Transcriptome comparison of winter and spring wheat responding to low temperature

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Abstract: Freezing tolerance in plants is a complex trait that occurs in many plant species during growth at low, non-freezing temperatures, a process known as cold acclimation. This process is regulated by a multigenic system expressing broad variation in the degree of freezing tolerance among wheat cultivars. Microarray analysis is a powerful and rapid approach to gene discovery. In species such as wheat, for which large scale mutant screening and transgenic studies are not currently practical, genotype comparison by this methodology represents an essential approach to identifying key genes in the acquisition of freezing tolerance. A microarray was constructed with PCR amplified cDNA inserts from 1184 wheat expressed sequence tags (ESTs) that represent 947 genes. Gene expression during cold acclimation was compared in 2 cultivars with marked differences in freezing tolerance. Transcript levels of more than 300 genes were altered by cold. Among these, 65 genes were regulated differently between the 2 cultivars for at least 1 time point. These include genes that encode potential regulatory proteins and proteins that act in plant metabolism, including protein kinases, putative transcription factors, Ca^{2+} binding proteins, a Golgi localized protein, an inorganic pyrophosphatase, a cell wall associated hydrolase, and proteins involved in photosynthesis.

Key words: wheat microarray, expression profile, plant transcription, cold-regulated genes, freezing tolerance, cold acclimation, winter hardiness, stress genes, gene regulation, wheat transcriptome.

Résumé : La tolérance au gel chez les plantes est un caractère complexe qu'on rencontre chez plusieurs plantes au cours de la croissance à de faibles températures (au-dessus du point de congélation), un phénomène appelé acclimatation au froid. Ce processus est régulé par un système multigénique qui produit une grande variation quant au degré de tolérance au gel parmi différents cultivars du blé. Les puces à ADN constituent une approche puissante et rapide en vue de l'identification de gènes. Chez des espèces telles que le blé, pour lesquelles le criblage à grande échelle de mutants ou la transgenèse ne sont pas présentement pratiques, la comparaison de différents génotypes constitue une approche essentielle pour l'identification de gènes clés dans l'acquisition de la tolérance au gel. Une puce à ADN a été constituée avec des inserts de clones ADNc amplifiés par PCR, ces clones provenant de 1 184 EST (étiquettes de gènes exprimés) et représentant 947 gènes. L'expression génique au cours de l'acclimatation a été comparée chez 2 cultivars présentant une tolérance au gel très contrastée. L'abondance de plus de 300 gènes était modifiée par le froid. Parmi ceux-ci, 65 gènes présentaient une régulation différente chez les 2 cultivars pour au moins 1 des temps d'échantillonnage. On y retrouve des gènes qui codent pour de possibles protéines régulatrices et des protéines impliquées dans le métabolisme dont des protéines kinases, des facteurs de transcription putatifs, des protéines liant le Ca^{2+}, une protéine localisée dans le Golgi, une pyrophosphatase inorganique, une hydrolase associée à la paroi cellulaire, ainsi que des protéines impliquées dans la photosynthèse.

Mots clés : puce à ADN pour le blé, profil d'expression, transcription chez les plantes, gènes régulés par le froid, tolérance au gel, acclimatation au froid, résistance au froid, gènes de stress, régulation génique, transcriptome du blé.

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Introduction

Low temperature (LT) is a major cause of crop damage, and the ability of plants to develop freezing tolerance (FT) during cold acclimation is a determining factor in their survival. Exposure of plants to LT produces morphological, biochemical, and physiological changes that are often highly correlated with plant FT and winter survival. There is a great deal of genetic variation both between and within species for the process of cold acclimation and in the ultimate degree of FT that can be achieved. Molecular and genetic analyses have demonstrated differential gene expression and the increased accumulation of several proteins during cold acclimation in several species. The complexity of the LT response creates a challenge to differentiate the genes responsible for LT acclimation and cold hardiness from those associated with metabolic adjustment to LT. Identifying the genes contributing to FT and understanding how they are regulated is important in establishing the appropriate strategy for the improvement of FT in crop species. To achieve this goal, global approaches to identifying the genetic components involved in this mulitgenic trait are essential. Microarray analysis to characterize gene expression profiles for large numbers of transcripts has been used to describe the response to environmental stresses in several species including *Arabidopsis* for cold, drought, and salt (Seki et al. 2001; Fowler and Thomashow 2002; Kreps et al. 2002), recovery from dehydration (Oono et al. 2003) and oxidative stress (Desikan et al. 2001); rice for response to salt stress (Kawasaki et al. 2001); and in maize for response to UV radiation (Casati and Walbot 2003). The large number of stress-regulated genes that were detected by these studies indicates that transcript profiling could readily be applied to comparative studies of cold hardy genetic stocks.

Wheat cultivars have a remarkable range of FT and thus are an excellent model to study this trait. Several LT responsive genes have been identified in wheat using differential screening (Houde et al. 1992; Chauvin et al. 1993; Danyluk et al. 1994; Danyluk et al. 1996; Danyluk 1997; N'Dong et al. 2002; Breton et al. 2003). However, these methods are limited and time consuming; moreover, they do not describe the global view of coordinated expression and the interactions among the expressed genes. To accelerate the pace of discovery, 1200 expressed sequence tags (ESTs) were generated from clones from a cDNA library of cold-acclimated winter wheat and used to construct a cDNA amplicon microarray. The LT-regulated gene expression profile of winter wheat 'Norstar', which has a lethal temperature for 50% of the seedlings (LT_{50}) of –19 °C, was compared with that of spring wheat 'Glenlea', which has an LT_{50} of –8 °C. The objective of this comparison was to differentiate genes associated with the development of FT from those associated with LT stress. We report changes in the expression pattern of a significant portion of the genes probed by the microarray over the course of 36 days of cold acclimation. More importantly, we identified a subset of genes with significant differences in regulation between the 2 cultivars. This subset includes genes with regulatory functions that may prove to play an important role in the acquisition of FT.

Materials and methods

Plant material and growth conditions

The spring wheat *Triticum aestivum* L. 'Glenlea', LT_{50} –8 °C, and the winter wheat *Triticum aestivum* L. 'Norstar', LT_{50} –19° C, were used in the microarray analysis. Further characterization of the expression of selected genes was done using *Triticum aestivum* L. 'Clair', LT_{50} –17 °C, a derivative of 'Norstar'. Seeds were germinated in moist sterilized vermiculite for 5 days in the dark and 2 days under artificial light. Cool-white fluorescent and incandescent lighting was combined to provide an irradiance of 250 µmol·m^{-2}·s^{-1}. The temperature was maintained at 25 ± 1 °C (15-h photoperiod) during the day and 20 ± 1 °C during the night. The relative humidity was 70% ± 5%. Seedlings were watered daily with a nutrient solution (0.5 g/L of N:P:K at a ratio of 20:20:20). At the end of this period, control plants were maintained under the same conditions of light and temperature for an additional 1 and 6 days. Cold acclimation was performed by subjecting germinated seedlings to a temperature of 6 ± 1 °C during the day (10-h photoperiod and 175 µmol·m^{-2}·s^{-1}) and 2 ± 1 °C during the night for 1, 6, and 36 days. Control seedlings grown 1 and 6 days beyond the 7 days of germination at 25 °C – 22 °C (day–night) are referred to as Control-1-day plants and Control-6-days plants, respectively. Based on seedling dry mass, Control-1-day plants and Control-6-days plants have the same phenological phase as seedlings that were cold acclimated for 6 and 36 days, respectively. Gene induction in plants that were cold acclimated for 1 and 6 days was measured by comparison with Control-1-day plants. Plants cold treated for 36 days were compared with Control-6-days plants. Plants were harvested 3 h before the end of the day cycle. The aerial part of 40–80 cold-treated seedlings, including the meristematic crown and leaves (10 g), was used for the extraction of total RNA according to a method described previously (Danyluk and Sarhan 1990).

cDNA library construction and EST sequencing

Ribonucleic acid from meristematic crown and leaves of 'Norstar' seedlings cold treated for 1 day was used for construction of a cDNA library in Lambda ZAP® II vector (Stratagene, La Jolla, California, USA) in random orientation and rescued as pBluescript SK⁻ plasmid by in vivo excision. Details of cDNA library construction are provided in the supplementary materials and methods.

Virtual subtraction was carried out to enrich cDNAs from low abundance mRNAs. Colonies were transferred into 96-well plates containing Luria–Bertani (LB) with 100 mg/L ampicillin and grown overnight at room temperature. Plates were replicated onto nylon membranes (Hybond-N., Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, USA) and grown on LB agar with 100 mg ampicillin/L overnight at 37 °C. Colonies were lysed, denatured, and neutralized according to standard methodology (Sambrook et al. 1989). First strand cDNA from 1 day cold-acclimated 'Norstar' was labeled with digoxigenin, and hybridization and detection were done according to the DIG High Prime DNA Labeling and Detection Starter Kit II instructions (Roche Molecular Bio-

chemicals, Mannheim, Germany). Approximately 30% of colonies showing the lowest hybridization signal were chosen for sequencing.

Plasmids were prepared with the QIAprep Miniprep Kit (QIAGEN, Mississauga, Ontario). Expressed sequence tags were generated by single-pass sequencing of cDNA inserts using either the dideoxynucleotide chain termination method (T7 sequencing kit, Amersham Pharmacia, Uppsala, Sweden) and gel electrophoresis, or the dye terminator sequencing kit (Beckman Coulter Inc. Fulerton, Calif.) and the Beckman CEQ2000 automated sequencer (Center for Structural and Functional Genomics, Concordia University, Montréal, Quebec). The quality of ESTs was assessed using phred/phrap software (Ewing and Green 1998; Ewing et al. 1998; www.phrap.org), and vector and low quality regions were trimmed using LUCY (The Institute for Genomic Research (TIGR), Rockville, Maryland, USA). The ESTs were queried against the TIGR gene indices using Wheat Version 6.0, Barley Version 6.0, Rice Version 12.0, and *Arabidopsis* Version 10.0 indices and annotated using the TIGR descriptions. Some additional annotation of differentially expressed genes was taken from GenBank nr data set. Independent members of gene families were scored as individual genes. Thus, some independent clones on the microarray may have similar annotations.

cDNA microarray

The cDNA inserts for each EST were amplified from the plasmids using a protocol from Hegde et al. (2000) with some modifications (details of the microarray construction are provided in the supplementary materials).[3] Clones were not rearranged from the original 96-well plates used for EST sequencing to avoid picking and tracking errors. After expression analysis, approximately 190 clones that showed changed expression during cold acclimation were chosen for verification by resequencing. Microarrays were spotted on Corning® CMT-GAPS 2 or UltraGAPS™ (Corning Life Sciences, Corning, N.Y.) glass slides using a Virtek arrayer robot (Virtek, Waterloo, Ontario) equipped with Telechem Stealth II quill pins (TeleChem International, Sunnyvale, Calif.) in a 24-pin configuration. The spots were spaced on a 0.3-mm grid. Each product was spotted 4 times, 2 spots side by side, in 2 locations. The target cDNA was generated by indirect labeling with the initial incorporation of aminoallyl-dUTP (Sigma, Oakville, Ontario) during reverse transcription and subsequent reaction with Cy-3™ or Cy-5™ mono-reactive ester dyes (Amersham Pharmacia, Uppsala, Sweden). For each pair of comparisons, 3 to 6 replicate hybridizations were carried out. For each experiment, at least 1 dye-swap slide was done in which the labeling of control and treatment target cDNA with Cy-3 and Cy-5 were reversed. (The details of the labeling protocols and hybridization protocols are provided in the supplementary materials.[3])

Microarray scanning and data analysis

The slides were scanned on an Axon 4000b scanner (Molecular Devices, Sunnyvale, Calif.). The images were saved as multichannel TIFF files and the images were analyzed using the GenePix 3.0 software (Molecular Devices). Low quality spots including misshapen spots and those obscured by dust motes, scratches, or buffer spots were flagged and subsequently disregarded in the analysis. The statistical analysis was done using the GeneSpring software package version 5.0.3 (Silicon Genetics, Redwood City, California, USA). The 4 replicate spots on each slide were averaged. The dye-swap normalization method, on a per spot basis, was first used in applicable experiments. Furthermore, all slides were normalized, on a per chip basis, using the Lowess regression method. Variances were calculated using cross-gene error model and on-chip replicates. Changes of transcript levels during cold acclimation were determined by comparing the treatment to control plants for each genotype. This comparison used the multiple testing correction of the Benjamini-Hochberg false discovery rate procedure with a cutoff of 0.05 (Benjamini and Hochberg 1995). Then the transcript levels in the 2 cultivars were compared directly by comparison of target cDNAs tested with the hypothesis that there were no differences in expression in the 2 cultivars (WT/ST = 1). Gene expression in control plants was not measured directly. (The arithmetic calculation of those values based on other comparisons is presented in the supplementary materials.)[3]

Clustering analysis was performed using the Genesis software (Sturn et al. 2002). The analysis included genes that had altered expression during at least 1 time point and those genes that had valid data for all time points in at least 1 cultivar. Genes with the same functional annotation that were not members of the same contig were kept as independent sets of values. The analysis included 215 and 136 genes from the winter and spring data sets, respectively; 125 of the genes were common to both data sets. Data were averaged for the replicates for each feature of each genotype at each time point, and clustering was based on the 3 expression ratio values at the 3 time points of 1, 6, and 36 days. Expression values for the same gene measured in each of the 2 cultivars were entered into the analysis as independent sets of values in a single clustering analysis to detect pattern similarity and differences between the 2 cultivars. An average-linkage hierarchical clustering method that uses Euclidian distances was employed.

RT-PCR measurement of transcript level of selected genes

Total RNA samples were treated with DNAse I (Ambion Inc., Austin, Texas, USA). RNA samples were quantified by spectrometer, and 5 µg of RNA was used for reverse transcription using Invitrogen Superscript II and oligo-dT primer (Invitrogen Canada, Burlington, Ontario) according to the manufacturer's recommendation. One-fiftieth volume of each cDNA was used for PCR amplification by *Taq* DNA Polymerase (MBI Fermentas, Burlington, Ontario) under the following conditions: 94 °C for 2 min; followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s. The primers for each gene are listed in supplementary Table S3.[3] To

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[3] Supplementary data for this article are available on the Web site or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0S2, Canada. DUD 4007. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

rule out gDNA contamination, PCRs were run with untranscribed RNA samples as template. The ubiquitin cDNA was amplified as an internal control (Yan et al. 2003).

Results and discussion

EST annotation and microarray design

High quality EST sequences were generated by single-pass sequencing of 1187 cDNA clones from a cDNA library made from crown and shoots of LT-treated wheat seedlings. Annotation for the wheat ESTs derived from BLAST queries against the TIGR gene indices or the GenBank nr database assigned a functional description to 783 ESTs. Of these, 386 ESTs were classified either as without functional descriptions or as unknown, hypothetical, putative, ESTs or open reading frames. Contig assembly within our EST set and clustering with wheat tentative contigs (TCs) in the TIGR database indicated the EST collection contained 801 singletons and 147 contigs that in total provided a unigene set of 948 genes. We constructed a wheat microarray with PCR amplified inserts from 1187 ESTs without rearrangement of the clones from the original sequencing master plates; this was done to avoid tracking errors. DNA sequences for ESTs used in these studies are presented in supplementary Table S1.[3] Approximately 50% of spots were detected with significant signals above background. Resequencing of approximately 190 selected clones of cold-regulated genes indicated accurate clone identification and tracking.

Gene regulation during cold acclimation

Both highly cold-tolerant and less tolerant cultivars increase cold tolerance during cold acclimation. In a effort to detect genes that may play important roles in cold acclimation and increased freezing tolerance, we have directed our analysis to the detection of genes that are differently regulated in the 2 cultivars. Gene expression was measured in cultivars 'Norstar' (LT_{50} –19 °C) and 'Glenlea' (LT_{50} –8 °C) after 1, 6, and 36 days of cold acclimation. Microarray hybridizations were analyzed with 2 approaches: (*i*) measurement of gene induction and repression by comparison of target cDNAs from cold-acclimated and nonacclimated control plants, and (*ii*) direct comparison of pairs of target cDNAs derived from 'Norstar' and 'Glenlea' that had been cold acclimated for 6 or 36 days. The experimental design, shown in Fig. 1, allowed a thorough comparison of the gene expression profiles of the 2 cultivars. Direct comparison of mRNA populations from 1 cultivar taken at 2 time points or comparison of the 2 cultivars with samples taken from identical treatments were used to derive most of the expression data, but the design allowed additional data to be deduced by calculation. For example, ratio of expression levels in control plants of the 2 cultivars was calculated from ratio of gene induction between 1 and 6 days in the 2 cultivars divided by the ratio of expression between the 2 cultivars measured at 6 days of cold acclimation.

There were 337 genes with altered levels of expression that were statistically significant with $P < 0.05$ and that had changes in expression greater than a threshold of 1.5 or less than 0.66 for at least 1 time period. Genes meeting these criteria are the basis of subsequent analysis; detailed data for these genes are listed in supplementary Table S2[3] and sum-

Fig. 1. The experimental design is based on the series of pairwise comparisons. Numbers indicate the number of days plants were grown at the indicated experimental temperatures. Before day 1 of the experiment, all plants had been germinated and grown for 7 days at 24 °C. N, winter wheat 'Norstar'; G, spring wheat 'Glenlea'.
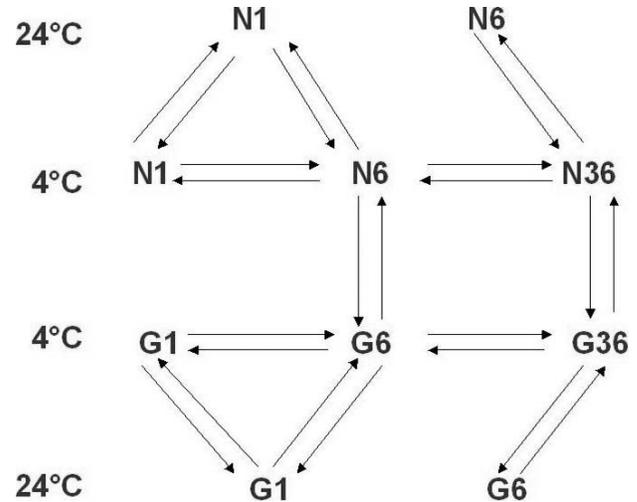

Table 1. Genes with changes in expression 1.5-fold or more and P-value < 0.05 in the winter wheat 'Norstar' and the spring wheat 'Glenlea' after 1, 6, and 36 days of cold acclimation at 4 °C.

Days of treatment	Upregulated genes		Downregulated genes	
	'Norstar'	'Glenlea'	'Norstar'	'Glenlea'
1	74	44	78	32
6	90	66	115	60
36	57	45	77	27

marized in Table 1. The use or choice of threshold values is somewhat debatable since even small changes in gene expression may be biologically important, especially for regulatory genes. The choice of higher thresholds used elsewhere is influenced by higher levels of statistical confidence associated with larger changes in RNA signals; however, there are exceptions to the trend to have lower P-values associated with larger changes in gene expression. The 1.5 and 0.66 threshold values for data presented in supplementary Table S2[3] were chosen to give an ample presentation of the data; P-values for each expression ratio are also given in the table.

In the winter wheat cultivar 'Norstar', levels of the transcripts of 309 genes were altered during the cold acclimation. Among these genes, 170 were significantly changed at a single time point, 96 were changed at 2 time points, and 43 had altered expression at all 3 time points. In the spring wheat cultivar 'Glenlea' levels of 166 gene transcripts were altered during cold acclimation. Among these genes, 88 were significantly changed at a single time point, 48 were changed at 2 time points, and 30 had altered expression at all 3 time points. The higher number of genes with altered expression that were detected in winter wheat is partially due to the greater degree of experimental replication carried out with 'Norstar'. Thus, more genes with relatively small

Table 2. Genes with different induction in winter and spring wheat at 1 day of cold acclimation. W/S, winter/spring; LT, low temperature.

Clone	TIGR accession No.	Annotation	Control W/S	Spring induction	Winter induction	Induction W/S
J470	TC85079	LEA14-A	5.69	4.56*	11.55*	2.53*
J517	TC91471	LT 101 putative membrane protein	4.90	7.72*	4.50*	0.58*
J275	TC87867	ESI3/LT 101 LT and salt induced	4.92	9.93*	5.08*	0.51*
J330	TC87536	LEA/RAB/COR cold-responsive protein	2.60	14.59*	6.85*	0.47*
J666	TC69676	WCOR14a — cold-regulated protein	2.60	14.44*	5.55*	0.38*

Note: TIGR accessions are the most similar sequence in the TIGR Wheat Gene Index, V6. Control W/S is the relative level of mRNA in control winter wheat plants compared with control spring wheat plants. It is calculated indirectly by dividing the relative level of mRNA in the 2 cultivars by the relative rate of induction at 6 days of accumulation.

*Statistical significance criteria is P-value ≤ 0.05.

changes in expression could be classified as statistically significant changes. The large proportion of genes that were seen to be regulated by cold treatment is somewhat higher than the approximately 25% of the genes of *Arabidopsis* that were seen to have changes in mRNA levels of 2-fold or more when the plants are treated with LT alone (Kreps et al. 2002). This is not surprising since the cDNA library that was the source of clones for this array was derived from cold-treated plants and is expected to be enriched for cold-induced genes. In addition, the high proportion of genes whose altered expression was detected at only 1 of 3 time points indicates that the number of genes classified as regulated by cold is highly dependent on the number of times plants are sampled during the course of cold acclimation.

Differences in gene regulation in spring and winter wheat

To compare gene expression between the winter and spring cultivars, first gene expression was evaluated by comparing up- and down-regulation at 1, 6, and 36 days acclimation. Here, cDNA from cold-treated plants was compared with cDNA from appropriate controls from each cultivar. Then, direct comparisons were made by hybridizing cDNA from 'Norstar' with cDNA from 'Glenlea' plants acclimated to cold for the same period. The first approach can detect genes that have different levels of induction or repression in the 2 cultivars that are not necessarily classified as induced or repressed in the individual cultivar analysis. For example, genes that are slightly downregulated in 1 cultivar and slightly upregulated in the other cultivar may not be scored as significantly altered in individual comparisons but may be significantly different in a comparison of the 2 cultivars. A total of 87 genes were differentially regulated in the 2 genotypes for at least 1 time point. Data from these comparisons are shown in Tables 2, 3, and 4.

At 1 day of cold acclimation, 5 genes had significant differences in upregulation in the 2 cultivars; 1 was more strongly induced in winter wheat and 4 were more strongly induced in spring wheat (Table 2). After 6 days of cold acclimation, 57 genes had significant differences in expression between the 2 cultivars (Table 3). Among these, 37 genes were more strongly induced or had higher steady-state levels of mRNAs in winter wheat than in spring wheat. After 36 days of cold acclimation, 41 genes had different levels of expression or changes of expression in the 2 cultivars (Table 4). Among these, 14 were more strongly induced or expressed in winter wheat, than in spring wheat. Twenty-

four genes had a winter induction / spring induction value less than 0.66; the trend of this group of genes is that they were strongly upregulated or near control levels in spring wheat and at control levels or downregulated in the winter cultivar.

The majority of genes that were differentially regulated between the cultivars were different at only 1 time period. Only 15 genes had different expression in the 2 cultivars at 2 time periods, and 1 gene (J275) was differentially expressed at all 3 time periods. This suggests that a more thorough sampling during the time course of cold acclimation would likely detect additional differences. The majority of the genes that had significant differences in expression in the 2 cultivars had somewhat parallel patterns of expression, in that they were up- or down-regulated in both cultivars, but had greater changes in 1 of the cultivars.

Direct comparison of target cDNA from the 2 cultivars after 6 and 36 days of cold acclimation detected differences in expression that are not detected by comparison of gene induction and repression in the 2 cultivars as measured by comparison of control and cold-acclimated plants. This may be the case if the levels of expression are not the same in control plants for the 2 cultivars and because of reduced experimental variance since the comparison is based on 2 sample sets rather than the 4 sets that are required to compare gene induction between the 2 cultivars. Though many of the same genes were detected by the 2 methods of comparison, the direct comparison of the 2 cultivars allowed the detection of an additional 14 differentially expressed genes in the 2 cultivars at 6 days, 8 of these were more strongly expressed in 'Norstar' than in 'Glenlea' (Tables 3 and 4). Four additional genes that were differentially expressed in the 2 cultivars were detected at 36 days of cold treatment. All of the 4 genes were more strongly expressed in 'Glenlea' than in 'Norstar'.

The majority of transcripts that were seen to be more strongly induced in 1 cultivar also had higher levels of mRNA levels in that cultivar, though this was not universally the case. For example, several genes including J302, J517, J275, J666, and J854 have higher levels of induction in spring wheat than in winter wheat after 6 days of acclimation but have higher expression in winter wheat than in spring wheat, presumably because they had higher basal levels of expression in the nonacclimated controls on day 1. This indicates that the relative level of expression in nonacclimated controls in the 2 cultivars is also different. The relative level of gene expression of the control plants of the

Table 3. Genes with different induction or expression in winter and spring wheat at 6 days of cold acclimation. W/S, winter/spring; LT, low temperature.

Clone	TIGR accession No.	Annotation	Control W/S	Spring induction	Winter induction	Induction W/S	Expression W/S
C95	TC87317	Actin depolymerization factor	1.05	3.76*	12.14*	3.23*	3.40*
E31_F	TC92493	COR39 protein	1.19	6.52*	20.50*	3.14*	3.75*
J374	TC87737	Dehydrin WZY1–1	1.63	7.85*	22.81*	2.91*	4.74*
J619	TC87319	WCOR719 cold	0.83	2.91*	7.49*	2.57*	2.14*
J806	TC68452	Lipid transfer protein	0.75	5.99*	14.40*	2.41*	1.79*
J470	TC85079	LEA14-A	ND	ND	13.46*	ND	6.67*
C36	TC72617	WCOR80 cold acclimation	ND	ND	4.74*	ND	3.58*
J642	TC79747	Unknown protein	ND	ND	6.85*	ND	2.87*
J822	TC89914	Guanine regulatory protein	ND	ND	7.02*	ND	2.49*
J657	TC65078	Gamma-thionin precursor	ND	ND	2.86*	ND	2.35*
J733	TC94499	Golgi-localized protein	ND	ND	ND	ND	2.04*
J459	TC85842	Probable acclimation protein	1.24	7.49*	14.65*	1.96	2.43*
C272	TC65072	Gamma-thionin precursor	0.71	1.03	1.98*	1.93*	1.37
C213	TC73110	Unknown protein	0.80	1.92*	3.64*	1.90*	1.52
JB074	TC65862	Phosphoethanolamine methyltransferase	1.20	4.45*	8.40*	1.89*	2.26*
J339	TC93659	WCS120 protein	2.45	6.04*	11.32*	1.87*	4.59*
J765	TC69449	Chitinase 2	1.47	3.74*	6.88*	1.84*	2.70*
B60_F	TC67796	WCOR825 cold-acclimation protein	0.73	1.16	2.09*	1.81*	1.32
C197	TC87822	Unknown protein	0.89	1.05	1.82*	1.74	1.55*
C55	TC67115	Cold-acclimation protein	1.33	1.55	2.65*	1.70	2.26*
E25_F	TC83987	Unknown protein	ND	0.91	1.55*	1.70*	ND
J862	TC88307	LT-induced lipocalin	0.69	1.38*	2.34*	1.69*	1.18
C161	TC96342	LT-induced protein	1.27	11.75*	19.92*	1.69	2.15*
J882	TC63551	Inorganic pyrophosphatase	0.68	1.57*	2.63*	1.68*	1.15
C290	TC92066	Ice-recrystallization inhibition protein	0.93	1.50*	2.42*	1.61*	1.51
J680	TC79239	Ice-recrystallization inhibition protein	1.33	5.19*	8.17*	1.57*	2.10*
C108	TC93229	Unknown protein F15K9.6	1.16	2.12*	3.29*	1.55*	1.81*
C205	TC64307	Ubiquitin	0.74	1.09	1.67*	1.52*	1.12
C28	TC92066	Ice-recrystallization inhibition protein	1.74	21.93*	30.70*	1.40*	2.43*
C196	TC67656	Remorin 2	1.13	1.18	1.62*	1.38	1.56*
J885	TC92901	Cold-acclimation protein	1.93	13.91*	18.70*	1.34*	2.59*
C29	AL821437	Cold-acclimation protein	1.72	3.17*	3.60*	1.14	1.95*
J458	TC65326	Rubisco small subunit	0.60	0.54*	0.60*	1.10	0.66*
C465	TC66723	Unknown protein	0.52	0.77	0.73*	0.95	0.49*
JB080	TC85188	Lipid transfer protein	0.63	0.73*	0.63*	0.85	0.54*
C653	TC63643	Carbonic anhydrase	0.75	0.37*	0.32*	0.85	0.64*
J517	TC91471	Blt101.2. LT-induced protein	2.43	4.81*	3.87*	0.81*	1.96*
J275	TC87867	ESI3 LT, NaCl-induced protein	2.33	4.99*	4.02*	0.80*	1.87*
JB018	TC65343	Ribulose-1,5-bisphosphate carboxylase	0.77	0.44*	0.33*	0.76	0.59*
J302	TC87869	ESI3 LT, NaCl-induced protein	2.84	5.87*	4.42*	0.75*	2.14*
C302	CA630452	Hypothetical protein	2.13	5.87*	4.42*	0.75	1.60*
J883	TC64211	Carbonic anhydrase	0.96	0.58*	0.39*	0.68	0.66*
E32_F	TC65820	Nonspecific lipid-transfer protein	1.14	3.33*	2.21*	0.66*	0.76
C86	TC84168	Glycine rich protein	1.92	12.79*	8.27*	0.65*	1.24
C170	TC63101	Hypothetical protein	1.40	1.57*	0.81*	0.52*	0.72
F29_R	TC93925	LRK19 receptor-like kinase	1.28	2.17*	1.12	0.52*	0.66
S15_F	CA698312	Putative aspartate—tRNA ligase	ND	1.26	0.65*	0.51*	ND
J893	CA621935	Alpha-soluble NSF attachment protein	ND	1.28	0.63*	0.50*	ND
J851	TC64697	C13 endopeptidase NP1 precursor	ND	1.30	0.63*	0.49*	ND
C75	BQ170273	Peptidase-like protein	1.40	2.03*	0.98	0.48*	0.68
J279	TC72165	Unknown Triticae protein	1.51	1.98*	0.90	0.46*	0.69*
J666	TC69676	WCOR14a, cold-responsive protein	3.67	18.85*	8.41*	0.45*	1.64*
A02_F	TC70414	Cold-regulated protein	2.28	1.95*	0.75*	0.39*	0.88
J442	TC233441	E2 Ubiquitin ligase	2.37	1.73*	0.62*	0.36*	0.85

Table 3 (*concluded*).

Clone	TIGR accession No.	Annotation	Control W/S	Spring induction	Winter induction	Induction W/S	Expression W/S
J342	TC68241	Hypothetical protein	2.35	1.66*	0.43*	0.26*	0.61
J626	TC63138	Cell wall-associated hydrolase	3.10	1.73*	0.41*	0.24*	0.73
J854	TC87536	LEA/RAB-related COR	7.20	12.55*	2.64*	0.21*	1.51*

Note: TIGR accessions are the most similar sequence in the TIGR Wheat Gene Index, V6. Control W/S is the relative level of mRNA in control winter wheat plants compared with control spring wheat plants. It is calculated indirectly. Induction of W/S was calculated as the rate of induction or repression in winter wheat divided by the rate of induction or repression in spring wheat. Expression W/S is the relative level of mRNA in winter wheat compared with spring wheat; it was measured directly.

*Statistical significance criteria is P-value ≤ 0.05.

2 cultivars was calculated arithmetically from other hybridization measurements and is included in Tables 3 and 4.

Cluster analysis

Cluster analysis was used to further characterize differences in gene expression between the freezing-tolerant winter wheat and the less freezing-tolerant spring wheat. Genes with detectable expression for all 3 time points in winter wheat and significant up- or down-regulation for at least 1 time point were used for cluster analysis. Clustering included 215 genes that were characterized in winter wheat and 136 genes that were characterized in spring wheat; the results are available as supplementary data in Figs. S1 and S2, respectively.[3] To facilitate the detection of differences and similarities of gene expression in the 2 cultivars, the combined data sets for the 2 cultivars, which included 125 common genes, were clustered together. The results are available as supplementary data in Fig. S3.[3] Genes in the combined data set from winter and spring wheat were grouped into 23 clusters. Those genes whose expression patterns differed in 'Norstar' and 'Glenlea' are readily observed since they did not cluster together in supplementary Fig. S3.[3] The analysis of coclustering of the 128 genes for which complete data exists for both cultivars revealed that 48 genes show highly similar patterns of expression in the 2 cultivars in that they were grouped into the same cluster. Fifty-three genes showed similar patterns of expression but had some quantitative differences at 1 or more time periods. They were grouped into different clusters that had somewhat similar profiles. More striking differences in patterns of expression were observed for 21 genes that were grouped into distinct clusters with clearly different patterns of expression; these are shown in Fig. 2. The observed qualitative or quantitative changes in expression patterns strongly suggest that important differences exist in cold signaling pathways between cultivars and these genes represent candidates for the elucidation of these pathways. Below is a brief discussion of genes with important differences in expression in the 2 cultivars and their possible role in the cold acclimation process.

Signal transduction and novel genes

Many genes encoding regulatory type proteins (C176, C288, C338, C652, F29_R, J293, J621, J713, J770, J772, J822, J840, J878, J900, J904, J925, S31_F, S42_F,) had altered expressions in cold-treated plants, some of which had significantly different expressions in the 2 cultivars. The majority of these genes had fewer than 2-fold changes in ex-

pression, though a few of the genes in this class had high levels of upregulation. The quantitative nature of microarray analysis is well suited to the detection of subtle changes of expression, since such changes are easily overlooked in classical screening methods such as differential screening of cDNA clones, differential display, and northern analysis. In the case of signal transduction components, subtle changes in cellular levels can be significant since the signal is amplified downstream. The level of transcript for J900, which encodes a calcium-binding EF-hand protein, was upregulated 2.9- and 9.0-fold at 36 days of cold acclimation in spring and winter wheat, respectively. Levels of F29_R, which encodes an LRK10-like protein kinase, were induced 3.2-fold in winter wheat at 36 days of cold acclimation and transcript levels for C288, which encodes a protein kinase, were 2.8-fold higher in winter wheat at 6 days of LT treatment. Although a number of other genes in this class, including protein kinases, protein phosphatases, transcription factors, RNA-binding protein, a 14–3–3 homolog, and a Ras-related GTP-binding protein, had altered expression in at least 1 cultivar for at least 1 time point (supplementary Table S2[3]), significant differences were not detected between the 2 cultivars. Regulatory genes with differential expression between the 2 cultivars are high priority candidates for further characterization.

Photosynthesis and carbon fixation

A large number of genes encoding proteins involved in photosynthesis and carbon fixation had altered expressions in response to cold treatment. A number of these genes were differentially expressed in the winter and spring cultivars. Many of these genes are members of gene families whose members showed similar patterns of expression. There is a tendency for photosynthesis genes to be downregulated by LT, and the most accentuated differences between spring and winter wheat occur after 36 days of growth at LT when photosynthesis-related genes tend to be downregulated in winter wheat relative to spring wheat. Genes, encoding the rubisco small subunit, carbonic anhydrase, and PSI antenna proteins are repressed in winter wheat after 36 days of cold acclimation, whereas they are either induced or expressed at control levels in spring wheat (Table 4 and supplementary Table S2[3]). Chlorophyll a/b-binding proteins, in contrast with other genes encoding proteins involved in photosynthesis, were upregulated after 1 and 6 days of cold treatment in both cultivars. They remained significantly upregulated in the spring cultivar at 36 days, whereas in the winter cultivar

Table 4. Genes with different induction or expression winter and spring wheat at 36 days of cold acclimation. W/S, winter/spring; LT, low temperature.

Clone	TIGR accession No.	Annotation	Control W/S	Spring induction	Winter induction	Induction W/S	Expression W/S
J339	TC93659	WCS120 protein	1.78	2.15*	11.63*	5.41*	9.62*
C29	AL821437	Cold-acclimation protein	0.98	5.24*	20.29*	3.87*	3.80
J900	BQ161796	Calcium-binding EF-hand protein	0.47	2.64*	8.82*	3.34*	1.56
C161	TC96342	LT induced protein	1.70	14.80*	47.56*	3.21*	5.46
J619	TC87319	Actin depolymerization factor	ND	1.32	3.96*	3.01*	ND
J374	TC87737	Dehydrin WZY1–1	3.42	2.41*	7.20*	2.98*	10.20*
J459	TC85842	Probable acclimation protein	2.10	11.93*	34.35*	2.88*	6.06
E31_F	TC92493	COR39 cold-regulated gene	ND	2.43	6.84*	2.82*	ND
F29_R	TC93925	LRK10 receptor-like kinase	0.34	1.23	3.23*	2.63*	0.89
J536	TC84127	Rubisco large subunit	ND	0.77	1.67	2.18*	ND
C274	TC93229	Expressed protein	ND	1.20	2.43	2.02*	ND
J680	TC79239	Ice recrystalization inhibitor	1.23	4.62*	8.85*	1.91*	2.36
J275	TC87867	ESI3/Blt 101 LT, NaCl induced	1.65	2.14*	3.58*	1.67*	2.76
S06_F	TC91686	Lipoxygenase	0.45	0.93	0.96	1.03	0.47*
C09	BU979312	Unknown protein	0.65	0.87	0.87	1.00	0.64*
J625	BJ276977	Expressed protein	0.55	0.81	0.77	0.96	0.53*
J377	TC69422	Plastid ribosomal protein CL9	0.62	0.77	0.50*	0.65	0.40*
J330	TC87536	LEA/RAB-protein	3.40	5.82*	3.50*	0.60*	2.04
J597	TC65328	Rubisco small subunit	0.48	0.43*	0.23*	0.52*	0.25*
J324	TC64862	Rubisco small subunit	0.81	0.99	0.50*	0.51*	0.41*
J773	TC65728	HMG1/2-like protein	1.42	1.22	0.62	0.51*	0.72
S09_F	TC82882	LHCI-680 PS I antenna protein	1.00	1.31*	0.62*	0.47*	0.47*
J883	TC64211	Carbonic anhydrase	0.49	0.94	0.42*	0.45*	0.22*
JB036	TC64840	Rubisco small subunit	1.10	1.05	0.47*	0.45*	0.49*
C301	TC68028	PE-PGRS homolog MAG24–1	0.93	0.72	0.32*	0.44	0.41*
J382	TC87552	RRM-containing protein	ND	ND	0.70*	ND	0.42*
J884	TC70334	Peroxidase	1.10	2.04	0.85	0.42*	0.46
J649	TC90819	Hypothetical protein	0.94	0.85	0.35	0.41*	0.39*
J458	TC65326	Rubisco small subunit	0.71	0.54*	0.21*	0.40*	0.28*
J393	TC64827	Hypothetical protein	1.23	0.99	0.36*	0.36*	0.44*
JB025	TC84155	Probable heat shock protein	1.33	1.08	0.37*	0.34*	0.46*
C26	TC83130	LHCI-680 PS I antenna protein	1.59	1.17	0.38*	0.33*	0.52*
JB018	TC65343	Rubisco small subunit	0.84	0.68	0.21*	0.31*	0.26*
C77	TC63643	Carbonic anhydrase	0.83	1.00	0.31*	0.31*	0.25*
S41_F	TC86043	Chlorophyll a/b-binding protein	1.57	4.16*	1.09	0.26*	0.41*
B34_F	TC83306	Chlorophyll a/b-binding	ND	2.26*	0.57*	0.25*	ND
F33_F	TC86043	Chlorophyll a/b-binding protein	ND	2.93*	0.71*	0.24*	ND
C177	TC63105	Chlorophyll a/b-binding protein	1.97	2.08*	0.46*	0.22*	0.43*
J444	TC63552	Chlorophyll a/b-binding protein	1.81	4.64*	0.97	0.21*	0.38*
J676	TC83195	Latex-abundant protein putative	2.06	3.85*	0.78	0.20*	0.42
B24_F	TC86051	Chlorophyll a/b-binding protein	ND	5.75*	0.77	0.13*	ND

Note: TIGR accessions are the most similar sequence in the TIGR Wheat Gene Index, V6. Control W/S is the relative level of mRNA in control winter wheat plants compared with control spring wheat plants. It is calculated indirectly. Induction W/S was calculated as the rate of induction or repression in winter wheat divided by the rate of induction or repression in spring wheat. Expression W/S is the relative level of mRNA in winter wheat compared with spring wheat; it was measured directly.

*Statistical significance criteria is P-value ≤ 0.05.
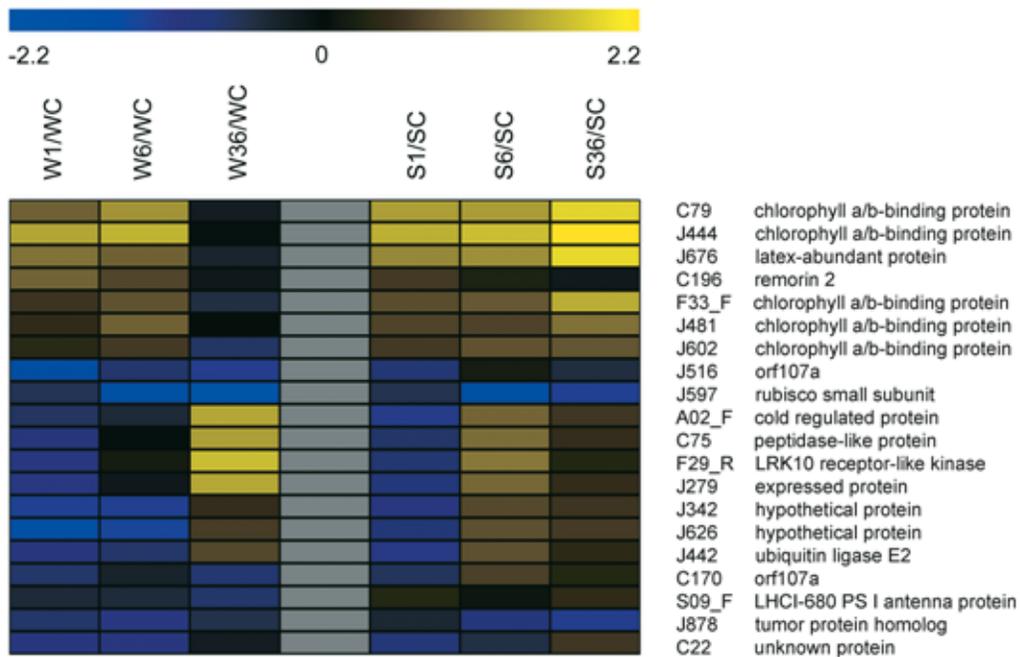
these genes returned to control levels after 36 days of cold acclimation.

A shift of warm-grown plants to LT can result in an imbalance between the light energy absorbed through photochemistry and the energy utilized through metabolism (Huner et al. 1998). This imbalance is the result of the light-harvesting and O_2 generation reactions that are temperature independent and the kinetics of the carbon fixation pathway that is slowed by LT. The excess level of light excitation can

lead to oxidative damage due to a higher level of unutilized electrons. Excess electrons can damage photosystem subunits, particularly the PSII D1 reaction center, leading to an increased production of reactive oxygen species.

Transcripts for light-harvesting complex chlorophyll-binding proteins of the outer antenna of PSII and the inner antenna of PSI have been shown to be regulated by cold treatment in *Arabidopsis*; however, their patterns of regulation were quite different from those observed in our experi-

Fig. 2. Selected genes that showed distinct clustering patterns in samples from winter and spring wheat. Columns 1–3 show the expression ratios for treatment/control of the winter wheat 'Norstar' treated at 4 °C for 1, 6, and 36 days. Columns 5–7 show the induction ratio of the spring wheat 'Glenlea' treated at 4 °C for 1, 6, and 36 days, respectively. Values are based on $\log_2$ ratios.
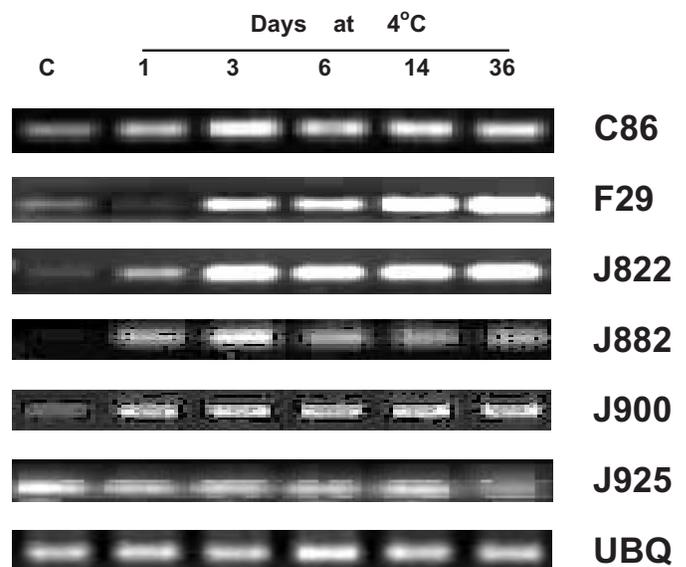

ments (Strand et al. 1997). Though the experimental conditions and the days of sampling in the *Arabidopsis* work were not identical to the experiments reported here, the comparison of patterns of expression indicates a different response to cold in these 2 species and between the 2 wheat cultivars. In *Arabidopsis*, these genes show a rapid decline in transcript levels at 1 day of cold treatment and a partial and gradual recovery over 21 days (Strand et al. 1997). In wheat, transcripts show little change after 1 and 6 days of acclimation, but at 36 days the spring wheat shows a small increase in transcript levels, whereas the winter wheat shows a reduction of transcripts for these genes. The biochemical significance of the decreased gene expression for photosynthesis-related genes in winter wheat warrants further investigation. It may reflect a decrease in the light-harvesting apparatus that would reduce the imbalance between light-harvesting and dark reactions; it may also reflect reduced damage and consequently reduced recycling of proteins of the photosynthetic apparatus of winter wheat compared with spring wheat.

Oxidative stress

In our study, peroxidase (J884) transcript levels are more strongly upregulated in spring wheat than in winter wheat at 1 day and at 36 days (Table 4 and supplementary Table S1[3]). Peroxidase plays a role in detoxification of peroxide, an active oxygen species that is enhanced by cold stress, and changes in peroxidase expression in cold-stressed plants has been previously described in several species (e.g., Anderson et al. 1995). The disruption of photosynthesis by cold increases the reduction state of components of the photosynthetic apparatus that contributes to increased reactive oxygen species. A peroxidase was shown to be transiently induced early in cold treatment and was again upregulated at 7 days of cold

Fig. 3. Gene expression profile for selected genes. Transcript levels of cold-regulated genes C86, a glycine-rich protein; F29, a receptor-like kinase; J822, guanine nucleotide regulatory protein; J882, an inorganic pyrophosphatase; J900, a calcium-binding EF-hand protein; J925, a GTP-binding protein measured by RT-PCR. Seedlings of freezing-tolerant winter cultivar 'Clair' were either kept at 22 °C (C) or grown at 4 °C for the periods indicated. Their total RNAs were extracted and used for RT-PCR with gene-specific primers as described in Materials and methods.

acclimation in *Arabidopsis* (Fowler et al. 2002). Though longer term treatments were not included in the study, the *Arabidopsis* pattern is most similar to that observed in spring wheat catalase, which also detoxifies hydrogen perox-

ide. In winter wheat, the catalase is slightly repressed at 6 days, though it is not statistically different from spring wheat catalase. These patterns show a similarity between spring wheat and *Arabidopsis* and suggest that winter wheat has evolved additional responses to avoid oxidative stress. The decreased turnover of components of the photosynthetic apparatus may also reflect this adaptation. The data presented here are suggestive since peroxidases are encoded by a gene family of at least 9 members in *Arabidopsis* (Ostergaard et al. 1998) and the gene expression patterns differ among gene family members (Fowler et al. 2002). Further studies are necessary to fully describe the response of peroxidase genes during cold acclimation in wheat.

Previously known cold-regulated genes

Microarray analysis identified a large number of genes with relatively strong gene induction during cold acclimation; in some cases genes showed greater than 20-fold induction. Many such genes are seen in clusters 1 to 6 for winter wheat in supplementary Fig. S1[3] and include a large number of the previously described cold acclimation-induced genes. These include WCS120 (Houde et al. 1992, Saran et al. 1997), actin depolymerizing factor-like (Danyluk et al. 1996), WCOR615, WCOR80, WCOR825 (Danyluk 1997), J330 a cold-responsive late embryogenesis abundant (LEA)/RAB-related protein (Tsuda et al. 2000), phosphoethanolamine methyltransferase (Frenette Charron et al. 2002), WCOR14a (N'Dong et al. 2002), and WCOR413 (Breton et al. 2003). Expression patterns for these genes are similar to what was previously obtained by northern blot analysis, thus supporting the accuracy of the microarray analysis. Other strongly induced genes in these clusters such as the ice-recrystalization inhibitor protein were previously described in carrot (Worrall et al. 1998) and in wheat (Tremblay et al. 2005).

Validation of microarray data for selected cold-regulated transcripts

Several genes that were observed to have altered transcript levels and differential expression between the 2 cultivars in the microarray analysis were selected for confirmation of transcript changes by RT-PCR. The genes include C86, F29, J822, J900, and J925. Their BLAST-based annotations are glycine rich protein, a receptor-like kinase, guanine nucleotide regulatory protein, calcium-binding EF-hand protein, and Ras-related GTP-binding protein, respectively. All but J822, which was downregulated, were found to be up-regulated in the more freezing-tolerant cultivar (Table 1). The majority of the selected clones are from genes that have not been previously characterized as being cold-regulated proteins in wheat and represent genes that showed a range of changes in transcript levels by microarray analysis from less than 2-fold to more than 5-fold. Transcript levels were measured over a time course of cold acclimation of 1, 6, and 36 days in 'Norstar'-derived winter cultivar 'Clair', LT_{50} −19 °C.

As can be seen in Fig. 3, the pattern of expression revealed by RT-PCR is in general agreement with that of the microarray. However, in the case of J822, the micorarray data predicted strong increases before day 6 and a weaker increase after, whereas the RT-PCR data showed a reverse trend. In light of the potential regulatory roles of these genes, we are conducting further studies that may clarify this discrepancy.

These results together with those obtained in previous studies with known cold-regulated genes strongly support the reliability of the data obtained by microarray analysis.

Conclusions

Microarray analysis led to the discovery of 43 novel genes that were differentially regulated between spring and winter cultivars. These genes and their products are good candidates for functional analysis using bioinformatic, biochemical, and genetic approaches. In addition, a large number of novel cold-regulated genes were detected that were not necessarily differentially regulated between the 2 cultivars. The identification of this large number of novel genes using transcription profiling demonstrates that microarray analysis for genotype comparisons is an extremely useful approach to characterizing differences in gene expression associated with superior freezing tolerance.

Acknowledgements

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