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ORIGINAL PAPER

Induction of DREB2A pathway with repression of E2F, jasmonic acid biosynthetic and photosynthesis pathways in cold acclimation-specific freeze-resistant wheat crown

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Abstract Winter wheat lines can achieve cold acclimation (development of tolerance to freezing temperatures) and vernalization (delay in transition from vegetative to reproductive phase) in response to low non-freezing temperatures. To describe cold-acclimation-specific processes and pathways, we utilized cold acclimation transcriptomic data from two lines varying in freeze survival but not vernalization. These lines, designated freeze-resistant (FR) and freeze-susceptible (FS), were the source of crown tissue RNA. Well-annotated differentially expressed genes ($p \leq 0.005$ and fold change≥ 2 in response to 4 weeks cold acclimation) were used for gene ontology and pathway analysis. "Abiotic stimuli" was identified as the most enriched and unique for FR. Unique to FS was "cytoplasmic components." Pathway analysis revealed the "triacylglycerol degradation" pathway as significantly downregulated and common to both FR and FS. The most enriched of FR pathways was "neighbors of DREB2A," with the highest positive median fold change. The "13-LOX and 13-HPL" and the "E2F" pathways were enriched in FR only with a negative median fold change. The "jasmonic acid biosynthesis" pathway and four "photosynthetic-associated" pathways were enriched in both FR and FS but with a more negative median fold change in FR than in FS. A pathway unique to FS was "binding partners of LHCA1," which was enriched only in FS with a significant negative median fold change. We propose that the DREB2A, E2F, jasmonic acid biosynthesis, and photosynthetic pathways are critical for discrimination between cold-acclimated lines varying in freeze survival.

Keywords Gene ontology (GO) · Pathway · Cold acclimation · Vernalization · Freeze survival · Winter wheat · DREB2A · Jasmonic acid · E2F.

Introduction

Winter wheat lines can achieve cold acclimation (development of tolerance to freezing temperatures) and vernalization (delay in transition from vegetative to reproductive phase) in response to low non-freezing temperatures. During cold acclimation, plants undergo biochemical changes to enhance their ability to withstand freezing temperatures and desiccation stress (Guy 1990; Olien and Clark 1993; Hughes and Dunn 1996; Thomashow 1999; Xin and Browse 2000; Wisniewski et al. 2003).

In both barley and wheat, a number of investigators have reported on genes that respond to cold treatment over different time scales (Sutton et al. 1992; Houde et al. 1992; Chauvin et al. 1993; Danyluk et al. 1994; Zhang et al. 1993; Gana et al. 1997; Han 1997; Monroy et al. 2007). Most of the studies vary in the length of time of cold acclimation, the tissue type studied, and the growth medium used. Su et al.

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(2010) described a MYBS3-dependent pathway conferring cold tolerance on rice after 4 °C for 72 h. Fowler and Thomashow (2002) performed the cold acclimation studies leading to the identification of multiple regulatory pathways, including the C-repeat binding factor (CBF) cold response pathway, using seedlings cold-acclimated for 0.5, 1, 4, 8, 24 h, and 7 days. Christova et al. (2006) limited their studies to 14 days. Laudencia-Chingcuanco et al. (2011) grew their plants hydroponically and cold-acclimated at 6 °C for up to 98 days. Ganeshan et al. (2011) performed cDNA-AFLP to compare low-temperature response between crown and leaf tissues for up to 70 days and determined that the regulatory pathways were different. Our study focused not on perception and signaling but on the identification of long-term cold-acclimation crown processes and pathways of winter wheat lines varying in freeze survival and grown in specified soil and watering conditions (Kenefick et al. 2002).

Interpretation of the association of low-temperature responsive genes to freeze survival has been confounded by the fact that low temperature also plays a significant role in vernalization of many winter plants. Gulick et al. (2005) performed a microarray analysis between the spring wheat *Triticum aestivum* L 'Glenlea' with a LT_{50} of −8 °C and winter wheat *T. aestivum* L 'Norstar' with a LT_{50} of–19 °C. The results obtained, although valuable, did not allow for a selection of low-temperature-regulated genes that are unassociated with the vernalization requirements of those lines. (Dhillon et al. 2010a, 2010b) utilized two independent diploid wheat lines, *Triticum monococcum* mutants, varying in period of time to flowering in the spring due to deletions of chromosomal regions within the major vernalization gene. The line that flowered the latest in response to a long photoperiod was found to be more freeze-tolerant and expressed high transcript levels for CBF transcription factors and some of the target cold-responsive (*COR*) genes. Dhillon et al. (2010a, 2010b) also reported on the low level of both *CBF* and *COR* transcripts during low-temperature treatment of the line that flowered earlier (spring-type). However, because the two lines examined were different with respect to both freeze tolerance and vernalization requirements, the cold-acclimation-specific genes and processes that are associated with freeze resistance have still to be described. Therefore, unless the experimental systems are designed to normalize for vernalization, the cold-regulated networks described may also apply to vernalization and not cold acclimation.

To contribute to the identification of cold-acclimation-specific processes in temperate cereals, we utilized two hexaploid winter wheat *T. aestivum* cv. 'Winoka' mutant lines that exhibit similar vernalization requirements but differ in freeze survival. The lines are designated FR (freeze-resistant) and FS (freeze-susceptible). We have successfully completed comparative cold acclimation transcriptomic analyses between the two mutant lines (Sutton et al. 2009) with RNA from crown tissue, the most freeze-resistant part of the plant (Chen et al. 1983; Gusta and Weiser 1972; Olien 1967). When the crown meristematic tissue is destroyed by freezing, the plants are unable to resume growth in the spring (Winfield et al. 2010).

Our initial comparison of the crown transcriptomics data from cold-acclimated FR and FS lines focused on the *CBF* cluster within the frost locus on the long arm of chromosome 5, as well as on several *COR* genes. The results were presented and discussed and revealed six *CBF* genes that were significantly differentially expressed between FR and FS (Sutton et al. 2009). Several other gene expression studies have utilized RNA from wheat and barley crown tissue (Janská et al. 2011; Pearce et al. 1998; Skinner 2009; Winfield et al. 2010). The research presented in this paper differs from those referenced above in that we performed cold acclimation on plants at the fourth leaf stage and under light intensity a minimum of threefold lower than the other studies.

Utilizing the wheat crown transcriptomic dataset (Sutton et al. 2009), we generated gene ontology (GO) terms based on similarities to the *Arabidopsis* classifications. Those terms were used for the identification of processes and pathways that are differentially expressed or enriched between FR and FS in response to long-term cold acclimation. Because cold-acclimated FR has greater freezing survival than cold-acclimated FS, we propose that the identification of pathways that differentiate 4-week cold-acclimated FR and FS lines are critical to deciphering the mechanisms that discriminate between cold-acclimated lines varying in freeze survival.

Materials and methods

Plant materials

Generation of FR and FS 'Winoka' mutant lines

These lines were derived from azide mutagenesis of the hard red winter wheat cv. 'Winoka' (Sutton et al. 2009; Wells et al. 1969). An average of two replications of M5 lines in the Northern Uniform Winter Hardiness Nursery at Casselton, ND, in 1988 identified Winoka as having 35 % freeze survival. The winter wheat SD_{16029} demonstrated 75 % freeze survival and was designated FR, and the winter wheat SD_{16169} demonstrated 30 % freeze survival, and, thus relative to FR, SD_{16169} was designated FS. These two mutant winter wheat lines share with Winoka the need for vernalization before heading is achieved. They also exhibit similar levels of expression of the *VRN* genes in response to the

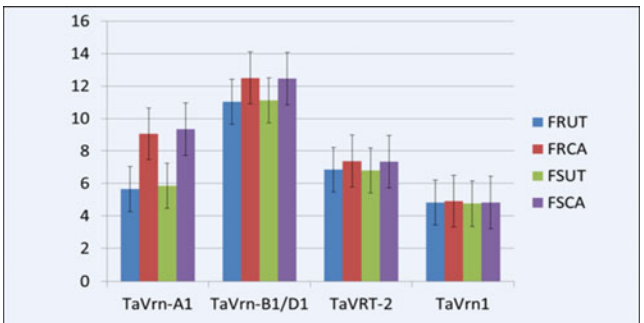
low-temperature treatment (Fig. 1). The expression data for the *VRN* genes was obtained from the transcriptomic studies (Sutton et al. 2009).

Growth conditions

Plants were grown in the green house at 22–28 °C and soil water maintained at 0.3–0.44 kgH_2Okg^{-1}. Plants were transferred to the 2 °C cold room for cold acclimation after reaching the fourth leaf stage. Since light prior to and during cold acclimation plays a significant role on freeze survival (Gray et al. 1997), plants were allowed to accumulate photosynthate prior to cold acclimation by supplementing sunlight in the greenhouse with low-pressure sodium vapor lamps that provided a photosynthetic photon flux density (PPFD) of 150 µmol $m^{-2}s^{-1}$ for a 14-h photoperiod. To replicate the conditions of autumn that result in cold acclimation, plants transferred to 2 °C were exposed to a shortened photoperiod of 11.5 h and a low light intensity of 4 µmol $m^{-2}s^{-1}$ supplied from cool-white fluorescent lamps. We have previously validated that there was no further plant development during the cold-acclimation conditions, since plants remained at the four-leaf stage, and there was no change in dry weight (Kenefick et al. 2002).

Transcriptomics

RNA isolation and processing of the Affymetrix wheat microarrays were as described (Sutton et al. 2009). The RNA samples were from untreated and 4-week cold-acclimated crown tissue. There were two biological replicates consisting of three pots of four plants for each treatment. The microarray data have been deposited at the Plant Expression database http://www.plexdb.org/ website accession number TA22.



	FRUT	FRCA	FR_fold	FR_p	FSUT	FSCA	FS_fold	FS_p	Accession
TaVrn-A1	5.65	9.06	3.41	0.004	5.86	9.35	3.49	0.005	AY747601
TaVrn-B1/D1	11.04	12.50	1.46	0.004	11.12	12.46	1.35	0.001	AY747604
TaVRT-2	6.84	7.38	0.54	0.039	6.79	7.35	0.56	0.012	DQ022679
TaVrn1	4.82	4.92	0.10	0.116	4.76	4.82	0.07	0.662	AB281427

Fig. 1 Similar expression of *VRN* genes in FR and FS mutant lines

The data have also been deposited in the GEO database, accession # GSE14697 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dlqnpqqecacwgba&acc=GSE14697.

Statistical methods for identifying differentially expressed genes

All analyses were based on the Affymetrix GenChip Manual (Affymetrix Inc.) using the statistical program R 2.12.0 (http://www.R-project.org) with affy, gcRMA Bioconductor packages (Irizarry et al. 2003) and log-transformed for the pre-processing steps. Expression measurements were calculated according to the model developed by Bolstad et al. (2003) and Irizarry et al. (2003). Two-sample *t*test with equal variances was applied to detect significantly expressed genes. A cutoff of $p \leq 0.005$ and fold change ≥2 was used to define differentially expressed genes in response to cold acclimation.

Arabidopsis Gene IDs (AGIs) for wheat genes on array AGIs for the normalized and processed wheat probes present on the 61K Affymetrix wheat array were identified by comparing the whole gene consensus wheat sequences (obtained from Affymetrix) to the *Arabidopsis* protein database (TAIR 10) using BlastX. The top BlastX hits for all genes with *E*-values less than E-5 were recorded, and the functional ontologies from the associated *Arabidopsis* genes were used for the various gene set and sub-network analyses.

Gene ontology (GO) analysis

The GO analysis was performed using different programs: Pathway studio 8.1 http://www.ariadnegenomics.com/); GOstat by Tim Beißbarth (http://gostat.wehi.edu.au/cgi-bin/goStat.pl; and the PANTHER Classification System (http://www.pantherdb.org/) programs. Gene set enrichment analysis and Fisher's exact test were used to identify the statistically significantly enriched GO terms (Beißbarth and Speed, 2004; Mi et al. 2005; Young et al. 2010; Mi et al. 2010; PANTHER 7.0). GO terms were considered as significantly enriched if they had a *p* value≤0.001 and at least five genes within the GO category. GO IDs were converted to GO terms and vice versa as per http://www.sigenae.org/index.php?id=168. In order to more specifically identify the processes that were relevant, we identified the ancestor and children relationships within the GO terms using the AmiGO program (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi).

Pathway analysis

Pathway Studio was used to identify the statistically significantly enriched pathways (Bogner et al. 2011; Pathway studio Desktop 7.1 training manual). Pathways with a *p*

value≤0.005 and at least five genes were considered as significantly enriched in FR and FS.

Results

Cold-acclimation regulated genes

Of the 61K Affymetrix wheat probe set, only 31,568 wheat genes passed the hybridization analyses as good probes. Of these, only 17,887 were similar enough to *Arabidopsis* genes to obtain probable functional associations for ontological analysis (Electronic supplementary material, File 1). The application cutoff of $p \leq 0.005$ and fold change ≥2 identified 2,850 and 3,540 genes as differentially expressed in response to the cold-acclimated FR and FS lines, respectively (Electronic supplementary material, File 2). Based on comparison with the total 31,568 genes that passed the hybridization as good probes, we calculated that approximately 10 % of the genes were cold-regulated in both FR and FS. However, a comparison with the 17,887 AGIs revealed cold regulation of 15.9 % and 19.8 % for FR and FS, respectively. Further analyses identified 1,254 genes as upregulated and 1,596 genes as downregulated in the cold-acclimated FR line. In the cold-acclimated FS line, 1,610 genes were upregulated and 1,930 were downregulated (Fig. 2). Among the common upregulated genes were those encoding the transcription factor MYB 51 (~8.3-fold); a number of LEA proteins including LEA3 (~6.5-fold), dehydrins such as Wdhn13 (~6-fold), and protein phosphatase 2C-like (~6-fold). These genes are highlighted in green (Electronic supplementary material, File 2).

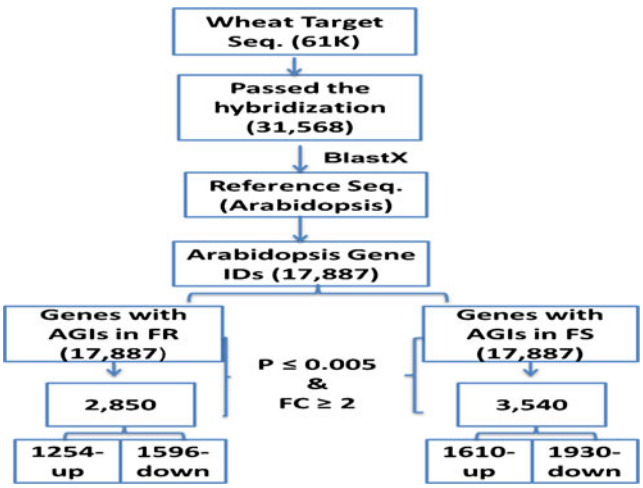


Fig. 2 Flow chart to identify cold acclimation-responsive differentially expressed genes and results. Of the 61K Affymetrix wheat probe set, only 31,568 wheat genes passed the hybridization analyses as good probes. Of these, AGIs were available for only 17,887 genes by BlastX against TAIR10 *Arabidopsis* protein database with an *E*-value cutoff 10E-5. The application of cutoff $p \leq 0.005$ and fold change ≥2 identified 2,850 and 3,540 genes as expressed in the cold-acclimated FR and FS mutant lines, respectively. *FC*=fold change

GO analyses

Only the significantly differentially expressed genes ($p \leq 0.005$ and fold change ≥2) were used for the GO analysis. The results of the significantly enriched GO terms in FR and FS identified by all three GO analysis programs (Pathway Studio, GO stat, and PANTHER) are displayed in Fig. 3. Application of a cutoff of $p \leq 0.001$ and a minimum of five genes resulted in a reduction of GO IDs in FR from 583 to 55 and from 735 to 65 in FS. Of these, a total of 38 GO terms were identified as common in both FR and FS (Electronic supplementary material, File 3). A total of 17 and 27 GO terms were identified as unique and significantly enriched in FR and FS, respectively (Electronic supplementary material, File 4). Of the 17 GO terms defined as unique in FR, the classifications were as follows—11 biological processes, 1 cellular component, and 5 molecular functions. In FS, the 27 GO terms were classified as 13 biological processes, 5 cellular components, and 9 molecular functions.

Classification of GO terms unique to FR or FS

The top five GO terms with the lowest *p* values for FR and FS are depicted in Fig. 4. Four of the five GO terms for FR belonged to the biological process category. The fifth term belonged to the molecular functions category. As shown, "response to abiotic stimulus," (GO:0009628) with a *p* value of 1.59E-09, was the major biological process,

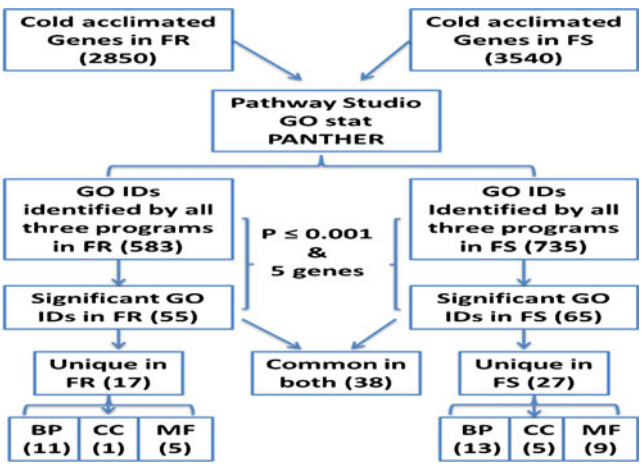


Fig. 3 GO terms enriched in FR and FS in response to cold acclimation using all three programs. The 583 GO terms are identified by all three programs in FR, and 735 GO terms are identified by all three programs in FS. Of these, 17 GO terms are identified unique in FR, 27 GO terms identified in FS, and 38 GO terms identified common in both FR and FS. *CA*=cold acclimation, *BP*=biological processes, *CC*=cellular components, *MF*=molecular functions

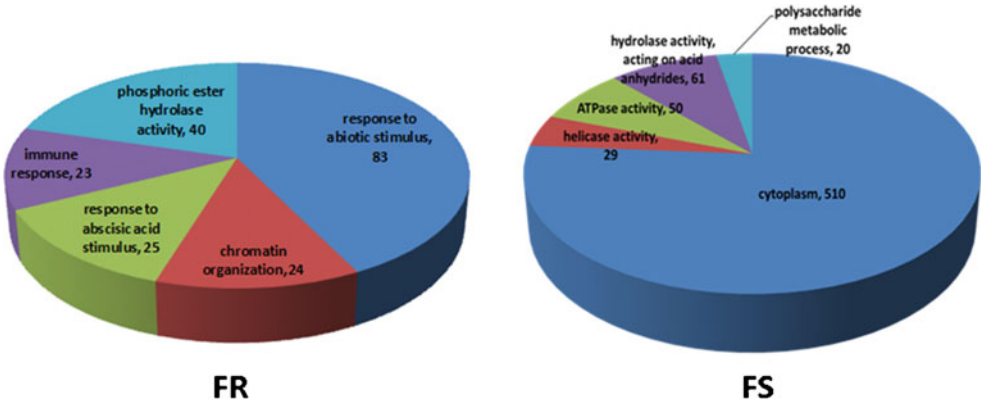


Fig. 4 Top five highly significant GO terms in **a** FR and **b** FS. **a** The highest number of genes (83) corresponds to response to "abiotic stimulus" in FR, and **b** the highest number of genes (510) corresponds to "cytoplasm" in FS

containing 83 genes. "Phosphoric ester hydrolase activity," (GO:0042578), *p* value of 0.00012, of the molecular functions category contained 40 genes. GO terms: "chromatin organization" (GO:0006325), *p* value 1.75E-08; "response to ABA stimulus" (GO:0009737), *p* value 1.7E-08; and "immune response" (GO:0006955), *p* value 8.13E-05 contained 24, 25, and 23 genes, respectively.

Among the top five GO terms in FS with the lowest *p* values: "cytoplasm" (GO:0005737), *p* value 7.9E-28, 510 genes, belonged to the "cellular components" classification. The other four terms are: "hydrolase activity acting on acid anhydrides" (GO:0016817), *p* value 7.13287E-06, 61 genes; "ATPase activity" (GO:0016887), *p* value 4.7E-06, 50 genes; "helicase activity" (GO:0004386), *p* value 3E-09, 29 genes; and "polysaccharide metabolic process" (GO:0005976), *p* value 1.50351E-05, 20 genes.

Pathway analysis

As depicted in Fig. 5, of the 2,850 cold-acclimated FR genes, 25 pathways were identified, and of the 3,540 cold-acclimated FS genes, 28 pathways were identified (Electronic supplementary material, File 5). A filter with

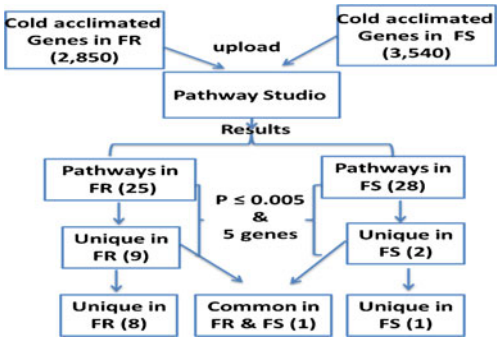


Fig. 5 Highly enriched ($p \leq 0.005$ and presence of at least five genes) pathways in FS using Pathway Studio. A total of 25 pathways were identified in FR and 28 in FS. Of these, nine pathways in FR and two pathways in FS passed the threshold ($p \leq 0.005$ and presence of at least five genes)

a cutoff of $p \leq 0.005$ and at least five genes present identified nine pathways as unique for FR, two as unique for FS, and one (triacylglycerol degradation) as common to both. A list of the unique pathways for FR and FS is presented in Table 1. The median fold change and *p* values for the pathways are provided. As observed (Electronic supplementary material, File 5), all FS *p* values are >0.005, whereas all FR *p* values are <0.005, and thus, the pathways listed in Table 1 are considered unique for FR.

In terms of levels of fold change, "neighbors of DREB2A" had the highest positive median fold change of 20.34 for FR compared with 4.32 for FS. All other pathways in both FR and FS displayed negative median fold changes. "Binding partners of LHCA1" was the most cold-acclimation downregulated pathway, with a median fold change of −18.2 in FS. Pathway "binding partners of LHCA1" was not significantly cold-acclimation-regulated in FR, and so it was designated not detected, "nd." The two most downregulated pathways in FR were "neighbors of ribulose 1, 5-bisphosphate carboxylase/oxygenase (RuBisCo)" (−16.44-fold) and the "13-LOX and 13-HPL pathway" (−16.09-fold). The "13-LOX and 13-HPL pathway" was not cold-acclimation-regulated in FS, and so it was designated "nd." However, the pathway "neighbors of RuBisCo" was downregulated in FR (−16.14) and in FS (−5.13) but at a *p* value of 0.0021 (FR) compared with 0.017 (FS).

The pathway "neighbors of E2F" was not detected as cold-acclimation-regulated in FS and so was also listed as "nd." However, in FR, it was downregulated with a fold change of −9.94. The "photorespiration" pathway was also not cold-acclimation-regulated in FS. However, in FR, it was downregulated with a median fold change of −6.66.

Among the FR unique pathways listed in Table 1, "jasmonic acid biosynthesis" was detected in both FR and FS with median fold changes of −8.06 and −6.67, respectively. However, the *p* values varied, with FR at 0.0006 and FS at 0.021. The higher FS *p* value resulted in this pathway being filtered out, and thus the "jasmonic

Table 1 Pathways identified for FR and FS

Enriched pathways	FR		FS	
	No. of genes	Median change	No. of genes	Median change
Neighbors of DREB2A	5	20.345*	6	4.320**
Photosynthesis	20	−5.742*	21	−4.377**
Photosynthesis, light reaction	11	−5.751*	11	−4.377**
Photorespiration	6	−6.66*	nd	
Jasmonic acid biosynthesis	10	−8.065*	9	−6.676**
Neighbors of E2F	7	−9.939*	nd	
13-LOX and 13-HPL pathway	5	−16.096*	nd	
Neighbors of RuBisCo	5	−16.440*	5	−5.127**
Binding partners of LHCA1	nd		5	−18.212*

Median change is the median fold change (non-log) for genes identified by ontology. In cases where more than one representative of a given gene was present, the gene with the lowest *p* value was chosen

nd not detected

*p value < 0.0050; **p value < 0.05

acid biosynthesis" pathway is considered enriched only for FR.

The pathway considered as unique to FS is "binding partners of LHCA1." This pathway was found not to be cold-acclimation-regulated in FR, and so it was listed as "nd" while in FS it was downregulated with a median fold change of −18.212.

Discussion

Cold-acclimation-regulated genes

It was of interest to know whether the percentage of cold-acclimation-regulated genes we obtained agreed with those reported by others. We determined that the results of 15.9 % and 19.8 % for FR and FS, respectively, obtained from comparison with the total AGIs are higher than that reported by others. However, the lower percentage of 10 % obtained when the calculation was performed with all good probes on the array was due to the fact that the total number of genes from AGIs was 13,681 less than that of the total number of good probes on the array. However, the 10 % value was more consistent with other studies. Hannah et al. (2005) reported that 10 % of *Arabidopsis* genes are cold-regulated. Houde et al. (2006) reported that 8.4 % of wheat genes are cold-regulated. The presence of common FR and FS cold-acclimated genes (encoding: the MYB51 transcription factor; some LEAs and dehydrins; and the protein phosphatase 2C-like proteins) reveals that these genes are responsive to cold. However, individually, they do not appear to be involved in long-term cold-acclimation-induced differential freeze survival between the two lines, since they are not differentially expressed between the two lines. We proposed that a better understanding of the relationship between cold-acclimated gene expression and freeze survival can be achieved by comparing gene ontologies GO and pathways between the lines varying in freeze survival.

Gene ontologies of FR versus FS in response to cold acclimation

The large number of significantly over-represented ontologies common among the cold-regulated genes in FR and FS reflects the fact that these lines are very similar. Both lines were derived from the same cv "Winoka." We believe that the common GO terms (Electronic supplementary material, File 3) will aid in deciphering the default mechanisms of cold acclimation and vernalization for cereals in general. These GO terms included some such as: GO:0006979 (response to oxidative stress); GO:0005985 (sucrose metabolic process), and GO:0009266 (response to temperature stimulus). However, in this study, we focused on the GO terms and pathways unique or highly enriched to FR and to FS. These will provide significant insights into differential freeze survival via cold-acclimation-specific processes and not vernalization processes, since these lines share the same vernalization requirement.

GO terms identified for FR, such as "response to abiotic stimulus," were listed among those described from EST studies for the cold-acclimated freeze-resistant winter wheat cv. "Norstar" (Houde et al. 2006). The second GO term unique to FR, "chromatin organization," has been previously reported by Laudencia-Chingcuanco et al. (2011) in their genome-wide study using the same Affymetrix wheat array with RNA from cold-acclimated crown tissue of different lines with neutralized spring vernalization. "Response to ABA stimulus" has long been implicated as distinguishing between freeze resistance among plants. Such studies include our research on the differential expression of the ABA-regulated gene *HVA1* in barley between the freeze-resistant cv. "Dicktoo" and the less freeze-resistant cv. "Winter Malt" (Sutton et al. 1992).

The unique FR cellular component GO term, "mitochondrial part," corroborates the role of mitochondria in cold acclimation. Lee et al. (2002) reported on the lesion in the

mitochondrial electron transfer chain in the cold-sensitive *Arabidopsis* mutant *frostbite1*.

The unique FS cellular GO term "Cytoplasm" (510 genes) is too broad to gain any insight into the cold responses of FS as it pertains to freeze susceptibility.

Go term "phosphoric ester hydrolase activity," which is unique to FR molecular functions, contains the child term phosphatase. Sharma et al. (2005) listed the cold-signal-mediating phosphatases in plants. The phosphatases associated with phosphoric ester hydrolase activity were not listed among them. The long-term cold acclimation (4 weeks) of our study would not necessarily result in the identification of enzymes involved in perception and early signaling. "Phosphoric ester hydrolase activity" was also identified as over-represented in studies on both salt stress in *Arabidopsis* (Sakamoto et al. 2008) and acute drought stress in mosquito (Wang et al. 2011). These reports suggest that "phosphoric ester hydrolase activity" is a highly evolutionarily conserved process related to osmotic stress such as desiccation, salt, and freezing stress. It is not clear at this point the unique role of this process in FR.

"Chromatin organization" which was also listed as a unique term for FR has previously been shown to be involved in plant response to stress. Zhu et al. (2008) suggested that HOS15 serves as a repressor of gene expression important to cold tolerance through histone deactylation. This and other epigenetic changes, which result in repression of gene expression, fit the hypothesis of a developed FR cold-acclimation state in which metabolism, growth, and development are quiescent.

The last of the FR five unique GO terms "immune response" reflects the crosstalk between abiotic and biotic stress, since plant immune response is usually described in terms of pathogen response (Kwon et al. 2008). In particular, our results reflect an association between immune response and higher freeze tolerance, since this term did not appear among the terms unique to FS.

In the case of FS, the unique GO terms corresponding to cytoplasmic cellular components, helicase and ATPase activities, reflect a higher level of metabolic processes in cold-acclimated FS than in FR. The DEAD-box ATPase/RNA helicase (FL2-5A4/At3g01540) was classified by Seki et al. (2002) as responsive to cold. However, Fowler and Thomashow (2002) did not observe such a cold response. Gusta et al. (2005), in the review of DEAD-box helicases and ABA, reflected on a positive correlation between helicase activity and cold acclimation as deduced from the work of Gong et al. (2005) who demonstrated an essential role for these enzymes in mRNA nucleo-cytoplasmic export. We propose that such a role for the helicases would be important at the onset and not the later stage of cold acclimation. Such activity could serve to reduce the level of cold acclimation as reflected by the higher activity in FS than FR.

Pathways of FR and FS in response to cold acclimation

The most important dataset from our studies are those resulting from the pathway analysis. As with the GO analysis data, we can validate our results by comparison with previous research. Pathways involved in photosynthesis are downregulated in response to cold acclimation in both FR and FS, since cold acclimation was performed under low PPFD. Kosmala et al. (2009) in studying proteins involved in photosynthesis reported on the depression of photosynthesis during cold acclimation and the differential regulation of the relevant proteins between high freeze-tolerant and low freeze-tolerant plants. The significantly downregulated "photorespiration" and "neighbors of RuBisCo" pathways would suggest less energy production and thus a lower level of activity within the FR crown tissue at advanced cold acclimation compared with the FS crown tissue. Reduced photosynthesis in FR lines may result in protection from photo-oxidative stress commonly associated with chilling-damaged chloroplast membranes. Janská et al. (2011) reported on some induction of genes involved in photosynthesis in the crown tissue. Such induction of photosynthetic genes in the non-photosynthetic crown tissue was also observed by Skinner (2009). Our results demonstrated repression of the pathway to a greater extent in FR than FS. These results do not necessarily contradict that of Janská et al. (2011) or Skinner (2009), since those studies were performed with plants at different stages of development and cold-acclimated under different light intensities than ours. Janská et al. (2011) performed cold acclimation when barley plants were at the second leaf stage under light intensity of 120 μmol $m^{-2}s^{-1}$. Skinner (2009) performed cold acclimation on wheat plants at the third leaf stage and under 250 μmol $m^{-2}s^{-1}$ light intensity. Additionally, the focus of the Skinner (2009) study was on the post-cold acclimation transcriptomic changes in the crown. As described previously (Sutton et al. 2009), our study was performed with wheat plants at the fourth leaf stage with cold acclimation under the low light intensity of 4 μmol $m^{-2}s^{-1}$. The most relevant variable is probably the light intensity which was 62.5-fold less in our wheat study than that of Skinner (2009).

Pathway "neighbors of E2F" is under the control of E2F transcription factors, which were first described in animal cells and demonstrated to be involved in cell cycle regulation and growth. Ramirez-Parra et al. (1999) were the first to clone a plant *E2F* and to demonstrate its similarity to the animal E2F protein structure. del Pozo et al. (2002), de Jager et al. (2001), and Mariconti et al. (2002) demonstrated that *E2F* is cell-cycle-regulated as transcripts accumulated in the S phase. GUS activity under the control of the 5' upstream

region of *E2F* revealed that *E2F* is expressed in actively dividing cells as well as shoot and root meristem (del Pozo et al. 2002). At present, we can only assume that the *E2F* downregulation in response to cold acclimation in FR crown tissue reflects a strong association between cold-acclimation-induced cell quiescence and level of freeze survival.

The last of the enriched pathways identified as downregulated in FR were "13-LOX and 13-HPL" and "jasmonic acid biosynthesis." Lipoxygenases (LOX) and hydroperoxide lyases (HPL) are involved in the biosynthesis of jasmonic acid. The fact that 13-LOX and 13-HPL pathway was significantly downregulated in FR and not cold-acclimation-responsive in FS suggests that this pathway may play a critical role in distinguishing between lines varying in freeze survival.

Of all the cold acclimation pathways identified, only "neighbors of DREB2A" was significantly enriched and over-represented in FR as compared with FS, and thus more significantly linked to freeze survival. Kume et al. (2005) isolated two wheat DREB1 homologs they designated *WCBF2*. This gene was found to be upregulated in early and late cold acclimation and by drought but not ABA. *WCBF2* was also mapped to chromosome 5. Egawa et al. (2006) isolated a DREB2 homolog designated *WDREB2*. They described the generation of three transcripts due to alternative splicing and the induction of all three transcripts by low temperature treatment. In response to cold acclimation, *WDREB2* transcripts increased and remained high until 63 days. The *Arabidopsis DREB2A* has been reported to be induced by dehydration (Liu et al. 1998) resulting from freezing (review, Sharma et al. 2005). Our results and those of Egawa et al. (2006) reflect cold-acclimation induction of *WDREB2A* without freezing-induced dehydration. However, since cold acclimation also includes dehydration, we cannot rule out that *WDREB2A* is not also drought-regulated as described by Egawa et al. (2006).

In summary, we propose that the cold-acclimation-enriched pathways function to reduce energy, shut-down metabolism, inhibit cell division, and maintain cell quiescence needed to withstand freeze stress. The degree to which one line is able to achieve those functions would appear to confer a higher degree of freeze survival.

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