damage to buds, based on artificial freezing tests. Each data point is the average damage score of eight Coos Bay (CB; —◇—) or eight Yakima (YK; —■—) seedlings for each test temperature.

Table A1. Regression lines for Douglas-fir seedling cold damage measured using artificial freeze tests. Statistical tests indicate whether the intercepts and slopes are significantly different from zero, and whether the predicted damage is significantly different between the Coos Bay (CB) and Yakima (YK) populations.

Population	Date[†]	N	Mean (%)	Intercept		Slope		Predicted percent damage (SE)	P-value (CB vs. YK)
				Estimate (SE)	P-value	Estimate (SE)	P-value		
Needles (relative conductivity)									
CB	19 Sep	12	85.0	78.0 (7.0)	<0.001	-1.2 (1.1)*	0.313	86.1 (2.1)	<0.001
YK	19 Sep	12	62.0	16.9 (7.0)	0.025	-7.5 (1.1)*	<0.001	69.5 (2.1)	
CB	26 Oct	12	70.2	40.3 (6.6)	<0.001	-5.0 (1.1)*	0.000	75.2 (2.0)	<0.001
YK	26 Oct	12	13.5	9.7 (6.6)	0.155	-0.6 (1.1)*	0.557	14.2 (2.0)	
CB	4 Nov	12	37.2	-13.6 (5.7)	0.027	-8.5 (0.9)*	<0.001	45.6 (1.8)	<0.001
YK	4 Nov	12	6.9	5.5 (5.7)	0.344	-0.2 (0.9)*	0.822	7.1 (1.8)	
CB	24 Nov	12	22.1	-70.8 (17.4)	0.001	-11.6 (2.1)*	<0.001	10.5 (4.1)	0.381
YK	24 Nov	12	13.4	28.8 (21.6)	0.198	1.5 (2.1)*	0.478	18.0 (7.3)	
CB	14 Jan	12	79.1	19.7 (8.5)	0.031	-1.6 (0.2)	<0.001	74.4 (1.1)	<0.001
YK	14 Jan	12	45.3	-35.2 (8.5)	0.001	-2.1 (0.2)	<0.001	38.9 (1.1)	
CB	8 Mar	12	52.1	-49.0 (18.4)	<0.001	-12.6 (1.3)	<0.001	39.4 (2.6)	<0.001
YK	8 Mar	12	31.4	-47.6 (18.4)	0.001	-9.9 (1.5)	<0.001	20.3 (2.6)	
CB	29 Mar	12	68.9	12.4 (10.5)	0.251	-9.4 (1.7)	<0.001	78.4 (3.2)	<0.001
YK	29 Mar	12	37.1	-25.2 (10.5)	0.026	-10.4 (1.7)	<0.001	47.5 (3.2)	
CB	15 Apr	12	78.3	57.3 (5.2)	<0.001	-3.5 (0.8)*	0.001	81.8 (1.6)	<0.001
YK	15 Apr	12	46.7	-6.7 (5.2)	0.214	-8.9 (0.8)*	<0.001	55.6 (1.6)	
CB	17 May	12	87.9	55.5 (28.8)	0.068	-5.4 (4.6)	0.256	93.4 (8.9)	0.886
YK	17 May	12	80.7	15.7 (28.8)	0.592	-10.8 (4.6)	0.030	91.5 (8.9)	

Table A1 cont. Regression lines for Douglas-fir seedling cold damage measured using artificial freeze tests.

Population	Date[†]	N	Mean (%)	Intercept		Slope		Predicted percent damage (SE)	P-value (CB vs. YK)
				Estimate (SE)	P-value	Estimate (SE)	P-value		
Needles (visual assessment)									
CB	19 Sep	24	68.8	36.9 (13.3)	0.008	-5.3 (2.1)	0.017	74.1 (4.1)	0.197
YK	19 Sep	24	58.3	9.6 (13.3)	0.476	-8.1 (2.1)	0.001	66.5 (4.1)	
CB	26 Oct	24	51.5	-8.5 (14.8)	0.566	-10.0 (2.4)*	0.000	61.5 (4.5)	<0.001
YK	26 Oct	24	2.9	0.1 (14.8)	0.994	-0.5 (2.4)*	0.844	3.4 (4.5)	
CB	4 Nov	24	19.6	-17.9 (7.8)	0.026	-6.3 (1.3)*	<0.001	25.8 (2.4)	<0.001
YK	4 Nov	24	1.0	-1.8 (7.8)	0.822	-0.5 (1.3)*	0.710	1.5 (2.4)	
CB	24 Nov	24	14.6	-32.9 (11.8)	0.008	-5.9 (1.5)*	0.000	8.6 (2.8)	0.622
YK	24 Nov	24	2.1	14.6 (14.7)	0.325	1.3 (1.5)*	0.392	5.8 (4.9)	
CB	14 Jan	24	57.9	-146.3 (30.4)	<0.001	-5.4 (0.8)*	<0.001	41.8 (4.0)	0.058
YK	14 Jan	24	38.8	-63.4 (30.4)	0.043	-2.7 (0.8)*	0.002	30.7 (4.0)	
CB	8 Mar	24	61.0	-47.7 (20.8)	0.026	-13.6 (2.5)	<0.001	47.4 (4.9)	<0.001
YK	8 Mar	24	40.4	-115.8 (20.8)	<0.001	-19.5 (2.5)	<0.001	20.9 (4.9)	
CB	29 Mar	24	61.0	15.1 (15.7)	0.340	-7.7 (2.5)	0.004	68.7 (4.8)	0.196
YK	29 Mar	24	47.1	-28.9 (15.7)	0.072	-12.7 (2.5)	<0.001	59.7 (4.8)	
CB	15 Apr	24	44.0	35.5 (12.7)	0.007	-1.4 (2.0)	0.493	45.4 (3.9)	<0.001
YK	15 Apr	24	17.9	-21.5 (12.7)	0.097	-6.6 (2.0)	0.002	24.5 (3.9)	
CB	17 May	24	66.9	0.3 (10.6)	0.977	-11.1 (1.7)*	<0.001	78.0 (3.3)	0.737
YK	17 May	24	58.8	-47.2 (10.6)	<0.001	-17.7 (1.7)*	<0.001	76.4 (3.3)	

Table A1 cont. Regression lines for Douglas-fir seedling cold damage measured using artificial freeze tests.

Population	Date[†]	N	Mean (%)	Intercept		Slope		Predicted percent damage (SE)	P-value (CB vs. YK)
				Estimate (SE)	P-value	Estimate (SE)	P-value		
Stems (visual assessment)									
CB	19 Sep	24	87.1	51.5 (13.9)	0.001	-5.9 (2.2)	0.011	93.0 (4.3)	<0.001
YK	19 Sep	24	53.8	1.3 (13.9)	0.929	-8.8 (2.2)	0.000	62.5 (4.3)	
CB	26 Oct	24	51.7	42.3 (9.5)	<0.001	-1.6 (1.5)	0.314	53.2 (2.9)	<0.001
YK	26 Oct	24	4.2	-0.5 (9.5)	0.957	-0.8 (1.5)	0.613	4.9 (2.9)	
CB	4 Nov	24	37.1	-51.0 (10.9)	<0.001	-14.7 (1.8)*	<0.001	51.8 (3.4)	<0.001
YK	4 Nov	24	0.6	-2.2 (10.9)	0.842	-0.5 (1.8)*	0.791	1.1 (3.4)	
CB	24 Nov	24	6.3	-23.8 (9.2)	0.013	-3.8 (1.1)*	0.002	2.5 (2.2)	0.550
YK	24 Nov	24	1.9	12.8 (11.4)	0.268	1.1 (1.1)*	0.337	5.2 (3.8)	
CB	14 Jan	24	31.3	-125.5 (37.0)	0.002	-4.1 (1.0)	0.000	18.9 (4.9)	0.137
YK	14 Jan	24	14.4	-61.6 (37.0)	0.103	-2.0 (1.0)	0.045	8.4 (4.9)	
CB	8 Mar	24	42.7	-81.0 (22.0)	0.001	-15.5 (2.7)	<0.001	27.2 (5.2)	0.501
YK	8 Mar	24	33.5	-56.5 (22.0)	0.014	-11.3 (2.7)	0.000	22.3 (5.2)	
CB	29 Mar	24	75.6	43.8 (12.5)	0.001	-5.3 (2.0)	0.012	80.9 (3.9)	<0.001
YK	29 Mar	24	47.5	0.6 (12.5)	0.961	-7.8 (2.0)	0.000	55.3 (3.9)	
CB	15 Apr	24	72.1	3.7 (12.1)	0.765	-11.4 (2.0)	<0.001	83.5 (3.7)	<0.001
YK	15 Apr	24	45.6	-28.4 (12.1)	0.024	-12.3 (2.0)	<0.001	58.0 (3.7)	
CB	17 May	24	58.8	3.4 (8.7)	0.696	-9.2 (1.4)*	<0.001	68.0 (2.7)	0.806
YK	17 May	24	53.8	-25.9 (8.7)	0.005	-13.3 (1.4)*	<0.001	67.0 (2.7)	

Table A1 cont. Regression lines for Douglas-fir seedling cold damage measured using artificial freeze tests.

Population	Date[†]	N	Mean (%)	Intercept		Slope		Predicted percent damage (SE)	P-value (CB vs. YK)
				Estimate (SE)	P-value	Estimate (SE)	P-value		
Buds (visual assessment)									
CB	19 Sep	15	98.0	99.2 (10.2)	<0.001	0.2 (1.7)	0.905	97.7 (3.5)	<0.001
YK	19 Sep	24	20.8	17.1 (8.3)	0.047	-0.6 (1.3)	0.643	21.5 (2.6)	
CB	26 Oct	24	37.3	4.5 (10.0)	0.655	-5.5 (1.6)*	0.001	42.8 (3.1)	<0.001
YK	26 Oct	24	0	0 (10.0)	1.000	0 (1.6)*	1.000	0.0 (3.1)	
CB	4 Nov	24	6.7	-7.4 (7.2)	0.307	-2.3 (1.2)	0.048	9.0 (2.2)	0.006
YK	4 Nov	24	0	0 (7.2)	1.000	0 (1.2)	1.000	0.0 (2.2)	
CB	24 Nov	24	0	0 (5.2)	1.000	0 (0.6)	1.000	0.0 (1.2)	0.008
YK	24 Nov	24	3.1	15.6 (6.5)	0.020	1.3 (0.6)	0.056	6.9 (2.2)	
CB	14 Jan	24	99.2	89.7 (11.0)	<0.001	-0.3 (0.3)*	0.388	98.4 (1.5)	0.003
YK	14 Jan	24	95.4	52.7 (11.0)	<0.001	-1.1 (0.3)*	0.000	92.0 (1.5)	
CB	8 Mar	24	2.1	4.6 (27.7)	0.870	0.3 (3.4)	0.927	2.4 (6.5)	0.006
YK	8 Mar	21	36.9	-17.4 (29.7)	0.561	-6.8 (3.6)	0.069	30.1 (7.0)	
CB	29 Mar	24	46.2	25.5 (20.6)	0.221	-3.4 (3.3)	0.304	49.6 (6.3)	<0.001
YK	29 Mar	23	92.9	62.2 (21.0)	0.005	-5.2 (3.4)	0.136	98.6 (6.7)	
CB	15 Apr	23	72.2	13.6 (19.4)	0.488	-9.5 (3.1)	0.004	80.0 (5.7)	0.028
YK	15 Apr	24	94.0	66.6 (18.5)	0.001	-4.5 (3.0)	0.135	98.3 (5.7)	
CB	17 May	24	66.7	17.0 (9.6)	0.084	-8.3 (1.5)	<0.001	74.9 (3.0)	0.202
YK	17 May	24	74.0	35.5 (9.6)	0.001	-6.4 (1.5)	0.000	80.4 (3.0)	

[†] Test temperatures varied by date (see Table 1).

* Populations have different slopes (p-value ≤ 0.05; see Table A2).

Table A2. Tests for homogeneity of slopes between the Coos Bay seedlings and Yakima seedlings, when damage was regressed on artificial freezing test temperature.

Date	Test[†]	Tissue	P-value	Slopes[‡]
19 Sep 04	RC	Needles	<0.001	D
26 Oct 04	RC	Needles	0.009	D
4 Nov 04	RC	Needles	<0.001	D
24 Nov 04	RC	Needles	<0.001	D
14 Jan 05	RC	Needles	0.092	ND
8 Mar 05	RC	Needles	0.690	ND
29 Mar 05	RC	Needles	0.179	ND
15 Apr 05	RC	Needles	<0.001	D
17 May 05	RC	Needles	0.417	ND
19 Sep 04	VA	Needles	0.359	ND
26 Oct 04	VA	Needles	0.007	D
4 Nov 04	VA	Needles	0.002	D
24 Nov 04	VA	Needles	0.001	D
14 Jan 05	VA	Needles	0.021	D
8 Mar 05	VA	Needles	0.106	ND
29 Mar 05	VA	Needles	0.167	ND
15 Apr 05	VA	Needles	0.080	ND
17 May 05	VA	Needles	0.009	D
19 Sep 04	VA	Stems	0.379	ND
26 Oct 04	VA	Stems	0.720	ND
4 Nov 04	VA	Stems	<0.001	D
24 Nov 04	VA	Stems	0.004	D
14 Jan 05	VA	Stems	0.128	ND
8 Mar 05	VA	Stems	0.274	ND
29 Mar 05	VA	Stems	0.386	ND
15 Apr 05	VA	Stems	0.736	ND
17 May 05	VA	Stems	0.047	D
19 Sep 04	VA	Buds	0.705	ND
26 Oct 04	VA	Buds	0.020	D
4 Nov 04	VA	Buds	0.157	ND
24 Nov 04	VA	Buds	0.172	ND
14 Jan 05	VA	Buds	0.037	D
8 Mar 05	VA	Buds	0.161	ND
29 Mar 05	VA	Buds	0.714	ND
15 Apr 05	VA	Buds	0.254	ND
17 May 05	VA	Buds	0.395	ND

[†] Damage measured by relative conductivity (RC) or visual assessment (VA).

[‡] Slopes are different (D) or not different (ND) between the Coos Bay and Yakima seed sources at $p \leq 0.05$.

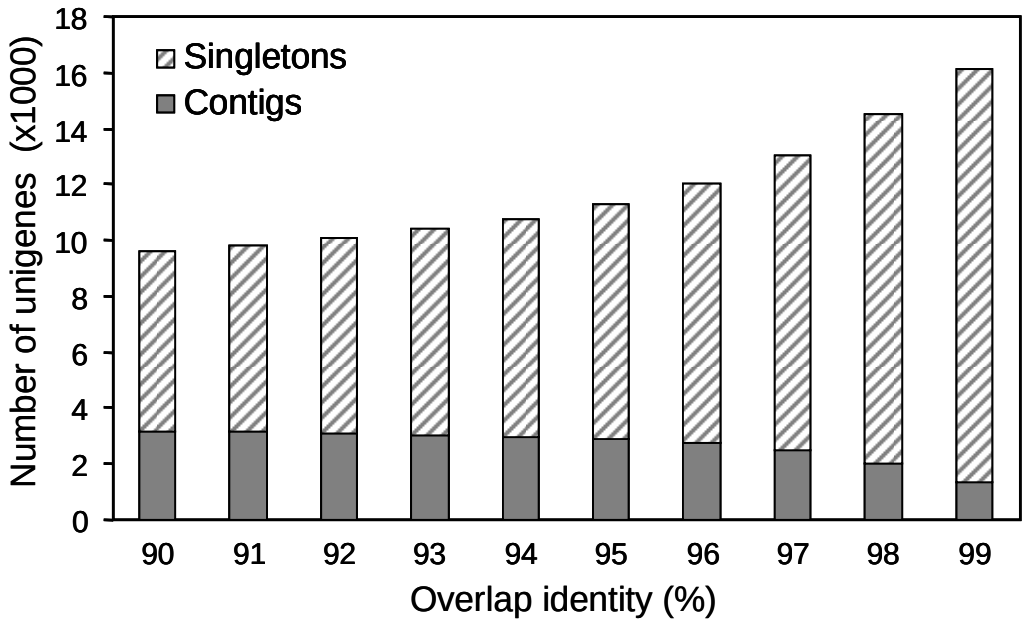

Figure A5. CAP3 unigene sets of Douglas-fir ESTs from four seedling libraries. Each unigene set is composed of contigs (i.e., consensus sequences formed from two or more overlapping ESTs) and singletons (i.e., individual ESTs). The contig set generated with an overlap identity of 94% was used to design 60-mer oligonucleotide probes for oligonucleotide microarray analysis.

Appendix A. NimbleGen criteria for oligonucleotide selection.

Currently we select longer oligos 25-70mers based on the composite scores of the 24mer probes that make up the longer oligo. For example, a 36mer is selected on the 13 overlapping 24mer probes.

Each 24mer probe is checked for uniqueness in two ways. The first is a simple frequency count. We count the exact number of times a 24mer probe appears in the genome of interest. The second check is what we call a careful uniqueness test. Each 24mer is compared to the target genome and given a pass/fail score for uniqueness. The criterion is that each 24mer must be at least 3 mismatches away from any other 24mer in the target genome. The comparison is not a simple 3 mismatches, however. Since we know that mismatches at the end of a 24mer are less important than mismatches in the center, we place a vector of weights onto the length of the 24mer, giving full weight to mismatches in the core 8 bp (12 bp for large eukaryotic genomes) and less weight to mismatches at the end. The weight vector is trapezoidal in shape:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1	1	2	2	2	4	4	4	4	4	4	4	4	4	4	4	4	2	2	2	1	1	1

To pass the uniqueness threshold, the sum of the mismatch weights must be greater than 10. So 3 mismatches between positions 7 and 18 would be considered unique. Six mismatches in positions 1 to 6 would not meet the uniqueness criterion (1+1+1+2+2+2 = 9). Mismatch oligos are also evaluated (standard mismatches occur at positions 6 and 12 for 24mers).

The long oligo is also subjected to a set of heuristics based on base pair composition. We attempt to avoid oligos that have long runs (>5) of homopolymers or that are too GC rich or AT rich. We also calculate a self-annealing score, comparing the oligo to its reverse complement and eliminate oligos that are more than ~60% self-complementary. All of these rules are combined into a single pass/fail score.

To select long oligos, we combine the scores from these various measure and calculate a rank score. When choosing multiple oligos from a given region, we select the first oligo, and then recalculate the rank score, adding a positional weight based on other selected probes. Once a probe has been selected in a given region, its neighbor's scores are decreased so that the tendency is to pick evenly spaced probes.

The selection criteria for a probe (in the design file) will contain a value like:

rank: 01;uniq: 11;freq: 13;nimble: 1

For this example, 36mers were being selected, and thus there are 13 24mer probes in the final 36mer.

The rank indicates a rank order for the set of probes selected for that region. For a set of 10 probes, a rank of 1 would indicate the best probe and a rank of 10 would indicate the worst probe in the set.

The uniq score indicates how many of the probes were carefully unique (as described above). Higher scores are better. A score of 13 would be the best you could achieve for a 36mer. A zero would indicate that none of the probes were carefully unique.

The freq score indicates the sum of the frequency counts for the 24mer probes. Lower scores are better. While you could achieve a score of 0 if you were comparing to a genome other than the one the probe was derived from, the minimum score is normally equal to the number of 24mer probes. For the case of a 36mer, the best score would be a 13.

The nimble score will be a 0 or 1, indicating whether or not the long oligo passed our "NimbleCheck" base pair composition/self-complementary rules. One equals pass, zero equals fail.

Table A3. Differentially expressed Douglas-fir unigenes in buds.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-105-A02	57.67	0	0	-3.92	-0.52	1.55	0.18	2.56	2.82
73-6-148-E01	70.34	0	0	-3.89	-0.72	1.06	-0.03	2.80	3.02
rc_WP_MD_053_A06	61.97	0	0	-1.46	-1.34	0.15	-1.33	1.94	2.27
73-6-144-E04	44.38	3.4E-07	4.1E-04	1.09	1.44	-0.35	0.45	-0.66	-0.94
Contig191	51.59	3.4E-07	4.1E-04	-0.50	-0.30	1.62	-1.19	1.65	1.98
Contig776	51.38	3.4E-07	4.1E-04	-2.47	-1.10	0.18	-0.50	2.70	2.93
Contig1393	49.70	3.4E-07	4.1E-04	-1.90	-0.67	1.02	-1.26	1.52	0.98
rc_WP_MD_042_B06	36.66	1.9E-06	2.0E-03	-1.70	-1.25	0.05	-0.74	0.64	2.61
rc_73-6-257-G09	35.37	2.4E-06	2.0E-03	0.80	-1.00	1.75	-1.14	0.87	0.35
rc_WP2_CDB_028_E03	35.28	2.4E-06	2.0E-03	-3.45	-0.16	1.37	-0.36	2.03	2.18
rc_WP_MD_021_A12	34.27	2.9E-06	2.2E-03	-1.45	-0.69	1.16	-1.44	1.56	1.66
73-6-200-H10	33.68	3.6E-06	2.5E-03	-1.24	-0.64	1.23	-0.58	1.20	0.69
rc_WP_MD_010_C03	33.37	4.1E-06	2.6E-03	-2.32	-0.35	0.88	-0.60	1.78	2.31
73-6-107-G01	31.64	5.4E-06	3.3E-03	0.11	0.76	-0.66	0.92	-0.57	-0.58
73-6-222-B07	29.86	8.2E-06	4.1E-03	-1.35	-0.29	0.96	-1.01	1.41	1.76
Contig1168	29.59	8.3E-06	4.1E-03	0.98	1.79	1.01	0.49	-1.52	-1.52
Contig1609	29.94	8.2E-06	4.1E-03	-1.82	-0.66	0.92	-0.92	1.39	2.20
73-6-210-D05	28.33	1.0E-05	4.5E-03	0.25	1.00	-0.74	0.65	-0.35	-0.65
Contig340	28.69	9.7E-06	4.5E-03	-0.14	-0.35	0.88	-0.33	1.64	1.90
rc_WP_MD_023_D09	27.15	1.3E-05	5.6E-03	-2.33	-1.09	0.00	-0.84	1.71	2.77
73-6-301-H03	26.45	1.5E-05	5.9E-03	-0.32	0.39	-0.95	0.47	-0.65	-0.24
Contig312	26.58	1.5E-05	5.9E-03	-0.96	-0.62	1.37	-1.02	0.78	0.78
Contig1714	25.82	1.8E-05	6.6E-03	0.55	-0.40	1.04	-1.22	0.76	0.45
rc_WP2_CDB_045_A06	24.94	2.3E-05	8.2E-03	-0.85	-0.43	0.39	-0.42	0.99	0.96
rc_WP2_CDB_012_C08	24.71	2.4E-05	8.2E-03	-2.75	-1.13	0.61	0.45	1.91	2.00
Contig1263	23.89	3.0E-05	9.6E-03	0.93	-0.21	1.16	-1.19	0.55	0.27

Table A4. Differentially expressed Douglas-fir unigenes in stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-136-F10	59.09	0	0	0.05	-0.95	1.02	-0.26	1.74	1.41
73-6-210-F02	55.45	0	0	3.42	2.69	1.97	0.92	-3.08	-3.03
Contig146	83.91	0	0	3.29	2.88	2.09	1.29	-2.95	-3.45
Contig385	77.59	0	0	-1.83	-0.84	0.36	-0.49	2.08	1.69
rc_WP_MD_023_D09	95.81	0	0	-1.90	-1.94	-0.07	-1.10	3.07	2.98
rc_WP_MD_053_A06	77.95	0	0	-2.11	-2.46	-0.01	-0.81	3.51	2.73
73-6-250-G04	45.07	5.5E-07	6.5E-04	1.72	2.76	1.45	1.93	-2.17	-1.01
Contig2869	44.30	9.1E-07	9.5E-04	-2.92	-1.05	0.53	0.27	2.20	2.74
Contig1868	41.20	1.3E-06	1.2E-03	-0.21	-1.31	0.59	-0.56	1.40	1.17
73-6-222-B07	37.29	2.5E-06	1.6E-03	-0.91	-1.57	0.20	-0.61	2.03	1.61
73-6-251-H10	35.83	2.7E-06	1.6E-03	1.64	1.87	0.89	0.73	-2.45	-1.80
Contig1301	37.40	2.5E-06	1.6E-03	-0.44	-0.74	0.58	-0.32	1.30	0.96
Contig1393	36.49	2.5E-06	1.6E-03	-1.63	-1.19	-0.11	-0.74	2.34	1.29
Contig2467	38.03	2.2E-06	1.6E-03	-2.61	-1.08	0.29	-0.79	2.42	3.51
Contig331	34.90	3.3E-06	1.7E-03	-0.48	-0.88	0.86	0.16	1.16	1.51
Contig719	35.41	3.1E-06	1.7E-03	-1.00	0.16	0.97	-0.57	2.23	1.69
rc_WP_MD_010_C03	33.99	3.8E-06	1.9E-03	-1.97	-1.23	0.34	0.04	2.49	2.23
rc_WP_MD_021_A12	33.25	4.9E-06	2.3E-03	-1.47	-1.48	1.00	-0.29	2.29	1.00
73-6-105-A02	29.58	8.9E-06	2.5E-03	-2.12	-0.50	-0.30	-0.24	2.61	2.38
73-6-261-E02	30.55	8.4E-06	2.5E-03	-0.24	-1.01	0.77	-0.53	0.64	0.60
Contig191	31.67	6.2E-06	2.5E-03	-0.91	-1.46	0.83	0.10	2.41	2.31
rc_73-6-132-D07	29.44	9.1E-06	2.5E-03	-0.63	-1.03	0.52	-0.69	1.34	0.55
Contig1168	29.53	8.9E-06	2.5E-03	1.31	1.62	1.84	1.14	-2.25	-1.94
Contig147	30.35	8.7E-06	2.5E-03	-0.03	-0.71	0.60	-0.70	1.39	0.41
Contig1549	30.05	8.9E-06	2.5E-03	-1.15	-1.05	0.49	-0.07	1.88	1.24
Contig1664	31.10	7.1E-06	2.5E-03	-0.93	-0.99	-0.51	-0.58	1.50	1.34

Table A4 cont. Differentially expressed Douglas-fir unigenes in stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
Contig196	29.52	8.9E-06	2.5E-03	0.21	-1.12	0.71	-0.21	1.64	0.62
Contig2086	31.14	7.1E-06	2.5E-03	0.41	-0.76	0.91	-0.33	1.75	1.05
Contig265	31.87	6.2E-06	2.5E-03	2.35	1.95	0.87	1.16	-1.97	-1.38
rc_WP2_CDB_047_D01	30.12	8.9E-06	2.5E-03	-1.76	-1.51	-0.17	-0.75	2.71	1.37
rc_73-6-220-G07	29.27	9.5E-06	2.6E-03	-0.48	-0.78	0.48	-0.23	1.07	0.78
Contig2134	27.94	1.2E-05	3.1E-03	-0.60	-1.01	0.45	-0.68	1.37	0.43
rc_WP_CD_047_G11	27.80	1.2E-05	3.1E-03	-1.84	-1.48	-1.94	-0.32	3.48	2.71
73-6-153-E03	26.82	1.4E-05	3.2E-03	-1.90	-0.92	-0.71	-0.46	2.25	3.35
Contig371	26.81	1.4E-05	3.2E-03	-0.93	-0.53	0.36	0.15	1.79	1.60
Contig1266	26.67	1.4E-05	3.2E-03	-2.12	-0.90	0.27	-0.24	1.76	2.60
rc_WP2_CDB_007_D10	26.64	1.4E-05	3.2E-03	-3.69	-0.74	0.89	1.08	2.92	3.29
rc_WP_CD_014_H04	26.46	1.5E-05	3.2E-03	-1.43	-1.03	0.35	-0.80	1.73	1.00
rc_WP_CD_045_G07	25.70	1.7E-05	3.6E-03	1.84	1.57	1.58	1.03	-2.06	-2.29
Contig1019	25.44	1.8E-05	3.7E-03	0.06	-1.25	0.77	-0.17	2.04	0.91
rc_WP_CD_042_D11	25.32	1.9E-05	3.8E-03	-1.13	-0.62	-0.06	-0.72	2.00	0.28
73-6-267-H03	24.80	2.1E-05	3.8E-03	0.37	1.67	-0.24	0.17	-1.19	-0.21
73-6-279-H07	24.80	2.1E-05	3.8E-03	-0.21	-1.51	-0.14	-0.61	1.54	0.77
rc_WP_CD_038_E01	24.87	2.1E-05	3.8E-03	-1.27	-0.90	0.53	-0.38	2.01	1.55
Contig1109	24.58	2.2E-05	3.8E-03	-1.14	-0.80	0.70	-0.50	1.46	1.16
Contig1609	25.16	2.0E-05	3.8E-03	-2.32	-0.69	0.32	-0.13	2.28	1.87
Contig1714	24.93	2.0E-05	3.8E-03	-0.60	-1.66	0.11	-0.16	2.19	1.10
rc_WP2_CDB_014_H07	24.58	2.2E-05	3.8E-03	0.65	1.02	0.00	0.39	-1.09	-0.87
Contig2167	24.35	2.3E-05	4.0E-03	-0.40	-0.58	0.25	-0.36	1.85	1.05
rc_Contig1675_2	24.04	2.5E-05	4.3E-03	-0.97	-0.64	0.78	-0.35	1.47	1.51
rc_WP2_CDB_045_A06	23.72	2.9E-05	4.8E-03	-2.04	-0.86	0.22	0.27	2.13	1.10
rc_WP2_CDB_028_E03	23.53	3.1E-05	5.0E-03	-3.30	0.09	0.87	-0.93	2.69	2.24

Table A4 cont. Differentially expressed Douglas-fir unigenes in stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-282-A02	23.17	3.4E-05	5.1E-03	-1.45	-0.05	1.31	0.68	1.64	1.88
Contig410	23.37	3.2E-05	5.1E-03	1.27	1.70	0.38	1.35	-1.65	-0.12
rc_73-6-163-B11	23.17	3.4E-05	5.1E-03	-1.32	-0.04	1.13	0.10	1.88	2.07
rc_WP2_CDB_043_C12	23.33	3.3E-05	5.1E-03	-0.14	-0.98	0.52	-0.69	1.52	0.13
73-6-291-D09	22.83	3.8E-05	5.7E-03	-0.68	0.01	0.60	-1.04	1.12	1.67
Contig2486	22.59	4.1E-05	5.8E-03	2.48	1.30	0.56	0.77	-1.83	-1.31
Contig2632	22.56	4.1E-05	5.8E-03	-0.83	-0.44	0.58	0.14	1.46	1.39
Contig937	22.63	4.1E-05	5.8E-03	-0.56	-0.48	-0.38	-1.10	1.26	1.47
73-6-222-B11	22.36	4.4E-05	5.9E-03	-0.23	-1.00	0.46	-0.02	1.19	0.67
rc_73-6-224-G11	22.35	4.4E-05	5.9E-03	-0.42	-0.71	0.52	-0.08	1.47	0.69
Contig90	22.46	4.3E-05	5.9E-03	-0.68	-1.18	0.43	-0.24	1.46	0.58
73-6-155-E12	21.96	4.9E-05	6.1E-03	2.75	1.73	0.66	1.05	-1.48	-1.65
Contig1065	22.03	4.9E-05	6.1E-03	-0.96	-0.86	0.73	-0.13	1.92	1.38
Contig1764	22.12	4.7E-05	6.1E-03	-0.50	-0.51	0.58	-0.36	1.37	1.03
Contig2318	22.02	4.9E-05	6.1E-03	-0.19	-0.59	0.54	-1.27	1.17	0.68
Contig2841	21.97	4.9E-05	6.1E-03	1.85	1.37	1.42	1.01	-1.54	-1.73
rc_73-6-110-F10	21.54	5.6E-05	6.7E-03	-0.76	-1.75	0.47	0.19	2.36	0.60
rc_73-6-157-H09	21.48	5.7E-05	6.7E-03	0.25	-0.79	0.23	-0.35	1.31	-0.06
73-6-202-A11	21.47	5.7E-05	6.7E-03	0.44	1.05	-0.48	0.14	-0.99	-0.62
rc_WP_MD_034_A12	21.50	5.7E-05	6.7E-03	-0.28	-0.77	0.67	-0.45	1.29	0.57
73-6-163-D06	21.42	5.9E-05	6.7E-03	-0.58	-1.51	0.27	-0.22	1.80	0.51
Contig2200	21.33	6.0E-05	6.7E-03	-1.04	-1.07	0.12	-0.47	1.76	0.75
rc_WP2_CDB_045_B04	21.27	6.1E-05	6.7E-03	-0.89	-0.93	-0.35	-1.02	2.60	0.05
rc_WP_CD_013_E10	21.35	6.0E-05	6.7E-03	-0.73	-1.65	-0.33	-0.16	1.76	1.21
73-6-118-B04	20.94	6.7E-05	7.1E-03	1.36	1.30	0.37	0.10	-1.56	-0.50
73-6-148-E01	20.87	6.8E-05	7.1E-03	-2.59	-0.94	0.03	-0.18	1.95	2.80

Table A4 cont. Differentially expressed Douglas-fir unigenes in stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-211-E09	21.00	6.6E-05	7.1E-03	0.16	-1.67	1.12	-0.04	1.39	1.41
73-6-269-F05	20.84	6.9E-05	7.1E-03	0.09	-1.52	0.20	0.01	2.04	0.19
rc_WP_CD_043_E08	20.87	6.8E-05	7.1E-03	1.77	1.06	0.09	-0.03	-1.55	-1.28
rc_WP_CD_051_G11	20.76	7.0E-05	7.2E-03	-0.57	-0.98	0.08	-0.56	1.75	0.19
Contig907	20.66	7.2E-05	7.3E-03	-0.12	-0.56	0.64	0.27	1.14	1.56
rc_WP2_CDB_006_G07	20.55	7.5E-05	7.5E-03	1.52	0.08	-0.43	0.89	-1.24	-0.60
Contig1516	20.52	7.6E-05	7.5E-03	-0.23	-0.48	0.80	-0.57	0.60	0.74
rc_WP_MD_032_E10	20.50	7.7E-05	7.5E-03	-0.77	-1.04	0.10	-0.59	0.78	1.18
Contig1302	20.30	8.0E-05	7.6E-03	-0.61	-1.25	0.72	0.08	0.75	1.12
rc_WP_MD_023_G06	20.29	8.0E-05	7.6E-03	-0.59	-0.92	0.42	-0.05	1.08	0.79
Contig1082	20.04	8.6E-05	8.1E-03	-3.85	-0.15	1.45	1.37	2.55	2.21
73-6-112-C04	19.85	9.1E-05	8.1E-03	0.24	0.88	-0.35	0.08	-0.90	-0.39
73-6-200-H10	19.94	8.9E-05	8.1E-03	-0.95	-1.03	-0.25	-0.53	1.51	1.12
rc_73-6-123-A10	19.94	8.9E-05	8.1E-03	0.08	-1.21	0.46	-0.36	1.98	0.59
rc_WP_MD_020_H01	19.86	9.1E-05	8.1E-03	-0.80	-1.07	-0.82	-0.90	2.32	1.24
Contig2733	19.98	8.8E-05	8.1E-03	-0.48	-0.79	0.54	0.02	1.16	0.75
73-6-131-H08	19.77	9.3E-05	8.2E-03	-0.50	-0.62	-0.17	-0.48	1.95	1.02
Contig2299	19.59	9.7E-05	8.4E-03	-0.38	-0.79	0.50	-0.25	1.32	-0.06
73-6-202-E04	19.23	1.1E-04	9.3E-03	-0.93	-0.81	-0.09	-0.36	1.05	1.03
Contig2279	19.23	1.1E-04	9.3E-03	-0.62	-1.02	0.43	-0.47	0.85	0.53
Contig2523	19.10	1.1E-04	9.5E-03	0.50	1.08	-0.63	0.22	-1.03	-1.13
Contig827	19.06	1.1E-04	9.5E-03	1.34	1.28	0.62	0.43	-1.06	-1.08
Contig264	19.00	1.2E-04	9.6E-03	-0.03	-1.36	0.04	-0.15	0.63	0.29

Table A5. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
rc_WP_MD_053_A06	62.07	0	0	-1.78	-1.90	0.07	-1.07	2.73	2.50
Contig1393	56.76	0	0	-1.77	-0.93	0.45	-1.00	1.93	1.13
rc_WP_MD_010_C03	50.02	0	0	-2.14	-0.79	0.61	-0.28	2.13	2.27
Contig191	46.47	0	0	-0.70	-0.88	1.22	-0.54	2.03	2.14
rc_WP_MD_023_D09	45.29	0	0	-2.12	-1.51	-0.04	-0.97	2.39	2.87
rc_73-6-163-B11	43.81	0	0	-1.38	-0.10	0.94	-0.03	2.12	2.25
rc_WP_MD_021_A12	42.27	0	0	-1.46	-1.09	1.08	-0.87	1.92	1.33
Contig1609	40.90	0	0	-2.07	-0.67	0.62	-0.52	1.83	2.04
rc_WP2_CDB_014_H07	40.37	0	0	1.00	0.87	-0.05	0.38	-1.08	-0.92
rc_WP2_CDB_028_E03	39.39	0	0	-3.38	-0.03	1.12	-0.64	2.36	2.21
rc_WP2_CDB_007_D10	35.15	0	0	-3.87	-0.52	0.80	1.10	2.99	3.24
rc_WP2_CDB_006_B08	34.90	0	0	-3.32	0.15	1.24	1.06	2.52	2.53
rc_WP2_CDB_012_C08	34.00	0	0	-2.85	-1.22	0.40	0.64	2.12	2.18
Contig2869	33.77	0	0	-2.75	-0.87	0.27	0.18	2.00	2.83
73-6-222-B07	31.93	0	0	-1.13	-0.93	0.58	-0.81	1.72	1.69
Contig1266	31.85	0	0	-2.42	-1.06	-0.04	-0.06	1.99	2.71
Contig1168	31.41	0	0	1.14	1.71	1.43	0.82	-1.88	-1.73
73-6-153-E03	31.39	0	0	-1.93	-1.03	-0.58	-0.10	2.18	3.27
73-6-301-H03	30.35	2.3E-07	1.0E-04	-0.21	0.38	-0.68	0.16	-0.62	-0.34
73-6-282-A02	29.32	4.6E-07	2.0E-04	-1.40	0.19	1.09	0.44	1.64	1.97
73-6-136-F10	28.59	7.0E-07	2.3E-04	0.26	-0.29	1.34	-0.31	1.09	0.95
rc_WP_MD_030_F10	27.82	7.0E-07	2.3E-04	-0.65	-0.30	0.47	-0.53	1.11	0.38
rc_WP2_CDB_042_A09	27.80	7.0E-07	2.3E-04	-0.83	-0.77	-0.46	-0.78	0.78	1.65
rc_WP_MD_042_B06	27.39	7.0E-07	2.3E-04	-1.61	-1.17	-0.03	-0.44	0.67	2.41
Contig1409	27.27	7.0E-07	2.3E-04	0.66	-0.53	0.82	0.33	0.91	1.13

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
Contig146	27.21	7.0E-07	2.3E-04	3.27	2.35	1.30	1.10	-2.03	-2.79
Contig791	26.97	9.3E-07	2.9E-04	-0.72	0.13	0.65	0.35	1.75	1.90
Contig2467	26.89	1.2E-06	3.3E-04	-2.72	-0.64	-0.19	-0.80	2.47	3.34
Contig1569	26.88	1.2E-06	3.3E-04	0.11	-0.52	0.87	-0.80	1.08	0.13
Contig147	26.57	1.2E-06	3.3E-04	0.41	-0.30	0.86	-0.96	0.92	0.24
73-6-107-G01	26.07	1.9E-06	5.0E-04	0.47	0.83	-0.57	0.39	-0.84	-0.46
73-6-267-H09	25.17	3.0E-06	6.5E-04	-0.27	0.21	-0.60	0.57	-0.68	-0.06
rc_WP_CD_045_G07	24.88	3.0E-06	6.5E-04	1.84	1.44	0.97	0.97	-1.35	-2.14
rc_WP2_CDB_006_G07	24.82	3.0E-06	6.5E-04	1.74	0.09	-0.52	0.52	-1.08	-0.63
rc_WP2_CDB_045_A06	24.80	3.0E-06	6.5E-04	-1.44	-0.65	0.30	-0.07	1.56	1.03
73-6-216-D02	24.02	3.0E-06	6.5E-04	1.10	1.03	0.09	1.03	-0.69	-0.67
Contig1714	23.78	3.0E-06	6.5E-04	-0.02	-1.03	0.58	-0.69	1.47	0.78
rc_WP_MD_006_B08	23.73	3.0E-06	6.5E-04	-0.22	-0.47	0.44	-1.05	0.90	0.76
Contig1400	23.69	3.0E-06	6.5E-04	-1.66	0.16	1.13	0.66	1.67	1.39
Contig1608	23.26	3.5E-06	7.1E-04	-1.62	-0.62	-0.27	-0.06	1.99	1.79
Contig1019	23.26	3.5E-06	7.1E-04	0.27	-0.56	1.23	-0.55	1.48	0.71
rc_WP_CD_012_B10	23.14	4.0E-06	7.7E-04	1.09	0.09	0.81	-0.78	0.70	0.51
Contig1868	23.14	4.0E-06	7.7E-04	-0.07	-0.65	0.93	-0.82	1.06	0.99
73-6-105-A02	22.73	4.4E-06	8.2E-04	-3.02	-0.51	0.62	-0.03	2.58	2.60
rc_WP_MD_054_D06	22.68	4.4E-06	8.2E-04	-1.23	-0.94	-0.84	-0.44	1.50	1.55
Contig2086	22.55	4.9E-06	8.7E-04	0.46	-0.13	1.30	-0.33	1.28	0.64
rc_73-6-157-H09	22.52	4.9E-06	8.7E-04	0.31	-0.46	0.51	-0.70	0.91	0.07
rc_WP2_CDB_032_H12	22.36	5.3E-06	9.3E-04	-1.96	-1.13	-0.18	-0.29	1.56	2.16
Contig694	22.32	5.6E-06	9.4E-04	0.04	-0.71	0.58	-0.61	0.82	1.19
Contig2279	22.07	5.6E-06	9.4E-04	0.10	-0.80	0.48	-0.79	0.63	0.18
73-6-122-C11	21.81	6.5E-06	1.1E-03	0.07	0.34	-0.48	0.67	-0.71	-0.18

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-200-H10	21.76	6.5E-06	1.1E-03	-1.10	-0.84	0.49	-0.56	1.35	0.90
Contig1301	21.39	7.7E-06	1.2E-03	0.06	-0.33	0.46	-0.60	0.98	0.76
Contig1263	21.35	7.9E-06	1.2E-03	0.34	-0.26	0.78	-0.80	0.87	0.40
rc_WP_MD_055_H07	21.24	8.1E-06	1.2E-03	0.13	-0.82	0.73	-0.32	1.10	0.45
rc_WP_CD_010_D07	21.08	8.1E-06	1.2E-03	-1.56	-1.12	-0.72	-0.77	1.97	2.61
Contig95	20.99	8.8E-06	1.3E-03	0.44	-0.71	0.55	-0.54	1.00	0.48
73-6-148-E01	20.98	8.8E-06	1.3E-03	-3.24	-0.83	0.55	-0.11	2.38	2.91
Contig1113	20.92	9.1E-06	1.3E-03	-0.18	-0.05	0.44	-0.53	0.93	1.04
Contig926	20.90	9.3E-06	1.3E-03	0.72	-0.31	1.01	-0.24	0.93	0.41
rc_73-6-257-G09	20.63	1.1E-05	1.5E-03	0.38	-1.10	0.84	-0.54	1.49	0.37
Contig2289	20.62	1.1E-05	1.5E-03	0.37	-0.53	0.62	-0.62	0.95	0.71
73-6-251-H10	20.52	1.2E-05	1.6E-03	1.63	1.30	0.20	0.62	-1.85	-1.37
rc_WP2_CDB_003_H11	20.45	1.2E-05	1.6E-03	-0.15	-0.18	0.09	-0.28	0.27	0.58
Contig1333	20.18	1.5E-05	1.9E-03	0.06	-0.46	0.33	-0.35	0.68	0.52
73-6-127-F02	20.12	1.5E-05	1.9E-03	-0.30	0.13	-0.39	0.28	-0.52	-0.13
Contig340	20.03	1.5E-05	1.9E-03	-0.16	-0.18	0.71	0.07	1.41	1.55
rc_WP_CD_043_E08	19.92	1.6E-05	2.0E-03	2.06	0.67	-0.48	-0.03	-1.34	-1.04
73-6-156-B08	19.86	1.6E-05	2.0E-03	0.15	-0.03	0.95	-0.99	0.97	0.44
rc_WP2_CDB_035_B10	19.35	1.9E-05	2.3E-03	0.34	-0.23	0.60	-0.38	0.75	0.19
Contig196	19.30	1.9E-05	2.3E-03	0.31	-0.61	0.91	-0.27	1.33	0.56
Contig2863	19.21	2.0E-05	2.4E-03	-0.17	-0.19	0.86	-0.84	0.97	-0.25
Contig2889	19.16	2.1E-05	2.4E-03	-0.03	-0.34	0.22	-0.43	0.34	0.19
rc_WP_MD_037_B09	19.07	2.2E-05	2.4E-03	-1.40	-0.97	-0.92	-0.96	1.63	2.62
rc_WP_MD_016_C07	19.07	2.2E-05	2.4E-03	0.03	-0.54	0.78	-0.17	0.53	0.70
rc_WP2_CDB_016_D03	19.05	2.2E-05	2.4E-03	-1.52	0.03	0.59	-0.26	0.94	1.38
73-6-210-F02	19.04	2.2E-05	2.4E-03	3.45	2.04	1.10	0.74	-2.07	-2.35

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
rc_WP_CD_042_A12	19.01	2.3E-05	2.4E-03	-0.09	0.12	-0.32	0.50	-0.70	-0.31
rc_WP2_CDB_033_A11	18.97	2.3E-05	2.5E-03	-0.07	-0.73	0.38	-0.85	0.90	0.41
Contig1102	18.92	2.4E-05	2.5E-03	-0.24	0.04	-0.35	0.33	-0.72	-0.43
rc_73-6-224-G11	18.87	2.4E-05	2.5E-03	-0.12	-0.34	0.81	-0.33	1.05	0.63
rc_WP_CD_035_C10	18.81	2.5E-05	2.6E-03	-1.25	-0.82	-0.33	0.28	1.30	1.04
73-6-279-H07	18.75	2.6E-05	2.6E-03	-0.18	-0.88	0.33	-0.71	0.97	0.40
Contig1214	18.63	2.8E-05	2.8E-03	0.57	-0.47	0.96	-0.99	0.82	0.28
rc_73-6-132-D07	18.45	3.1E-05	3.1E-03	-0.27	-0.45	1.06	-0.92	0.86	0.03
rc_WP_CD_036_F03	18.36	3.3E-05	3.2E-03	-0.35	-0.50	0.55	-0.42	1.01	0.62
Contig385	18.35	3.3E-05	3.2E-03	-1.15	-0.60	0.29	-0.46	1.45	1.19
rc_WP_CD_013_E10	18.34	3.4E-05	3.2E-03	-0.45	-1.28	-0.26	-0.31	1.14	1.14
rc_73-6-110-F10	18.08	3.7E-05	3.5E-03	-0.38	-1.15	1.05	-0.41	1.71	0.56
Contig2489	17.94	4.0E-05	3.5E-03	0.19	-0.53	0.43	-0.56	0.52	0.48
Contig2	17.91	4.0E-05	3.5E-03	0.20	-0.85	0.78	-0.43	1.04	0.47
Contig1431	17.89	4.0E-05	3.5E-03	0.09	-0.82	0.75	-0.50	0.54	-0.25
rc_WP_CD_052_D02	17.88	4.0E-05	3.5E-03	-0.24	-0.70	-0.52	-0.60	1.13	-0.22
Contig315	17.87	4.1E-05	3.5E-03	0.10	0.37	-0.08	0.19	-0.14	0.00
73-6-110-C03	17.85	4.1E-05	3.5E-03	-0.25	0.52	-0.32	0.64	-0.45	-0.03
rc_WP2_CDB_043_C12	17.84	4.1E-05	3.5E-03	0.04	-0.54	1.06	-0.77	0.79	-0.01
rc_WP_MD_038_F04	17.83	4.1E-05	3.5E-03	0.39	-0.25	1.04	-1.14	0.67	0.35
Contig1167	17.83	4.1E-05	3.5E-03	-0.15	-0.13	0.44	-0.46	0.40	0.15
rc_73-6-234-E02	17.68	4.3E-05	3.7E-03	0.54	0.21	1.33	-0.50	0.89	0.30
Contig1562	17.62	4.4E-05	3.7E-03	0.04	-0.23	0.69	-0.25	0.83	0.74
rc_WP_CD_038_E01	17.61	4.5E-05	3.7E-03	-0.67	-0.31	0.86	-0.86	1.32	1.10
Contig719	17.59	4.5E-05	3.7E-03	-0.36	0.38	1.11	-0.32	1.59	1.31
73-6-269-F05	17.56	4.5E-05	3.7E-03	0.17	-0.88	0.73	-0.42	1.39	0.26

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
rc_73-6-123-A10	17.54	4.5E-05	3.7E-03	0.43	-0.76	1.04	-0.60	1.38	0.49
Contig1389	17.46	4.7E-05	3.8E-03	-0.52	-0.22	0.19	-0.22	0.98	1.14
rc_WP_MD_049_B10	17.39	4.9E-05	3.8E-03	0.48	-0.34	1.21	-0.27	1.40	0.85
Contig1121	17.38	4.9E-05	3.8E-03	0.12	-0.57	0.58	-0.53	0.51	-0.11
Contig2200	17.36	5.0E-05	3.8E-03	-0.67	-0.66	0.67	-0.65	1.03	0.50
rc_73-6-256-F01	17.36	5.0E-05	3.8E-03	-0.09	-0.92	0.58	-0.27	0.87	0.27
Contig1551	17.35	5.0E-05	3.8E-03	-0.08	-0.40	0.75	-0.62	0.83	0.34
rc_WP2_CDB_033_H09	17.31	5.0E-05	3.8E-03	0.18	-0.23	0.65	-0.77	0.63	0.19
73-6-211-E12	17.30	5.1E-05	3.8E-03	0.24	0.30	-0.65	0.68	-0.53	0.09
rc_WP_MD_036_D04	17.28	5.2E-05	3.8E-03	0.08	-0.60	0.59	-0.73	0.87	0.08
73-6-292-D01	17.22	5.2E-05	3.8E-03	0.14	0.60	-0.45	1.17	-0.55	-0.02
73-6-120-E09	17.20	5.2E-05	3.8E-03	-0.36	0.22	-0.31	0.75	-0.51	-0.01
Contig287	17.19	5.2E-05	3.8E-03	0.21	-0.33	0.68	0.00	0.92	0.80
73-6-211-E09	17.05	5.5E-05	3.9E-03	0.80	-0.84	1.16	-0.86	0.94	1.17
73-6-123-B08	17.04	5.5E-05	3.9E-03	-0.03	-0.48	0.16	-0.59	0.11	0.44
Contig1958	17.03	5.6E-05	3.9E-03	1.14	-0.31	0.94	-0.19	0.64	0.09
Contig2053	17.03	5.6E-05	3.9E-03	0.57	0.48	0.94	0.05	1.14	0.76
rc_73-6-242-E06	16.98	5.9E-05	4.1E-03	-0.89	0.02	0.83	0.08	1.78	1.74
Contig264	16.96	5.9E-05	4.1E-03	-0.14	-0.90	0.08	-0.25	0.49	0.20
Contig666	16.93	6.0E-05	4.1E-03	-0.06	-0.95	0.12	-0.52	1.09	1.18
rc_WP_MD_021_D04	16.87	6.2E-05	4.2E-03	-0.54	-0.55	-0.13	-0.54	0.97	0.66
rc_WP2_CDB_026_C12	16.78	6.4E-05	4.2E-03	-2.22	0.24	2.27	0.21	1.23	1.58
Contig2754	16.78	6.4E-05	4.2E-03	0.41	-0.33	0.49	-0.29	0.87	0.42
73-6-254-B04	16.76	6.4E-05	4.2E-03	0.10	0.32	-0.60	0.59	-0.46	0.00
Contig1540	16.75	6.4E-05	4.2E-03	-0.26	-0.56	0.58	-0.85	0.86	0.20
73-6-248-A07	16.68	6.6E-05	4.3E-03	1.29	1.36	-0.22	0.44	-0.81	-0.05

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
rc_WP_CD_004_A07	16.66	6.8E-05	4.4E-03	-0.32	0.54	-0.50	0.34	-0.63	-0.14
rc_WP_CD_050_D11	16.65	6.9E-05	4.4E-03	-1.46	-0.97	-0.69	-1.04	1.15	2.37
Contig1348	16.63	6.9E-05	4.4E-03	0.45	-0.53	0.89	-0.50	0.73	0.21
Contig265	16.61	7.0E-05	4.4E-03	2.03	1.44	0.05	1.37	-0.82	-0.87
73-6-150-B01	16.54	7.2E-05	4.4E-03	0.83	0.94	-0.50	1.01	-0.76	-0.07
rc_WP_MD_023_G06	16.53	7.2E-05	4.4E-03	-0.14	-0.51	0.36	-0.33	0.76	0.50
rc_WP2_CDB_013_A05	16.51	7.2E-05	4.4E-03	0.97	1.04	0.35	0.01	-1.30	-1.31
Contig1483	16.50	7.3E-05	4.4E-03	0.48	0.44	-0.52	0.35	-0.68	-0.39
Contig1090	16.46	7.4E-05	4.5E-03	-0.22	-0.55	0.18	-0.33	0.86	0.47
Contig1737	16.42	7.5E-05	4.5E-03	0.25	-0.20	0.73	-0.33	0.82	0.48
Contig1365	16.41	7.5E-05	4.5E-03	0.85	-0.01	-0.16	0.43	-0.61	-0.83
rc_WP_MD_018_A08	16.38	7.7E-05	4.5E-03	0.50	-0.53	0.43	-0.46	0.72	0.03
Contig2263	16.37	7.7E-05	4.5E-03	0.34	0.02	0.96	-1.18	0.81	-0.07
rc_73-6-224-F07	16.26	7.9E-05	4.7E-03	-0.45	0.36	-0.77	0.83	-0.46	-0.12
rc_WP2_CDB_047_F03	16.24	8.0E-05	4.7E-03	0.24	-0.47	0.75	0.04	0.76	0.37
rc_WP_MD_049_H09	16.23	8.0E-05	4.7E-03	-3.55	0.42	1.42	1.09	2.59	2.61
Contig176	16.20	8.2E-05	4.7E-03	0.34	-0.44	0.44	-0.29	0.63	0.64
Contig1549	16.15	8.3E-05	4.8E-03	-0.66	-0.50	0.79	-0.43	1.18	0.83
Contig1849	16.11	8.6E-05	4.9E-03	-0.07	-0.49	0.69	-0.49	0.48	0.56
73-6-106-A07	16.07	8.8E-05	4.9E-03	0.20	0.47	-0.43	0.87	-0.71	-0.12
Contig1298	16.06	8.9E-05	5.0E-03	0.22	0.40	-0.17	0.28	-0.47	-0.11
Contig1563	16.04	9.0E-05	5.0E-03	0.00	-0.27	0.64	-0.06	0.98	0.77
rc_WP2_CDB_001_D05	16.01	9.0E-05	5.0E-03	0.27	-0.53	0.79	-0.65	0.59	0.02
73-6-104-E11	15.98	9.3E-05	5.1E-03	0.12	0.49	-0.60	0.71	-0.64	0.22
Contig549	15.97	9.4E-05	5.1E-03	-0.04	-0.35	0.47	-0.58	0.69	0.21
Contig860	15.93	9.6E-05	5.1E-03	0.11	-0.53	0.45	-0.67	0.44	0.20

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
Contig1588	15.92	9.7E-05	5.1E-03	0.18	-0.42	0.56	-0.36	0.85	0.24
Contig2134	15.92	9.7E-05	5.1E-03	-0.06	-0.52	0.56	-0.87	0.77	0.23
73-6-130-B07	15.91	9.7E-05	5.1E-03	0.09	0.93	-0.29	1.30	-0.43	0.19
73-6-212-C05	15.90	9.7E-05	5.1E-03	-0.20	0.30	-0.55	0.23	-0.44	-0.11
rc_73-6-223-D12	15.89	9.8E-05	5.1E-03	0.04	-0.31	0.37	-0.67	0.58	0.13
73-6-267-H03	15.89	9.8E-05	5.1E-03	0.14	0.85	-0.46	0.70	-0.83	-0.02
rc_WP2_CDB_011_F10	15.82	1.0E-04	5.2E-03	-2.48	-0.16	0.86	0.34	1.76	1.81
rc_WP_MD_017_H06	15.82	1.0E-04	5.2E-03	-0.85	-0.36	-0.41	-0.69	1.05	-0.04
Contig869	15.82	1.0E-04	5.2E-03	-0.35	0.03	0.76	-0.49	0.61	0.42
rc_WP2_CDB_016_B07	15.77	1.0E-04	5.2E-03	0.10	0.22	-0.49	0.08	-0.79	-0.25
rc_WP2_CDB_038_H02	15.71	1.1E-04	5.4E-03	-0.10	-0.87	0.19	-0.52	0.63	0.29
Contig200	15.71	1.1E-04	5.4E-03	0.85	0.05	0.87	-0.04	1.14	0.87
73-6-107-C02	15.70	1.1E-04	5.4E-03	-1.53	-0.81	0.21	0.06	0.91	1.64
Contig1673	15.66	1.1E-04	5.4E-03	0.44	0.01	0.94	-0.59	0.58	-0.02
73-6-160-A05	15.61	1.1E-04	5.6E-03	-0.30	-0.63	0.21	-1.01	1.00	0.04
rc_WP_CD_020_A07	15.57	1.1E-04	5.6E-03	-0.25	0.33	-0.65	0.29	-0.59	-0.21
rc_WP_CD_025_F10	15.51	1.2E-04	5.8E-03	-0.56	0.00	-0.49	0.23	-0.34	-0.15
73-6-163-F04	15.49	1.2E-04	5.8E-03	0.48	-1.09	0.47	-0.23	1.04	0.72
73-6-134-A03	15.46	1.2E-04	5.9E-03	0.24	0.29	-0.34	0.86	-0.77	0.14
Contig1423	15.45	1.2E-04	5.9E-03	0.04	-0.42	0.83	-0.52	1.51	0.92
Contig1615	15.44	1.2E-04	5.9E-03	1.13	-0.06	0.49	-0.94	0.13	-0.39
Contig301	15.43	1.2E-04	5.9E-03	-0.03	-0.13	0.64	-0.54	0.54	-0.19
Contig2808	15.42	1.2E-04	5.9E-03	0.09	-0.34	0.79	-0.43	0.70	0.72
73-6-131-D06	15.41	1.3E-04	5.9E-03	0.15	-0.50	0.33	-0.67	0.60	-0.31
rc_73-6-148-A03	15.37	1.3E-04	6.0E-03	0.43	-0.41	0.80	-0.42	1.06	0.13
Contig4	15.32	1.3E-04	6.1E-03	0.10	-0.21	0.75	-0.19	0.92	0.93

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-254-D10	15.30	1.3E-04	6.1E-03	-0.04	-0.51	0.19	-0.49	0.30	-0.04
rc_WP_MD_044_D09	15.28	1.3E-04	6.2E-03	0.05	-0.18	0.30	-0.45	0.58	0.47
rc_WP2_CDB_003_F12	15.26	1.4E-04	6.2E-03	0.32	-0.11	-0.56	0.23	-0.62	-0.30
rc_WP_MD_034_A12	15.25	1.4E-04	6.2E-03	0.13	-0.31	1.00	-0.62	0.89	0.24
rc_WP_CD_003_D05	15.23	1.4E-04	6.2E-03	-0.19	0.66	-0.44	0.73	-0.66	0.12
Contig1721	15.22	1.4E-04	6.2E-03	-0.12	-0.79	0.17	-0.65	0.87	0.56
rc_WP2_CDB_001_G04	15.21	1.4E-04	6.2E-03	0.07	-0.59	0.45	-0.59	0.68	-0.04
rc_WP_CD_020_F01	15.20	1.4E-04	6.2E-03	-0.26	0.25	-0.55	0.27	-0.61	-0.29
Contig2733	15.19	1.4E-04	6.2E-03	-0.01	-0.27	0.61	-0.33	0.79	0.43
Contig582	15.17	1.4E-04	6.3E-03	0.15	-0.31	0.73	-0.50	0.95	0.18
rc_WP2_CDB_028_G02	15.13	1.4E-04	6.3E-03	-0.01	-0.55	0.01	-0.35	0.42	-0.19
rc_WP2_CDB_021_E12	15.11	1.5E-04	6.3E-03	0.08	-0.25	1.01	-0.62	0.39	0.32
73-6-125-E08	15.10	1.5E-04	6.3E-03	0.32	-0.25	0.56	-1.06	0.41	-0.02
rc_WP2_CDB_003_G09	15.09	1.5E-04	6.3E-03	-1.40	-0.82	-0.42	-0.65	1.30	1.22
rc_WP_MD_032_A05	15.07	1.5E-04	6.4E-03	-0.87	-0.60	0.38	-0.25	0.94	0.50
rc_WP_MD_013_H06	15.06	1.5E-04	6.4E-03	-0.12	-0.38	0.77	-0.79	0.84	0.19
Contig363	15.04	1.5E-04	6.4E-03	0.89	-0.87	0.54	-0.24	0.69	0.37
rc_WP_MD_038_D08	15.00	1.5E-04	6.5E-03	-0.34	-0.32	0.51	-0.86	0.37	0.19
Contig1900	15.00	1.5E-04	6.5E-03	-0.27	-0.75	0.15	-0.66	0.81	0.24
73-6-112-C04	15.00	1.5E-04	6.5E-03	-0.31	0.45	-0.62	0.67	-0.62	-0.16
Contig2782	14.93	1.6E-04	6.6E-03	0.16	-0.18	0.53	-0.42	0.82	0.33
rc_73-6-135-H06	14.92	1.6E-04	6.6E-03	-0.15	0.24	-0.43	0.50	-0.71	-0.04
73-6-148-A09	14.86	1.7E-04	6.8E-03	0.17	-0.35	0.40	-0.77	0.22	0.10
Contig2589	14.80	1.7E-04	7.0E-03	0.35	-0.33	0.78	0.24	1.34	1.06
rc_WP2_CDB_012_B06	14.78	1.7E-04	7.0E-03	0.59	0.43	-0.27	0.73	-0.49	-0.10
73-6-109-A07	14.78	1.7E-04	7.0E-03	0.54	0.86	-0.46	1.15	-0.75	-0.05

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
73-6-202-E04	14.75	1.7E-04	7.0E-03	-1.17	-0.25	0.13	-0.76	1.05	1.18
Contig2001	14.73	1.7E-04	7.0E-03	0.55	-0.33	0.55	-0.59	0.56	0.22
rc_WP_MD_033_E04	14.71	1.8E-04	7.0E-03	-0.09	-0.42	0.60	-0.62	0.54	0.20
Contig2299	14.71	1.8E-04	7.0E-03	-0.06	-0.32	0.60	-0.42	0.86	-0.22
Contig1535	14.70	1.8E-04	7.0E-03	-0.40	0.27	-0.55	0.66	-0.49	-0.08
rc_WP2_CDB_031_G03	14.69	1.8E-04	7.0E-03	-0.19	0.21	-0.51	0.42	-0.59	-0.50
rc_WP_MD_022_H07	14.66	1.8E-04	7.0E-03	0.77	0.20	1.06	-0.88	0.52	0.21
Contig90	14.64	1.8E-04	7.1E-03	-0.14	-0.58	0.49	-0.56	0.90	0.29
Contig1771	14.61	1.8E-04	7.1E-03	-0.02	0.33	-0.55	0.61	-0.30	-0.16
Contig2167	14.60	1.8E-04	7.1E-03	-0.12	-0.23	0.35	-0.44	1.18	0.87
73-6-103-H12	14.59	1.8E-04	7.1E-03	-0.05	0.47	-0.53	0.49	-0.40	-0.17
rc_WP_CD_012_F07	14.55	1.9E-04	7.1E-03	-0.29	0.36	-0.56	0.50	-0.36	-0.10
rc_WP_CD_050_G08	14.55	1.9E-04	7.1E-03	-0.05	-0.45	0.29	-0.23	0.55	0.49
Contig11	14.55	1.9E-04	7.1E-03	0.46	-0.13	0.93	-1.01	0.50	0.12
Contig2901	14.54	1.9E-04	7.1E-03	0.16	-0.54	0.38	-0.68	0.60	0.54
Contig2111	14.53	1.9E-04	7.1E-03	0.01	-0.37	0.40	-0.26	0.29	0.55
Contig2318	14.51	1.9E-04	7.1E-03	0.39	-0.45	1.08	-1.15	0.32	0.37
Contig996	14.51	1.9E-04	7.1E-03	-0.15	0.74	1.38	0.46	1.39	1.39
73-6-295-B02	14.51	1.9E-04	7.1E-03	-0.01	0.80	-0.39	0.85	-0.59	-0.10
Contig2039	14.49	1.9E-04	7.2E-03	-1.09	-0.64	0.13	-0.26	1.01	0.88
Contig2817	14.48	1.9E-04	7.2E-03	0.04	-0.22	0.75	-0.46	0.96	0.52
73-6-202-D08	14.43	2.0E-04	7.3E-03	-0.41	0.25	-0.39	0.27	-0.42	-0.11
Contig1676	14.40	2.0E-04	7.4E-03	0.54	-0.24	0.93	-0.62	0.67	-0.03
rc_WP_CD_050_H10	14.39	2.0E-04	7.4E-03	0.34	-0.67	0.69	-0.60	0.45	-0.13
Contig75	14.38	2.1E-04	7.4E-03	0.31	-0.28	0.44	0.60	1.29	0.99
rc_WP_CD_021_F03	14.37	2.1E-04	7.4E-03	0.20	0.11	0.53	-0.60	0.40	0.50

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
Contig2738	14.37	2.1E-04	7.4E-03	0.06	-0.34	0.62	-0.52	0.87	0.84
73-6-291-D09	14.37	2.1E-04	7.4E-03	-0.28	0.18	0.33	-0.77	0.83	1.40
rc_WP_CD_042_D11	14.33	2.1E-04	7.5E-03	-0.90	-0.41	0.48	-0.76	1.05	0.05
rc_WP_MD_029_B06	14.30	2.2E-04	7.6E-03	0.17	-0.29	0.98	-0.37	0.69	0.35
rc_WP2_CDB_041_G12	14.26	2.2E-04	7.7E-03	0.33	0.64	0.20	0.40	-0.69	-0.54
rc_WP_MD_028_G05	14.26	2.2E-04	7.7E-03	-0.15	-0.33	0.26	-0.38	0.43	0.34
Contig1486	14.24	2.2E-04	7.7E-03	1.21	0.96	0.49	0.87	-0.66	-0.79
Contig1076	14.21	2.3E-04	7.8E-03	-0.38	-0.49	0.48	-0.71	0.34	-0.41
73-6-141-A11	14.20	2.3E-04	7.8E-03	0.75	1.16	-0.31	1.26	-0.81	0.12
rc_WP_MD_009_G06	14.19	2.3E-04	7.8E-03	0.15	-0.45	0.56	-0.26	0.63	0.30
Contig1238	14.19	2.3E-04	7.8E-03	0.79	0.10	0.85	-0.49	0.64	0.14
Contig1764	14.18	2.3E-04	7.8E-03	0.01	-0.28	0.56	-0.23	1.19	0.71
rc_WP_MD_034_G04	14.15	2.3E-04	7.8E-03	-0.14	-0.02	0.20	-0.19	0.52	0.35
73-6-145-E03	14.15	2.3E-04	7.8E-03	-0.46	0.13	-0.55	0.33	-0.45	-0.18
rc_WP_CD_008_D06	14.15	2.3E-04	7.8E-03	0.41	-0.33	0.50	-0.30	0.74	0.22
rc_WP_CD_014_H04	14.13	2.3E-04	7.8E-03	-1.04	-0.51	0.33	-0.65	0.99	0.62
Contig1831	14.13	2.3E-04	7.8E-03	0.45	-0.04	0.41	-0.46	0.68	0.60
73-6-286-H02	14.07	2.4E-04	8.0E-03	-0.24	0.36	-0.46	0.59	-0.47	-0.02
73-6-157-F04	14.05	2.4E-04	8.0E-03	-0.35	0.14	-0.43	0.31	-0.57	-0.25
Contig2264	14.05	2.4E-04	8.0E-03	-0.19	-0.57	0.38	-0.36	0.82	0.08
rc_WP2_CDB_047_D01	14.02	2.4E-04	8.0E-03	-0.97	-1.03	0.48	-0.83	1.57	0.58
rc_WP2_CDB_043_C09	14.01	2.4E-04	8.0E-03	-0.23	0.01	-0.48	0.29	-0.70	-0.21
Contig2864	13.99	2.5E-04	8.1E-03	-0.81	-0.66	-0.41	-0.60	1.51	0.78
rc_73-6-149-F04	13.98	2.5E-04	8.1E-03	0.41	-0.42	0.46	-0.33	0.72	0.99
rc_WP_MD_033_G04	13.97	2.5E-04	8.1E-03	-0.09	-0.32	0.21	-0.34	0.63	0.51
Contig369	13.95	2.5E-04	8.1E-03	0.73	-0.21	0.62	-0.69	0.54	0.14

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
rc_WP_MD_023_A02	13.91	2.6E-04	8.3E-03	0.34	0.33	-0.44	0.60	-0.32	-0.04
rc_WP2_CDB_024_C06	13.86	2.6E-04	8.4E-03	0.32	0.56	-0.14	0.59	-0.29	0.08
rc_WP_MD_029_G03	13.84	2.6E-04	8.4E-03	0.44	-0.29	0.48	-0.30	0.07	-0.04
73-6-149-A06	13.84	2.6E-04	8.4E-03	-0.29	0.63	-0.19	0.63	-0.60	-0.29
rc_WP2_CDB_019_E11	13.84	2.6E-04	8.4E-03	-0.42	0.35	-0.47	-0.10	-0.58	-0.24
Contig100	13.83	2.7E-04	8.4E-03	-0.34	-0.42	0.34	-0.20	0.95	0.77
Contig1624	13.81	2.7E-04	8.5E-03	0.15	-0.71	0.26	-0.17	0.90	0.73
Contig2049	13.78	2.7E-04	8.5E-03	0.23	0.16	0.78	-0.36	0.62	0.34
rc_WP_CD_042_E01	13.74	2.8E-04	8.7E-03	0.20	0.46	-0.34	0.38	-0.61	-0.25
rc_73-6-218-E01	13.71	2.8E-04	8.7E-03	-0.06	-0.23	0.82	-1.05	1.03	0.22
rc_WP2_CDB_014_A10	13.71	2.8E-04	8.7E-03	-0.17	-0.48	0.54	-0.49	0.45	0.28
rc_WP2_CDB_005_B04	13.70	2.8E-04	8.7E-03	0.42	0.06	1.11	-0.85	0.94	0.44
73-6-274-B09	13.69	2.8E-04	8.7E-03	0.15	-0.25	0.53	-0.36	0.62	0.26
rc_WP2_CDB_022_G02	13.67	2.8E-04	8.7E-03	0.13	-0.26	0.49	-0.54	0.60	0.44
rc_WP_MD_031_E05	13.64	2.9E-04	8.8E-03	0.48	0.31	0.84	-0.26	0.91	0.45
73-6-147-D10	13.64	2.9E-04	8.8E-03	0.42	0.63	-0.40	0.96	-0.61	0.13
rc_WP_MD_050_G05	13.64	2.9E-04	8.8E-03	0.17	-0.20	0.51	-0.27	0.66	0.29
Contig2637	13.64	2.9E-04	8.8E-03	0.33	-0.41	0.68	-0.40	0.80	0.30
rc_WP2_CDB_043_C10	13.63	2.9E-04	8.8E-03	0.34	-0.34	0.48	-0.23	0.62	0.40
Contig1354	13.62	2.9E-04	8.8E-03	0.28	-0.62	0.49	-0.50	0.58	-0.06
Contig331	13.60	3.0E-04	8.9E-03	-0.17	-0.29	0.87	0.09	0.93	1.12
rc_WP_MD_049_E03	13.57	3.0E-04	9.0E-03	-0.31	-0.06	-0.62	0.38	-0.68	-0.31
73-6-146-H11	13.57	3.0E-04	9.0E-03	-0.43	0.15	-0.60	0.26	-0.50	-0.11
rc_WP_MD_029_D12	13.53	3.1E-04	9.1E-03	-0.16	-0.55	0.34	-0.77	0.54	-0.12
Contig2464	13.52	3.1E-04	9.2E-03	0.04	-0.75	0.54	-0.44	0.50	-0.26
73-6-141-F06	13.50	3.1E-04	9.2E-03	-0.08	-0.25	0.68	-0.49	1.44	0.10

Table A5 cont. Differentially expressed Douglas-fir unigenes in combined analyses of buds and stems.

	F_s								
Unigene	obs *F*	Pvalperm	FDR adj. Pvalperm	CB Sep	CB Oct	CB Nov	YK Sep	YK Oct	YK Nov
Contig793	13.48	3.1E-04	9.2E-03	0.17	0.13	0.73	-1.13	0.82	0.08
Contig2153	13.44	3.2E-04	9.4E-03	-0.45	0.27	-0.50	0.24	-0.49	-0.17
rc_73-6-297-D02	13.38	3.3E-04	9.6E-03	-0.02	-0.12	0.20	-0.34	0.44	0.12
73-6-276-C06	13.36	3.4E-04	9.7E-03	0.04	0.71	-0.49	0.38	-0.60	-0.24
73-6-163-D06	13.35	3.4E-04	9.7E-03	-0.21	-0.84	0.75	-0.65	1.01	0.23
rc_WP_CD_002_C03	13.35	3.4E-04	9.7E-03	0.22	-0.17	0.88	-0.52	0.65	0.59
Contig713	13.34	3.4E-04	9.7E-03	-0.14	-0.79	0.64	-0.50	0.55	0.00
rc_WP2_CDB_042_F08	13.34	3.4E-04	9.7E-03	-0.96	-0.59	0.12	-0.61	0.53	0.30
Contig471	13.31	3.4E-04	9.8E-03	-0.02	0.21	-0.50	0.87	-0.45	0.05
rc_WP_CD_046_D01	13.30	3.4E-04	9.8E-03	0.07	-0.43	0.82	-0.69	1.20	0.10
73-6-202-A11	13.29	3.5E-04	9.8E-03	-0.07	0.57	-0.68	0.45	-0.60	-0.29
Contig344	13.27	3.5E-04	9.9E-03	0.40	-0.70	0.83	-0.61	1.13	0.26
Contig1867	13.26	3.5E-04	9.9E-03	-0.39	-0.64	0.24	-0.52	0.79	0.77
rc_WP_MD_038_F02	13.26	3.5E-04	9.9E-03	-0.01	-0.12	0.45	-0.69	0.65	0.11
rc_WP2_CDB_040_D12	13.25	3.5E-04	9.9E-03	0.15	0.41	-0.40	0.56	-0.69	-0.27
rc_WP2_CDB_047_B02	13.24	3.6E-04	9.9E-03	-0.08	-0.66	0.29	-0.28	0.83	0.51
rc_WP2_CDB_001_E12	13.23	3.6E-04	9.9E-03	0.15	0.11	-0.45	0.10	-0.65	-0.10
rc_WP_CD_032_G10	13.22	3.6E-04	9.9E-03	-0.01	-0.48	0.65	-0.77	0.77	-0.08
rc_WP_CD_021_C09	13.22	3.6E-04	9.9E-03	-0.87	-0.72	0.11	-0.96	0.84	1.43
rc_73-6-246-F04	13.21	3.6E-04	9.9E-03	0.11	-0.59	1.14	-0.71	0.71	0.25
rc_WP_MD_051_F03	13.20	3.6E-04	9.9E-03	-0.49	-0.37	0.25	-0.58	0.55	0.10
rc_WP_CD_009_E02	13.19	3.6E-04	9.9E-03	-0.38	-0.61	0.21	-0.53	0.89	0.05
73-6-146-A11	13.19	3.6E-04	9.9E-03	0.05	0.41	-0.38	0.38	-0.45	-0.19
Contig1663	13.18	3.6E-04	9.9E-03	0.36	-0.24	0.42	-0.43	0.42	0.01
73-6-141-E02	13.17	3.7E-04	9.9E-03	0.77	0.47	0.86	0.11	1.10	0.55