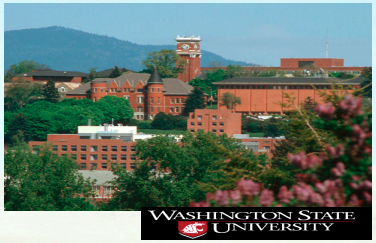
Transcriptional Response of Cold-Acclimated Winter Wheat to Potentially Damaging Temperatures

Daniel Z. Skinner and Brian S. Bellinger

U.S. Department of Agriculture and Department of Crop & Soil Sciences

Washington State University, 209 Johnson Hall, Pullman, WA 99164, USA



Abstract

The ability of cold-acclimated wheat plants to survive freezing to several degrees below 0°C varies among wheat genotypes. While the transcriptional regulation of the cold acclimation process above 0°C has been studied extensively, very little is known of the changes in gene expression that may occur as the temperature decreases to potentially damaging levels well below 0°C. Using microarray and quantitative real - time PCR analysis, this study evaluated transcriptomic variation of cold-acclimated winter wheat plants as the temperature was lowered to -10°C, and then was either maintained at -10°C or was lowered further to -12°C. Expression levels of a total of 423 genes were significantly altered in these treatments; genes upregulated outnumbered those downregulated by about a 9: 1 ratio. Many of the most strongly responsive genes apparently were involved in transcription regulation or signal transduction. Most genes responded similarly in the very freezing tolerant cultivar Norstar and in the moderately freezing tolerant Tiber, but some genes responded in opposite fashion in the two cultivars. These results suggested that wheat crowns actively adapt as the temperature declines below 0°C to potentially damaging levels, and that genetic variation for this ability exists among cultivars.

Materials and Methods

The crowns (meristematic regions) of the plants were subjected to controlled freezing episodes as follows. The soil was washed from the plants using ice water, the residual caryopsis, roots and shoots were removed, and six crowns (about 5mm in length) were placed in 2ml microcentrifuge tubes and held on ice as samples were prepared. One tube was frozen immediately in liquid nitrogen and stored at -80°C (Treatment 1, Fig. 1). Other tubes containing six crowns were transferred to a computer-controlled low temperature alcohol bath (Model F25 MV controlled by EasyTemp software, Julabo Inc., Vista, CA) at 0°C. The temperature was lowered at a rate of 1.5°C/h. Tubes were removed from the alcohol bath and frozen in liquid nitrogen when they had been exposed to one of three conditions: when the temperature reached -10°C (Treatment 2, Fig. 1); when the temperature reached -10°C and was held at -10°C for 1.3hr (Treatment 3, Fig. 1); or when the temperature reached -12°C (Treatment 4, Fig. 1). Because the rate of temperature decline was 1.5°C/h, the time required to cool from -10°C to -12°C was 1.3h, hence, plant crowns in treatments 3 and 4 (Fig. 1) were exposed to subfreezing temperatures for equal amounts of time. Each temperature treatment was replicated three times on plants grown independently at different times. A duplicate set of tubes with the plants trimmed to about 2cm length was included with two of the trials to determine whether the temperature treatments were fatal to the plants. These plants were removed from the freezing bath, thawed overnight on ice, then planted into moist Vermiculite in closed plastic boxes, and incubated at 25°C with a 16h photoperiod. All plants survived the freezing treatment and regrew a leaf within 5 days.

To extract RNA, the six crowns that had been frozen in liquid nitrogen and stored at -80°C were homogenized in liquid nitrogen using a mortar and pestle; RNA was extracted using 1.5ml of Trizol reagent and the standard protocol (Invitrogen, Carlsbad, CA). RNA was quantified with UV spectrophotometry and stored at -80°C. About 40µg RNA was recovered from six plant crowns.

Global gene expression levels were assayed with the GeneChip® Wheat Genome Array (Affymetrix, Santa Clara, CA). This array contains 61,127 probe sets representing 55,052 transcripts for all 42 wheat chromosomes (http://www.affymetrix.com). RNA labeling and hybridization to the Affymetrix arrays, and post-hybridization scanning and data pre-processing was conducted by the Washington State University Biotechnology Core Facility. The data files were further analyzed using "Flexarray" software, available from Genome Quebec (http://genomequebec.mcgill.ca/FlexArray). The microarray data were normalized using the "Robust Multi-chip Average" (RMA) method and probe sets showing significant differences in hybridization intensity among treatments were sought using the empirical Bayes analysis due to Wright and Simon (algorithm EB Wright & Simon) available in the Flexarray software. The GenBank identifiers of the sequences the probesets were designed to identify were obtained from the GeneChip information file provided by Affymetrix. Tentative identifications of genes of interest were sought through BLASTN searches of the GenBank nonredundant database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In the event the BLASTN algorithm returned only genes of unknown function, translated searches (BLASTX and TBLASTX) were also performed.

Quantitative real-time PCR (qPCR) was used to confirm expression level changes of selected genes. The Protoscript II kit (New England Biolabs, Ipswich, MA, USA) was used to synthesize cDNA. The cDNA synthesis reactions were initiated with 1μg RNA that had been treated with RNase-free DNase I according to the manufacturers protocol (Ambion, Austin, TX, USA) and were primed with the anchored oligo dT primers provided in the Protoscript II kit. Synthesis was according to the manufacturers protocol except that the reactions were allowed to proceed for 100 min instead of 60 min. The completed 20μl cDNA syntheses were diluted to 50μl with nuclease-free water and stored at -20°C. This template cDNA was diluted 1:10 prior to use and 1μl of the resulting solution was used as template for qPCR. The PCRs were conducted on an Applied Biosystems (Foster City, CA USA) 7300 Real-Time PCR System using 25μl preparations that consisted of 1X Go-Tag® Colorless Master Mix (Promega, Madison, WI, USA), 1μl cDNA, 200μM each primer, 0.85X SYBR Green I (Invitrogen, Carlsbad, CA, USA), and 300nM ROX dye (Roche, Indianapolis, IN, USA) as an internal fluorescence standard. The qPCR software available with the instrument was used to determine the C_t, the fractional cycle number at which the fluorescence intensity reached an arbitrary threshold, using the threshold determined by the software. Relative fold change was determined using the delta-delta C_t method as described by Dussault and Pouloit (Biol Proced Online 8:1–10, 2006) using the Cbf1B gene primers (Vagujfalvi et al., Mol Genet Genom 274:506-514, 2005) as an endogenous control. This gene was found to express to very similar levels among the treatments during the course of this study.

Results

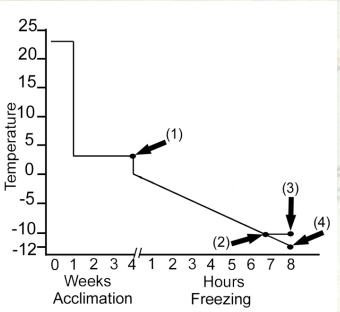


Figure 1. Temperature and time of exposure over the life of wheat plants used to assess changes in gene expression during the freezing process. RNA was extracted from crown tissue at the four indicated time points.

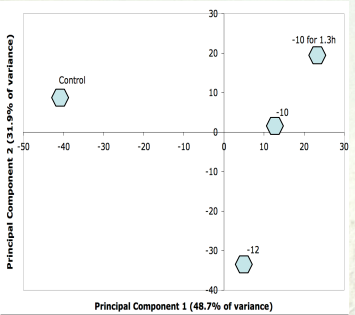


Figure 2. Principal component analysis plot of transcriptome expression in crown tissue of winter wheat cultivar Tiber plants exposed to the indicated freezing conditions.

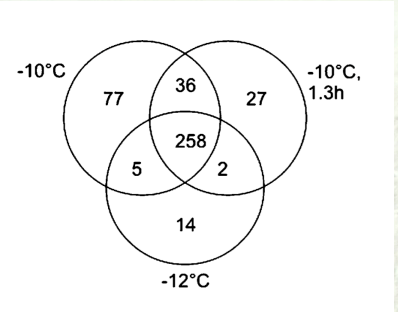


Figure 3. Venn diagram of genes responding specifically or in common to three freezing conditions in crown tissue of winter wheat cultivar Tiber plants. Numbers indicate the total number of genes responding significantly, including those that were upregulated and those that were downregulated.

Table 1. Sixty-eight wheat (*Triticum aestivum*) genes that were indicated from Affymetrix microarray analysis as upregulated at least 5-fold as cold-acclimated wheat plants were exposed to freezing treatments shown in Figure 1.

Number of genes	Probe set bases, (GenBank accession)	Related to
4	AF181661.1, BJ272451.1, BQ162281.1, CA498188.1	Calcium trafficking
1	BE516383.1	dehydrogenase
1	BQ169503.1	F-box
6	CK192888.1, CK193135.1, CK194114.1, CK195062.1, CK200985.1, CK214385.1	glycosyltransferase
1	AL828817.1	Heat shock
2	CA730802.1, CK200303.1	Histone
2	CA731347.1, CA615595.1	Z. mays long-cell
2	BF199967.1, BJ230300.1	Lyase
3	CK214034.1, BQ172227.1, CD872545.1	Membrane protein
6	AL829285.1, BQ168712.1, BT008938.1, CA604824.1, CA639632.1, CK207520.1	Phosphatase, kinase
1	BJ321266.1	Ribosomal
3	BJ315637.1, CA666137.1, CA730299.1	Cold induced
1	BQ801759.1	tubulin
7	BJ278480.1, BQ483742.1, BQ802663.1, CA652153.1, CK211677.1, CK213889.1, D16416.1	Zn-finger protein
7	BQ801587.1, CD453611.1, CD492203.1, CK211510.1, CK211571.1, CK214542.1, CK214676.1	*Cbf* transcription factors
3	CA624333.1, CD935984.1, CK163938.1	WRKY transcription factors
18	AL828936, AL830288, BJ212029, BJ316004, BJ316053, BQ802439, CA484005, CA593923, CA604124, CA609153, CA621140, CA625729, CA652933, CA727379, CD937104, CD938201, CK212624, CK214945	Unknown

Table 2. Expression level changes of five genes in crown tissue of winter wheat cultivars Tiber or Norstar in response to the three freezing treatments indicated in Figure 1.

Gene	Putative function	Temperature treatment; frozen to °C	Expression fold change relative to control in cultiva r	
			Norsta r	Tibe r
BQ172277	Unknown	- 1 0	4.3±1.6	2.6±0.89
		-10 for 1.3 hours	14.0±5.2	3.2±0.71
		- 1 2	3.9±1.4	2.0±0.17
CA614891	Unknown	- 1 0	-1.8±0.2	4.7±1.7
		-10 for 1.3 hours	-1.8±0.2	-2.3±0.1
		- 1 2	-4.2±0.1	-1.4±0.1
CK211276	Chlorophyll a/b binding protein	- 1 0	-1.7±0.2	-1.6±0.1
		-10 for 1.3 hours	-1.2±0.02	-1.5±0.1
		- 1 2	-2.0±0.2	-1.6±0.2
CK212505	Chlorophyll a/b binding protein	- 1 0	1.9±0.4	2.7±0.7
		-10 for 1.3 hours	3.4±0.2	1.5±0.2
		- 1 2	1.7±0.1	5.5±0.9
CK216032	light-inducible protein	- 1 0	30.4±10.2	-4.5±0.1
		-10 for 1.3 hours	58.1±39.9	-1.5±0.3
		- 1 2	9.9±7.0	-4.6±0.1

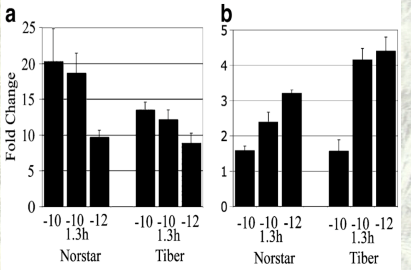


Figure 4. Diagram of expression fold-changes relative to the control of two C-repeat binding protein (*Cbf*) genes in crown tissue of winter wheat cultivar Norstar or Tiber plants exposed to three freezing conditions. (a) *Cbf7*, (b) *Cbf2c1*.

Discussion

The results presented here demonstrated that cold-acclimated wheat plants effect large changes of the transcriptome in crown tissue during the process of freezing and that lowering the temperature to from -10 to -12°C over 1.3 hours resulted in considerable differences in genes expression compared to incubating the crowns at -10°C for 1.3 hours (Fig. 2). Relative to the control, the expression levels of 423 genes changed significantly (Fig. 3); genes that were upregulated outnumbered genes that were downregulated by about a 9:1 ratio. Nearly half of the most strongly upregulated genes were involved in signal transduction or transcription regulation, including *Cbf* genes (Table 1). Most of the *Cbf* genes were strongly upregulated as the temperature was decreased to -10°C, then expression declined as the temperature was held at -10°C or was further reduced to -12°C. An example (*Cbf7*) is shown in Fig. 4a. *Cbf2c1* behaved in opposite fashion; it was moderately upregulated as the temperature was decreased to -10°C, but then continued to increase in expression level as the temperature was held at -10°C or was further lowered to -12°C (Fig. 4b). This result extends the influence of the *Clf* regulon to include freezing stress response in addition to cold acclimation in wheat. ¶ The finding of increased expression of genes encoding chlorophyll a/b binding protein in crown tissue frozen to -12°C (Table 2) was unexpected. Satoh et al. (Plant and Cell Physiol. 42:906-911, 2001) reviewed chlorophyll a/b binding-like proteins and concluded that, although certain of these proteins are capable of binding chlorophyll, their relatively low affinity for chlorophyll and characteristic stress-inducibility "suggested little contribution to photosynthesis," but rather, suggested a "protective role during stress conditions." ¶ Overall, these results suggested that a significant part of the ability of wheat plants to survive freezing may be determined by their ability to adjust the transcriptome in crown tissue as the temperature is decreased to potentially damaging levels. Because about 25% of the responsive genes identified in this study were not significantly similar to any genes of known function, and genes encoding chlorophyll binding-like proteins were upregulated in the nonphotosynthetic crown tissue, it may be that responses to subfreezing stresses involve processes that have not yet been described.