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Regulatory gene candidates and gene expression analysis of cold acclimation in winter and spring wheat

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Abstract Freezing tolerance in plants develops through acclimation to cold by growth at low, above-freezing temperatures. Wheat is one of the most freezing-tolerant plants among major crop species and the wide range of freezing tolerance among wheat cultivars makes it an excellent model for investigation of the genetic basis of cold tolerance. Large numbers of genes are known to have altered levels of expression during the period of cold acclimation and there is keen interest in deciphering the signaling and regulatory pathways that control the changes in gene expression associated with acquired freezing tolerance. A 5740 feature cDNA amplicon microarray that was enriched for signal transduction and regulatory genes was constructed to compare changes in gene expression in a highly cold-tolerant winter wheat cultivar CDC Clair and a less tolerant spring cultivar, Quantum. Changes in gene expression over a time course of 14 days detected over 450 genes that were regulated by cold treatment and were differentially regulated between spring and winter cultivars, of these 130 are signaling or regulatory gene candidates, including: transcription factors, protein kinases, ubiquitin ligases and GTP, RNA and calcium binding proteins. Dynamic changes in transcript levels were seen at all periods of cold acclimation in both cultivars. There was an initial burst of gene activity detectable during the first day of CA, during which 90% of all genes with increases in transcript levels became clearly detectable and early expression differential between the two cultivars became more disparate with each successive period of cold acclimation.

Keywords Cold acclimation · Regulatory gene · Gene expression profile · Wheat

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Introduction

In nature, low temperatures in the fall normally begin before freezing temperatures occur and plant cells sense these changes and initiate a genetically regulated program that leads to an increased capacity to survive freezing (Thomashow 1999). Although the precise mechanism for low temperature sensing and acclimation is not well understood, the characterization of cold regulated genes has given important insight into the signaling pathways and the regulation of genes associated with cold acclimation. An alteration of membrane fluidity appears to be the primary sensing event followed by an influx of calcium into

the cytosol (Monroy and Dhindsa 1995), and the activation of a signaling cascade of transducers, some of which are protein kinases. Two well-characterized protein kinases that participate in these cascades are CDPKs (Martin and Busconi 2001) and MAPK (Teige et al. 2004). Although the ultimate primary targets of the cold signaling cascade have not been identified, they are likely to be downstream transcription factors such as *ICE1* (Chinnusamy et al. 2003), a constitutively expressed MYC-like bHLH transcription factor. ICE1 in turn regulates the cold-induced expression of CBF transcription factors belonging to the AP2 family. Overexpression of CBF3 has been shown to regulate a large portion of cold-modulated gene expression and to lead to alteration of physiological markers of cold acclimation at room temperature (Cook et al. 2004). However, close to one third of the genes induced by cold are not responsive to CBF regulation (Vogel et al. 2005), suggesting that other regulatory elements may synergistically contribute to mounting the cold response.

Although low temperature sensing and transduction may be common to many plant species, the ultimate response is not. Among plant species and cultivars of the same species there is wide range of freezing tolerance that develops upon cold acclimation (Thomashow 1999). For species such as wheat, which has complex ploidy and is currently not easily approachable by mutant screening and transformation, a promising approach is the identification of key genes by comparative transcriptome analysis of genotypes with contrasting responses to cold acclimation (Gulick et al. 2005). Winter cultivars develop a stronger freezing tolerance than spring cultivars, but require a period of exposure to low temperature before flowering (Fowler et al. 1996). Genetic control of this vernalization requirement involves an epistatic interaction between two Vrn loci. Vrn1, which encodes a MADS-box transcription factor that is hypothesized to induce flower initiation, has increased levels of expression in winter wheat after vernalization, but is constitutively expressed in spring wheat (Yan et al. 2003; Danyluk et al. 2003). Vrn2, which encodes a zinc finger-CCT domain transcription factor, is a repressor of flower initiation and is down-regulated by vernalization in winter cultivars (Yan et al. 2004). Inheritance of dominant spring alleles of Vrn1 in wheat has an inverse relationship with the maximum acquired freezing tolerance (Fu et al. 2005). The Vrn alleles are thought to control not only the transition from vegetative to reproductive growth, but also to control genes affecting the expression of low temperature-induced genes associated with the acquisition of frost tolerance (Danyluk et al. 2003).

In a prior 1200 feature microarray analysis, we detected complex changes in gene expression in wheat during cold acclimation, pointing to distinct clusters of genes whose expression is differentially regulated between winter and spring wheat (Gulick et al. 2005) that included genes with potential signal transduction function. However, the small size of the array led to a lack of representation of transcription factors. It is clear that key transcription factors, such as CBF, those encoded by Vrn genes and other gene families implicated in flower induction, may play critical roles in the complex genetic interactions that regulate freezing tolerance. In this study we used a semi-dedicated cDNA amplicon micorarray with improved gene coverage, with 5740 features, one third of which are potential signal transduction genes. Our main objective was to discern critical differences in the expression of signal transduction genes between a winter and a spring wheat cultivar.

Experimental procedures

Plant material and growth conditions

Spring wheat *Triticum aestivum* L. cv Quantum, (LT50 of –8°C), and the winter wheat *T. aestivum* L. cultivars CDC Clair, (LT50 of –17°C), Jagger and Alabaskaya were germinated in a 1:1 mixture of vermiculite and soil for seven days with a 16 h photoperiod. White fluorescent and incandescent lighting was combined to provide an irradiance of 196 μmol m^{-2} s^{-1}. The temperature was maintained at 20 ± 1°C. Seedlings were cold acclimated by lowering the growth temperature to 4°C and plants were harvested after 6 h, 1, 3, 6, and 14 days. Three biological replicates of the experiment were grown for all microarray hybridizations. Control seedlings were grown for one additional day at 20 ± 1°C. The aerial part of 15 to 30 seedlings, including the meristematic crown and leaves (3–4 g), was used for the extraction of total RNA. Tissues were ground in liquid Nitrogen and homogenized in TRIZOL reagent (5 ml/g) according to the manufacturer's protocol (Invitrogen, Burlington, Ont).

LT_{50} determination

The LT_{50} of the winter wheat *Triticum aestivum* L. cv CDC Clair and the spring wheat *T. aestivum* L. cv Quantum was determined by controlled freezing and regrowth tests; details of which are presented in the supplementary materials.

Microarray construction and analysis

A cDNA amplicon microarray representing 5170 genes including 1630 genes with sequence similarity to genes involved in signal transduction and regulation was prepared from clones from *T. aestivum* identified in the Genome Canada supported Functional Genomics of

Abiotic Stress (FGAS) and NSF supported wheat EST sequencing program. Details of the microarray construction, hybridization and statistical analysis are provided in the supplementary materials.

Comparison to Arabidopsis microarray analysis

The EST sequences for the wheat microarray features were used for a blastx search for the highest scoring match in the Arabidopsis protein sequence database from TAIR and the AT identifier for the gene was recorded. The features from the wheat array were compared to Arabidopsis genes that were reported to be cold regulated in any of the four studies reported in three publications: Fowler and Thomashow (2002), Seki et al. (2002) and Oono et al. (2006). Wheat genes with a P value ≤ 0.01 for cold treatment effects or for cold treatment-by-cultivar interaction effects in ANOVA analysis were used to compare to the set of cold regulated genes identified in Arabidopsis.

RT-PCR measurement of transcript level of selected genes

Total RNA samples were treated with DNaseI (Ambion inc, Austin, Texas) and 5 μg of RNA was reverse transcribed using Invitrogen Superscript II and oligo-dT primer (Invitrogen, Burlington, Ont.) according to the manufacturer's protocol. A fiftieth volume of each cDNA was used for PCR amplification by Taq DNA Polymerase (MBI Fermentas, Burlington, Ont.) using the cycling conditions: 94°C, 2 min; 35 cycles of 94°C, 30 s; 57°C, 30 s; 72°C, 45 s. The primers for each gene are listed in Supplementary Table S4. To rule out gDNA contamination, control PCR reactions were done with RNA samples that had not been reverse transcribed.

Mapping of microarray features to chromosome bins

Chromosome locations for 2999 of the genes represented on the microarray were obtained from *T. aestivum* EST mapping programs with a number of features mapped to more than one locus. The NSF (UAS) wheat EST mapping program had mapped 2804 of these (Qi et al. 2004) and were they were identified with their corresponding GenBank ID or NSF sequence identifier (http://www.wheat.pw.usda.gov/cgi-bin/westsql/sql.cgi). These ESTs had been mapped by Southern blot analysis to bins corresponding to chromosomal deletion regions among a series of cytogenetic stocks of the wheat cultivar Chinese Spring. A sub-set of 165 genes from the FGAS project were mapped using the same series of cytogenetic stocks of the wheat cultivar Chinese Spring used by Qi et al. (2004). These included 19 nullisomic-tetrasomic lines, 23 ditelosomic lines and 96 deletion lines. Southern blotting and hybridization analysis was conducted using membranes with genomic DNA of the Chinese Spring wheat digested with the restriction enzyme *Dra*I as described by Faris et al. (2000) with minor modifications. In addition 30 features with intriguing expression patterns were virtually mapped by Blastn search with their sequences against the mapped database (http://wheat.pw.usda.gov/GG2/blast.sht) using a cut-off of 95% identity over a minimum of 200 bp.

Results and discussion

Gene expression during cold acclimation

To identify candidate genes associated with increased winter survival, we compared the gene expression profiles of a winter and a spring cultivar that differ in freezing tolerance by 10°C. cDNA clones were selected from the EST databases of the Genome Canada program Functional Genomics of Abiotic Stress (FGAS), and the National Science Foundation supported wheat EST program (Qi et al. 2004). We produced a cDNA amplicon microarray with 5740 features representing 5160 unique genes, including 1630 ESTS with high sequence similarity to several classes of regulatory and signal-transduction associated genes. Changes in gene expression during cold acclimation were compared in the winter wheat cultivar CDC Clair and the spring cultivar Quantum over a time course of cold acclimation at 4°C including five time points ranging from 6 h to 14 days. The dynamic development of low temperature tolerance has been extensively characterized in wheat and other Triticeae species and these studies show that development of low temperature tolerance is most rapid during the first 14 days of cold acclimation (Fowler et al. 1996, Prasil et al. 2004). A common-reference experimental design was used in which all hybridizations were carried out with a test sample compared to a common reference sample of RNA that was a mixture of control and cold acclimation samples (Fig. 1). This design facilitates multiple comparisons between sample sets (Simon and Dobbin 2003).

Thirty-three features that are annotated as previously identified cold regulated genes were seen to be significantly up-regulated at multiple time points in this study, indicating that the plant growth system and microarray detection methodology functioned correctly. Pair-wise comparisons between non-acclimated and cold-acclimated plants detected a large number of genes with altered levels of expression in each cultivar and significant differences in expression between the two cultivars. Complete data

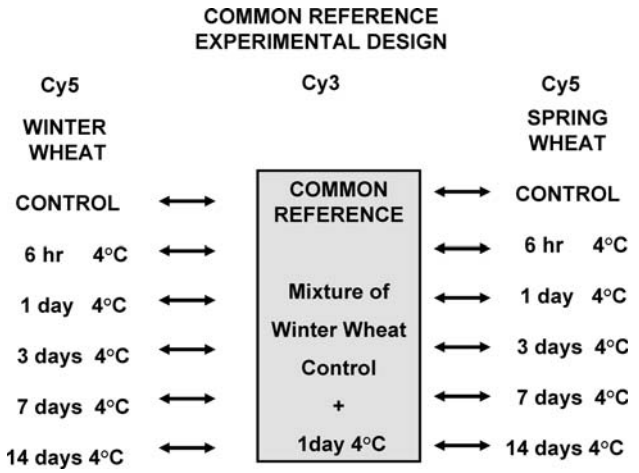

Fig. 1 Experimental design. A common reference design was used in which RNA from control plants and plants acclimated for 6 h, 1 day, 3 day, 7 day, and 14 day from each cultivar were compared to a common reference RNA that was a mixture of RNA from CDC Clair control plants and plants acclimated for 1 day at 4°C

including fold change, *P* values, and *q* values (False Discovery Rate) are presented in supplementary data, Table S2. Genes that had at least a 2-fold change of mRNA levels and *P* value $\leq$ 0.01 in a Student's *t*-test are summarized in Fig. 2. More than twice as many genes were up-regulated at 6 h of cold acclimation (CA) in the spring cultivar relative to the winter cultivar (Fig. 2A), however, by one day of CA, the winter cultivar had substantially more genes up-regulated than the spring cultivar. Throughout the time course up to 14 days of CA the number of up-regulated genes remained much higher in winter wheat. A similar pattern was seen for the down-regulated genes with the winter cultivar having substantially more genes down regulated than the spring cultivar throughout the CA time course. The number of newly detectable genes at each time point further showed the marked difference between the two cultivars after the initial response (Fig. 2 B). The great majority of genes that had significant changes in expression during 14 days of CA were detected within 24 h of the initiation of CA. The relative steady-state level of specific mRNAs was also compared between the two cultivars at each time point of CA. Differences between the two cultivars were detected before the onset of CA, with more genes showing a higher constitutive level of expression in the winter cultivar than in the spring cultivar (Fig. 3A). At 6 h there were more genes with a higher level of expression in spring wheat than in winter wheat but after 24 h the number of genes with higher levels of expression was greater in winter wheat with very large differences detected by 6 days of acclimation. Comparison of induction for genes on the array which were annotated as cold regulated genes based on Blastx hit in GenBank, which tend to be highly expressed and highly induced genes, showed higher

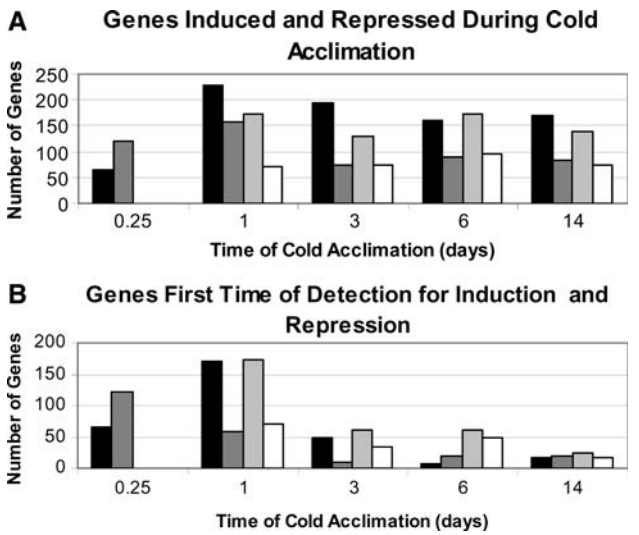

Fig. 2 (**A**) The number of genes with significant increase or decrease in RNA levels compared to the non-cold treated plants of the same cultivar. Genes with a *P* value $\leq$ 0.01 in a Student's *t*-test, and at a least a 2-fold change in RNA levels were counted at each time point. Color-coding of the bars: genes induced in CDC Clair: black; induced in Quantum: dark grey; repressed in CDC Clair: light grey; repressed in Quantum: white. (**B**) The number of genes detected as induced or repressed for the first time. The color/pattern coding is the same as in 2A

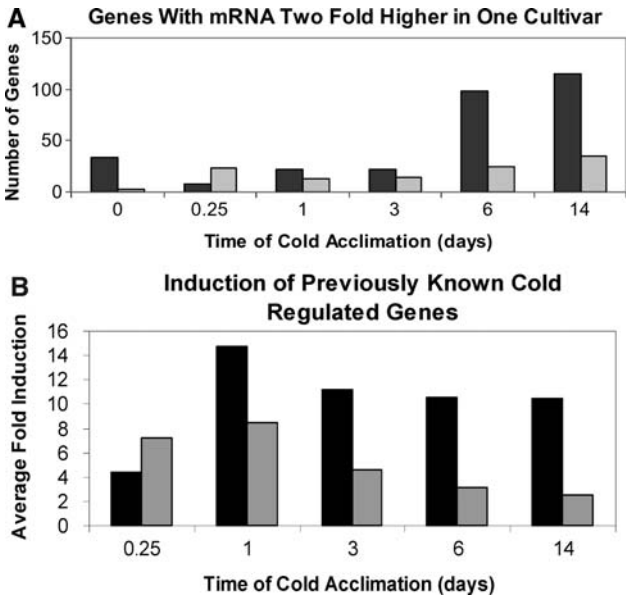

Fig. 3 (**A**) The number of genes that had transcript levels 2-fold higher in one cultivar than the other at each time point with ($P \leq 0.01$). The levels of RNA for the genes were compared directly between the two cultivars. Winter wheat cultivar CDC Clair, black; spring wheat cultivar Quantum, grey. (**B**) The average level of induction of a set of 43 genes that had been previously reported as cold regulated over the time course of cold treatment in the two cultivars

and more sustained induction in winter wheat than in spring wheat. (Fig. 3B).

To focus on genes with significantly different patterns of expression in response to CA in the two cultivars, data was analyzed by two-way ANOVA with cultivar and cold treatment as independent variables. Genes with a significant cultivar-by-treatment interaction effect are expected to be genes with changes in mRNA levels in response to cold treatment but with different response in the two cultivars. There were 441 genes with a P value $\leq$ 0.01 for a treatment by cultivar interaction effect and with at least a 2-fold change in expression relative to non-treated controls for at least one point. Features with a P value of 0.01 or less for the cultivar by treatment interaction have a false discovery rate (q value) of 0.038 (Storey and Tibshirani 2003). This set of genes is listed in supplementary materials, Table S3. Among these genes, 134 were annotated with regulatory and signal transduction functions. The 441 genes were analyzed by average linkage hierarchical clustering analysis (Eisen et al. 1998) and the global changes in the transcription profiles of the two cultivars were readily apparent. Selected clusters that best illustrate the differences in gene expression in the two cultivars are presented in Fig. 4 (global clustering is presented in supplementary figure S1). There is a tendency of a more marked and sustained up- and down-regulation of genes in the winter than in the spring cultivar. However, there are also some clusters with genes whose induction or repression is more marked in the spring than in the winter cultivar. For example, genes in cluster 1 are induced early, but transiently. Their transcript levels rise early, to higher levels in the winter than in the spring cultivar, but then decrease rapidly to basal levels. Most transcription factors detected as differentially expressed genes were found to follow this pattern of regulation. Common signaling and regulation networks may regulate genes with common patterns of regulation.

Sorting the data by functional classes indicates that both a stronger and a more sustained induction of genes in the winter cultivar than in the spring cultivar are evident in each functional group. Data for the signal transduction candidates can be found in Tables 1–3. Taken together, these results show that the initial response to CA, a burst of gene activity, is common to both cultivars, but the global transcriptional profiles of the two cultivars become markedly different during the time course of cold acclimation. This suggests that in the spring cultivar, the program initiated by CA is rapidly overridden by another regulatory program.

It should be noted that microarray features may detect the expression of more than a single gene since probes will cross hybridize with mRNAs with high sequence identity. *T. aestivum* is an allohexaploid species and thus, most genes are present as three homeologous copies with sequence identity in the range of 97–95%. Homeologs, ''orthologs'' from the three ancestral species, are expected to have similar patterns of expression for the great majority of genes, though there are numerous examples of tissue specific silencing of one or more homeologs (Bottley et al. 2006). The detection of closely related genes by a single microarray feature is expected with cDNA amplicon arrays as well as with most oligonucleotide based arrays, especially for polyploid species for which complete genome sequence is not available.

Comparison to Arabidopsis microarray analysis

The four sets of microarray analysis of gene expression in response to cold treatment in Arabidopsis reported a total of 1136 genes that were up or down regulated by cold treatment (Fowler and Thomashow 2002; Seki et al. 2002; Oono et al. 2006). A total of 262 of these genes had 580 matches on the wheat microarray due to the fact that multiple wheat EST sequences share the same best hit in the Arabidopsis database. Among the wheat microarray features with a $P \leq 0.01$ for treatment effects or treatment by cultivar interaction effects in ANOVA, 237 genes had a corresponding Arabidopsis gene that was reported to be cold regulated in Arabidopsis in at least one publication. These represent 139 unique genes in Arabidopsis since 98 of the wheat genes shared the same best hit in this set of Arabidopsis genes. These include a large number of transcription factors as well as RNA binding proteins and proteins involved in photosynthesis. The comparison of the wheat microarray results with those of Arabidopsis are incorporated into the supplementary Table S2. There are a number of limitations in making direct comparison of wheat microarray results to those of Arabidopsis including the divergence of DNA sequence between such distantly related species, differences in experimental design, and the different statistical cut-off and fold-change thresholds used in the different studies. Nevertheless there is remarkable similarity in the genes that have been reported to be cold regulated in the two species.

Analysis of differentially expressed genes by functional classes

Protein kinases

Protein kinases play a central role in sensing and integrating environmental signals. Thirty-four probes for protein kinase genes showed significant cultivar-by-cold treatment interaction effects during CA (Table 1). Among them there were representatives of four known protein kinase groups: receptor-like protein kinases, calcium-modulated protein kinases, MAPK, casein kinase, and ESTs

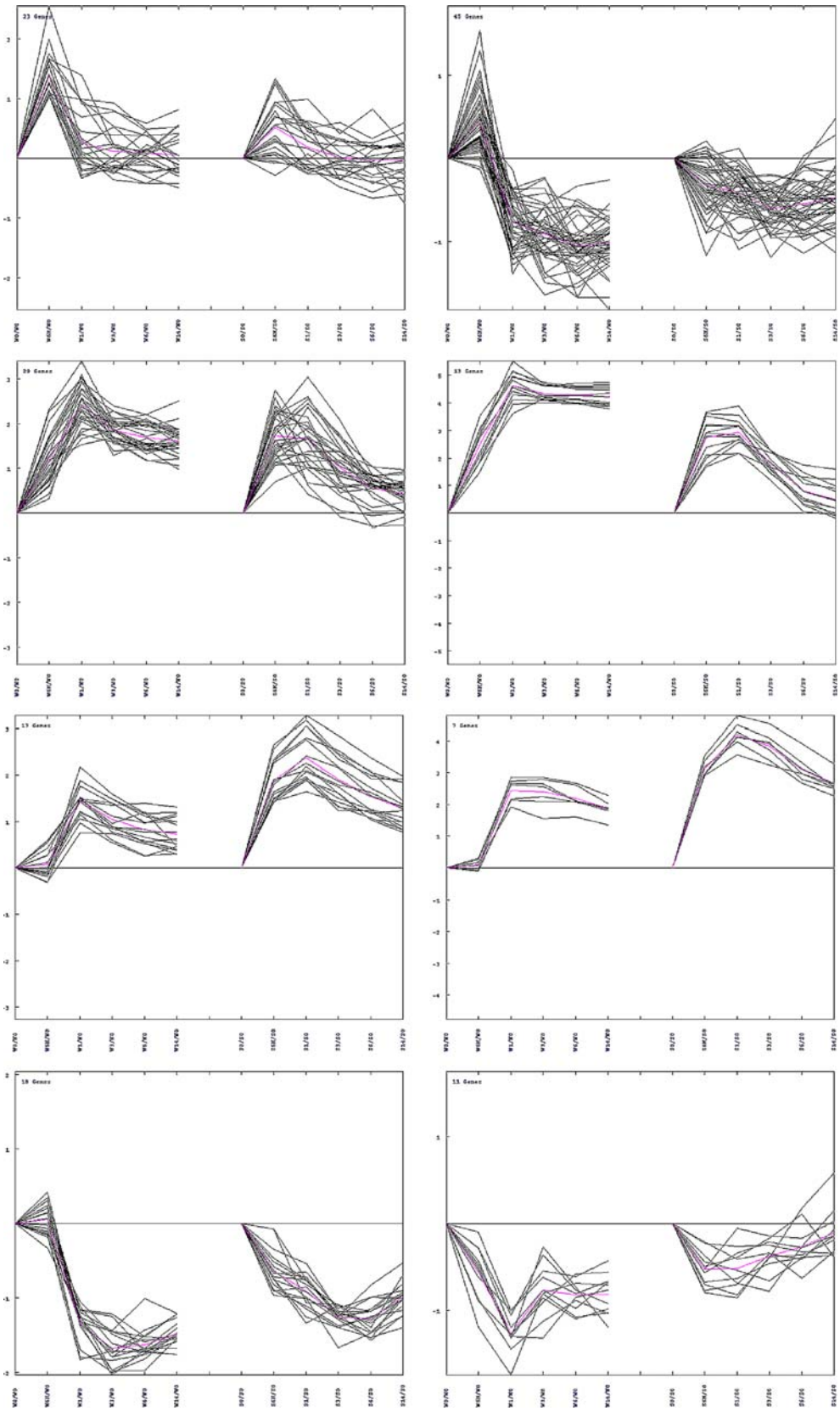

Fig. 4 Selected gene clusters demonstrate different patterns of expression in the winter cultivar CDC Clair and spring wheat cultivar Quantum during cold acclimation. Genes that likely had different patterns of expression in CDC Clair and Quantum were those that had a significant interaction effect between cultivar and treatment in the two-way ANOVA and had at least a 2-fold change relative to untreated controls for at least one time point. Hierarchical clustering was used to identify clusters of genes that had similar patterns of expression in the two cultivars

Table 1 Protein kinases differentially regulated in Clair and Quantum wheat cultivars during cold acclimation

ID	GB ID	Annotation	W0/S0	Winter Wheat, days 4°C					Spring Wheat, days 4°C				
				0.25	1	3	6	14	0.25	1	3	6	14
Tr004_D05	BE490430	casein kinase II B-su	1.23	1.88	2.28	2.17	2.08	2.30	1.97	1.93	1.57	1.44	1.60
Tr012_O18	CV769361	CBL-interacting PK	1.27	1.65	4.99	3.63	2.91	2.38	2.43	5.17	2.64	1.27	1.02
Tr012_N21	DR741590	Calcium-dependent PK	1.13	1.80	4.35	3.12	2.46	2.01	1.67	3.01	1.87	1.28	1.03
Tr013_F22	DR741744	CBL-interacting PK	0.81	1.98	0.98	1.71	1.29	1.57	1.23	1.83	2.81	2.53	2.36
Tr013_N24	DR741905	CBL-interacting PK	0.91	1.68	0.75	1.12	0.90	0.99	1.02	1.25	2.04	2.08	1.82
Tr012_A22	CV760865	CBL-interacting PK	1.01	2.08	0.80	0.92	0.93	0.85	1.73	0.99	1.26	1.12	1.20
Tr009_P20	BF478514	PK CDG1-like	0.66	1.44	0.51	0.29	0.33	0.37	0.90	0.59	0.43	0.44	0.60
Tr013_J14	DR741812	LRR transmembrane PK	1.58	2.34	17.5	29.4	21.4	24.1	7.44	14.7	15.9	11.8	9.13
Tr001_M19	CK197231	LRR transmembrane PK	1.21	2.88	16.4	27.1	19.6	20.4	5.13	10.8	8.00	6.68	5.58
Tr002_A03	CK161386	LRR transmembrane PK	0.78	8.48	28.2	18.4	15.9	13.6	8.99	8.94	3.18	1.21	0.88
Tr013_A12	DR741660	LRR transmembrane PK	1.06	0.73	0.41	0.70	0.63	0.92	0.67	0.59	0.84	1.01	1.05
Tr001_D05	CK201314	LRR transmembrane PK	0.91	3.00	9.4	14.4	11.6	10.5	3.47	5.86	4.06	4.14	3.32
Tr002_A10	CK201931	LRR transmembrane PK	2.06	2.79	10.3	14.2	10.2	10.9	7.54	11.2	8.34	6.63	6.32
Tr001_B19	CK201227	LRR transmembrane PK	0.95	3.34	8.40	12.7	8.00	9.51	4.22	4.56	3.73	3.56	3.66
Tr013_N12	CV781244	Receptor PK	1.41	1.30	7.4	12.8	11.1	14.1	2.06	5.24	5.86	6.02	4.14
Tr001_H22	CK196048	Receptor-like PK	1.08	1.74	6.87	4.86	3.81	4.32	3.83	1.97	1.30	1.03	1.04
Tr013_L15	DR741835	Receptor-like PK	0.62	1.15	0.41	0.29	0.35	0.43	0.61	0.59	0.42	0.56	0.69
Tr012_A04	DR733134	Wall-associated kinase	1.05	1.40	1.44	2.20	1.99	2.19	0.94	1.37	1.61	1.53	1.42
Tr013_M24	DR734723	Wall-associated kinase	0.90	2.47	2.97	1.79	2.00	1.65	2.79	2.33	1.26	0.88	0.78
Tr012_L13	CV766715	LRR transmembrane PK	1.13	1.21	1.66	1.84	2.22	2.62	1.37	1.51	1.37	1.27	1.97
Tr002_N16	BE591959	Receptor-like PK	1.16	1.61	2.33	1.73	1.73	1.91	1.63	1.33	1.30	1.23	1.34
Tr005_G07	BE403270	S-receptor kinase	1.53	0.41	0.50	1.01	0.99	1.52	1.08	0.88	0.68	0.93	1.39
Tr012_D17	DR741398	Receptor-like kinase	0.72	1.09	0.41	0.63	0.79	1.13	0.39	0.42	0.57	0.96	1.53
Tr016_I14	DY741574	LRR receptor PK	0.74	1.19	0.46	0.50	0.54	0.62	0.57	0.50	0.62	0.71	0.81
Tr001_B22	CK204397	Receptor PK	1.00	1.04	0.46	0.33	0.30	0.30	0.95	0.59	0.44	0.40	0.49
Tr001_K06	CK199849	MAP kinase 4	1.04	2.16	1.04	1.14	1.08	1.47	1.31	0.86	0.98	1.17	1.09
Tr002_L10	BG262240	PK	0.74	1.39	1.45	2.39	2.41	3.39	0.92	0.98	0.91	0.68	0.87
Tr013_P06	DR741928	PK	1.15	1.40	2.77	1.93	1.37	1.53	1.49	2.01	1.75	1.21	1.00
Tr014_N16	DY761342	PK	0.93	1.94	0.92	1.64	1.48	1.57	1.27	1.79	3.12	2.97	2.81
Tr006_K05	BE606438	PK	0.77	1.76	0.91	1.34	1.09	1.16	1.26	1.51	2.27	2.20	2.04
Tr014_I17	CV767912	PK	1.22	3.00	1.09	1.15	1.56	1.38	3.94	1.15	1.33	1.06	0.93
Tr014_O11	DR734156	PK	1.16	0.68	1.04	1.02	1.74	2.43	1.02	1.13	1.28	1.27	1.42
Tr001_G13	CK195470	PK	0.86	2.61	0.82	0.88	1.11	1.24	1.07	0.84	0.93	0.91	0.98
Tr012_H03	CV763951	PK	1.09	0.97	0.48	0.85	0.84	0.99	0.86	0.56	0.63	0.83	0.96
Tr003_E03	CK162128	Protein phosphatase	1.04	2.39	0.93	1.05	1.27	1.28	3.23	0.84	1.06	1.16	1.27
Tr013_A20	DR741667	Protein phosphatase	1.71	1.71	4.50	3.03	2.25	2.31	3.57	5.46	3.39	1.53	1.49
Tr013_K22	DR741860	Protein phosphatase	0.51	1.84	0.67	0.33	0.45	0.36	0.71	0.64	0.41	0.41	0.38
Tr013_F07	DR741713	Protein phosphatase	1.07	1.66	3.18	3.63	2.51	2.97	1.63	2.03	1.92	1.45	1.54

Genbank ID is the Genbank accession for the corresponding wheat EST for the feature. W0/S0 is the level of mRNA in the winter control relative to the spring control. Other numbers indicate induction or repression relative to the control for each cultivar

classified only as serine/threonine protein kinases. The most abundant class among those PKs were the receptor-like protein kinases. For the majority of these, transcript levels rose rapidly early in CA and remained up-regulated throughout the period of cold acclimation. There was a significant difference in the induction of these receptor-like protein kinases between the two cultivars. They were more strongly induced in the winter cultivar and transcripts remained elevated at the later time points in the winter cultivar. Most of the available evidence indicates that these protein kinases play important roles in plant development and disease resistance (Shiu et al. 2004). In light of their

Table 2 Transcription factors differentially regulated between Clair and Quantum wheat cultivars during cold acclimation

ID	GB ID	Annotation	W0/S0	Winter Wheat, days 4°C					Spring Wheat, days 4°C				
				0.25	1	3	6	14	0.25	1	3	6	14
Tr014_J01	DY761246	AP2 TF (CBF)	1.28	7.41	6.87	1.38	2.57	1.47	10.4	3.41	2.19	1.48	1.22
Tr014_L06	DY761332	AP2 TF(CBF)	0.86	4.66	0.95	1.01	1.18	1.26	6.93	0.91	1.01	0.66	0.63
Tr002_O04	CK194031	AP2 TF(CBF)	1.92	3.61	0.81	0.73	0.64	0.68	7.82	1.53	1.20	1.39	1.39
Tr002_K01	CK208992	AP2 TF	1.36	3.41	1.65	1.44	1.27	1.47	5.64	1.73	1.97	1.36	1.69
Tr003_B13	CK212603	AP2 TF(CBF)	2.07	2.66	0.67	0.44	0.51	0.46	6.88	1.57	1.03	1.29	1.17
Tr014_J09	DY761250	AP2 TF(EREBP)	0.86	2.42	0.97	0.84	1.10	0.93	0.96	0.88	0.78	0.94	0.75
Tr001_F24	CK205085	Ap2 TF	0.73	2.36	1.26	0.99	0.81	0.71	0.94	0.93	0.88	0.80	0.80
Tr014_L08	DY761333	AP2 TF(CBF)	0.93	2.28	0.73	0.89	1.19	1.21	3.06	0.75	0.80	0.53	0.45
Tr011_B14	DY761110	AP2 TF(ERF)	0.98	2.09	1.61	1.72	1.51	1.77	1.87	1.41	1.25	0.97	1.02
Tr014_P07	DY761287	AP2 TF(DRF3)	1.16	2.09	0.86	0.97	1.11	0.93	1.21	1.13	0.92	0.75	0.87
Tr002_E01	CK163316	AP2 domain TF	1.09	2.02	1.36	1.31	1.30	1.33	2.54	1.40	1.22	1.15	1.16
Tr011_F02	DY761124	AP2 TF(ERF1)	1.13	1.42	1.43	1.38	1.21	1.44	1.41	1.27	1.69	1.89	2.23
Tr014_N09	DY761276	AP+D192 TF(ERF3)	0.87	1.00	0.86	0.65	0.71	0.84	0.50	0.64	0.74	0.73	0.85
Tr002_I05	CK207591	EREBP-type TF	0.91	0.97	1.40	1.36	1.15	1.48	0.86	1.06	1.55	1.59	2.08
Tr012_A08	DR741345	ARF-Aux/IAA TF	1.16	1.81	2.64	4.92	3.41	3.76	1.28	2.46	2.83	2.16	2.06
Tr002_M15	CK210277	ARF-Aux/IAATF	0.67	1.39	0.66	0.36	0.34	0.41	0.65	0.83	0.54	0.45	0.53
Tr012_J05	CV765299	bHLH TF	1.82	0.69	0.53	0.42	0.45	0.44	0.88	0.79	0.54	0.47	0.52
Tr012_O04	DR741593	bHLH TF	1.49	0.41	0.53	0.48	0.44	0.48	0.65	0.72	0.44	0.33	0.34
Tr013_I08	DR741808	bHLH TF	1.39	0.38	0.40	0.38	0.62	0.45	0.55	0.47	0.42	0.32	0.38
Tr013_E19	DR741724	bHLH TF	1.89	0.39	0.42	0.52	0.45	0.48	0.80	0.81	0.51	0.50	0.55
Tr012_P15	DR741622	bHLH TF	1.57	0.72	0.64	0.55	0.59	0.64	0.68	0.74	0.50	0.50	0.64
Tr003_C17	CK161884	bZIP	0.85	2.41	0.84	1.02	0.98	0.93	1.66	0.90	0.96	0.55	0.70
Tr013_H18	DR741776	Dof zinc finger TF	1.04	2.65	1.17	1.44	1.05	1.20	1.62	1.42	1.52	1.26	1.52
Tr011_L06	DY761158	Homeodomain LZIP TF	1.27	3.03	3.20	1.13	1.58	1.14	3.30	1.69	1.33	1.43	1.04
Tr001_D20	CK204922	Homeobox TF	0.83	0.83	0.24	0.19	0.20	0.25	0.52	0.34	0.27	0.28	0.37
Tr012_G16	CV763584	Homeobox TF	0.99	0.82	0.48	0.78	0.59	0.53	0.86	0.75	0.79	0.65	0.80
Tr014_H04	DY761362	MADS-box	0.51	4.21	17.4	17.1	17.3	14.9	3.21	4.47	3.16	2.45	2.33
Tr012_K04	CV765903	MADS-box TF	0.83	2.71	2.95	1.57	1.74	1.66	2.13	2.01	0.95	0.68	0.77
Tr014_H18	DY761369	MADS-box TF	0.38	2.57	1.48	1.09	1.45	1.32	0.75	1.57	2.01	1.53	1.51
Tr014_H06	DY761363	MADS-boxTF	0.48	2.24	0.96	1.15	1.05	0.95	0.43	1.17	1.64	1.23	1.32
Tr013_E15	DR741720	MADS box TF	0.36	2.13	0.74	0.90	1.13	0.93	0.26	1.21	1.56	1.35	1.56
Tr014_D12	DY761319	MADS box TF	0.81	1.77	1.09	0.95	1.03	0.99	0.50	1.34	1.62	1.27	1.74
Tr013_L17	DR741837	MADS box TF	0.51	2.37	1.39	1.07	1.21	1.29	0.65	1.42	1.74	1.34	1.45
Tr014_D14	DY761320	MADS box TF	0.71	1.75	0.83	0.78	0.92	0.91	0.42	1.30	1.72	1.25	1.20
Tr014_H16	DY761368	MADS box TF	0.94	1.05	0.62	0.66	0.31	0.31	0.97	0.71	0.76	0.61	0.71
Tr014_B18	DY761311	MADS box TF	2.48	0.22	0.43	0.40	0.33	0.29	1.15	0.89	0.51	0.55	0.52
Tr017_B04	DY742590	c-myb-like TF	2.13	3.27	9.51	4.26	4.29	4.08	12.7	10.2	4.76	3.48	2.99
Tr012_G20	CV763706	MYB TF	3.29	2.38	0.57	0.21	0.15	0.11	5.36	1.21	0.67	0.35	0.51
Tr012_J21	CV765827	MYB TF	2.27	1.09	2.28	1.84	1.71	1.71	3.14	3.89	2.51	2.10	1.77
Tr003_D11	CK213356	NAC TF	1.09	5.05	1.32	1.02	1.47	1.65	4.65	1.47	1.24	1.01	0.80
Tr012_B02	CV760974	NAC TF	0.96	4.81	3.43	1.78	2.31	2.13	4.52	2.23	1.68	1.16	1.10
Tr003_J21	CK217339	NAC TF	0.81	3.10	1.96	1.45	1.30	1.03	2.37	1.21	1.16	1.16	1.14
Tr012_P20	CV770225	NAC TF	1.06	2.76	2.43	1.59	1.92	1.53	3.44	1.68	1.20	0.83	1.10
Tr013_L14	DR741855	NAC TF	0.66	2.22	1.09	0.82	0.91	0.84	1.47	0.54	0.51	0.59	0.61
Tr012_B03	CV760976	NAC TF	1.29	1.90	3.56	6.59	5.70	7.11	2.44	2.97	2.93	3.05	3.20

Genbank ID is the Genbank accession for the corresponding wheat EST for the feature. W0/S0 is the level of mRNA in the winter control relative to the spring control. Other numbers indicate induction or repression relative to the control for each cultivar

Table 3 Additional signaling and regulatory genes differentially regulated in Clair and Quantum wheat cultivars during cold acclimation

ID	GB ID	Annotation	W0/S0	Winter Wheat, days 4°C					Spring Wheat, days 4°C				
				0.25	1	3	6	14	0.25	1	3	6	14
Tr002_P09	DY742468	calcium binding protein	0.53	4.68	4.08	4.63	5.86	8.17	1.08	4.00	6.73	5.21	5.46
Tr001_H04	CK205139	Calmodulin	1.06	1.93	2.77	2.25	1.91	1.58	2.33	1.92	1.73	1.85	1.48
Tr001_F20	CK205047	Calmodulin	1.91	1.03	1.43	0.97	0.76	0.80	1.93	2.38	1.92	1.59	1.47
Tr008_N09	BF474927	calmodulin	0.91	1.39	0.99	0.96	2.08	1.13	1.09	0.82	0.74	0.78	0.68
Tr013_E13	DR741718	Calmodulin	1.21	0.59	0.51	0.64	0.46	0.57	0.72	0.82	0.80	0.73	0.99
Tr001_D06	CK204110	Calmodulin	1.21	1.15	0.48	0.58	0.52	0.53	1.10	0.69	0.81	0.93	0.89
Tr001_B14	CK204343	Calmodulin	3.05	0.32	0.50	0.57	0.50	0.31	3.11	0.81	0.77	0.71	0.97
Tr012_J20	CV765822	Calmodulin	0.76	1.11	0.40	0.29	0.31	0.39	0.79	0.69	0.44	0.44	0.51
Tr001_H02	CK205124	RNA recognition motif- p	6.02	1.05	7.21	7.26	6.36	4.89	11.6	27.5	23.3	15.1	9.71
Tr013_N04	DR741889	RNA-binding glycine-rich p	1.15	0.89	1.38	2.00	2.23	2.23	0.99	1.26	1.26	1.27	1.30
Tr008_O21	BE495896	pumilio/Puf RNA-binding p	0.95	3.97	1.23	0.84	1.17	1.04	1.91	1.99	1.33	1.78	1.17
Tr012_A06	DR741343	RNA-binding glycine-rich p	0.97	2.63	1.33	1.27	1.93	1.55	3.11	1.02	1.11	0.85	0.72
Tr010_C19	BF202303	MTD2-RNA binding protein	0.87	2.08	0.79	1.01	1.37	0.87	1.26	0.90	0.90	0.91	1.21
Tr002_C01	CK201194	RNA Binding Protein 45	0.88	0.50	0.80	1.30	1.04	1.01	0.45	0.78	0.69	0.67	0.89
Tr013_F20	DR741743	RNA-binding glycine-rich p.	1.26	0.93	0.50	0.65	0.57	0.63	0.85	0.84	0.84	0.80	0.78
Tr013_D21	DR741691	RNA-binding glycine-rich p.	2.38	0.81	1.69	1.67	1.43	1.31	2.70	3.14	2.68	2.27	2.36
Tr013_L13	CV779416	RNA-binding glycine-rich p.	1.36	0.73	2.06	2.41	3.25	2.97	1.24	2.03	1.60	1.17	1.52
Tr013_G04	DR741764	RNA-binding glycine-rich p.	1.91	0.46	0.78	1.00	0.81	0.70	1.16	1.36	1.16	1.04	1.16
Tr013_F11	DR741717	RNA-binding glycine-rich p.	2.16	1.00	2.73	2.55	2.62	2.48	2.80	4.20	3.36	2.38	1.97
Tr010_E20	BF146083	RNA-binding protein	0.63	1.98	0.56	0.60	0.46	0.54	0.54	0.82	0.81	0.71	0.71
Tr009_N14	BF146083	RNA-binding protein	0.66	1.90	0.67	0.56	0.53	0.49	0.58	0.88	0.64	0.62	0.53
Tr005_D21	BE496903	RNA-binding protein	0.66	1.70	0.57	0.51	0.51	0.47	0.65	0.76	0.76	0.62	0.63
Tr003_M09	CK206180	RNA recognition motif p.	0.70	1.51	0.67	0.44	0.51	0.48	0.63	0.74	0.44	0.61	0.72
Tr001_H08	CK205155	Kelch repeats protein	0.94	2.17	11.1	19.0	14.0	13.7	4.19	5.94	5.28	4.29	3.32
Tr001_D24	CK204928	TIR1/COI1 E3 ubiquitn lig.	1.51	1.83	1.43	3.20	3.12	4.72	2.25	1.36	2.14	2.28	2.64
Tr014_C06	CV773930	Eceriferum3 (CER3)	0.99	0.57	1.89	2.71	4.63	4.50	0.65	1.53	2.04	1.80	1.85
Tr014_A05	CV758796	cullin	2.89	0.94	3.41	2.62	1.97	1.99	3.47	5.31	4.47	2.93	2.48
Tr014_M04	CV779911	ubiquitin-conjugating enz.	1.07	1.67	1.80	2.36	1.96	1.91	1.54	1.60	1.71	1.34	1.32
Tr014_K07	CV768601	cullin	0.88	0.42	1.00	1.26	2.16	2.36	0.39	1.01	1.14	0.86	0.75
Tr009_P07	BF483259	F-box family protein	0.79	2.13	0.97	1.09	1.10	0.92	0.93	1.13	1.10	1.21	1.11
Tr014_E16	CV775650	F-box ubiquitin ligase	0.81	0.94	0.62	0.72	0.44	0.48	0.56	0.57	0.52	0.47	0.54
Tr014_G02	CV776318	F-box ubiquitin ligase	1.09	0.65	0.50	0.68	0.66	0.74	0.69	0.96	0.91	0.89	1.02
Tr014_I07	CV767155	ubiquitin-conjugating e.	1.00	0.82	0.44	0.59	0.51	0.62	0.67	0.79	0.86	0.82	0.91
Tr014_K11	CV768941	ubiquitin conjugating e.	1.09	0.54	0.41	0.55	0.47	0.50	0.58	0.55	0.77	0.78	0.88
Tr017_I03	DY742260	ubiquitin-conjugating e.	0.81	1.20	0.45	0.47	0.52	0.68	0.54	0.62	0.59	0.66	0.72
Tr014_G18	CV776801	ubiquitin-conjugating e.	0.76	1.11	0.43	0.37	0.25	0.22	0.53	0.66	0.59	0.44	0.39
Tr016_G14	DY741560	Zinc-finger protein	4.03	1.24	6.63	6.96	6.15	4.08	7.69	17.4	15.5	7.78	6.19
Tr013_C20	DR741702	Zinc finger protein	0.86	3.68	24.8	19.2	19.4	18.5	5.26	6.36	3.56	1.73	1.37
Tr003_C03	CK161637	C2H2 zinc finger protein	0.67	3.94	3.16	1.79	1.43	2.31	3.78	1.27	1.16	0.78	0.97
Tr003_N17	DR734368	C2H2 zinc finger protein	0.91	1.89	2.10	1.27	2.01	1.65	1.92	1.53	1.03	1.04	0.89
Tr002_A21	CK204293	C2H2 zinc finger protein	0.94	2.74	2.03	1.16	1.21	1.16	4.30	1.15	0.85	1.09	1.35
Tr010_E10	BF484144	Zinc finger (C2H2 type) p.	0.94	0.86	0.47	0.44	0.47	0.51	0.82	0.64	0.55	0.86	0.72
Tr004_L15	BE406351	GPI-anchored protein	1.32	1.21	4.06	2.08	1.68	1.46	2.47	1.72	0.93	0.64	0.89
Tr011_K14	BF483740	leucine zipper protein	0.78	1.90	2.60	1.80	1.84	1.43	0.90	1.56	1.62	1.61	1.21

Table 3 continued

ID	GB ID	Annotation	W0/S0	Winter Wheat, days 4°C					Spring Wheat, days 4°C				
				0.25	1	3	6	14	0.25	1	3	6	14
Tr005_C17	BE403597	small GTP-binding protein	1.23	1.11	0.97	1.33	1.18	1.68	1.01	1.29	1.68	1.80	2.10
Tr017_K19	DY741551	phosphatidylinositol 4-kinase	0.77	1.13	0.68	0.93	0.62	0.48	0.53	0.59	0.55	0.60	0.71
Tr014_L18	DY761337	developmental/GTP binding p.	0.77	0.93	0.40	0.37	0.35	0.43	0.53	0.55	0.41	0.45	0.57

Genbank ID is the Genbank accession for the corresponding wheat EST for the feature. W0/S0 is the level of mRNA in the winter control relative to the spring control. Other numbers indicate induction or repression relative to the control for each cultivar

marked differential regulation between the two wheat cultivars, their role in CA also appears to be important.

There was differential regulation by CA of two well-characterized protein kinases, MAPK and Casein kinase. Transcript levels for a variant of MAPK (Tr001_K06), a protein kinase recognized for its central role in stress signaling, increased significantly early in CA and remained at basal levels for the remainder of cold treatment. This up-regulation was detectable only in the winter cultivar at the time points that were assayed. The rapid activation of MAPK enzyme during abiotic stress has been described (Shou 2004; Teige et al. 2004) and in some cases enzyme activation is accompanied by delayed up-regulation of MAPK transcript levels (Teige et al. 2004). Although both cultivars sense low temperature and initiate CA, the higher transcript levels seen for MAPK in the 6 h treatment in winter wheat shows a differential dynamic of expression which would warrant a more detailed analysis of gene regulation at the earlier time points during cold treatment. Differences in expression for this central kinase suggest that activation of MAPK early in CA may be involved in the initial response to low temperature and might be important to the development of freezing tolerance. The report that tobacco MAPK has been shown to confer enhanced freezing tolerance to transgenic maize plants (Shou et al. 2004) supports this hypothesis. In addition, fourteen serine/threonine protein kinases had significant cultivar by treatment interaction effects, five of which had markedly different patterns of expression between the two cultivars. In all five cases, CA induced elevated transcript levels of these protein kinases but the dynamic of changes in expression were markedly different in the two cultivars.

Protein kinases and calcium binding proteins

Calcium has been clearly shown to be involved in signal transduction in response to low temperature (Monroy and Dhindsa 1995) in conjunction with a large number of proteins that either bind calcium or are regulated by calcium binding proteins. These calcium-binding proteins, which include calcium-modulated protein kinases, have been shown to participate in orchestrating calcium-directed signal transduction networks (Yang et al. 2004). A calcium-dependent protein kinase (Tr012_N21) had significantly increased transcript levels in both cultivars by 24 h of CA, but was more strongly induced in the winter cultivar than in the spring cultivar. Moreover, this induction was transient in the spring cultivar whereas it was sustained in the winter cultivar. The gene has a high sequence similarity to rice CDPK7, which has been demonstrated to play a central role in cold stress in rice (Saijo 2000). Among calcium-modulated protein kinases, the most marked differences between the two cultivars were detected for cold regulated protein kinases that interact with calcineurin B-like sensors.

Two calcineurin-interacting proteins (Tr013_N24, Tr013_F22), were up-regulated after three days in the spring but not in the winter cultivar and a third (Tr012_O18) showed early up-regulation in both cultivars but had sustained elevated expression only in the winter cultivar. CA also increased the levels of transcripts for six calcium binding proteins and decreased the levels of four others. The most marked differential down-regulation between the two cultivars could be observed for two calmodulins (Tr001_B14, Tr013_E13) whose transcript levels decreased more markedly in the winter than in the spring cultivar, and for another calmodulin (Tr008_N09) for which the transcript levels increased in the winter but not the spring cultivar.

Transcription factors

The relative transcript levels of 45 probes annotated as transcription factors, representing 10 major families, had significant cultivar by treatment interaction. Differentially regulated transcription factors included members of the MADS-box, the AP2, the NAC, the MYB, and zinc finger transcription factor gene families (Table 2). Most of these transcription factors were up-regulated early in CA and the very early induction response for these transcription factors was very similar in both cultivars. However by 24 h of CA, the level induction of many transcription factors became significantly higher in the winter than in the spring cultivar. The AP2 family of transcription factors has received special

attention in abiotic stress since some of its members, the CBF genes, have been shown by functional studies to modulate the regulation of a large number of stress-induced proteins (Cook et al. 2004) and the early induction of CBF by CA is well documented (Reviewed by Thomashow 1999). Our data indicates that CA induced elevated transcript levels of 17 representatives of this family.

Most of the inductions of AP2 TFs were very transient and nearly similar between the two cultivars. The cold-induced up-regulation of CBF genes in Arabidopsis has been shown to be under control of *ICE1*, a MYC-like transcription factor in the bHLH family of transcription factors (Chinnusamy et al. 2003). Five members of this gene family (Tr012_J05, Tr012_O04, Tr013_E19, Tr013_I08, Tr012_P12) had significant differences in changes in mRNA levels between the two cultivars. The genes were down-regulated with subtle differences between the cultivars. The repression could be detected very early in the winter cultivar, but only after three days in the spring cultivar. This may suggest that they have different functions than the *ICE1* gene, whose transcript levels are slightly up-regulated by CA (Chinnusamy et al. 2003).

Ten MADS-like probes were found to have significant cultivar by cold acclimation interaction effects. Members of the MADS family have been found to be involved in the transition from vegetative to reproductive growth, a transition that has a important effect on the capacity to CA in wheat (de Folter et al. 2005; Riechmann and Ratcliffe 2000). Our analysis reveals that one of the MADS-box transcription factors (Tr014_H04) was more highly induced in the winter than in the spring cultivar. Two other up-regulated MADS-box transcription factors had patterns of induction different from each other as well as different between the two cultivars. The transcript levels of the MADS-box transcription factor (Tr014_H18) were higher in spring wheat under control conditions; they were induced more strongly in the winter cultivar early in the acclimation period, but became more strongly induced in the spring cultivar after three days of CA. Two MADS-box transcription factors (Tr012_E15 and Tr013_L17) had higher levels in the spring cultivar in non-acclimated plants, and were transiently induced at 6 h in the winter cultivar but transiently repressed at the same time in the spring cultivar. Four other MADS-box transcription factors (Tr014_B18, Tr014_H16, Tr014_D12, Tr014_D14) were down regulated and had different patterns of repression in the two cultivars. During flowering, there are extensive interactions among MADS box family members to form heterodimers, adding versatility to the extent of regulation excerpted by these transcription factors (de Folter et al. 2005). These results suggest that in addition to TAVRT-1, other MADS-box gene family members play important roles in the regulation of CA.

Members of MYB, the largest transcription factor family in the Arabidopsis genome have been implicated in the regulation of a variety of growth and physiological processes, including abiotic and biotic stress responses (Riechmann and Ratcliffe 2000). Transcripts for three genes (Tr017_B04, Tr012_G20 and Tr012_J21) were initially more strongly expressed in the non-acclimated winter cultivar and were strongly induced in the spring cultivar within 6 h of cold treatment. Recent reports indicate that the rice Osmyb4 gene is induced by cold treatment and can enhance freezing tolerance when introduced into Arabidopsis (Vannini et al. 2004). A mutation in the MYB gene HOS10 of Arabidopsis; completely impairs the capacity for CA, and is the regulator of pathways other than those in the CBF regulon (Zhu et al. 2005). The wheat MYB genes identified in our study are strong candidates for genetic studies and characterization of their respective roles in CA.

Six members of the NAC/NAM transcription factor family had significant differential regulation between winter and spring wheat during CA. Most of the up-regulated genes had transient induction at 6 h and 24 h of cold induction, whereas one NAC transcription factor (Tr012_B02) maintained high levels of transcript up to 14 days in at least one cultivar. This gene family has only been identified in plants, and members of the family have been implicated in developmental pattern formation (Riechmann and Ratcliffe 2000; Olsen et al. 2005). Selective NAC genes have been shown to be induced by cold and other environmental stresses and to regulate stress inducible genes in Arabidoposis (Tran et al. 2004).

The Dof domain, zinc-finger transcription factors, which are found only in plants, has been shown to control diverse aspects of plant development and metabolism (Umemura et al. 2004), however, to our knowledge there is no report of a Dof domain transcription factor involved in cold acclimation. The Dof transcription factor (Tr013_H18) was transiently up-regulated early in CA in the winter wheat but was not induced above the two fold threshold in the spring cultivar. In addition, we detected a C2H2-type zinc-finger transcription factor (Tr010_E10), whose expression is more than 2-fold down regulated in the winter but not the spring cultivar. Another zinc-finger transcription factor (Tr016_G14) whose up-regulation was sustained in both cultivars, was induced earlier and more strongly in the spring than in the winter cultivar.

Two homeobox transcription factors were transiently down-regulated (Tr012_G16, Tr011_L06) during CA. A significant difference between the two cultivars was observed for one homeobox gene (Tr012_G16) that was induced only in the winter cultivar. During development, homeobox genes are required for cell-fate determination as well as pattern definition (Riechmann and Ratcliffe 2000). Members of this family have been shown to modulate salt

tolerance (Shin et al. 2004), and HOS9 has been shown to contribute to the regulation of freezing tolerance independently of the CBF regulon (Zhu et al. 2004).

Other regulatory genes

Nucleic-acid-binding proteins. Tagging of specific classes of transcripts by specific RNA-binding proteins has been suggested to be a general mechanism for control of the localization, translation, and decay of mRNAs during physiological and developmental programs (Simpson et al. 2004). Specifically, these proteins have been implicated in diverse aspects of post-transcriptional gene regulation, including RNA processing, export, localization, degradation, and translational control (Simpson et al. 2004). The relative transcript levels of 12 genes encoding RNA-binding proteins had significant cultivar by CA interaction effects.

Members of the C2H2 zinc finger super family of nucleic-acid-binding proteins were also differentially regulated by CA. These zinc finger proteins bind DNA and RNA and regulate a variety of signaling pathways in other organisms (Brown 2005). Four probes annotated as C2H2 zinc finger proteins were differentially regulated between the two cultivars. For two of them (Tr002_A21, Tr003_C03), up-regulation was detected early in both cultivars. However, up-regulation was transient in the spring cultivar, but more sustained in the winter cultivar. For another C2H2 zinc finger protein (Tr003_C03), significant up-regulation was biphasic in the winter but not in the spring cultivar.

Ubiquitin-proteosome. Regulation of proteolysis has been shown to play a crucial role in signaling pathways by modulating selective degradation of regulatory proteins and has been shown to play a role in plant development, hormonal and environmental signaling. Proteins are targeted for degradation via the sequential activity of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and an E3 ubiquitin-ligating enzyme (Hellman and Estelle 2002). In our analysis, we found that the relative transcript levels of eight E3 ubiquitin ligases and four E2 ubiquitin conjugating enzymes had significant changes in transcript levels and had significantly different patterns of expression in the two cultivars.

Other cold acclimation response genes

Several other classes of genes were found to be differentially regulated in the spring and winter cultivars. Expression data for genes involved in sugar metabolism, actin cytoskeleton, photosynthesis, known stress responsive genes, cell wall proteins and proteins of unknown function are discussed in supplementary materials and expression data is presented in Supplementary Table S3.

Confirmation of gene regulation during cold acclimation

The expression of 7 genes was analyzed by RT-PCR during cold acclimation in two winter wheat cultivars, Alabaskaya and Jagger, to confirm results obtained from the microarray. These included two NAC transcription factors, a cullin-like E3 ubiquitin ligase, three genes encoding ice recrystalization inhibitor proteins and an unknown protein. Seven of the genes showed increased mRNA levels during cold treatment and one showed repression, all of which was in agreement with the microarray data (Fig. 5, Table 1 and Table S3). In the case of Tr007_G17 both the microarray data and the RT-PCR show repression at day 1, however the RT-PCR data showed that expression levels returned to control levels by day 3 of cold treatment whereas the microarray data indicated repression continues through day 14 of the cold treatment (Table S3). The differences may be

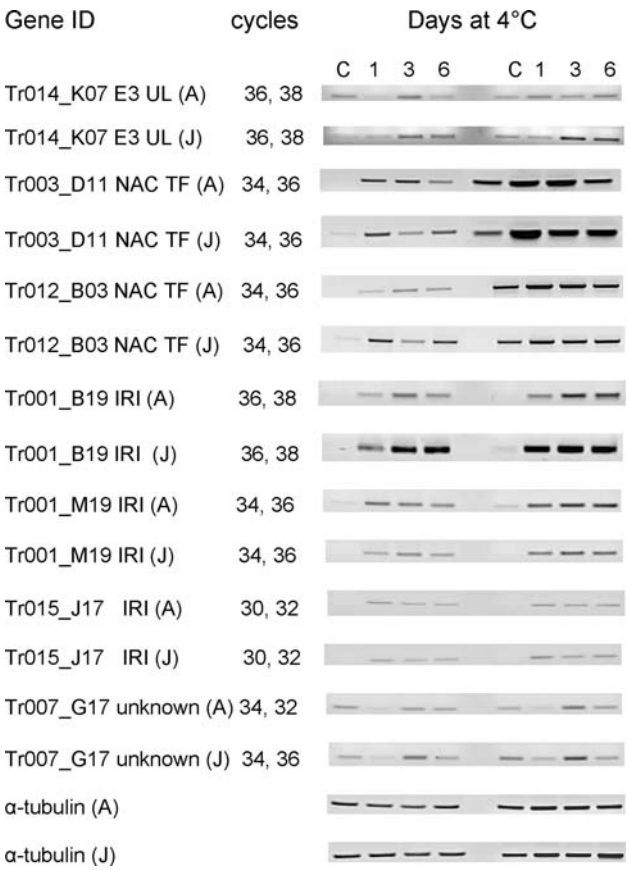

Fig. 5 The expression of selected genes measured by RT-PCR in winter wheat cultivars. Relative levels of mRNA were measured by reverse transcription-PCR in wheat cultivars Alabaskaya (A) and Jagger (J) in samples from control plants and plants cold acclimated for 1,3, 6 days. The genes characterized were Tr014_K07 a Cullin E3 ubiquitin ligase (E3 UL); Tr003_D11 a NAC transcription factor; Tr012_B03 a NAC transcription factor; three ice recrystallization inhibitor proteins (IRI), Tr001_B19, Tr015_J17, and Tr001_M19; and Tr007_G17 an unknown protein. The number of PCR cycles is indicated for sets of 4 samples in adjacent lanes

due to variation in expression between the different genotypes, as the pattern of expression seen by RT-PCR also differed slightly between the two cultivars. These are novel genes for which cold regulation has not previously been reported and thus full-length cDNA sequence was determined for five of the genes (Supplementary Materials).

The NAC transcription factors (Tr003_D11 and Tr012_B03), which were induced within 24 h of cold treatment, contained the conserved domain that is characteristic of NAC/NAM transcription factors. Among NAC transcription factors found in Arabidopsis they are most similar to ATAF1 and ATAF2.

The two genes, Tr001_B19 and Tr001_M19, are novel genes with high sequence similarity to cold regulated ice recrystallization inhibitor (IRI) proteins. Studies with winter rye have shown that members of this gene family protect plants from low temperature injury by inhibition of recrystallization of ice in the apoplast (Griffith et al. 2005). These are novel members of this gene family in wheat with 52%, 84% amino acid sequence similarity to previously identified ice recrystalization inhibitor proteins (Tremblay et al. 2005) and are strongly induced during cold acclimation (sequences in Supplementary Materials).

Genome distribution and chromosome location of cold-responsive genes

Chromosomal bin location of genes corresponding to 2999 microarray features for which data is available is listed in supplementary data Table S5. Supplementary Table S6 lists 185 genes corresponding to microarray features with significant cultivar by cold acclimation effects detected by ANOVA for which the chromosomal arm and bin position are available. The physical distribution among chromosome arms of these cold acclimation associated genes (supplementary data Fig. S2) indicates that not all variation in the distribution of significant cold acclimation associated features among chromosomes can be accounted for by the variation in the distribution of the set of mapped microarray features. The ratio of observed/expected number of features with significant cold acclimation-by-cultivar interaction per genome and per chromosome is based on the distribution of mapped features (Qi et al. 2004); it showed a 6% over-representation of cold acclimation genes in the D genome. The observed/expected ratio of gene distribution for each chromosome (Fig. 6) shows that the over-representation of genes in the D-genome is due mainly to chromosome 4D. The D genome is thought to make a significant contribution to environmental stress tolerance. *Triticum aestivum* is significantly more cold tolerant that the tetraploid wheat, *T. turgidum*, which lacks the D genome (Limin and Fowler 1985). Probes in the Southern blot analysis are expected to hybridize to all three

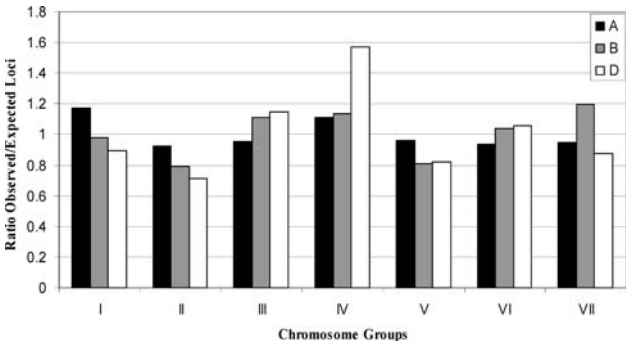

Fig. 6 Chromosomal distribution of genes with significant cold treatment by cultivar interaction effects. Ratio of the number of genes that mapped to each chromosome arm relative to expected values. Expected values are based on the chromosomal distribution of all mapped features on the microarray

genomes. This over representation may be due to differential gene duplication in the D genome or losses in the A and B genomes. This observation warrants further investigation, since it could also be an artefact of the ability to score loci in the mapping programs. Genes with significant cold acclimation-by-cultivar interaction, mapped in chromosome group 4 are listed in Supplementary Table S7.

Conclusion

We have studied the transcription profiles of winter and spring wheat during CA and found striking differences by four approaches: (1) comparison of the number of significantly regulated genes at each period of CA (2) comparison of genes clustered by similar patterns of expression, (3) measuring the quantitative differences of induction rates of a set of genes with a common sustained induction, and (4) comparison of gene regulation in the two cultivars by functional classes. These analyses repeatedly revealed that the initial response to CA, a burst of gene activity, is common to both cultivars. However, gene activity differs substantially soon thereafter and the global transcriptional profiles of the two cultivars become markedly different during cold acclimation. Whereas the CA-induced gene expression program proceeds in the winter cultivar, with genes displaying sustained induction and newly detectable genes later in CA, the spring cultivar has an initial burst of gene activity which subsequently slows and results in the decline of induced transcript levels and low gene expression later in CA. This pattern of expression suggests that the CA program is overridden by another regulatory program. Available evidence from other studies indicates that the overriding regulatory program of CA in spring wheat is the developmental control of the transition into a reproductive stage.

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References

Bottley A, Xia GM, Koebner RM (2006) Homoeologous gene silencing in hexaploid wheat. Plant J 47:897–906

Brown RS (2005) Zinc finger proteins: getting a grip on RNA. Curr Opin Struct Biol 15:94–98

Chinnusamy V, Ohta M, Kanrar S et al (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev 17:1043–1054

Cook D, Fowler S, Fiehn O et al (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proc Natl Acad Sci USA 101:15243–15248

Danyluk J, Kane NA, Breton G et al (2003) TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol 132:1849–1860

de Folter S, Immink RG, Kieffer M et al (2005) Comprehensive Interaction Map of the Arabidopsis MADS Box Transcription Factors. Plant Cell 17:1424–1433

Eisen MB, Spellman PT, Brown PO et al (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868

Faris JD, Haen KM, Gill BS (2000) Saturation mapping of a gene-rich recombination hot spot region in wheat. Genetics 154:823–835

Fowler DB, Limin AE, Shi-Ying Wang S-Y et al (1996) Relationship between low-temperature tolerance and vernalization response in wheat and rye. Can J Plant Sci 76:37–42

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14:1675–1690

Fu D, Szucs P, Yan L et al (2005) Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. Mol Genet Genomics 273:54–65

Griffith M, Lumb C, Wiseman SB, Wisniewski M, Johnson RW, Marangoni AG (2005) Antifreeze proteins modify the freezing process in planta. Plant Physiol 138:330–340

Gulick PJ, Drouin S, Yu Z et al (2005) Transcriptome comparison of winter and spring wheat responding to low temperature. Genome 48:913–923

Hellmann H, Estelle M (2002) Plant development: regulation by protein degradation. Science 297:793–797

Limin AE, Fowler DB (1985) Cold hardiness in *Triticum* and *Agelops* species. Can J Plant Sci 65:71–78

Martin ML, Busconi L (2001) A rice membrane-bound calcium-dependent protein kinase is activated in response to low temperature. Plant Physiol 125:1442–1449

Monroy A, Dhindsa RS (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell 7:321–331

Olsen AN, Ernst HA, Leggio LL et al (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci 10:79–87

Oono Y, Seki M, Satou M et al (2006) Monitoring expression profiles of Arabidopsis genes during cold acclimation and deacclimation using DNA microarrays. Funct Integr Genomics 6:212–234

Prasil IT, Prasilova P, Pankova K (2004) Relationships among vernalization, shoot apex development and frost tolerance in wheat. Ann Bot 94:413–418

Qi LL, Echalier B, Chao S et al (2004) A chromosome bin map of 16,000 expressed sequence tag loci and distribution of genes among the three genomes of polyploid wheat. Genetics 168:701–712

Riechmann JL, Ratcliffe OJ (2000) A genomic perspective on plant transcription factors. Curr Opin Plant Biol 3:423–434

Saijo Y, Hata S, Kyozuka J et al (2000) Over-expression of a single Ca2_-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J 23:319–327

Seki M, Narusaka M, Ishida J et al 2002 Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31:279–292

Shin D, Koo YD, Lee J et al (2004) Athb-12, a homeobox-leucine zipper domain protein from Arabidopsis thaliana, increases salt tolerance in yeast by regulating sodium exclusion. Biochem Biophys Res Commun 323:534–540

Shiu SH, Karlowski WM, Pan R et al (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. Plant Cell 16:1220–1234

Shou H, Bordallo P, Fan JB et al (2004) Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc Natl Acad Sci USA 101:3298–3303

Simon RM, Dobbin K (2003) Experimental design of DNA microarray experiments. Biotechniques Mar Suppl:16–21

Simpson GG, Quesada V, Henderson IR et al (2004) RNA processing and Arabidopsis flowering time control. Biochem Soc Trans 32:565–566

Storey JD, Tibshirani R (2003) Statistical significance for genome-wide studies. Proc Natl Acad Sci USA 100:9440–9445

Teige M, Scheikl E, Eulgem T et al (2004) The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. Mol Cell 15:141–152

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

Tran LS, Nakashima K, Sakuma Y et al (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16:2481–2498

Tremblay K, Ouellet F, Fournier J, Danyluk J, Sarhan F (2005) Molecular characterization and origin of novel bipartite cold-regulated ice recrystallization inhibition proteins from cereals. Plant Cell Physiol 46:884–891

Umemura Y, Ishiduka T, Yamamoto R et al (2004) The Dof domain, a zinc finger DNA-binding domain conserved only in higher plants, truly functions as a Cys2/Cys2 Zn finger domain. Plant J 37:741–749

Vannini C, Locatelli F, Bracale M et al (2004) Overexpression of the rice Osmyb4 gene increases chilling and freezing tolerance of Arabidopsis thaliana plants. Plant J 37:115–127

Vogel JT, Zarka DG, van Buskirk HA et al (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. Plant J 41:195–211

Yan L, Loukoianov A, Blechl A et al (2004) The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644

Yan L, Loukoianov A, Tranquilli G et al (2003) Positional cloning of the wheat vernalization gene VRN1. Proc Natl Acad Sci USA 100:6263–6268

Yang T, Chaudhuri S, Yang L et al (2004) Calcium/calmodulin up-regulates a cytoplasmic receptor-like kinase in plants. J Biol Chem 279:42552–42559

Zhu J, Shi H, Lee BH et al (2004) An Arabidopsis homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. Proc Natl Acad Sci USA 101:9873–9878

Zhu J, Verslues PE, Zheng X et al (2005) HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. Proc Natl Acad Sci USA 102:9966–9971