Global monitoring of autumn gene expression within and among phenotypically divergent populations of Sitka spruce (*Picea sitchensis*)

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Summary

- Cold acclimation in conifers is a complex process, the timing and extent of which reflects local adaptation and varies widely along latitudinal gradients for many temperate and boreal tree species. Despite their ecological and economic importance, little is known about the global changes in gene expression that accompany autumn cold acclimation in conifers.
- Using three populations of Sitka spruce (*Picea sitchensis*) spanning the species range, and a *Picea* cDNA microarray with 21 840 unique elements, within- and among-population gene expression was monitored during the autumn. Microarray data were validated for selected genes using real-time PCR.
- Similar numbers of genes were significantly twofold upregulated (1257) and downregulated (967) between late summer and early winter. Among those up-regulated were dehydrins, pathogenesis-related/antifreeze genes, carbohydrate and lipid metabolism genes, and genes involved in signal transduction and transcriptional regulation. Among-population microarray hybridizations at early and late autumn time points revealed substantial variation in the autumn transcriptome, some of which may reflect local adaptation.
- These results demonstrate the complexity of cold acclimation in conifers, highlight similarities and differences to cold tolerance in annual plants, and provide a solid foundation for functional and genetic studies of this important adaptive process.

Key words: adaptation, cold hardiness, genetic cline, microarray, real-time polymerase chain reaction (PCR), Sitka spruce (*Picea sitchensis*).

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Introduction

Temperate and boreal trees alternate between periods of active growth in summer, and dormancy in winter. Beginning in late summer, and in response to short days, cessation of shoot elongation leads to initiation of cold acclimation. This period is characterized by suspension of mitotic activity that can be reversed given favourable conditions, and is followed late in autumn by bud endodormancy, which is maintained throughout the winter period until appropriate chilling and heat sum requirements are met (Rohde & Bhalerao, 2007). Substantial cold hardiness is incompatible with growth, and tradeoffs exist in this annual cycle between competition for light resources and the need to acquire and maintain cold hardiness (Howe *et al.*, 2003). As a result, timing of entry into, and exit from dormancy is locally adaptive, and genetic clines observed in common gardens usually correspond to variation in climate of population origin along latitudinal or elevational gradients (Morgenstern, 1996). In woody plants, the period of autumn cold acclimation is typically divided into two phases (Weiser, 1970). Phase I, which overlaps with growth cessation and dormancy induction, is triggered by a critical

night length that is reached weeks or months before the first frost, and varies among families, populations and species (Weiser, 1970; Cannell & Sheppard, 1982; Cannell *et al.*, 1990; Aitken & Adams, 1996). Cold hardiness increases steadily until the first subfreezing temperatures occur. At this point, entry into phase II leads to further hardening, which is often rapid, and maximum cold hardiness is ultimately achieved (Weiser, 1970). As maximum cold hardiness often far exceeds minimum recorded winter temperatures, cold injury is most commonly observed in spring and autumn, before the onset of acclimation or after de-acclimation (Weiser, 1970; Aitken & Adams, 1996).

Although the phenotypic responses of conifers to long nights and low temperature have been well studied, little is known about how these environmental cues are integrated at the molecular level. Much of what we do know about the molecular basis for plant cold tolerance is based on studies of herbaceous annuals such as *Arabidopsis thaliana* and winter rye (*Secale cereale*). These species provide favourable starting points for the study of cold hardiness because they are experimentally tractable. However, in contrast to perennials, annuals typically acclimate fully in response to a low temperature cue, which makes the regulation of cold hardiness in these two systems fundamentally different. Despite this difference, few studies have focused on the suite of genes that contribute to cold hardiness in perennials. The recent completion of the *Populus trichocarpa* genome (Tuskan *et al.*, 2006), as well as successful efforts to sequence large numbers of spruce (*Picea* spp.) and pine (*Pinus* spp.) expressed sequence tags (ESTs) (Ralph *et al.*, 2006), afford new opportunities in the study of cold hardiness in forest trees. The first microarray study comparing the actively growing and dormant cambial meristem in *Populus tremula* reveals wide variation in gene expression between these two developmental states, some of which appears to be related to cold hardiness (Schrader *et al.*, 2004). In addition, a 1.5-K microarray was used recently to study the transcriptional response of apical buds in a Scots pine (*Pinus sylvestris*) provenance grown in three separate field sites along a latitudinal transect, and identified a number of candidate pine cold hardiness genes (Joosen *et al.*, 2006). Although these studies provide the first insights into the genomics of cold hardiness and dormancy, a global picture of the temporal regulation of autumn gene expression in a conifer has yet to be reported. Similarly, the degree to which adaptation to local climate is manifested at the level of gene expression is unknown.

Conifers are among the most economically and ecologically important terrestrial plant species worldwide, and are also evolutionarily distinct from model angiosperms, having diverged approx. 380 million yr ago (Kenrick & Crane, 1997). This evolutionary distance makes selection of candidate genes in conifers based on sequence comparisons alone very challenging, a problem that is confounded by the evolutionary expansion of multigene families in conifers (Kinlaw & Neale, 1997; Ahuja & Neale, 2005). In the face of a changing climate, existing variation in the genes involved in adaptation to local climate provides the foundation for short-term survival and long-term adaptation (Aitken, 1999). Conservation of these genetic resources is therefore crucial. In addition, rapid climate change may make marker-aided selection for traits related to local adaptation increasingly important in managed forests. Information on the genomic architecture of local adaptation to climate, including the number of genes involved, linkages among them, and pleiotropic effects on phenotypes will also shed greater light on the ability of populations to adapt rapidly to climate change than will assuming a purely biometric approach based on quantitative genetic variation (Aitken *et al.*, in press). In order to advance gene conservation and selective breeding efforts in an expedient, economic and rigorous way, we must first have an understanding of the genes involved. The goals of this study were threefold: to analyse the reorganization of the transcriptome in Sitka spruce during autumn cold acclimation; to identify transcripts that are differentially expressed among phenotypically divergent populations; and to combine these transcriptional data with functional data from model species to identify a list of candidate genes for Sitka spruce cold acclimation that will form the basis for a future candidate gene-based association study. We used the Treenomix *Picea* spp. cDNA microarray comprising 21 840 unique elements to assay temporal and among-population variation in gene expression of Sitka spruce seedlings. Sampling of foliage for RNA extraction and cold hardiness phenotyping was carried out at five time points between August and December (2004) within a population originating at the approximate geographical centre of the species range (Prince Rupert, British Columbia). In addition, northern (Valdez, Alaska, USA) and southern (Redwood, California, USA) peripheral populations, which have markedly different cold acclimation phenotypes, were sampled at early and late autumn time points. Microarray expression data were validated for selected genes using real-time PCR.

Materials and Methods

Plant material and tissue sampling

Foliage for RNA extraction and cold hardiness phenotyping was obtained from 4-yr-old Sitka spruce (*Picea sitchensis* (Bong.) Carr.) seedlings, which were grown from seed collected from natural populations spanning the species range in a raised-bed outdoor common garden in Vancouver, BC, Canada (49° N) (Mimura & Aitken, 2007). Needle tissues from current-year upper lateral shoots were collected at five time points between late summer and early winter 2004. Three of 17 available populations were chosen for sampling based on geographical location and previously characterized genetic clines: Valdez, Alaska, USA (61° N) (AK); Prince

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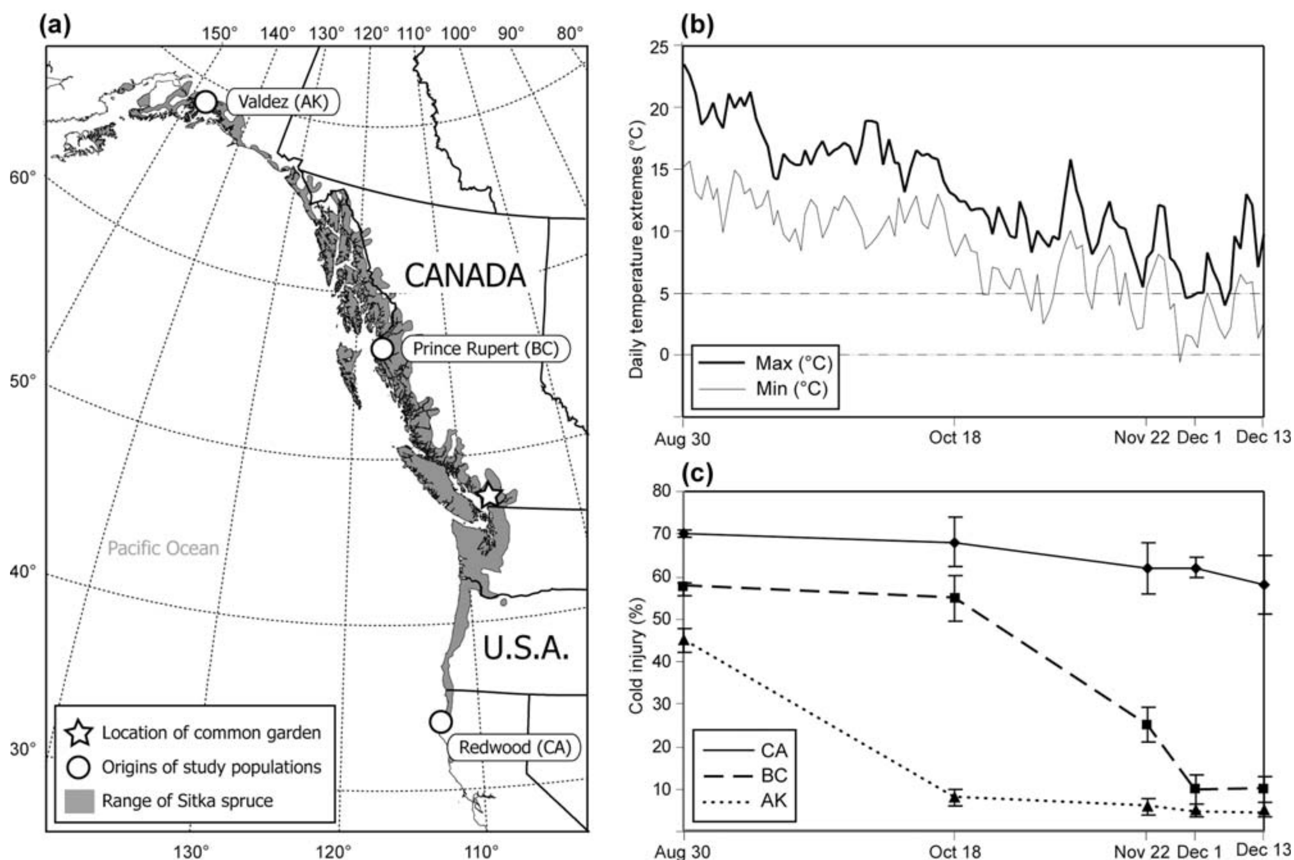

Fig. 1 (a) Distribution of Sitka spruce (*Picea sitchensis*), origins of study populations, and location of common garden. (b) Daily maximum and minimum temperatures measured at field site, located at Vancouver, British Columbia, Canada. Dashed lines indicate 5 and 0°C. (c) Mean percentage cold injury at –10°C (± SE) for study populations, measured on each of five sampling dates.

Rupert, British Columbia, Canada (54° N) (BC); Redwood, California, USA (41° N) (CA) (Fig. 1a). Needle samples (approx. 1 g) were taken from eight individuals in the BC population on each of the five dates (30 August, 18 October, 22 November, 1 December, 13 December). To compare among-population gene expression, eight individuals from the AK and CA populations, as well as eight additional individuals from the BC population, were also sampled on the second and fourth dates. The common garden contained eight experimental blocks, and seedlings from each population were sampled from most blocks on each sampling date. Tissues were flash frozen in an N_2 vapour tank immediately on collection, and subsequently stored at –80°C until processing.

To measure changes in gene expression triggered by both lengthening nights (phase I of cold acclimation) and the first subfreezing temperatures (phase II of cold acclimation), we chose sampling dates that spanned both these events. Cold acclimation begins in earnest when primary growth has ceased and terminal bud formation has commenced ('bud set') (Weiser, 1970). For the AK, BC and CA populations, the average dates of bud set in 2003 at the common garden site were 2 July, 28 August and 5 October, respectively. As a compromise between these divergent dates, we conducted our first sample collection on 30 August. The second time point was approx. 6 wk later, on 18 October, well before the arrival of the first freezing temperatures. We subsequently sampled just before the first frost event (which occurred on 27 November; sampling on 22 November), and 4 d after that frost (1 December). In order to capture any late responses, we conducted a final sampling 16 d after the first frost (13 December).

Cold hardiness phenotyping

Cold hardiness for each individual sampled for RNA at each time point was measured using electrolytic leakage as a proxy for cell death (Hannerz *et al.*, 1999). Briefly, four to five needles from the current year's growth of upper lateral shoots were cut into 0.5-cm segments and frozen in 0.2 ml water with a small amount of an ice nucleator (AgI). Samples were kept at 4°C overnight, then the temperature was reduced by 4°C h^{-1} and held for 1 h at the selected test temperature. Samples were then thawed overnight at 4°C. A control was kept at 4°C throughout this process. After freezing, the electrolytic conductivity of the solution was measured. Frozen and control samples were then heat-killed at 95°C and

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measured again. The following ratio expresses the result as an index of injury (I_t) (%):

$$I_t = \frac{100(R_t - R_o)}{(1 - R_o)} \qquad \text{Eqn 1}$$

where $R_t = L_t/L_k$, $R_o = L_o/L_d$. L_t is the conductance of leachate from the sample frozen at temperature *t*, L_k is the conductance of the leachate from the sample frozen at temperature *t* and then heat-killed, L_o is the conductance of the leachate from the unfrozen sample, and L_d is the conductance of the leachate from the corresponding heat-killed, unfrozen sample.

Freeze-testing temperatures were selected *a priori* for each sampling date based on cold hardiness in previous years (Mimura & Aitken, 2007). We measured cold hardiness in all three populations (CA, BC, AK) on all five dates, and tested at two temperatures on each date. These temperatures were: −8 and −11°C on 30 August, −10 and −14°C on 18 October, −10 and −20°C on 22 November, −15 and −25°C on 1 December, and −15 and −25°C on 13 December. To provide a graphical representation of cold hardiness phenotypes across the acclimation period, mean I_t for 20 seedlings at or interpolated/extrapolated to −10°C are illustrated in Fig. 1c. Actual values of I_t at each of two test temperatures for only those eight seedlings used for RNA extraction were used for statistical analyses of phenotypic variation. Phenotypic data were subject to ANOVA using the General Linear Model procedure of SAS (SAS, 1989). Degrees of freedom were inadequate to test both experimental block and biological seedling pool simultaneously, and preliminary ANOVAS indicated that variation among experimental blocks was not significant. Sources of variation tested in ANOVAS among populations included population (CA, BC, AK), sampling date (18 October, 1 December), test temperature nested within date, biological pool nested within date and population (two pools of four seedlings per population and sampling date), and population × date interaction. All effects were treated as fixed. In the analysis of temporal variation for the BC population, the model included sampling date, temperature within date (two temperatures), and biological pool nested within date (two pools of four seedlings per date). Reduced ANOVA models were also run on each sampling date separately with terms involving date deleted from the above models. Least-square phenotypic means were calculated for each population at each test temperature on each sampling date, and tests of differences between pairs of populations were conducted using a Bonferroni adjustment (SAS, 1989).

RNA extraction, experimental design and microarray hybridization

To decrease the effects of biological variance among individual seedlings within populations, equal amounts of foliar tissue were pooled from four individuals before RNA extraction. Two pools were collected at each time point for each population, and total RNA was extracted following a previously published protocol (Kolosova *et al.*, 2004). RNA quality was assessed by measuring spectral absorbance between 200 and 350 nm and by visual assessment on a 1% agarose gel. Because contaminants undetectable by these two methods can interfere with enzymatic manipulation, 5 µg total RNA were reverse-transcribed incorporating P-32 labelled dGTP. Resulting cDNA was run on a 1% alkaline buffer gel and visualized using a Storm phosphorimager (Amersham Biosciences, Piscataway, NJ, USA).

A factorial hybridization design with dye balance was chosen to assess gene expression among each of the five time points for the BC population (30 August (BC1), 18 October (BC2), 22 November (BC3), 1 December (BC4), 13 December (BC5); Fig. 2a). Among-population hybridizations were also conducted in a factorial fashion at the second and fourth time points (18 October (CA2, BC2, AK2), 1 December (CA4, BC4, AK4); Fig. 2b). Two biological replicates of this design were made for a total of 32 hybridizations. Hybridizations were performed using the Genisphere Array350 kit (Genisphere, Hatfield, PA, USA). Hybridization conditions were the same as in Ralph *et al.* (2006), except that 40 µg total RNA were used for each channel and hybridizations were incubated at 60°C. Complete details of cDNA microarray fabrication and quality control will be described elsewhere (S.G.R. and co-workers, unpublished). All microarray experiments were designed to comply with MIAME guidelines (Brazma *et al.*, 2001). Raw and normalized data, as well as TIFF images, have been uploaded to the Gene Expression Omnibus under series accession number GSE8370. Sequences for array clones can be found by searching National Center for Biotechnology Information (NCBI) using the clone IDs given in Tables 2, 3 and Table S1 in Supplementary material.

Microarray analysis

Slides were scanned and spot intensity was quantified using IMAGENE software (BioDiscovery, Inc., El Segundo, CA, USA). To correct for background intensity, the lowest 10% of median foreground intensities was subtracted from the median foreground intensities. Data were then normalized by variance stabilizing normalization to compensate for nonlinearity of intensity distributions (Huber *et al.*, 2002) To identify significant changes in gene expression, a linear mixed-effects model was fitted to the normalized intensities in the Cy3 and Cy5 channels of the 32 microarray slides. The model contained an adjustment for dye bias, an array effect indicating which Cy5/Cy3 pair was on each array, a treatment effect indicating sample population and time point, and a random effect to adjust for repeated measures on the same biological sample (Kerr *et al.*, 2000). *P* values were computed for each gene-by-treatment effect and *Q* values

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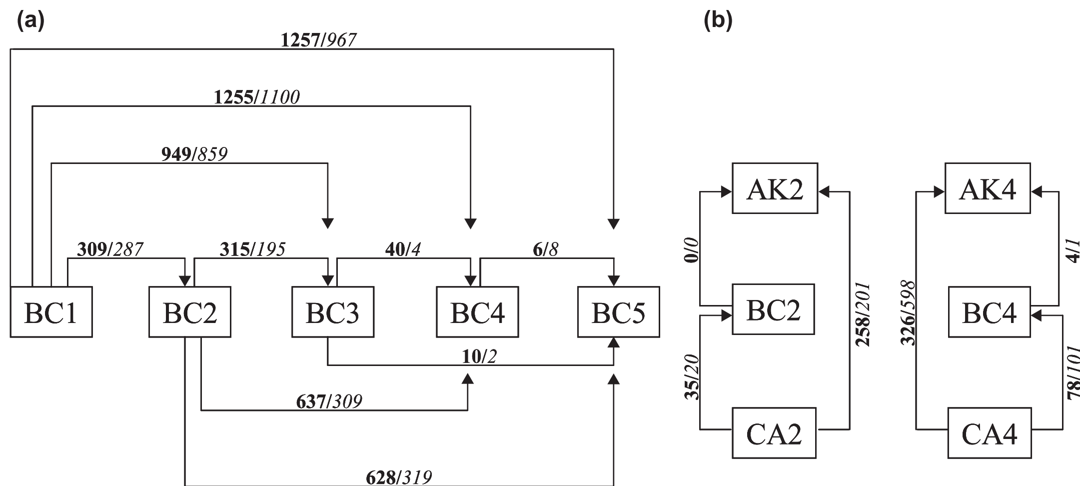

Fig. 2 Illustration of experimental design. Arrows indicate hybridizations that were made among (a) time points; (b) populations. Bold and italicized numbers indicate genes that were twofold up- and downregulated ($Q < 0.05$), respectively, between the time point at which the arrow originates and the time point at which it terminates.

were calculated to adjust for the false discovery rate (FDR) (Storey & Tibshirani, 2003). All the above statistical analyses of gene expression data were carried out using the R statistical package (http://www.r-project.org).

To identify themes in the time-series expression data, we used the CYTOSCAPE plug-in BiNGO to test for statistical overrepresentation of Gene Ontology (GO) terms within the GOSlim Plants ontology, among genes up- and downregulated twofold between the first and fifth time points in the BC population (Shannon *et al.*, 2003; Maere *et al.*, 2005). BiNGO uses a Fisher's exact test to compute the probability that the number of differentially expressed genes in each GO category could have occurred by chance, given the total number of genes on the microarray in that GO category. In our case, this involved comparing the nearest *Arabidopsis* homologs for all upregulated genes with all *Arabidopsis* homologs on the microarray. The FDR was used to correct for multiple testing, with a cutoff of 0.05.

Real-time PCR

To validate microarray expression data, real-time PCR was conducted for eight genes, chosen to represent a variety of biological functions and expression levels on the microarray. Genes chosen for validation of the array results include homologs to *EARLI1*, *GIGANTEA* (*GI*), *CAX1*, *PHYA*, *CBL2*, *LEA* and *MPK6*. Because of current interest in the role of *FLOWERING LOCUS T* (*FT*) in daylength-mediated growth-cessation responses in other species (see Discussion), we also used real-time PCR to determine the expression pattern of a spruce *FT*/*TFL1* homolog in our expressed sequence tag (EST) collection, although this transcript was not represented on the array. Gene expression was assayed across the five time points in the BC population. The same RNA pools (four individuals per pool per time point) were used as in the microarray component of the study. Before reverse transcription, 15 µg total RNA for each of the five BC time points was treated with DNaseI (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, to remove genomic DNA. RNA was then divided into three aliquots of 5 µg and cDNA synthesis was completed for each aliquot independently using Superscript II reverse transcriptase (Invitrogen) with an oligo dT_{12-18} primer. cDNA synthesis was assessed visually by gel electrophoresis before pooling of the three reactions.

Gene-specific primers were designed by aligning all BLAST matches in our EST collection with expect values (E-values) less than *E*-50 to the target sequence. Primers were then placed in non-conserved regions of each target sequence. Primer specificity was assessed by visual inspection on a 2% agarose gel (single product of expected length) and melting curve analysis. Primer sequences can be found in Table S2. Real-time PCR amplification conditions were identical to those described by Ralph *et al.* (2006), except that transcript abundance was normalized to translation initiation factor 5A (TIF5A, IS0013_F24, GenBank: DR448953) as its expression was invariant (data not shown).

Results

Cold hardiness

Highly significant ($P < 0.0001$) differences in cold hardiness across sampling dates within the central (BC) population (Table 1) were observed. Subfreezing temperatures were not observed at the study site until 27 November (Fig. 1b). However, both the central and northern populations (BC and AK) began developing cold hardiness well in advance of this date (Fig. 1c). This result agrees with the well established

Table 1 Results of ANOVA of index of injury (I_t) from freeze-testing of (a) the BC population on five test dates, and (b) the CA, BC and AK populations on October 18 and December 1

Source of variation	df	*F*	*P* > *F*
BC population only			
Date	4	**13.75**	**< 0.0001**
Temperature (date)	5	**2.66**	**0.0302**
Pool (date)	5	1.19	0.3257
Error	79		
CA, BC and AK populations			
Population	2	**80.43**	**< 0.0001**
Date	1	3.15	0.0799
Population × date	2	**6.03**	**0.0037**
Temperature (date)	2	**8.02**	**0.0007**
Population × temperature (date)	4	**3.69**	**0.0084**
Pool (population × date)	6	0.67	0.6722
Error	78		

role of night length in regulating phase I of cold acclimation. Cold hardiness increased steadily through our sampling period in northern populations, and wide among-population variation was observed. Population differences were highly significant ($P < 0.0001$) on both 18 October and 1 December (population $F = 22.28$ and 86.70, respectively; Table 1). On 18 October, the AK population had significantly higher hardiness (least-square mean $I_t = 20.3\%$ across test temperatures −10 and −14°C) than both BC and CA ($I_t = 57.4$ and 67.8%, respectively), while BC and CA did not differ significantly. By 1 December, all populations differed significantly, with least square mean I_t across test temperatures −15 and −25°C of 10.5% for AK, 36.8% for BC, and 78.4% for CA.

Temporal variation in gene expression within-population

Differentially expressed genes were selected using two criteria: fold-change for at least one contrast in our time-series of > 2.0×, and *Q* value < 0.05. To estimate the FDR, we calculated *Q* values (Storey & Tibshirani, 2003) and found, for example, the FDR for the BC5/BC1 contrast to be 5.6, 1.6 and 0.27% at $P = 0.05$, 0.01 and 0.001, respectively. Technical variance in our model generally exceeded biological variance, and although further biological replication may have been preferable, it would be unlikely to substantially enhance the accuracy of our gene expression estimates (Fig. S1). A complete list of all array elements with their annotations and associated gene-expression data is provided in Table S1. More extensive BLAST results from 26 public databases can be found in Table S4.

Similar numbers of genes were up- and downregulated twofold between the first (30 August) and fifth (13 December) time points in the central (BC) population. Out of 21 840 array elements, 1257 were upregulated and 967 downregulated (greater than twofold changes; $Q < 0.05$) (Fig. 2a). Many of these genes had no homology to *Arabidopsis*, including 549 of those upregulated and 387 of those downregulated. Expression patterns across the five BC time points show that transcripts for many genes began to increase by the second time point (Fig. 2a). Similarly, genes that were downregulated generally began to decrease between the first and second time points. Among 1257 genes twofold upregulated between the first and fifth time point, 309 were induced by the second time point (BC2/BC1), and 949 by the third (BC3/BC1). Few genes that were not activated early in the autumn were subsequently induced after the third or fourth time point (following subfreezing temperatures). In addition, most of the genes that were induced early plateaued in abundance by the fourth time point.

Ten 'GOSlim Plants' categories were overrepresented among genes upregulated in the BC population. These included, for example, 'response to stress' (45 genes), 'response to abiotic stimulus' (35 genes), 'membrane' (148 genes), and 'transport' (51 genes) (Fig. 3a; Table S3). Only three GO terms were overrepresented among genes twofold downregulated. These were 'plastid' (116 genes), 'thylakoid' (20 genes) and 'secondary metabolism' (19 genes) (Fig. 3b; Table S3).

Many of the induced genes were homologous to known or putative cold-response genes in herbaceous annuals. We categorized genes that were upregulated in our study into nine broad categories that reflect the most prevalent themes in the cold-acclimation literature. These are 'stress responsive', 'carbohydrate metabolism', 'lipid metabolism and cell wall-related', 'light signalling', 'calcium signalling', 'phytohormone signalling', 'transcriptional regulation', 'posttranscriptional regulation and protein turnover', and a 'miscellaneous' category for genes that did not easily fit into one of the above groups (Table 2). Stress-response genes were among the most strongly upregulated. These included many dehydrins and pathogenesis-related genes (e.g. chitinases, β-1,3-glucanases, thaumatin family); genes related to oxidative stress such as peroxidases and glutathione *S*-transferase; genes in the flavonoid/anthocyanin pathway (e.g. chalcone synthase, isoflavone reductase); and disease-responsive genes such as members of the dirigent, leucine-rich repeat and hevein families (Table 2; Table S1).

A number of carbohydrate metabolism genes were upregulated, including several involved in disaccharide synthesis (galactinol synthase, raffinose synthase, sucrose synthase, galactosyltransferase) and starch breakdown (α-amylase) (Table 2). Upregulated lipid metabolism genes included a sphingolipid desaturase, squalene synthase and lipid-transfer protein. The latter two were very strongly induced, with fold-increases of 16.9 and 34.4, respectively (BC5/BC1).

Signal transduction was one of the dominant themes among the genes upregulated in our study. It was unknown before we conducted this study whether signalling genes

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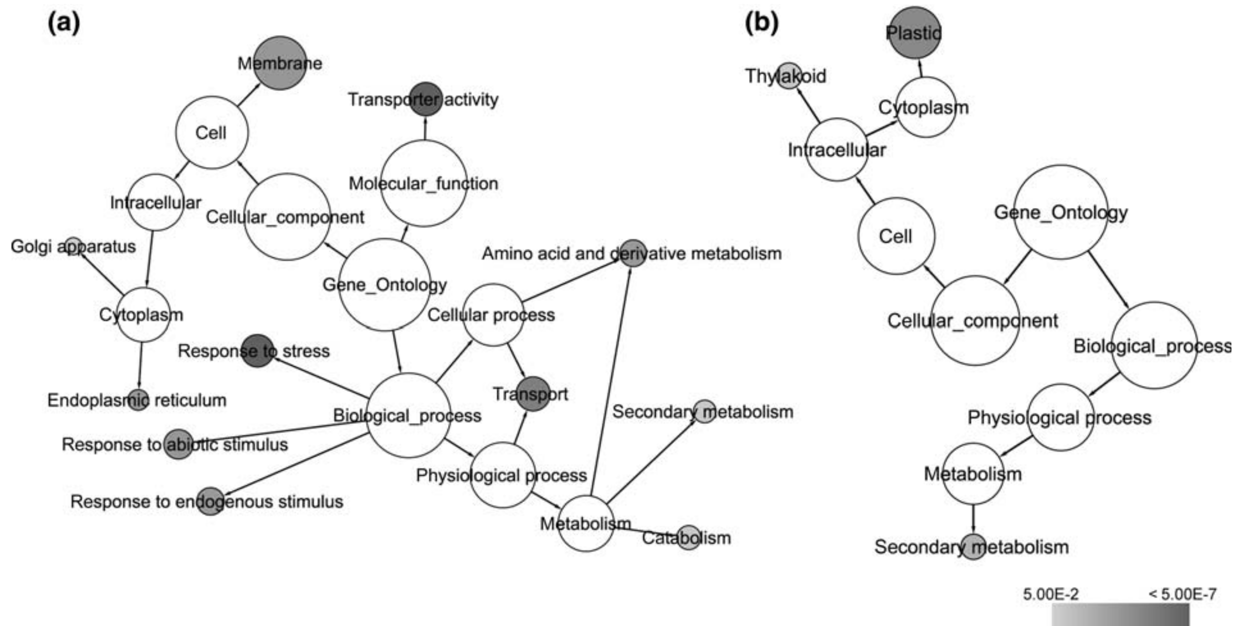

Fig. 3 Hierarchies of Gene Ontology (GO) terms statistically overrepresented (false discovery rate, FDR < 0.05) among (a) genes upregulated twofold between time points BC1 and BC5; (b) genes downregulated twofold between BC1 and BC5. Size of each circle indicates relative number of genes in each category; grey scale indicates FDR-adjusted *P* values. GO terms with no shading were not significant, and are included to illustrate the hierarchy for child terms that were significant.

upregulated in spruce during cold acclimation would parallel those identified so far in *Arabidopsis*. We observed generally small (1.5- to fivefold for BC5/BC1) but usually highly statistically significant increases in many signalling genes, some of which were homologous to genes with well defined roles in the cold-stress response of *Arabidopsis*. Others had less well understood functions. A small but significant increase in phytochrome A occurred between the first and second time points, whereas phytochrome B was downregulated across all sampling dates (Table 2; Table S1). *GIGANTEA*, a gene known to be downstream of phytochromes under certain environmental stimuli (e.g. photoperiodic flowering) (Huq *et al.*, 2000; Mizoguchi *et al.*, 2005), was upregulated fivefold. Our data also suggest a pivotal role for calcium signalling in spruce cold tolerance. Several genes involved in mediating the response to calcium were upregulated, including homologs to calmodulin 8, components of the calcineurin B-like (CBL) signalling module (CBL, CIPK), and the Ca^{2+}/H^+ antiporter *CAX1*. In addition, two annexin homologs and three C2 domain-containing genes were induced (Table 2; Table S1).

A large number of transcription factors (TF) were upregulated over the course of our study, including those belonging to bZIP, bHLH, leucine zipper, myb, *AP2*, CCAAT-box and NAC families (Table 2; Table S1). Although fold-change values were generally modest, associated *Q* values were typically very low. Targeted breakdown of cellular proteins and transcripts also appears to play a role in spruce cold acclimation. Several genes involved in ubiquitin-mediated protein degradation were induced, including members of the SCF complex (Kelch-repeat containing F-box, SKP1 interacting partner, COP10, Ring finger protein). A homolog to the RNA slicer Argonaute 1 (*AGO1*) was also moderately upregulated.

Among-population variation in gene expression

Among-population microarray hybridizations revealed substantial differential gene expression (Fig. 2b). Gene expression patterns corresponded to the genetic clines in cold hardiness-related traits among our study populations, with many genes upregulated in the northern (AK) and central (BC) compared with the southern (CA) population (Table 3; Fig. 2b). In the AK population, 326 genes were more than twofold more highly expressed than in the CA population at the fourth time point. These genes also tended to be more highly expressed in BC compared with CA, but generally did not differ significantly between AK and BC. Interestingly, the expression level for most of these 326 genes did not vary significantly in the CA population between the second and fourth time point (Table S1).

Many of the genes that were more highly expressed in the central and northern populations (BC and AK) relative to the southern CA population had annotations suggestive of involvement in cold hardiness. We grouped these genes in a similar fashion to the temporal component of our study, but collapsed some of the categories (e.g. 'carbohydrate metabolism' and 'lipid metabolism' together became 'metabolism') (Table 3). Examples of genes in the 'stress

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Table 2 Functional categorization of selected genes with > 2.0× fold-change for at least one contrast within the BC time series and $Q < 0.05$

Clone ID	BLASTX vs Arabidopsis	AGI code	*E* value	BLASTX vs NCBI (nonredundant)	NCBI accession	*E* value	Fold-change value			
							BC2/BC1	BC3/BC1	BC4/BC1	BC5/BC1
Stress-responsive										
WS0046_E20	Dehydrin	At4g38410	7.*E*-06	Dehydrin (*Picea abies*)	AAX92687	7.*E*-39	**7.6**	**21.1**	**20.6**	**25.6**
WS0047_B20	LEA	At1g01470	8.*E*-44	LEA (*Pseudotsuga menziesii*)	CAA10047	6.*E*-68	**8.7**	**20.6**	**20.9**	**17.7**
WS0097_C01	Hevein-like	At3g04720	1.*E*-49	Pathogenesis-related (*Capsicum chinense*)	BAD11073	1.*E*-51	*3.6*	**20.3**	**16.7**	**15.1**
WS0109_G23	ERD	At3g51250	9.*E*-10	Senescence-associated (*Hemerocallis* hybrid cultivar)	AAC34857	5.*E*-11	**3.4**	**9.5**	**12.9**	**14.9**
WS00930_N09	β-1,3-glucanase	At2g01630	3.*E*-58	Glucanase-like protein (*Thuja occidentalis*)	AAV66572	3.*E*-71	3.1	**8.2**	**13.2**	**13.9**
WS00923_I02	Osmotin	At4g11650	1.*E*-62	Thaumatin-like (*Pseudotsuga menziesii*)	AAQ84890	3.*E*-109	*3.4*	**6.9**	**13.9**	**10.8**
WS00924_G17	Glutathione *S*-transferase	At2g30860	3.*E*-58	Glutathione *S*-transferase (*Glycine max*)	AF243377	1.*E*-67	2.4	**6.4**	**10.8**	**9.5**
WS00715_B10	Thaumatin family	At1g19320	2.*E*-43	Thaumatin-like (*Pseudotsuga menziesii*)	AAV74248	2.*E*-63	*4.2*	**8.7**	**8.6**	**9.3**
WS00922_F20	Catalase	At4g35090	1.*E*-131	Catalase (*Zantedeschia aethiopica*)	AF207906	2.*E*-133	4.8	**7.0**	**7.3**	**7.9**
WS0024_K18	Chalcone synthase	At5g13930	1.*E*-12	Chalcone synthase (*Abies alba*)	ABD38596	2.*E*-17	2.0	**5.3**	**8.7**	**7.7**
WS0034_J13	RCI	At3g05880	1.*E*-12	Low temperature and salt responsive (*Solanum tuberosum*)	BAC23051	6.*E*-11	7.4	**9.0**	**10.0**	**7.3**
WS00922_B21	Basic chitinase	At3g12500	1.*E*-87	Endochitinase (*Musa acuminata*)	AF416677	6.*E*-94	2.1	**4.4**	**5.5**	**6.5**
WS00928_M02	Isoflavone reductase	At1g75290	5.*E*-36	Isoflavone reductase-like (*Cryptomeria japonica*)	AAK27264	6.*E*-42	**2.2**	**4.5**	**5.5**	**5.7**
WS00911_I09	Dirigent family	At1g64160	7.*E*-38	Dirigent-like protein (*Tsuga heterophylla*)	AF210071	2.*E*-69	**1.7**	**4.1**	**6.0**	**4.7**
WS0075_N02	Peroxidase	At1g71695	5.*E*-21	Peroxidase (*Picea abies*)	CAD92858	2.*E*-27	**2.4**	**3.3**	**3.4**	**4.2**
IS0014_D05	Glutathione peroxidase	At4g11600	6.*E*-57	Glutathione peroxidase-like (*Spinacia oleracea*)	O23814	8.*E*-58	**1.8**	**4.0**	**5.4**	**3.8**
WS00810_O07	Leucine-rich repeat	At1g15740	2.*E*-31	Leucine-rich repeat family (*Oryza sativa*)	NP_001047418	7.*E*-30	1.2	**2.4**	**2.4**	**3.1**
Carbohydrate metabolism										
WS00816_J13	Galactinol synthase	At1g60470	2.*E*-34	Galactinol synthase (*Ajuga reptans*)	CAB51533	1.*E*-32	**2.1**	**11.4**	**18.0**	**15.0**
WS0064_L12	Galactosyltransferase	At2g26100	5.*E*-44	β-1,3-glycosyltransferase-like (*Oryza sativa*)	CAD44839	1.*E*-36	**2.1**	**8.8**	**10.3**	**9.4**
WS0083_A08	Sugar transporter	At4g02050	7.*E*-27	Monosaccharid transporter (*Nicotiana tabacum*)	CAA47324	7.*E*-26	*1.7*	**4.9**	**6.9**	**5.5**
WS01028_M08	Sucrose synthase	At4g02280	2.*E*-86	Sucrose synthase (*Pinus taeda*)	ABR15470	5.*E*-102	*2.1*	**4.0**	**3.7**	**4.3**
WS00915_H03	Glycosyl hydrolase family 1	At1g26560	3.*E*-24	β-glucosidase (*Pinus contorta*)	AAC69619	7.*E*-28	1.4	**2.4**	**4.0**	**3.2**
WS01021_A02	Raffinose synthase	At5g20250	5.*E*-34	Raffinose synthase (*Oryza sativa*)	AAT77910	8.*E*-33	*1.4*	**2.3**	**3.1**	**2.7**
WS00917_K19	α-amylase	At1g69830	8.*E*-34	Plastid α-amylase (*Actinidia chinensis*)	AAX33233	9.*E*-32	**1.5**	**2.5**	**2.2**	**2.2**
Lipid metabolism and cell wall-related										
WS00939_C18	LTP (EARLI1)	At1g62500	9.*E*-26	Plant lipid transfer (*Medicago truncatula*)	ABO83639	5.*E*-27	3.0	**22.7**	**29.5**	**34.4**
WS0044_O08	PAL	At2g37040	8.*E*-24	PAL (*Equisetum arvense*)	AAW80639	8.*E*-48	**3.8**	**12.6**	**21.1**	**27.8**
WS01035_P02	Squalene synthase 1	At4g34640	2.*E*-17	Squalene synthase (*Panax notoginseng*)	ABA29019	4.*E*-19	1.6	**6.9**	**13.0**	**16.9**

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Table 2 *continued*

Clone ID	BLASTX vs Arabidopsis	AGI code	*E* value	BLASTX vs NCBI (nonredundant)	NCBI accession	*E* value	Fold-change value			
							BC2/BC1	BC3/BC1	BC4/BC1	BC5/BC1
WS00733_G19	COMT	At1g51990	4.*E*-10	*O*-methyltransferase, family 2 (*Medicago truncatula*)	ABD32718	2.*E*-10	**5.1**	**8.1**	**9.0**	**9.6**
WS00946_M24	Expansin	At4g38400	1.*E*-27	Expansin-like (*Solanum tuberosum*)	ABC47126	7.*E*-29	1.4	*2.6*	**3.8**	**5.8**
WS00930_N07	4-coumarate–CoA ligase	At3g21240	1.*E*-78	4-coumarate–CoA ligase (*Pinus taeda*)	P41636	1.*E*-100	1.7	**2.9**	**4.0**	**4.2**
WS00824_K23	δ-8 sphingolipid desaturase	At2g46210	4.*E*-07	Fatty acid desaturase (*Marchantia polymorpha*)	AAT85663	1.*E*-58	**1.9**	**3.3**	**3.2**	**3.9**
Calcium signalling										
WS0079_I12	CAX1	At5g01490	9.*E*-07	Ca^{2+}/H^+ exchanger (*Vigna radiata*)	BAA25753	4.*E*-05	1.5	**17.6**	4.6	**14.8**
WS01016_C12	Calcium-binding EF hand	At1g53210	6.*E*-46	Drought-induced protein (*Oryza sativa*)	BAD19956	2.*E*-46	**3.0**	**4.1**	**5.1**	**4.3**
WS00824_I18	C2 domain-containing	At5g11100	6.*E*-24	Ca^{2+}-dependent lipid-binding protein (*Oryza sativa*)	NP_001061492	4.*E*-25	**1.5**	**4.0**	**5.1**	**3.9**
WS00818_N13	Annexin	At5g10220	8.*E*-49	Annexin (*Zea mays*)	CAA66901	4.*E*-49	2.3	**3.0**	**4.6**	**3.8**
WS0055_B09	Calmodulin	At3g22930	2.*E*-39	Calmodulin (*Cryptomeria japonica*)	BAF31994	7.*E*-38	1.6	**1.8**	**2.6**	**3.1**
WS00925_D16	CBL	At5g55990	4.*E*-67	CBL (*Populus trichocarpa*)	ABO43662	6.*E*-66	0.9	1.7	1.9	**2.4**
WS00819_N13	CIPK	At1g30270	2.*E*-50	CIPK (*Populus trichocarpa*)	ABJ91220	3.*E*-50	1.3	**2.2**	**2.2**	**2.1**
Phytohormone signalling										
WS0074_E17	Dormancy/auxin-associated	At1g28330	8.*E*-15	Auxin-repressed protein (*Manihot esculenta*)	AAX84677	6.*E*-16	4.0	**15.9**	**12.2**	**13.4**
WS02610_F23	Auxin-downregulated	At3g22850	1.*E*-15	Hypothetical protein (*Vitis vinifera*)	CAN71784	1.*E*-16	**2.6**	**9.7**	**9.2**	**7.6**
WS00733_N14	Auxin efflux carrier	At5g01990	9.*E*-62	Auxin efflux carrier (*Oryza sativa*)	BAD73344	6.*E*-56	**2.8**	**5.6**	**7.6**	**6.5**
WS01011_O03	Auxin-responsive	At5g35735	1.*E*-22	Auxin-induced (*Oryza sativa*)	BAC75413	3.*E*-25	**2.3**	**3.1**	**4.7**	**4.4**
WS00937_G05	Auxin-responsive	At5g54510	9.*E*-84	Auxin-induced (*Pinus pinaster*)	CAJ14972	7.*E*-83	1.4	**4.1**	**3.2**	**4.4**
WS00918_P16	Gibberellin-response modulator	At1g14920	2.*E*-21	Gibberellic acid-insensitive protein (*Vitis vinifera*)	Q8S4W7	3.*E*-22	1.3	**4.4**	**3.4**	**3.6**
Transcription regulation										
WS00811_F18	NAM (NAC domain)	At1g52890	7.*E*-11	NAM protein (*Oryza sativa*)	NP_001049997	5.*E*-09	**4.8**	**12.2**	**13.2**	**15.0**
WS00925_L16	ERF	At1g50640	2.*E*-19	ERF (*Nicotiana tabacum*)	BAA76734	8.*E*-18	*1.9*	**5.8**	**12.8**	**9.0**
WS00728_K02	MYB family	At5g47390	5.*E*-20	MYB transcription factor (*Glycine max*)	ABH02845	2.*E*-20	*1.9*	**6.1**	**6.6**	**5.8**
WS00917_I23	AREB	At3g56850	4.*E*-24	AREB (*Oryza sativa*)	AAT77290	6.*E*-22	**1.9**	**2.6**	**2.7**	**2.7**
Posttranscriptional regulation and protein turnover										
WS00930_P10	Serine carboxypeptidase	At1g15000	3.*E*-18	Serine carboxypeptidase (*Oryza sativa*)	BAA94235	2.*E*-16	1.2	**10.1**	**22.4**	**14.0**
WS0076_I24	C3HC4-type zinc finger	At1g72310	4.*E*-23	RING-H2 (*Populus alba* × *Populus tremula*)	AAW33880	3.*E*-19	**1.9**	**2.1**	**2.9**	**3.1**
WS00716_J15	Ubiquitin-conjugating enzyme	At1g45050	7.*E*-08	Ubiquitin-conjugating enzyme (*Medicago truncatula*)	ABE89644	4.*E*-06	1.3	**2.0**	**1.6**	**2.8**

Table 2 *continued*

Clone ID	BLASTX vs Arabidopsis	AGI code	*E* value	BLASTX vs NCBI (nonredundant)	NCBI accession	*E* value	Fold-change value			
							BC2/BC1	BC3/BC1	BC4/BC1	BC5/BC1
WS00112_G14	Aspartyl protease family	At1g62290	1.*E*-37	Aspartic protease (*Oryza sativa*)	BAA06876	9.*E*-38	**1.5**	**2.0**	**1.8**	**2.1**
WS0091_H14	Kelch repeat-containing	At1g15670	4.*E*-23	Kelch repeat-containing F-box-like (*Oryza sativa*)	BAD25000	2.*E*-28	0.9	**1.9**	**1.9**	**2.0**
WS0084_C03	Argonaute (AGO1)	At1g31280	1.*E*-15	Piwi domain-containing (*Oryza sativa*)	AAS01930	1.*E*-14	*1.3*	**1.7**	**1.6**	**2.0**
Miscellaneous										
WS01012_F14	AAA-type ATPase	At4g28000	2.*E*-15	AAA ATPase (*Medicago truncatula*)	ABE83074	1.*E*-11	*2.1*	**12.6**	**15.8**	**30.6**
WS0063_I19	Phospholipase A2	At2g06925	5.*E*-15	Phospholipase A2 (*Nicotiana tabacum*)	BAD90927	3.*E*-15	*2.3*	**11.6**	**13.6**	**11.5**
WS0094_H16	ABC transporter	At1g67940	3.*E*-38	Multidrug resistance protein (*Cicer arietinum*)	BAA76420	2.*E*-34	1.9	**8.0**	**14.2**	**10.6**
WS0106_F09	Glutamate receptor family	At2g17260	5.*E*-07	Ligand gated channel-like (*Brassica napus*)	AF109392	7.*E*-05	1.7	**5.8**	**5.9**	**4.8**
WS0033_C24	Gigantea	At1g22770	3.*E*-11	Putative gigantea (*Picea abies*)	CAK26425	5.*E*-44	**2.7**	**4.1**	**4.3**	**4.9**
WS01039_D02	MAP kinase 6 (MPK6)	At2g43790	3.*E*-14	MAP kinase 2 (*Glycine max*)	AF329506	6.*E*-13	*1.7*	**2.5**	**2.4**	**2.8**
WS0021_H24	FK506-binding protein 15	At3g25220	2.*E*-56	Putative immunophilin (*Hordeum vulgare*)	CAD42633	2.*E*-55	**1.6**	**1.9**	**2.1**	**2.1**
WS0101_F05	Phytochrome A	At1g09570	2.*E*-09	Phytochrome N (*Pinus sylvestris*)	CAC11136	3.*E*-18	**2.2**	**1.6**	**1.4**	*1.3*
No hit										
WS00914_D20	No significant hit	N/A	N/A	N/A	N/A	N/A	**5.2**	**38.8**	**37.0**	**28.9**
WS0102_I01	No significant hit	N/A	N/A	N/A	N/A	N/A	**3.3**	**22.4**	**31.4**	**26.2**
WS00926_F21	No significant hit	N/A	N/A	Cold acclimation protein Picg6 (*Picea glauca*)	AAO63476	5E-04	**8.9**	**27.2**	**28.4**	**25.5**
WS0107_M21	No significant hit	N/A	N/A	N/A	N/A	N/A	**9.3**	**20.2**	**26.3**	**24.5**
WS0041_B09	No significant hit	N/A	N/A	N/A	N/A	N/A	**4.9**	**16.5**	**16.5**	**22.4**

Fold-change values are given as ratios of the second to fifth time points (BC2, 18 October; BC3, 22 November; BC4, 1 December; BC5, 13 December) to time point 1 (BC1, 30 August). Fold-change values in italics have $Q < 0.05$; those in bold $Q < 0.01$; all other fold-change values are not significant for the given contrast. Annotations are given for translated BLAST vs *Arabidopsis thaliana* and vs the nonredundant collection at the NCBI. In the case of the latter, the organism for each hit is given in parentheses.
ERD, early response to dehydrative stress; RCI, rare cold-inducible; NAM, no apical meristem; ERF, ethylene-response factor; AREB, ABA-response element-binding factor; COMT, caffeic acid *O*-methyltransferase; LTP, lipid transfer protein; PAL, phenylalanine ammonia lyase; CBL, calcineurin B-like protein; CIPK, CBL-interacting protein kinase; ABC, ATP-binding cassette; LEA, late embryogenesis abundant.

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Table 3 Functional categorization of selected genes with AK4/CA4 > 2.0 and *Q* < 0.05; untransformed fold-change values are given as ratios of northern (AK and BC) to southern (CA) population at the second (18 October 2004) and fourth (1 December 2004) time points

Clone ID	BLASTX vs *Arabidopsis*	AGI code	*E* value	BLASTX vs NCBI (nonredundant)	Accession number	*E* value	Fold-change value			
							AK2/CA2	AK4/CA4	BC2/CA2	BC4/CA4
Stress-responsive										
WS00923_A21	Terpene synthase/cyclase	At3g14490	5.*E*-10	(-)-limonene synthase (*Picea abies*)	AAS47694	3.*E*-66	*6.8*	**10.5**	4.4	*7.9*
WS00922_F20	Catalase 2	At4g35090	1.*E*-131	Catalase (*Prunus persica*)	CAD42909	5.*E*-132	**7.4**	**7.9**	*5.3*	*8.0*
WS0109_G23	ERD	At3g51250	8.*E*-10	Senescence-associated (*Medicago truncatula*)	ABE77914	6.*E*-09	**7.3**	**3.7**	**4.2**	*2.6*
WS0046_E20	Dehydrin	At4g38410	6.*E*-06	Dehydrin 1 (*Picea abies*)	AAX92687	7.*E*-39	**9.2**	*2.7*	**7.4**	2.2
WS00935_H19	Heat-shock protein	At1g56410	1.*E*-29	Cytosolic heat-shock 70 protein (*Spinacia oleracea*)	AAB88132	2.*E*-28	*2.2*	*2.2*	1.8	2.1
WS00811_K09	Leucine-rich repeat family	At1g80630	1.*E*-15	Cyclin-like F-box (*Medicago truncatula*)	ABE81084	3.*E*-11	**3.2**	**2.2**	2.0	*1.8*
Metabolism										
WS00931_D24	Lipase class 3	At3g14360	3.*E*-48	Lipase (*Ricinus communis*)	AAV66577	2.*E*-52	1.9	**11.1**	0.9	1.0
WS0064_L12	Galactosyltransferase family	At2g26100	4.*E*-44	Putative galactosyltransferease (*Hordeum vulgare*)	ABL11234	1.*E*-37	*2.3*	**3.4**	1.6	*3.0*
WS00928_N23	UDP-glucosyl transferase	At2g36970	6.*E*-39	UDP-glucuronosyl/UDP-glucosyltransferase (*Medicago truncatula*)	ABE90468	2.*E*-38	**3.7**	**2.9**	1.6	1.9
WS00733_G19	COMT	At1g51990	4.*E*-10	*O*-methyltransferase, family 2 (*Medicago truncatula*)	ABD32718	2.*E*-10	*3.4*	*2.6*	2.1	2.4
WS0099_O10	Malate oxidoreductase	At2g13560	5.*E*-40	Putative malate dehydrogenase (*Oryza sativa*)	NP_001059700	6.*E*-42	**3.3**	*2.2*	*2.8*	2.1
WS0044_O07	Family II lipases	At1g71120	3.*E*-13	Lipolytic enzyme (*Medicago truncatula*)	ABO83318	2.*E*-11	*2.0*	**2.1**	1.0	1.6
Signal transduction										
WS0106_F09	Glutamate receptor family	At2g17260	5.*E*-07	Ligand gated channel-like protein (*Brassica napus*)	AF109392_1	7.*E*-05	2.4	**5.0**	1.6	2.3
WS0063_I19	Phospholipase A2	At2g06925	5.*E*-15	Phospholipase A2 (*Nicotiana tabacum*)	BAD90927	3.*E*-15	*3.1*	*3.3*	1.6	*3.4*
IS0012_C06	Diacylglycerol acyltransferase	At3g51520	6.*E*-57	Diacylglycerol acyltransferase (*Medicago truncatula*)	ABO83262	1.*E*-50	*2.0*	**2.7**	1.3	1.7
WS00928_I04	AMP-dependent synthetase	At3g16910	2.*E*-82	AMP-binding enzyme (*Oryza sativa*)	ABF95517	3.*E*-80	**2.9**	**2.5**	1.4	*1.7*
WS0086_C05	Calcineurin B-like protein	At2g31800	2.*E*-11	Ankyrin protein kinase (*Brassica napus*)	AAT94403	2.*E*-09	*1.6*	**2.3**	1.1	*1.7*

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Table 3 *continued*

Clone ID	BLASTX vs *Arabidopsis*	AGI code	*E* value	BLASTX vs NCBI (nonredundant)	Accession number	*E* value	Fold-change value			
							AK2/CA2	AK4/CA4	BC2/CA2	BC4/CA4
Transcriptional regulation and protein turnover										
WS0101_P19	Serine carboxypeptidase	At5g08260	7.*E*-11	Putative carboxypeptidase D (*Oryza sativa*)	BAD25095	4.*E*-07	**5.3**	**4.7**	2.6	*3.2*
WS00112_G14	Aspartyl protease family	At1g62290	1.*E*-37	Aspartic protease (*Oryza sativa*)	BAA06876	9.*E*-38	**1.7**	**2.4**	1.5	*1.7*
WS0109_C08	Metalloprotease	At1g17870	7.*E*-71	Hypothetical protein (*Vitis vinifera*)	CAN73523	2.*E*-72	**2.4**	**2.3**	1.5	1.7
WS00933_K20	Metallo-β-lactamase family	At4g33540	2.*E*-11	β-lactamase-like (*Anabaena variabilis*)	YP_321165	7.*E*-12	**2.2**	**2.2**	2.0	*1.9*
Miscellaneous										
WS01011_B24	ACT domain-containing	At1g12420	1.*E*-23	Amino acid-binding ACT (*Medicago truncatula*)	ABE90521	3.*E*-27	1.6	**9.1**	1.3	**3.8**
WS01012_F14	AAA-type ATPase	At4g28000	2.*E*-15	AAA ATPase, central region (*Medicago truncatula*)	ABE83074	1.*E*-11	**8.1**	**6.4**	3.0	*4.7*
WS00946_G15	PPR	At3g02650	8.*E*-44	PPR repeat-containing protein-like (*Oryza sativa*)	BAC99540	2.*E*-41	3.6	**6.2**	2.0	1.2
WS00937_G05	Auxin-responsive	At5g54510	9.*E*-84	Auxin-induced (*Pinus pinaster*)	CAJ14972	7.*E*-83	*2.9*	**4.2**	1.5	2.1
WS0078_P14	Lateral organ boundaries domain	At2g28500	6.*E*-14	LOB domain protein 1, putative (*Solanum demissum*)	AAT40528	4.*E*-13	*2.6*	**3.6**	1.8	2.2
WS00922_D24	Acyl-CoA binding protein	At5g53470	2.*E*-33	Membrane acyl-CoA binding protein (*Agave americana*)	AAT81164	9.*E*-33	**3.5**	**3.0**	1.8	*2.1*
WS0071_O08	ABA-responsive	At5g13200	2.*E*-51	ABA-responsive (*Oryza sativa*)	ABA98234	5.*E*-46	**4.0**	*2.7*	2.1	1.8
WS0101_F10	Cytochrome P_{450} family	At5g36110	1.*E*-55	Cytochrome P_{450} (*Medicago truncatula*)	ABC59076	9.*E*-63	*2.2*	**2.5**	1.2	*2.3*
No hit										
WS00923_F16	No significant hit	N/A	N/A	N/A	N/A	N/A	11.9	*13.6*	1.1	1.8
WS0107_L16	No significant hit	N/A	N/A	N/A	N/A	N/A	*5.5*	**21.3**	1.2	1.8
WS00927_G12	No significant hit	N/A	N/A	Late embryogenesis abundant protein (*Picea glauca*)	AAB01550	2.*E*-44	2.4	**12.8**	1.3	*4.7*

Fold-change values given in italics have associated Q value < 0.05; those in bold Q < 0.01; all other fold-change values are not significant for the given contrast.
ERD, early response to dehydrative stress; TPR, tetratricopeptide repeat; PPR, pentatricopeptide repeat; COMT, caffeic acid *O*-methyltransferase.

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responsive' category that varied among populations include an early response to dehydrative stress (*ERD*), a dehydrin and a catalase. Several carbohydrate and lipid metabolism genes were also differentially expressed among populations, including a galactosyltransferase, a UDP-glucosyl transferase and a lipase. Among the genes involved in signal transduction and transcriptional regulation that were differentially expressed among populations were a CBL-like protein, calmodulin-binding protein and a phospholipase A2.

Real-time PCR validation of microarray results

The genes chosen for microarray expression validation using real-time PCR (*CAX1*, *EARLI1*, *CBL2*, *PHYA*, *GI*, *MPK6*, *LEA*) generally had patterns of expression consistent with the microarray results (Fig. 4). All these genes were upregulated between the first and subsequent time points, and whereas some peaked in expression early (e.g. *MPK6*), others reached maximum expression late (e.g. *EARLI1*). One particularly interesting gene was *CAX1*, which, according to the microarray results, peaked in expression at the third and fifth time points with a decrease in relative expression at the fourth time point (Table 2). Although the decrease from time point three to four was not significant (Table S1), our real-time data revealed the same pattern, thus this cyclical expression does not appear to be an artefact. Real-time PCR measurements also suggest that a gene in our EST collection similar to *FLOWERING LOCUS T*/*TERMINAL FLOWER 1* (*FT*/*TFL1*) was downregulated strongly between BC1 and subsequent time points.

Discussion

Cold hardiness

Phenotypic measurements of cold hardiness throughout our sampling period reinforce the importance of night length in determining the onset of cold acclimation in both BC and AK populations, but suggest that the CA population does not respond to this cue, despite experiencing the shortest daylength of its native environment on 20 November in Vancouver. Conversely, the AK population may not have a low-temperature requirement for entry to deep (phase II) cold acclimation, as this population achieved maximum cold hardiness well before the first frost. Measurements of cold injury taken at −25°C in the AK population showed similar and minimal levels of injury on 22 November, 1 December and 13 December (data not shown).

Temporal within-population gene expression during cold acclimation

The winter period is one of bud dormancy and suspension of vegetative growth. Cold acclimation is inextricably linked to the concomitant processes of growth cessation and bud dormancy induction, and the development of cold hardiness depends crucially on being preceded by bud set. Although this makes it difficult or impossible to separate autumn gene expression changes related to these three processes in this study, the candidate genes we have identified, whether they are involved directly in cold hardiness, or indirectly through the establishment of the dormant state, are nevertheless relevant cold hardiness-related candidate genes.

Whereas repression of a range of cellular processes accompanies the period of cold acclimation and dormancy induction, low-temperature stress necessitates induction of a complex suite of traits. These cold-adaptive processes require a large investment of resources for gene expression and metabolic remodelling during a period when energy production via photosynthesis is decreasing (Clapham *et al.*, 2001; Oquist *et al.*, 2001). As a result, not only would it be inefficient to continue expressing genes for processes such as growth and reproduction, the limited energy available means that downregulation of these genes may be crucial for survival. Results of our microarray study through the period of cold acclimation demonstrate this redirection of resources. Gene Ontology categories statistically overrepresented among differentially expressed genes in our time series reveal that metabolic remodelling and stress response are dominant themes among upregulated genes (Fig. 3a). Interestingly, both 'transport' and 'transporter activity' were overrepresented, suggesting that subcellular protein and metabolite targeting are important components of cold acclimation. By contrast, the downregulated autumn transcriptome was enriched in genes localized to the chloroplast and thylakoid, suggesting a redirection of resources away from photosynthesis. (Fig. 3b). Although most upregulated genes were induced early in our time course, many were not significantly upregulated until the third time point (BC3) (Fig. 2a; Table 2). Because the seedlings experienced chilling (> 0°C but < 5°C) temperatures after the second time point but before the third (Fig. 1b), a role for low, above-freezing temperatures in enhancing night length-mediated transcription is possible. This pattern would also be congruent with positive feedback on expression of these genes following the initial signal (long nights). Separation of these environmental cues will require further study in controlled environments, and this work is in progress. By contrast, we did not observe substantial changes in gene expression following the first subfreezing temperatures, which occurred between time points three and four, suggesting that the subfreezing temperature cue for phase II of cold acclimation is not manifested at the level of large-scale gene expression, and may trigger a more subtle response.

Stress-response genes induced during cold acclimation

Plant stress-response pathways often share both signalling components and response genes (Xiong *et al.*, 2002). In some cases, apparently disparate stressors involve common

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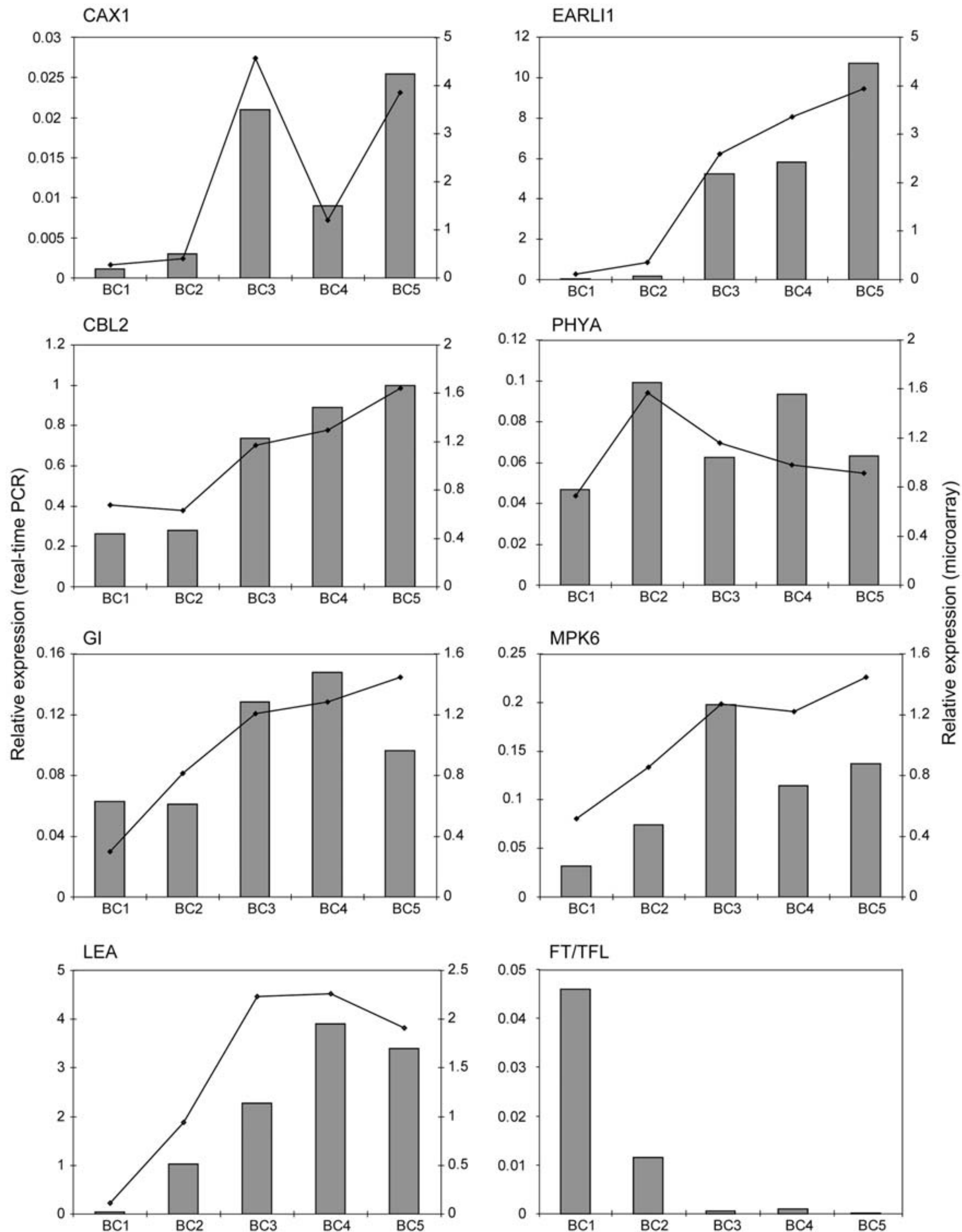

Fig. 4 Real-time PCR analysis of gene expression among five time points in the BC population for *CAX1* (WS0079_I12), *EARLI1* (WS00939_C18), *CBL2* (WS00925_D16), *PHYA* (WS01021_F05), *GIGANTEA* (WS0033_C24), *MPK6* (WS01039_DO2), *LEA* (WS0047_B20), *FT/TFL* (WS02738_CO3). Bars indicate real-time PCR expression measurements (relative to the housekeeping gene *TIF5A*); lines indicate corresponding microarray data (given as natural log difference from mean of the five BC time points). Note that because *FT/TFL1* was not represented on the microarray, it has only real-time PCR expression measurements.

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mechanisms of damage. For example, ice propagation in the apoplast draws water out of the cell, and in this way mimics dehydration stress (Zwiazek *et al.*, 2001). Dehydrins are one class of genes that respond to both these stresses. It is thought that dehydrins function as chaperones to stabilize cellular macromolecules and prevent their coagulation as water leaves the cell (Close, 1997). In addition, *in vitro* studies suggest a role for dehydrins in stabilizing cellular membranes (Koag *et al.*, 2003). Putative dehydrins were strongly upregulated (up to 30-fold) over the course of this study (Table 2; Table S1). Several genes with less-understood roles in the dehydration response were also induced, including an early response to the dehydration (*ERD*) gene and members of the osmotin family.

Whereas dehydrins are thought to function as intracellular molecular chaperones as water is drawn from the cell, apoplastic antifreeze proteins limit extracellular ice propagation by adhering to the surface of nascent ice crystals and ice nucleators. Plant antifreeze proteins are homologous to three classes of pathogenesis-related protein (chitinases, β-1,3-glucanases and the thaumatin family; Hon *et al.*, 1995), and our expression results revealed candidate antifreeze genes in each of these categories (Table 2). Many of these genes were induced strongly by the second time point in the BC populations, indicating that they are probably regulated by photoperiod. In order to verify antifreeze activity, *in vitro* assays must be performed, as it is not known what sequence motifs, if any, differentiate antifreeze genes from pathogenesis-related genes (Hiilovaara-Teijo *et al.*, 1999; Griffith & Yaish, 2004).

Generation of radical oxygen species is another stress brought on by low temperatures, and damage to nonacclimated plants can be profound (e.g. degradation of lipid membranes) (Kendall & McKersie, 1989; McKersie, 1991). We observed substantial allocation of resources to transcription of genes that protect against oxidative damage. Upregulated genes include peroxidases, catalases, glutathione peroxidases and glutathione *S*-transferases (Table 2; Table S1). In addition, several genes involved in flavonoid/anthocyanin biosynthesis were upregulated (e.g. chalcone synthase, isoflavone reductase). Induction of the flavonoid biosynthetic pathway could result in production of flavonoids that scavenge radical oxygen species, or of anthocyanins that block their generation.

Carbohydrate and lipid-metabolism genes induced during cold acclimation

Many studies have addressed changes in carbohydrate composition in conifers during the autumn, and point to an increase in nonreducing disaccharides such as sucrose and raffinose (Pomeroy *et al.*, 1970), and breakdown of polysaccharides, particularly starch (Alscher *et al.*, 1989). Several explanations for these changes have been proposed. First, release of energy from starch could compensate for decreased photosynthetic activity; second, an increase in disaccharides provides free hydroxyl groups to replace hydrogen bonds with cellular macromolecules lost upon cellular dehydration; third, increasing intracellular solutes compensates for the osmotic potential generated across the plasma membrane upon extracellular ice formation; and finally, increased osmolarity in the cytoplasm depresses the intracellular freezing point (Zwiazek *et al.*, 2001). We observed a fivefold increase in galactinol synthase, which catalyses the first step in raffinose synthesis, and a 2.7-fold increase in raffinose synthase itself (Table 2). Several clones with high homology to both sucrose synthase and α-amylase were also upregulated.

Cellular membranes are the primary site of freezing injury (Levitt, 1980; Ziegler & Kandler, 1980), and dehydration-related damage is in part a function of lipid composition (Uemura & Steponkus, 1994). The ratio of saturated to unsaturated membrane lipids decreases during acclimation (Lynch & Steponkus, 1987; Uemura *et al.*, 1995). Genes that may be involved in membrane remodelling were upregulated during our time course, including a delta-8 sphingolipid desaturase and several fatty acid synthases (Table 2). A lipid transfer protein similar to the vernalization-responsive *Arabidopsis* gene *EARLI1* showed a 34-fold increase (Wilkosz & Schlappi, 2000). In addition, we observed a 17-fold increase in squalene synthase 1. This result is interesting in light of a study of winter rye and spring oat (*Avena sativa*), which showed the former to have nearly fourfold more free sterols in the plasma membrane than the latter (Uemura & Steponkus, 1994).

Signal transduction genes induced during cold acclimation

Phytochromes are thought to be the primary regulators of night length-mediated bud set and initiation of autumn cold acclimation in perennials (Howe *et al.*, 1996; Horvath *et al.*, 2003), Interestingly, phytochromes have also been implicated recently in upstream regulation of the cold-stress machinery in *Arabidopsis*, suggesting a link between annual and perennial cold acclimation (Benedict *et al.*, 2006). This role has been demonstrated directly in hybrid aspen trees (*Populus tremula* × *tremuloides*), wherein overexpression of *PHYA* blocked growth cessation and cold acclimation under short days (Olsen *et al.*, 1997). Three features on our microarray are phytochrome homologs, two belonging to the *PHYA* subfamily and one to the PHYB subfamily (Table 2; Table S1). We noted a transient increase of one of the *PHYA*-like genes, with an expression peak on the second time point and subsequent decline. The second *PHYA*-like gene and the *PHYB*-like gene were both downregulated across all five time points (Table S1). A homolog to *GI* was also upregulated fivefold during our study. Recently shown to be involved in the cold-stress response in *Arabidopsis* (Cao *et al.*, 2005), *GI* is better known as a key component of the photoperiodic flowering pathway (Mizoguchi *et al.*, 2005). In the latter role, *GI* coordinates circadian expression of *CONSTANS* (*CO*) and *FT* (Mizoguchi *et al.*, 2005). The upregulation of *GI* during cold acclimation

in spruce suggests that it is uncoupled from the *FT*/*CO* regulon, particularly in light of a recent study which showed that downregulation of *FT* is necessary for seasonal growth cessation in hybrid aspen (Bohlenius *et al*., 2006). In contrast to this observed upregulation of *GI*, an *FT*/*TFL1* homolog identified in our EST collection was found to be strongly downregulated between time points one and five (see Real-time PCR). This sustained downregulation of *FT*/*TFL1* may reflect the latest stages of *FT* downregulation in response to critical night length (growth cessation), or it may suggest a specific role for *FT* in cold acclimation in conifers. In contrast to these results, Gyllenstrand *et al.* (2007) found a putative Norway spruce (*Picea abies*) *FT* homolog to be upregulated by short days. Further study is needed to elucidate the role of this gene in growth cessation and possibly cold acclimation processes in conifers.

Elevation of cytosolic calcium is a first step in cold signalling in herbaceous annuals (Xiong *et al*., 2002). It has even been suggested that membrane calcium channels could be the elusive temperature sensor, as calcium influx occurs very early in the cold response, and changes in membrane fluidity as a function of decreased temperature could trigger these channels (Plieth *et al*., 1999; Xiong *et al*., 2002). As calcium plays a pivotal role in numerous other signalling cascades, and because multiple calcium transients may be necessary for cold signalling itself (Xiong *et al*., 2002), restoration of resting calcium levels following the initial signal is crucial. *CAX1*, a vacuolar Ca^{2+}/H^{+} antiporter, performs this function in *Arabidopsis* (Catala *et al*., 2003). Upregulation of *CAX1* following the initial calcium influx results in attenuation of the cold-signalling machinery by downregulation of genes in the C-repeat binding factor/dehydration-responsive element-binding factor (CBF/DREB) pathway (Catala *et al*., 2003). Our data reveal periodic expression of a *CAX1* homolog, with expression peaks occurring at the third and fifth time points, and reduced expression at the fourth time point. This pattern would be congruent with a role for *CAX1* in regulating both night length and freezing temperature-induced cold acclimation. Many calcium-binding proteins were similarly induced over the BC time course, including calcineurin B-like (CBL) genes and CBL-interacting protein kinases (CIPKs) (Table 2). CBL proteins interact specifically with CIPKs upon calcium influx (Shi *et al*., 1999), and in *Arabidopsis* the CBL/CIPK-signalling module is a crucial component of cold-stress signalling (Albrecht *et al*., 2003; Cheong *et al*., 2003). Other calcium-related genes upregulated in our study include a MAP kinase (*MPK6*), several annexins, a C2-domain-containing gene, and two calmodulin genes.

Transcriptional regulation of gene expression during cold acclimation

Transcription factors are among the most studied cold-responsive genes. The CBF/DREB subfamily of *APETELA* (*AP2*) domain transcription factors, and their upstream regulator, *ICE1*, are essential for acclimation to low temperature in *Arabidopsis* (Stockinger *et al*., 1997; Jaglo-Ottosen *et al*., 1998; Chinnusamy *et al*., 2003). Although no CBF/DREB annotated transcription factors were induced over the course of our study at the twofold threshold, several *AP2* domain-containing genes were upregulated, including ethylene response element binding factors (*ERF*) (Table 2; Table S1). The role of ethylene in perennial cold acclimation has not been well studied, but in winter rye, antifreeze proteins accumulate in response to ethylene treatment (Yu *et al*., 2001). We observed significant upregulation of four *ERF*s, one of which increased ninefold between our first and fifth time points. Upregulation of *ERF*-like genes have also been observed during ecodormancy in leafy spurge (*Euphorbia esula*) (Horvath *et al*., 2006). Because the transcription factors regulating antifreeze gene expression have not been established, it will be interesting to investigate the possible role of these ERFs in regulating expression of spruce antifreeze genes.

Protein turnover and posttranscriptional gene silencing during cold acclimation

Molecular genetic studies of cold acclimation typically focus on genes that are induced. However, upon cold treatment, gene expression related to a range of cellular processes is repressed (Fowler & Thomashow, 2002), presumably in part because resources normally allocated to expression of those traits are needed to mount the stress response. In addition, targeted breakdown of cellular proteins that inhibit cold signalling under normal growing conditions may be important to the integration of abiotic cues governing cold acclimation. Indeed, expression of ubiquitin-conjugating enzymes is a crucial component of cold acclimation in *Arabidopsis* (Schwechheimer *et al*., 2002; Yan *et al*., 2003), and we observed induction of several such genes (Table 2; Table S1). We also noted upregulation of Argonaute 1 (*AGO1*), which directs posttranscriptional gene silencing by cleaving target mRNAs after binding small RNAs (siRNAs and miRNAs) (Brodersen & Voinnet, 2006). miRNAs are expressed in response to abiotic stresses including drought, cold and high salinity (Sunkar & Zhu, 2004; Borsani *et al*., 2005; Brodersen & Voinnet, 2006), and the induction of *AGO1* therefore suggests that it may also be worth investigating the role of small RNAs in spruce cold acclimation.

Genes with no homology

Although it is essential to view our results in the light of functional data from model plants, it is also important to remember that many conifer expressed genes have no homology to genes in angiosperm sequence databases. Many of the spruce ESTs sequenced thus far fall into this category

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(S.G.R. and co-workers, unpublished), and some of these were induced very strongly (up to 38-fold) during the course of our study (Table 2). In some cases, similarity to stress-inducible genes in other conifers was found through searches of the nonredundant collection of Genbank (e.g. WS00926_F21). However, in these cases little direct functional data exists. Given the approx. 380 million yr of evolution separating gymnosperms and angiosperms, some of these may be conifer-specific cold hardiness genes, and although they are among the most difficult to study, they may also be novel and therefore among the most interesting.

Among-population variation in gene expression

Global gene expression profiles among genetically distinct taxa (populations or species) have been described for both plants and animals, and although widespread variation exists, methods to separate neutral from adaptive variation are in their infancy (Rifkin *et al.*, 2003; Khaitovich *et al.*, 2004; Yanai *et al.*, 2004; Lemos *et al.*, 2005; Lai *et al.*, 2006). While some studies suggest that the vast majority of expression divergence among taxa is the result of genetic drift (Khaitovich *et al.*, 2004, 2005), others have found extensive hallmarks of selection (Rifkin *et al.*, 2003; Gilad *et al.*, 2006; Whitehead & Crawford, 2006a). One difficulty in evaluating these competing claims stems from the differences in evolutionary distance among the taxa compared within each study, where properties of among-taxa expression variation, and of the experimental method itself, depend strongly on the biological system (Whitehead & Crawford, 2006b). It has therefore been suggested that comparisons will be most fruitful when made within a species or between closely related species (Whitehead & Crawford, 2006b). In addition, closely related taxa are more likely to share similar ecological contexts, which facilitates comparisons of expression variation with trait variation (Whitehead & Crawford, 2006a, 2006b).

We have shown significant among-population differential gene expression along the latitudinal range of a single species, Sitka spruce. The clinal nature of this variation corresponds to a well studied genetic cline in cold hardiness, bud phenology and growth (Mimura & Aitken, 2007). Because cold hardiness comprises many component traits, differential gene expression could be expected to contribute to adaptive variation in cold hardiness in a variety of ways. For example, expression variation early in the autumn could explain differences in timing of cold acclimation, whereas genes that are expressed more strongly in northern relative to southern populations in early winter could contribute to differences in maximum cold hardiness. We observed both these patterns. Some of the genes already discussed were expressed more highly at the second time point in BC and AK populations than in the CA population. Surprisingly, many of the genes that were up-regulated across the BC time series did not exhibit population differences at either time point. There were, however, many genes that showed clinal expression patterns. There were 326 genes (among which 169 were annotated) more than twofold more highly expressed in the AK compared with the CA population at the fourth time point (Fig. 2b; Table 3). These genes also tended to be more highly expressed in BC compared with CA, particularly at the fourth time point, and did not vary to nearly the same extent between AK and BC as they did between CA and the other two populations.

Annotations for the genes that were more highly expressed in the AK relative to the CA population suggest roles for some of them in cold tolerance. There were several genes involved in dehydrative stress tolerance, as well as genes with roles in both carbohydrate and lipid metabolism (Table 3). As in the temporal component of our study, many genes with no known homology were differentially expressed among populations. A subset of these can be found in Table 3. The variation in expression levels of these genes was particularly striking at the fourth time point, where they were 13–21-fold more abundant in the AK population relative to the CA population.

Finally, it is important to consider neutral divergence as a possible alternative explanation to the adaptive significance we have proposed for the among-population differential gene expression we observed. It has already been shown that population differentiation for cold hardiness in Sitka spruce ($Q_{st} = 0.89$) is much stronger than for neutral microsatellite markers ($R_{st} = 0.09$; $F_{st} = 0.11$), reflecting strong divergent selection despite high gene flow (Mimura & Aitken, 2007). If genetic drift were driving gene expression divergence, we would expect to see the same strength and pattern of differentiation at neutral marker loci. However, R_{st} values show that, at neutral microsatellite loci, central British Columbia and northern California populations are substantially more similar to each other than to southern Alaska populations (Mimura & Aitken, 2007). Much of the among-population gene expression differentiation we observed suggests more similar expression in BC and AK, and divergent gene expression between these more northern provenances and their counterpart from California, particularly at the fourth time point. This is reflected in the late-autumn cold hardiness of these populations, where the least-squares means for cold injury for BC and AK (10.8 and 36.8%, respectively) suggest a relatively high degree of cold hardiness, whereas for CA a least-squares mean of 78.4% suggests significant cold injury at the test temperatures. Together, these patterns provide additional evidence that the gene expression differences we observed are adaptive rather than the result of selectively neutral evolutionary processes.

Real-time PCR

The performance of the custom-built Treenomix *Picea* cDNA microarray used in this study has been evaluated previously using real-time PCR, and was found to be accurate even at

moderate fold-differences (S.G.R. and co-workers, unpublished). As this validation effort used tissue of clonal origin, but we employed seedlings grown from seed collected in wild populations, we chose to use real-time PCR to ensure the reliability of our results. Real-time PCR measurements of gene expression generally coincided well with our microarray results. Although microarrays can be susceptible to cross-hybridization, leading to artefactual results, each gene we validated using the more sensitive, quantitative technique of real-time PCR had an expression pattern similar to that measured on the array (Fig. 4).

Conclusions

To the best of our knowledge, our study is the largest transcriptional profile of cold acclimation in a conifer, and provides many candidate genes for molecular dissection of this process. Until now, very little expression data existed in the literature connecting conifer genes to cold stress-inducible homologs in model systems. As such, our study informs not only functional and population genomic studies of cold hardiness in spruce, but also those in other conifers for which substantial EST resources exist (e.g. *Pinus taeda*, *Pseudotsuga menziesii*). The candidate genes presented here will form the basis for a population genetic survey of nucleotide diversity, and ultimately lead to an association study linking genetic and phenotypic variation in cold hardiness. By undertaking targeted functional studies, and by characterizing the patterns of variation in the genes presented here, we can begin to understand the genetic networks and molecular adaptations that govern adaptation to local climate in general, and cold hardiness in particular.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Boxplots of technical and biological variance estimates for all gene expression measurements.

Table S1 Fold-change and *Q* values for all array elements. Annotations (BLASTX vs *Arabidopsis*) are given for genes with expect values < 1E-5

Table S2 Primers for real-time PCR

Table S3 Statistically overrepresented Gene Ontology terms in the GOSlim Plant ontology among genes up- and down-regulated twofold between time points BC1 and BC5

Table S4 BLAST hits for 21.8 K microarray elements from 26 public databases

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