Molecular Breeding (2005) 15: 145–156
DOI 10.1007/s11032-004-3978-9

Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. IV. Cold-hardiness QTL verification and candidate gene mapping

Nicholas C. Wheeler[1,*], Kathleen D. Jermstad[2], Konstantin Krutovsky[2], Sally N. Aitken[3], Glenn T. Howe[4], Jodie Krakowski[3] and David B. Neale[2,5]
*[1]Molecular Tree Breeding Services, LLC, 21040 Flumerfelt Rd. SE, Centralia, WA 98531, USA; [2]Institute of Forest Genetics, Pacific Southwest Research Station, USDA Forest Service, Davis, CA 95616, USA; [3]Department of Forest Sciences, Faculty of Forestry, University of British Columbia, 2424 Main Mall, Vancouver, BC, Canada V6T 1Z4; [4]Department of Forest Science, Oregon State University, Corvallis, OR 97331-5752, USA; [5]Department of Environmental Horticulture, University of California, Davis, CA 95616, USA; *Author for correspondence (e-mail: nicholascollins@earthlink.net; phone: +1-360-278-3092)*

Received 27 January 2004; accepted in revised form 29 September 2004

Key words: Association Genetics, Candidate Gene, Cold-hardiness, Douglas-fir, MAS, QTL, Verification

Abstract

Quantitative trait locus (QTL) analyses are used by geneticists to characterize the genetic architecture of quantitative traits, provide a foundation for marker-aided-selection (MAS), and provide a framework for positional selection of candidate genes. The most useful QTL for breeding applications are those that have been verified in time, space, and/or genetic background. In this study, spring cold-hardiness of Douglas-fir foliar tissues was evaluated in two clonally replicated (n = 170 and 383 clones) full-sib cohorts derived from the same parental cross in two different years (made 5 years apart). The cohorts were established in widely separated forest test sites and tissues were artificially freeze tested using different cold injury assessment methods. Four of six unique QTL detected for spring cold-hardiness in needles of Cohort 1 were tentatively verified in the second cohort. Four additional QTL were detected in Cohort 2, two on linkage groups (LGs) not previously represented in the smaller cohort. In total, 10 unique QTL were identified across both cohorts. Seventeen of twenty-nine putative cold-hardiness candidate genes (Douglas-fir ESTs) placed on the Douglas-fir linkage map locate within the 95% confidence intervals of spring needle cold-hardiness QTL from the two cohorts and thus represent priority targets for initiating association mapping in Douglas-fir.

Introduction

Most traits of interest to forest tree breeders are considered to be quantitatively inherited (i.e. controlled by the collective action of many genes with small effects resulting in continuous or quantitative variation). Traditional analyses of quantitatively inherited traits rely on phenotypic means, variances and co-variances to estimate genetic parameters like variance components, heritabilities, and genetic correlations (Falconer and MacKay 1996). While useful, standard quantitative genetic methods, as typically applied in forest tree breeding, reveal little about the actual genes contributing to phenotypic variance. In the late 1980s, developments in DNA marker technology

and genome mapping resulted in the ability to construct genetic linkage maps and characterize and map individual Mendelian factors controlling quantitative traits in plants (Paterson 1998). A considerable body of literature on quantitative trait locus (QTL) mapping in forest trees has since evolved (Sewell and Neale 2000).

The molecular dissection of quantitative traits is conceptually straight-forward: statistical analyses identify correlations between phenotypes and genotypes (molecular markers) using pedigrees segregating for the traits of interest. The detection of a statistical association is viewed as evidence for a QTL (Lander and Botstein, 1989; Sewell and Neale 2000). QTL analyses are remarkably informative, revealing the (1) number and genome location of QTL affecting a trait, (2) magnitude of effect of each QTL, (3) mode of gene action at each QTL, (4) interactions among QTL (epistasis), and (5) parental source of beneficial QTL alleles (Bradshaw 1996). This information has three potential applications in forest trees:

- Marker-aided selection (MAS): The efficiency of selection may be improved (relative to phenotypic selection alone) by basing selection on specific QTL alleles (Williams and Neale 1992; OMalley and McKeand 1994; Johnson et al. 2000; Wilcox et al. 2001).
- Genetic architecture: QTL mapping provides a detailed understanding of the genetic architecture of complex traits (Edwards et al. 1987; Bradshaw 1996).
- Positional selection of candidate genes: Putative candidate genes may be identified by co-location of QTL and ESTs, or genes of known function on a genetic map (Frewen et al. 2000; Brown et al. 2003; Neale and Savolainen 2004).

The most useful QTL for breeding are those repeatedly detected or verified across time, space or genetic backgrounds. Verification is necessary to substantiate a biological basis for observed marker-trait associations, to predict QTL expression at a given age or in a particular environment (Brown et al. 2003), and may provide improved estimates of the magnitude of QTL effects.

For temperate woody plants, adaptation to winter cold involves complex genetic, physiological and developmental processes consisting of a suite of individual adaptive traits. The genetics of cold-hardiness in Douglas-fir has been well-documented using traditional quantitative and genecological test populations and artificial freeze tests (Aitken and Adams 1995, 1996, 1997; Anekonda et al. 1998, 2000a, b; Aitken and Hannerz 2000; O'Neill et al. 2000, 2001). Collectively, these studies suggest that, for the most part, variation in the timing of cold-hardiness development in the fall (acclimation) and loss of cold-hardiness in the spring (deacclimation) are under the control of different suites of genes. Variation in spring cold-hardiness is under relatively strong genetic control (h^2 = 0.36–1.00) and deacclimation appears to be synchronized in all tissues (needles, buds, and stems). Variation in fall cold-hardiness is under modest genetic control (h^2 = 0.09–0.39) and individual tissues acclimatize differentially (reviewed in Jermstad et al. 2001b). In Douglas-fir, spring and fall cold-hardiness usually exhibit weak to moderate negative genetic correlations.

QTL studies support these genetic interpretations. In a clonally replicated study of a large, full-sib Douglas-fir family, Jermstad et al. (2001b) observed 11 fall cold-hardiness QTL and 15 spring cold-hardiness QTL. QTL for each trait were located on four linkage groups (LGs), but only one QTL was common to both traits. Several QTL were associated with spring cold-hardiness in all three shoot tissues, supporting quantitative genetic studies suggesting that stem tissues are synchronized during deacclimation. Co-location of fall cold-hardiness QTL for different tissues was observed only once, on LG #2. The cumulative proportion of phenotypic variation explained for any given trait seldom exceeded 10% (Jermstad et al. 2001b).

Verification of the findings of Jermstad et al. (2001b) is necessary to assess the robustness of the genetic architecture of cold-hardiness previously described in Douglas-fir and to provide further guidance for selection of putative candidate genes. Brown et al. (2003) defined QTL verification as the repeated detection, at a similar position on the genetic map, of a QTL controlling a trait under more than one set of experimental conditions. To satisfy these conditions, a new, significantly larger cohort of the original detection population was created and assessed for spring cold-hardiness testing. The objectives of this study were to (1) conduct QTL analyses on a larger, independent cohort of the detection population to verify previously detected QTL and to identify putative new QTL controlling cold-hardiness; (2) improve

estimates of the percentage of phenotypic variation explained (PVE) by the QTL; and (3) identify putative positional candidate genes underlying cold-hardiness phenotypes by genetic mapping and co-location with QTL.

Materials and methods

Mapping populations

Two mapping populations (Cohort 1 and Cohort 2) were generated from a single, three-generation out-bred pedigree segregating for the timing of vegetative bud flush (Figure 1 in Jermstad et al. 2003; Table 1). The controlled cross producing Cohort 1 was made in 1993. Over 300 progeny from that cross were clonally propagated by rooted cutting in 1994 and 224 of these clones were out-planted to permanent, replicated field test sites in 1995 (clonal replicates are hereafter referred to as ramets). Of these, 170 clones were tested for spring cold-hardiness (Jermstad et al. 2001b). The second, significantly larger cohort (408 genotypes planted, 383 tested for cold-hardiness) was created in 1998, propagated by cutting in 1999 and field established in 2000 (Jermstad et al. 2003). This cohort was originally created to dissect QTL by environment interactions under controlled greenhouse and nursery conditions. Test trees were subsequently out-planted to the field at age 2. All field tests were established as incomplete randomized block

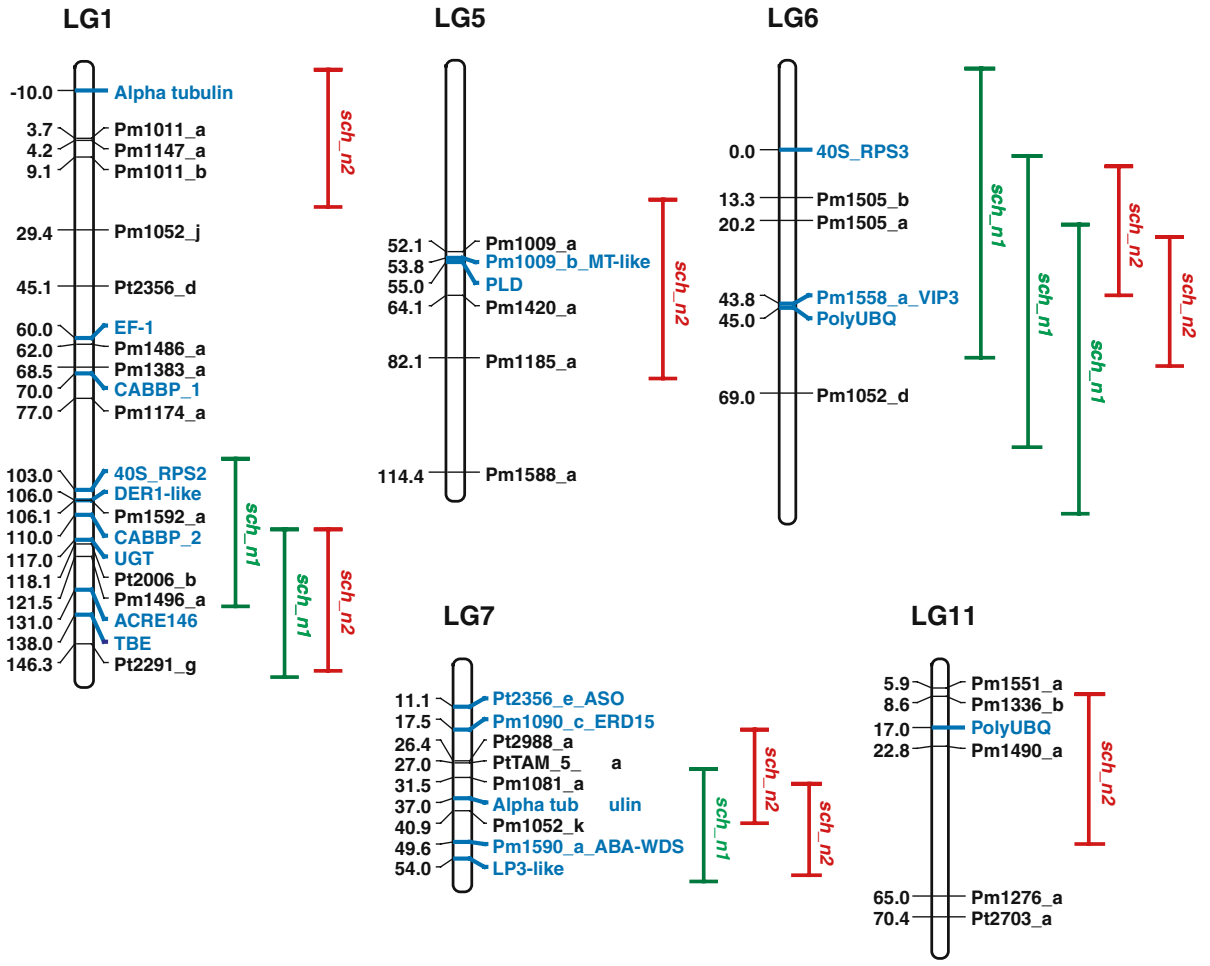

Figure 1. Unique QTL for spring needle cold-hardiness are presented with 95% CI bars on the consensus map for Cohorts 1 (sch_n1) and 2 (sch_n2). Framework markers used for QTL mapping and candidate genes, noted in blue, bold type, are indicated.

Table 1. Mapping population size, location and characteristics, assessment methods and traits tested.

Characteristic	Cohort 1	Cohort 2
Year propagated	1993	1998
Year test site established	1995	2000
Test location	Twin harbors	Longview
Damage assessment	Visual	Conductivity
Year evaluated	1997	2003
Tissue scored/ QTL name	Needles – sch_n1	Needles – sch_n2
Test temperatures (°C)	−12, −14, −16, −18	−11, −16
Number of clones freeze tested	170	383

designs with four blocks per site and either 2-ramet (Cohort 2) or 3-ramet (Cohort 1) plots. In both trials, blocking was perpendicular to slope.

Sampling

Cohort 1: For spring cold-hardiness trials, four 5-cm-long lateral shoot tips were harvested from each of two ramets in a clonal plot in each of two field blocks (four ramets per genotype), wrapped in damp towels, then placed in a plastic bag within a cooler (2–4 °C) for transport (Jermstad et al. 2001b).

Cohort 2: A single twig (last 15 cm of a first-order lateral branch) was collected from the east side of the second whorl from each of two ramets in a clonal plot in each of two field blocks (four ramets per genotype). Twigs from a single plot were combined, misted, and placed in a plastic bag together for transport in a cooler (2–4 °C). If one ramet within a plot was missing, two twigs from the remaining ramet were collected or a second ramet from another block was sampled.

Cold treatment

Cohort 1: Cold-hardiness testing used the artificial freezing methods and visual scoring of detached shoot tips described in Aitken and Adams (1997) and Anekonda et al. (2000a). Freeze-testing occurred within 48 h of field collection. Shoot tips from second-order shoots were wrapped in damp cheesecloth and aluminum foil prior to being stored overnight at −2 °C. The temperature was then slowly lowered in a programmable Forma Scientific 8359 chest freezer (Forma Scientific Inc., Marietta, OH, USA) until the first test temperature was reached, and held constant for 1 h before ramping down to subsequent test temperatures (four test temperatures; Table 1). At the end of each test temperature, one shoot tip from each test tree was removed, stored at 2 °C overnight, and then held for 7 days in the dark at room temperature to allow cold injury symptoms to develop. Test temperatures were selected based on results from preliminary trials run the week prior to testing.

Cohort 2: Freeze testing of needle segments evaluated using electrolytic leakage followed the methods of Hannerz et al. (1999). Specifically, 15–20 healthy needles were cut from each test twig between 5 and 10 cm from the terminal bud, and diced into 5 mm transverse segments. One segment from each of five needles from each of the paired twigs was placed in each of three capped vials along with a trace of silver iodide and 0.2 ml distilled, deionized water (dH_2O). The vials represented two test temperatures and an unfrozen control treatment. After preparation, vials were stored at 2 °C overnight until freezing. Freezing was conducted in a programmable Tenney T20C-3 freezer with a Watlow 942 control unit (Lunaire Ltd., Williamsport, PA, USA). Test temperature ramping occurred as noted above, and one set of vials was removed after each test temperature was achieved and stored at 2 °C (Table 1). Two hours after removal from the freezer, 3.5 ml dH_2O was added to each vial. Vials were subsequently stored at 2 °C for 24 h prior to processing. Two test temperatures (−11 and −16 °C) were evaluated.

Quantifying cold injury and cold injury traits

Cohort 1: Needle tissue was evaluated for cold injury symptoms using visual assessment (scored from 1 to 10) of tissue necrosis (Anekonda et al. 2000a). Phenotypic values were then calculated by averaging over replication. The highest and lowest test temperatures produced insufficient variation in cold injury to detect differences among clones (Anekonda et al. 2000b). Cold injury scores for the remaining two temperatures tested were highly correlated among clones; therefore, scores were averaged across test temperatures and these values were assessed in QTL scans.

Cohort 2: Freeze injury was determined by measuring electrolytic conductivity in vials containing frozen or control needle samples after one hour on a shaker using a Cole-Parmer 1481-61 conductivity meter (Cole-Parmer Co., Chicago, IL, USA). Thereafter, samples were placed in a water bath at 95 °C for 1.5 h to kill all tissues. Following a 24 h incubation period, samples from both test temperatures and the controls were again shaken for 1 h, and then retested to determine maximum conductivity. An index of cold injury was calculated following Flint et al. (1967). Specifically, the Index of Injury (I) for a given temperature (t) is defined by:

$$I_t = 100(R_t - R_0)/(1 - R_0)$$

where,

$$R_t = L_t/L_k; \qquad R_0 = L_0/L_d;$$

I_t is the index of injury (percent) resulting from exposure to temperature t, R_t is the relative conductivity from the sample exposed to temperature t, R_0 is the relative conductivity of the unfrozen control sample, L_t is the conductance of leachate from the sample frozen at temperature t, L_k is the conductance of leachate from the sample frozen at temperature t and then heat killed, L_0 is the conductance of leachate from the unfrozen control sample, and L_d is the conductance of leachate from the corresponding heat-killed unfrozen control sample.

Index of injury scores were determined for each replication and test temperature, and clone mean scores were averaged across replications by test temperature. Index scores were subsequently normalized to a mean of 0 and a variance of 1.0. QTL scans were conducted on normalized data from each test temperature (−11, −16 °C), the average of the normalized values for the two test temperatures, and on a selection index that uses data from both test temperatures weighted according to the heritabilities (Falconer and MacKay 1996).

Genotypic data, linkage maps and QTL genome scans

Linkage maps were constructed for both cohorts using 72–74 evenly spaced and informative RFLP markers as described by Jermstad et al. (1998, 2001a, b, 2003). For the current map, segregation data from both cohorts were combined and linkage analysis was performed using JoinMap version 1.4 (Stam and van Ooijen 1995). Map length is ca. 900 cm with an average marker density of ca. 12 cm. The Kosambi function was used to estimate map distances, and LOD thresholds of 4.0 and 0.1 were used for grouping markers into LGs and for ordering markers, respectively. The current map consists of 15 LGs, two less than previously reported (Jermstad et al. 2003). LGs were consolidated due to improved linkage estimates provided by a larger segregating population. Douglas-fir has 13 chromosomes. Previously reported LGs 1 and 10 were combined (LG 1 here) and previously reported LGs 12 and 16 were combined (LG 11 here). The multi-marker interval mapping approach of Knott et al. (1997) was used to scan individual LGs for the presence of QTL at 5 cm intervals following both 1- and 2-QTL models (Knott et al. 1997; Jermstad et al. 2001a, 2003; Sewell et al. 2000, 2002). For each model, the mapping software provided F-statistics for the most likely QTL on each LG, as well as sum of squares (SS), degrees of freedom (DF), and the effects for the parental main and interaction components. Critical thresholds of the F distribution probabilities for suggestive and significant QTL were established at $p \leq 0.01$ and $p \leq 0.005$, respectively, as described previously (Jermstad et al. 2001a, b, 2003) and all QTL meeting either level of significance were reported. For each QTL, the proportion of phenotypic variance explained (PVE) was estimated following Knott et al. (1997). Consistent with Sewell et al. (2000, 2002), unique QTL are defined here as any grouping of QTL influencing the same trait that map within ca. 15 cm of one another. The linkage map was drawn using MapChart 2.1 (Voorrips 2002).

Confidence intervals (95%) for QTL were estimated using the method of Darvasi and Soller (1997) where $CI = 530/Nv$, N being the sample size and v representing the proportion of phenotypic variance explained by the QTL.

Candidate gene selection and mapping

EST markers previously used for Douglas-fir QTL and comparative genetic mapping

(Jermstad et al. 1998; Krutovsky et al. 2004) were used to select putative candidate genes that may be responsible for the QTL effects found in the study. Selection was based on (1) co-location with QTL, (2) the functional role assigned to the annotated EST sequences, and (3) available data on differential expression at the mRNA level. A brief description of selected candidate genes located on LGs with cold-hardiness QTL is presented in Table 2 (more detailed information is available at http://dendrome.ucdavis.edu/dfgp/supplemental/html). We focused on genes involved in a broad array of biochemical pathways.

PCR amplification primers were designed using the GeneRunner program (http://www.generunner.com/) and individual Douglas-fir ESTs or contig assemblies of EST sequences (http://dendrome.ucdavis.edu/dfgp). PCR amplification was performed as described in Krutovsky et al. (2004). Genotypic data for segregating EST markers were obtained primarily by denaturing gradient gel electrophoresis (DGGE) according to Temesgen et al. (2001). A number of candidate genes were mapped previously by RFLP analysis (Jermstad et al. 1998). All candidates were mapped using an array of 96 progeny.

Fisher's exact test (2 tailed) and the hypergeometric distribution analysis were used to determine if the co-location of candidate genes and QTL was coincidental or non-random (Feller 1968; Spiegel 1992). Both analyses assume normal distribution for both QTL and candidate genes which is likely but difficult to substantiate.

Results and discussion

QTL detection

Cohort 1: Detection of spring cold-hardiness QTL in Cohort 1 was previously reported in Jermstad et al. (2001b) and is reviewed here to demonstrate verification (Figure 1). The number of QTL reported here (6) is less than reported in Jermstad et al. (2001b) because some of the latter were combined using the current definition of a unique QTL. Also, QTL are reported for spring cold-hardiness on three rather than four LGs as previously reported, because of the consolidation of LGs 1 and 10 in the new map.

Cohort 2: Mean clonal index of injury scores ranged from 0 to 27% and 20 to 81% for the −11 and −16 °C test temperatures, respectively, and were distributed nearly normally. Test temperature mean scores, averaged across all clones, were 10.2 and 46.9, respectively (for −11 and −16 °C), though differences among replications were statistically significant at $p \leq 0.05$ (8.6 vs. 11.5 and 31.1 vs. 62.3 for replicates 1 and 2 at test temperatures −11 and −16 °C, respectively). The larger cold injury values for replicate 2 might be attributable to field effects (replication 1 was near the bottom of the slope and may have been exposed to cold air drainage, thus inducing greater acclimation), experimental effects (replications were treated on different days), or both. Though not large ($r < 0.20$), correlations among clones for index of injury scores between replications were significant ($p \leq 0.01$) for both test temperatures. The correlation across test temperatures for clonal means was much higher ($r = 0.47$, $p < 0.001$).

Broad-sense heritability (H^2) for cold injury scores ranged from 0.25 for the normalized −11 °C data to 0.32 for the weighted index score. These compare favorably with the broad-sense heritability of cold injury scores noted for needles in Cohort 1 (0.45), and suggests that variation in spring cold-hardiness is under moderate genetic control.

Twenty-two QTL on five LGs were detected for spring needle cold-hardiness for all traits tested (Table 3), though many are repeated observations of the same QTL (e.g. injury at different test temperatures). Thus, only eight unique QTL are reported here for Cohort 2 (Figure 1). In total, 10 unique QTL for needle cold-hardiness (spring) are identified between the two cohorts.

QTL verification

Four of the eight unique cold-hardiness QTL observed for needle tissue in Cohort 2 co-locate with spring cold-hardiness QTL for needles in Cohort 1 (Table 4). Two of the remaining unique QTL in Cohort 2 were located on LGs not previously identified as having cold-hardiness QTL, one QTL was found at the opposite end of LG1 from previously identified QTL, and the other was identified by a 2-QTL model as being very close to a verified QTL on LG 7. In some instances, such as

Table 2. Douglas-fir cold-hardiness candidate genes, map locations by LG, and descriptions of similar genes from other species[a].

Priority[b]	LG	Douglas-fir candidate gene clones	Similar genes from other species			
			Abbreviation	Name of gene product	Function of gene product	Gene expression reference[c]
1	1	PtIFG_2006_a; PtIFG_2006_b	CABBP2	Chlorophyll *a*/*b*-binding protein type 2	Component of the photosynthetic light-harvesting complex	Dubos et al. (2003)
1	1	estPpINR_RN01G08_b	DER1-like	Unknown protein with DER1-motif	Degradation of misfolded proteins in the yeast endoplasmic reticulum	Binh and Oono (1992)[d]
2	1	estPaTUM_PA0006_a; PmIFG_1592_a	40S-RPS2	40S ribosomal protein S2	Aids in protein synthesis as a structural component of ribosomes	–
2	1	estPpINR_RS01G05_a	ACRE146	Avr9/Cf-9 rapidly elicited protein	ACRE proteins are induced by fungal pathogens and other stresses	–
2	1 7	estPmIFG_102G09_c estPmIFG_102G09_b	Alpha tubulin	Alpha tubulin	Major constituent of microtubules and cytoskeleton	–
2	1	estPpINR_AS01D10_b	TBE	Thiazole biosynthetic enzyme	Biosynthesis of the thiamine precursor thiazole	–
1	1	PmIFG_1162_a	UGT	Uridine diphosphate glycosyltransferases	Transfer of glycosyl residues from activated nucleotide sugars to aglycones	–
1	4 5	estPtIFG_1626_a (= estPtIFG_1165_a) PmIFG_1009_a (= PtIFG_8473 and estPmIFG_014A07_a); PmIFG_1545_a (= PtIFG_1165_a)	MT-like	Metallothionein-like protein	May be involved in metal homeostasis, heavy metal detoxification, and protection from free radicals	Dubos et al. (2003)
2	5	estPtIFG_0739_a	PLD	Phospholipase D	Phospholipid metabolism associated with signal transduction and stress response	–
2	6	estPmIFG_ 202A06_a	40S-RPS3a	40S ribosomal protein S3a	Aids in protein synthesis as a structural component of ribosomes	–
2	6 11	estPpINR_AS01A06_a; estPmIFG_154C01_a PmIFG_1278_b	PolyUBQ	Polyubiquitin	Protein degradation and proteolysis	–
2	6	PmIFG_1558_a	VIP3	Prefoldin subunit 4	Regulates protein folding and may protect proteins from freezing	–
1	7	estPmIFG_150CO1_a	LP3-like	Water deficit inducible protein	Dehydrin	Chang et al. (1996)
1	7	PmIFG_1590_a; estPmIFG_143D03_a; estPmIFG_150C01_a	ABA-WDS	Drought and cold inducible protein	Unknown	Pang et al. unpublished
1	7	PmIFG_1090_c	ERD15	Dehydration-induced protein	Unknown	Kiyosue et al. (1994)

[a]Separate candidate genes that probably belong to the same gene family are listed as a single entry with multiple map locations.
[b]High-priority candidates (=1) have been implicated via their map locations near cold-hardiness QTL (Figure 1) plus gene expression information for similar genes in other species (see *Gene expression reference*). Medium-priority candidates (=2) have been implicated based on their map locations alone.
[c]A citation indicates that the expression of the gene in other species changes in response to cold or drought, whereas a dash indicates that evidence of this type does not exist.
[d]It also has similarity to a cold-induced protein in rice.

Table 3. Spring cold-hardiness QTL trait association for LGs in Cohort 2 based on normalized data from each test temperature (−11, −16 °C), the average of the normalized data from each test temperatures, and on a selection index that gave equal weighting to the two test temperatures.

LG[a]	Model[b]	Position (cm)	Trait*			
			−11 °C	−16 °C	Mean	Index
1	1 QTL	0	**			
	2 QTL	0				
		5	**			
	2 QTL	0				
		130				**
5	2 QTL	50				
		70			*	*
6	1 QTL	23		**		
	1 QTL	33			*	
	2 QTL	38				
		48		*		
7	1 QTL	46		*		
	2 QTL	31				
		46		**	**	**
11	2 QTL	21				
		26		**		

* and ** represent chromosome-wide significance at $p < 0.05$ and 0.01, respectively.
[a]LG 1 in Cohort 2 is a condensation of LGs 1 and 10 from Cohort 1.
[b]Models were calculated as per Knott et al. (1997).

on LGs 6 and 7 (Figure 1), bud and stem cold-hardiness QTL from Cohort 1 similarly co-locate with needle cold-hardiness QTL from both cohorts (QTL not shown here, see Jermstad 2001b). Unfortunately, the lack of cold injury data for buds and stems in Cohort 2 made comparisons for these specific tissues impossible, but the frequent co-location of QTL for all three tissues implies they may be under similar genetic control. While quantitative genetic data support the hypothesis that deacclimation in all three tissues is under the control of the same suite of genes (Aitken and Adams 1997; O'Neill et al. 2000, 2001; Anekonda et al. 2000a), QTL may not always be detected for all tissues. Most genetic variation in cold-hardiness appears to be a function of timing of gene action (Howe et al. 2003). A point-in-time sampling may not catch identical gene expression in all tissues, although it demonstrates differences among genotypes for the same tissue.

The verification of all genomic regions containing spring cold-hardiness QTL for needles in this experiment is impressive, especially considering that (1) QTL were detected in different cohorts of the same cross made in two separate matings 5 years apart, (2) the field experiments were growing on very different test sites and were sampled 6 years apart, and (3) very different methods were used to assess cold-induced damage. The detection of additional QTL in Cohort 2 is likely evidence of the increased power of the second experiment which had over twice as many clones as the first experiment (Table 1). The proportion of phenotypic variation explained in Cohort 2 (for the same four QTL) was substantially less than in Cohort 1 (Table 4; 15.2% for Cohort 2 vs. 24.9% for Cohort 1), but interestingly, the estimated proportion of genetic variance explained was nearly identical for the two experiments (55% in Cohort 1; 52.4% in Cohort 2). The phenotypic results are consistent with the predictions of Beavis (1994) regarding the effect of sample size on QTL detection and estimation of size of effects. Maximum likelihood methods that simultaneously detect QTL and estimate parameters (e.g. PVE), such as the one used here, may be subject to selection bias and thus are prone to overestimation of QTL effects (Ball 2001). Bayesian approaches to QTL detection and parameter estimation adjust for

Table 4. Summary of spring cold-hardiness QTL and corresponding candidate genes verified by detection in Cohorts 1 and 2.

LG	Trait	Interval[a]	Cohort 1		Cohort 2		Candidate genes
			F value[b]	PVE[c]	*F* value[b]	PVE[c]	
1	sch_n1; sch_n2	Pm1496_a–Pt2291_g	5.60**	7.5[d]	3.29**	3.5[d]	ACRE146; TBE: UGT
6	sch_n1; sch_n2	Pm1505_b–Pm1505_a	7.72**	3.8	4.65**	2.8	VIP3; PolyUBQ; 40S_RPS3
6	sch_n1; sch_n2	Pm1505-a–Pm1558_a	7.72**	3.8[d]	2.99*	3.0[d]	VIP3; PolyUBQ; 40S_RPS3
7	sch_n1; sch_n2	Pm1081_a–Pm1052_k	4.68**	9.8[d]	4.99**	5.9[d]	Alpha tubulin; LP3–like; ABA-WDS

[a]Marker intervals containing the predicted location of QTL.
[b]* and ** represent chromosome-wide significance at $p < 0.05$ and 0.01, respectively.
[c]Percentage of the phenotypic variance explained by a QTL or pair of QTL.
[d]Verified QTL represent 1 of 2 QTL detected by the two-QTL model.

selection bias and may replace the need for independent verification trials if the only application is to improve parameter estimates (Ball 2001). In our case, the extreme range of environmental variation to which Douglas-fir is exposed, and the modest size of the original population argued strongly in favor of a larger, independent verification population. While such verification efforts may not always be desirable or economic from an applied tree improvement perspective, they are of great utility in defining the genetic architecture of a quantitatively inherited trait.

Co-location of cold-hardiness QTL and candidate genes

Twenty-nine ESTs representing potential cold tolerance candidate genes mapped to the Douglas-fir linkage map. Of these, 20 occurred on LGs exhibiting spring cold-hardiness QTL in needle tissues, and 17 of these fell within the 95% CI of the 6 QTL regions exhibiting 10 overlapping QTL (Figure 1). These candidate genes are involved in a broad spectrum of physiological processes that could potentially influence cold-hardiness Table 2). Given the average 95% CI of 37.4 cm observed in Cohort 2 QTL, both Fisher's exact test ($p = 0.025$) and the hypergeometric distribution test ($p < 0.0001$) reject the null hypothesis of coincidental co-location of candidate genes and QTL. In short, it is highly probable that at least some of these candidate genes are controlling variation in spring cold-hardiness. Though co-location of QTL and candidate genes provides a valuable means of prioritizing genes for association studies it by no means implies a definitive association.

Brown et al. (2003) mapped 18 candidate genes related to lignin biosynthesis and cell wall structure in loblolly pine. Several of these genes co-located with QTL controlling wood property traits and early association analyses suggest some of these genes directly control traits of interest, with the proportion of variation explained roughly equivalent to QTL analyses (G. Brown, pers. comm.). Frewen et al. (2000) mapped two candidate genes (PHYB2 and ABIIB) to genomic regions with QTL controlling bud flush and bud set in poplar. These genes may indirectly influence spring and fall cold-hardiness, respectively. In barley, 13 putative candidate genes have mapped directly adjacent to a major cold tolerance QTL region on chromosome 7 (Dr. T. Chen, http://www.cgrb.orst.edu/mcb/faculty/chen/).

QTL, candidate genes and applied tree improvement

Over the last decade more than two dozen studies have been published describing the genetic dissection of economic, adaptive and/or physiological traits in forest trees using the QTL mapping approach (reviewed in Sewell and Neale 2000). A preliminary description of the number, size of effect, and location of QTL in conifers has emerged from these studies. In general, relatively few QTL (2–6) of modest effect ($< 10\%$ of phenotypic variation explained per QTL), and low-resolution map location are identified for most traits. QTL verification was a component of only four previous studies (Wilcox et al. 1997; Frewen et al. 2000; Brown et al. 2003; Jermstad et al. 2003). Without verification, both within and across pedigrees, QTL have rather limited utility for MAS, though it has been argued that given appropriate experimental conditions, verification is not required for all within-family selection schemes (S. Carson, unpublished). No doubt the size and expense of experiments required to detect and verify QTL is a significant obstacle to their widespread use. The single greatest hurdle to MAS using QTL is the inability to verify, and thus predict, QTL effects among different pedigrees. In out-crossing species of ancient lineage and large effective population sizes, like most forest trees, this is primarily a function of linkage equilibrium in the population, as predicted by Strauss et al. (1992). That is, even if QTL are segregating in different pedigrees, the linkage relationship between QTL and marker may not be in the same phase for each pedigree. The linkage phase would have to be confirmed for every pedigree prior to selection, an expensive endeavor. While a few studies have shown MAS using QTL could be economically feasible for within-family selection in forest trees (Johnson et al. 2000; Wilcox et al. 2001), it seems unlikely it will play a significant role in those forest industry tree improvement programs that rely on relatively large breeding and production populations. Clearly, a more precise and flexible MAS approach is needed. Association genetics may provide such an option.

Association genetics (also called linkage disequilibrium mapping or association mapping) is a population genomics approach to complex trait dissection that aims to identify the specific genes responsible for phenotypic differences among individuals, and characterize molecular variants within those genes that associate with alleles that contribute to phenotypic differences. Association genetics was pioneered in humans (Risch 2000; Cardon and Bell 2001), has been applied in maize (Thornsberry et al. 2001), and is anticipated to be widely applied to plants in the near future (Rafalski 2002; Neale and Savolainen 2004). Though there are alternative approaches to conducting association studies (Rafalski 2002), the most efficient and cost-effective approach in forest trees is the candidate gene method (Neale and Savolainen 2004). Candidate genes may be identified by known function, available in genomics databases, by genetic expression studies, or by co-location of unknown genes or ESTs with known QTL. In this study, we have identified candidate genes based on their known function and potential role in plant cold tolerance and placed them on a QTL map. The co-location of several of these candidate genes with verified QTL for cold-hardiness in Douglas-fir provides added confidence that these loci should be targeted for association mapping, work that is currently underway. In addition to having applied tree breeding utility, association studies have great potential for the study of population ecological genomics, facilitating study of the relationships between genotypes and environments in populations without pedigree information (Howe et al. 2003).

Acknowledgements

We thank Joanna Warren and Val Wyant-Wheeler for assistance in field collections and the entire Aiken lab for timely assistance in sample preparation cold testing. We are grateful to Weyerhaeuser Company for allowing us to sample the Cohort 2 population. This research was supported by the United States Department of Agriculture Cooperative State Research, Education, and Extension Service – National Research Initiative Competitive Grants Program, #97-35300–4623.

References

Aitken S.N. and Adams W.T. 1995. Screening for cold-hardiness in coastal Douglas-fir. In: Potts B.M., Borralho N.M.G., Reid J.B., Cromer R.N., Tibbits W.N. and Raymond C.A. (eds), Eucalypt Plantations: Improving Fibre Yield and Quality. Proc. CRC/IUFRO Conference, 19–24 Feb. 1994, Hobart, Australia. CRC for Temperate Hardwood Forestry, Hobart, Australia, pp. 321–324.

Aitken S.N. and Adams W.T. 1996. Genetics of fall and winter cold-hardiness of coastal Douglas-fir in Oregon. Can. J. Forest Res. 26: 1828–1837.

Aitken S.N. and Adams W.T. 1997. Spring cold hardiness under strong genetic control in Oregon populations of *Pseudotsuga menziesii* var. *menziesii*. Can. J. Forest Res. 27: 1773–1780.

Aitken S.N. and Hannerz M. 2000. Genecology and gene resource management strategies for conifer cold hardiness. In: Bigras F.J. and Columbo S.J. (eds), Conifer Cold Hardiness. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 23–53.

Anekonda T.S., Adams W.T. and Aitken S.N. 1998. Influence of second flushing on genetics assessment of cold hardiness in coastal Douglas-fir (*Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco). Forest Ecol. Manage. 111: 119–126.

Anekonda T.S., Adams W.T. and Aitken S.N. 2000a. Cold-hardiness testing for Douglas-fir tree improvement programs: guidelines for a simple, robust and inexpensive method. West. J. Appl. Forest 15: 129–136.

Anekonda T.S., Adams W.T., Aitken S.N., Neale D.B., Jermstad K.D. and Wheeler N.C. 2000b. Genetics of cold-hardiness in a cloned full-sib family of coastal Douglas-fir. Can. J. Forest Res. 30: 837–840.

Ball R.D. 2001. Bayesian methods for quantitative trait loci mapping based on model selection: appropriate analysis using the Bayesian information criterion. Genetics 159: 1351–1364.

Beavis W.D. 1994. The power and deceit of QTL experiments: lessons from comparative QTL studies. In: Proc. 49th Annual Corn and Sorghum Indus. Res. Conf., pp. 304–312.

Binh L.T. and Oono K. 1992. Molecular cloning and characterization of genes related to chilling tolerance in rice. Plant Physiol. 99: 1146–1150.

Bradshaw H.D. 1996. Molecular genetics of *Populus*. In: Stettler R.F., Bradshaw H.D., Heilman P.E. and Hinckley T.M. (eds), Biology of *Populus* and its Implications for Management and Conservation, Part 1, Chapter 8. NRC Res. Press, Nat. Res. Coun. Can., Ottawa, ON, pp. 183–199.

Brown G.R., Bassoni D.L., Gill G.P., Fontana J.R., Wheeler N.C., Megraw R.A., et al. 2003. Identification of quantitative trait loci influencing wood property traits in loblolly pine (*Pinus taeda L.*) III. QTL verification and candidate gene mapping. Genetics 164: 1537–1546.

Cardon L.R. and Bell J.L. 2001. Association study designs for complex diseases. Nat. Rev. Genet. 2: 91–99.

Chang S., Puryear J.D., Dias M.A.D.L., Funkhouser E.A., Newton R.J. and Cairney J. 1996. Gene expression under water deficit in loblolly pine (*Pinus taeda*): Isolation and characterization of cDNA clones. Physiol. Plantarum 97: 139–148.

Darvasi A. and Soller M. 1997. A simple method to calculate resolving power and confidence interval of QTL maplocation. Behavior Genetics 27(2): 125–132.

Dubos C., Le Provost G., Pot D., Saline F., Lalane C., Madur D., Frigerio J.M. and Plomion C. 2003. Identification and characterization of water-stress-responsive genes in hydroponically grown maritime pine (*Pinus pinaster)* seedlings. Tree Physiol. 23: 169–179.

Edwards M.D., Stuber C.W. and Wendel J.F. 1987. Molecular marker-facilitated investigations of quantitative trait loci in maize. I. Numbers, genomic distribution and types of gene action. Genetics 116: 113–125.

Falconer D.S. and MacKay T.F.C. 1996. Introduction to Quantitative Genetics, 4th ed. Longman Scientific and Technical, Essex, UK.

Feller W. 1968. The hypergeometric series §2.6. In: An Introduction to Probability Theory and its Applications, Vol. 1, 3rd ed. Wiley, New York, pp. 41–45.

Flint H.L., Boyce B.R. and Beattie D.J. 1967. Index of injury – a useful expression of freezing injury to plant tissues as determined by the electrolytic method. Can. J. Plant. Sci. 47: 229–230.

Frewen B.E., Chen T.H.H., Howe G.T., Davis A., Rohde J., Boerjan W., et al. 2000. Quantitative trait loci and candidate gene mapping of bud set and bud flush in *Populus*. Genetics 154: 837–845.

Hannerz M., Aitken S.N., King J.N. and Budge S. 1999. Effects of genetic selection for growth on frost hardiness in western hemlock. Can. J. Forest Res. 29: 509–516.

Howe G.T., Aitken S.N., Neale D.B., Jermstad K.D., Wheeler N.C. and Chen T.H.H. 2003. From genotype to phenotype: unraveling the complexities of cold adaptation in forest trees. Can. J. Bot. 81: 1247–1266.

Jermstad K.D., Bassoni D.L., Jech K.S., Ritchie G.A., Wheeler N.C. and Neale D.B. 2003. Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. III. QTL by environment interactions. Genetics 165: 1489–1506.

Jermstad K.D., Bassoni D.L., Jech K.S., Wheeler N.C. and Neale D.B. 2001a. Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. I. Spring bud flush. Theor. Appl. Genet. 102: 1142–1151.

Jermstad K.D., Bassoni D.L., Wheeler N.C., Anekonda T.S., Aitken S.N., Adams W.T. and Neale D.B. 2001b. Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas-fir. II. Spring and fall cold-hardiness. Theor. Appl. Genet. 102: 1152–1158.

Jermstad K.D., Bassoni D.L., Wheeler N.C. and Neale D.B. 1998. A sex-averaged genetic linkage map in coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var '*menziesii*') based on RFLP and RAPD markers. Theor. Appl. Genet. 97: 797–802.

Johnson G.R., Wheeler N.C. and Strauss S.H. 2000. Financial feasibility of marker-aided-selection in Douglas-fir. Can. J. Forest Res. 30: 1942–1952.

Kiyosue T., Yamaguchi-Shinozaki K. and Shinozaki K. 1994. ERD15, a cDNA for a dehydration-induced gene from *Arabidopsis thaliana*. Plant Physiol. 106: 1707.

Knott S.A., Neale D.B., Sewell M.M. and Haley C.S. 1997. Multiple marker mapping of quantitative trait loci in an outbred pedigree of loblolly pine. Theor. Appl. Genet. 94: 810–820.

Krutovsky K.V., Troggio M., Brown G.R., Jermstad K.D. and Neale D.B. 2004. Comparative mapping in the Pinaceae. Genetics 168: 447–461.

Lander E.S. and Botstein D. 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121: 185–199.

Neale D.B. and Savolainen O. 2004. Association genetics of complex traits in conifers. Trends Plant Sci. 9: 325–330.

O'Malley D.M. and McKeand S.E. 1994. Marker-assisted selection for breeding value in forest trees. Forest Genet. 1: 207–218.

O'Neill G.A., Adams W.T. and Aitken S.N. 2001. Quantitative genetics of spring and fall cold hardiness in seedlings from two Oregon populations of coastal Douglas-fir. Forest Ecol. Manage. 149: 305–318.

O'Neill G.A., Aitken S.N. and Adams W.T. 2000. Genetic selection for cold hardiness in coastal Douglas-fir seedlings and saplings. Can. J. Forest Res. 30: 1799–1807.

Paterson A.H. (ed.) 1998. Molecular Dissection of Complex Traits. CRC Press LLC, Boca Raton, FL.

Rafalski A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 5: 94–100.

Risch N.J. 2000. Searching for genetic determinants in the new millennium. Nature 405: 847–856.

Sewell M.M., Bassoni D.L., Megraw R.A. and Wheeler N.C. 2000. Identification of QTL influencing wood property traits in loblolly pine (*Pinus taeda* L.). I. Physical wood properties. Theor. Appl. Genet. 101: 1273–1281.

Sewell M.M., Davis M.F., Tuskin G.A., Wheeler N.C., Elam C.C., Bassoni D.L. and Neale D.B. 2002. Identification of QTL influencing wood property traits in loblolly pine (*Pinus taeda* L.). II. Chemical wood properties. Theor. Appl. Genet. 104: 214–222.

Sewell M.M. and Neale D.B. 2000. Mapping quantitative traits in forest trees. In: Jain S.M. and Minocha S.C. (eds), Molecular Biology of Woody Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 407–424.

Spiegel M.R. 1992. Theory and Problems of Probability and Statistics. McGraw-Hill, NY, pp. 113–114.

Stam P.J. and van Ooijen J.W. 1995. JoinMap^{TM} Version 2.0: Software for the Calculation of Genetic Linkage Maps. CPRO-DLO, Wageningen, Germany.

Strauss S.H., Lande R. and Namkoong G. 1992. Limitations of molecular-marker-aided selection in forest tree breeding. Can. J. Forest Res. 22: 1050–1061.

Temesgen B., Brown G.R., Harry D.E., Kinlaw C.S., Sewell M.M., et al. 2001. Genetic mapping of expressed sequence tag polymorphism (ESTP) markers in loblolly pine (*Pinus taeda* L.). Theor. Appl. Genet. 102: 664–675.

Thomashow M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 571–599.

Thornsberry J.M., Goodman M.M., Doebley J., Kresovich S., Nielsen D. and Bucker E.S. IV 2001. *Dwarf8* polymorphisms associate with variation in flowering time. Nat. Genet. 28: 286–289.

Voorrips R.E. 2002. MapChart: software for the graphical presentation of linkage maps and QTL. J. Hered. 93: 77–78.

Wilcox P.L., Richardson T.E. and Carson S.D. 1997. Nature of quantitative trait variation in *Pinus radiata*: insights from

QTL detection experiments. In: Burdon R.D. and Moore J.M. (eds), Proc. IUFRO'97: Genetics of Radiata Pine. FRI Bull. No. 203. Rotorua, New Zealand, pp. 304–312.

Wilcox P.L., Carson S.D., Richardson T.E., Ball R.D., Horgan G.P. and Carter P. 2001. Cost-benefit analysis of marker based selection in seed orchard production populations of *Pinus radiata*. Can. J. Forest Res. 31: 2213–2224.

Williams C.G. and Neale D.B. 1992. Conifer wood quality and marker-aided selection: a case study. Can. J. Forest Res. 22: 1009–1017.