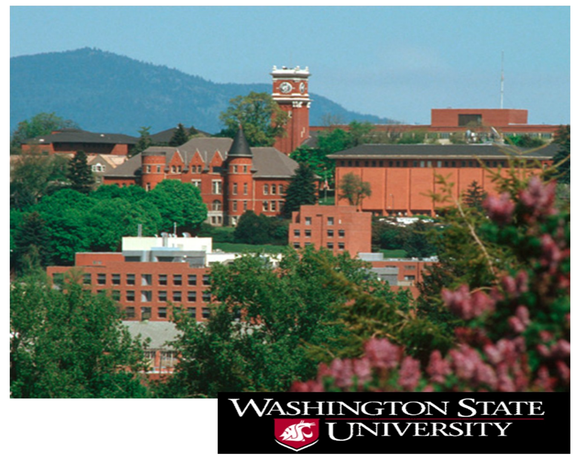
Phenomic and Transcriptomic Response of Winter Wheat to a Mild Freeze-Thaw Cycle

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

With the onset of the winter season, winter wheat seedlings in the field are likely to be exposed to several episodes of mild freezing followed by thawing at low, positive temperatures. The impact of these freeze-thaw cycles on the ability to survive the winter was evaluated in 22 winter wheat varieties. Seedlings were cold acclimated at +4C for five weeks, then were tested for freezing tolerance with or without a mild freeze-thaw pre-freezing treatment. Plants exposed to the freeze-thaw treatment were frozen at -3C for 24 h, then allowed to thaw at +4C for 24 h, then were frozen to potentially damaging temperatures. The freeze-thaw treatment resulted in significantly greater survival of most of the varieties. Affymetrix microarray analysis revealed that hundreds of genes were upregulated by the pre-freezing treatment. During the thaw portion of the freeze-thaw treatment, most of the genes upregulated during the freeze portion were downregulated, but many other genes were strongly upregulated. These transcription cascades revealed the cold acclimation process was stimulated by the freeze-thaw treatment and that broad-scale transcriptomic adjustment continued for at least 48 hours after thawing at +4C commenced. These results provide new insights into the cold response process and freezing tolerance of winter wheat.

Materials and Methods

Plant material and freezing survival tests

The 22 winter wheat cultivars included in this study are indicated in Table 1. Plants were grown in 6–container packs (Model 1020, Blackmore Co., Belleville, MI, USA) in Sunshine Mix LC1 planting medium consisting of 70-80% Sphagnum peat moss, coarse grade perlite, gypsum, Dolomitic lime to adjust the pH to 5-7, and a proprietary wetting agent (Sun Gro Horticulture, Bellevue, WA, USA). Plants were germinated and grown at 22C in a growth chamber (Model E15, Conviron, Pembina, ND) under cool, white fluorescent lights with a 16-hour photoperiod until the seedlings reached the three–leaf stage. The plants were then transferred to 4C with a 12-hour photoperiod for 35 days to induce cold acclimation prior to freezing survival tests. Prior to freezing, plants were counted, the flats were drenched with ice water containing 10mg/L Snowmax (Johnson Controls, Centennial, CO, USA) and allowed to drain until drainage had essentially ceased, a layer of crushed ice was placed on the soil surface, and freezing was carried out in a programmable freezer (model LU–113, Espec Corp., Hudsonville, MI, USA). Snomax is a commercial product used in the snow-making industry and results in uniform ice nucleation at about -3–C (Skirvin et al., 2000).

The plants were frozen to potentially damaging temperatures after one of three treatments: (1) no subzero pre-freezing treatment; (2) a 16 hour pre-freezing period at -3C followed by freezing to potentially damaging temperatures; and (3) a freeze/thaw cycle of -3–C for 24 hours followed by +4C for 24 hours, followed by 16 hours at -3C, prior to freezing to potentially damaging temperatures. The temperature in the crown region of the plants in each container was recorded every 2min using temperature probes. Following freezing, the plants were held at 4C for 24 h, and then were moved to a greenhouse. Survival was scored as the proportion of plants that had regrown after 5 weeks. The temperature record was parsed with a computer script to determine the cooling rate from -4C (indicating the end of the -3C pre-freezing treatment) to the minimum temperature, the minimum temperature experienced by the plants, the amount of time within 0.5C of that minimum temperature, and the warming rate as the temperature returned to 0C as the freezing episode ended. Two global measures of the freezing process, the total time frozen, and degree minutes, also were calculated. Total time frozen was calculated as the number of minutes the temperature measured in the crown zone was ≤0C. Degree minutes were calculated as the temperature multiplied by the time at that temperature, summed over the freezing episode (Chen and Walker, 1993), i.e. the area bounded by the temperature trace and the 0C line. The data were analyzed to evaluate the significance of the impact of each of these freezing process components on survival using logistic regression. A cluster analysis and a canonical discriminant analysis was performed using the logistic regression-generated odds ratios associated with the four freezing process components (cooling rate, minimum temperature, time and minimum temperature, and warming rate), and the two global measures of freezing exposure (degree minutes and total time frozen) as predictor variables.

RNA extraction and microarray interrogation

Plants were collected at the end of the 3 wk cold-acclimation period (control), after 24 hours at -3C (subzero acclimation, SZA [Herman et al., 2006]), after 24 hours of thawing at +3C following the subzero acclimation, and after 48 hours of thawing at +3C following subzero acclimation.

To extract RNA, six crowns about 5 mm in length 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 -80C. 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. Three independent sets of RNA samples were analyzed for each temperature treatment. The data files were further analyzed using "Flexarray" software (Blazejczyk et al., 2007). 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 functions of genes of interest were sought through BLASTN searches of the GenBank nonredundant database (http://blast.ncbi.nlm.nih.gov). In the event the BLASTN algorithm returned only genes of unknown function, translated searches (BLASTX and TBLASTX) were also performed.

Results

Table 1

Least-squares means of percent survival of winter wheat plants frozen to potentially damaging temperatures after prefreezing to -3°C for 16 hours (PF) or after a 48-hour freeze-thaw cycle followed by prefreezing (FTP)

	Prefreezing treatment		
Cultivar	PF	FTP	Significance[a]
Norstar	66.9	66.5	
Froid	59.6	59.4	
Kestrel	54.8	57.1	
Centurk	44.3	47.2	
Eltan	43.7	41.8	
Tiber	43.4	39.1	
Trego	43.2	43.3	
Wanser	41.4	42.4	
Munster	37.9	37.9	
Edwin	35.3	35.3	
2145	34.1	37.8	
2137	32.7	37.9	
Finley	29.0	26.7	
Masami	28.9	40.2	**
Overley	25.2	27.0	
Lewjain	19.7	28.1	**
Jagger	19.7	26.5	*
Farnum	18.8	28.0	*
Rely	12.0	28.9	**
Madsen	8.0	18.4	*
Tubbs	7.6	25.5	**
Hiller	6.4	18.1	*

[a]Statistical significance of the difference of the two means for the indicated cultivar. * P=0.05; ** P=0.01; blank = not significant.

Figure 1

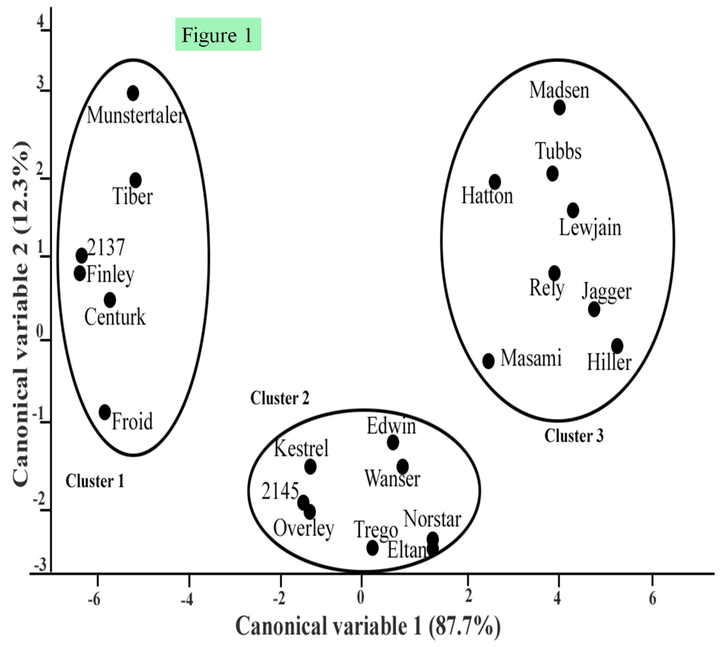
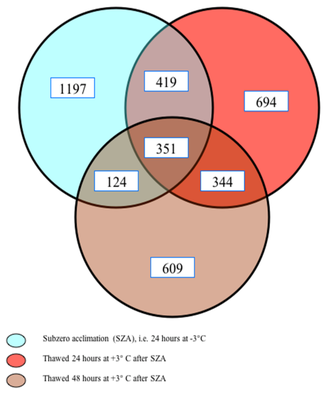
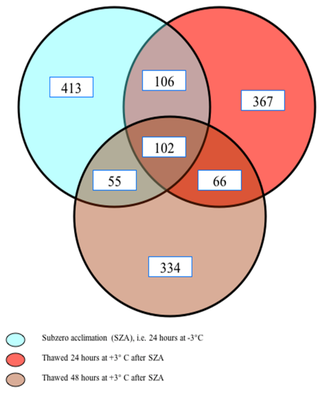


Figure 2a



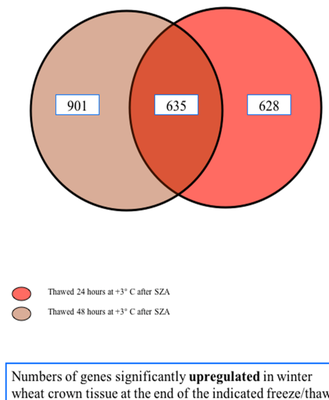
Numbers of genes significantly **upregulated** in winter wheat crown tissue at the end of the indicated freeze/thaw treatment relative to nonfrozen, cold-acclimated plants

Figure 2b



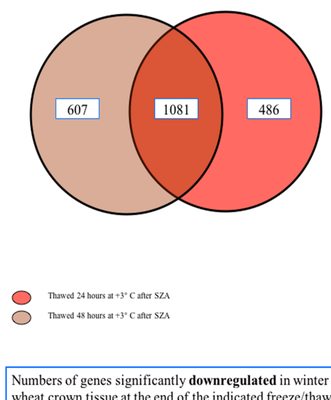
Numbers of genes significantly **downregulated** in winter wheat crown tissue at the end of the indicated freeze/thaw treatment relative to nonfrozen, cold-acclimated plants

Figure 2c



Numbers of genes significantly **upregulated** in winter wheat crown tissue at the end of the indicated freeze/thaw treatment relative to plants exposed to subzero acclimation

Figure 2d



Numbers of genes significantly **downregulated** in winter wheat crown tissue at the end of the indicated freeze/thaw treatment relative to plants exposed to subzero acclimation

Survival of many the cultivars clearly was enhanced by a 48 hour freeze (to -3C) -thaw cycle prior to freezing to potentially damaging temperatures. These results indicated that previously cold-acclimated wheat plants initiate further acclimation to freezing stress as the temperature declines below 0C. The results reported here suggest the effects of this subzero acclimation continue to develop for some length of time if the plants are returned to above-freezing conditions, resulting in enhanced freezing tolerance. Canonical discriminant analysis classification of the 22 winter wheat cultivars based on the logistic regression-generated odds ratios associated cooling rate, minimum temperature, time and minimum temperature, and warming rate; and two global measures of freezing exposure (degree minutes and total time frozen) as predictor variables resulted in three clusters of cultivars (Fig. 1). The average survival of the cultivars within the clusters were 40, 42, and 17% for clusters 1-3, respectively. Canonical variables 1 and 2 accounted for the percentage of variation indicated on the respective axis titles.

To further investigate the impact of the freeze-thaw cycle, microarray analysis was carried out on the crown tissue of plants exposed to subzero acclimation (SZA, a 16 hour period at -3C), and plants exposed to SZA followed by thawing at +3C for 24 or 48 hours.

Many more genes were upregulated than downregulated in response to subzero acclimation and after 24 hours of thawing at +3C following subzero acclimation compared to nonfrozen plants (Fig. 2a and 2b). Several hundred genes were uniquely upregulated 24 hours after the plants were moved to +3C following subzero acclimation, and hundreds more had upregulated after 48 hours (Fig. 2b). Over 1000 genes significantly downregulated during the thawing phase; some that were downregulated at 24 hours apparently were no longer downregulated at 48 hours (Fig. 2d), possibly indicating cyclical regulation.

Discussion

The results presented here demonstrated that wheat plants that had been cold-acclimated at +3C acquired significantly greater freezing tolerance if they were exposed to -3C for 24 hours (subzero acclimation, described by Herman et al., 2006), and then gained even more freezing tolerance if they were allowed to thaw at +3C for 24 hours before freezing to potentially damaging temperatures. Expression levels of hundreds of genes were significantly increased on exposure to -3C, and hundreds more were upregulated as the plants thawed at +3C for 24 or 48 hours (Fig. 2). Cultivars differed in their responses to components of the freezing process and formed distinct clusters based on the impact of the components on survival (Fig. 1). Taken together, these observations indicated that wheat plants actively adjust their transcriptome in response to freezing to -3C, and continue to adjust as the plants thaw at +3C; in general, these adjustments lead to greater freezing tolerance (Table 1).

References

Blazejczyk M, Miron M, Nadon R (2007) FlexArray: A statistical data analysis software for gene expression microarrays. Genome Quebec, Montreal, Canada, URL http://genomequebec.mcgill.ca/FlexArray

Chen, C.P., and V.K. Walker. 1993. Increase in cold-shock tolerance by selection of cold resistant lines in Drosophila melanogaster. Ecol. Ent. 18:184-190.

Herman EM, Rotter K, Premakumar R, Elwinger G, Bae R, Ehler-King L, Chen SX, Livingston DP (2006) Additional freeze hardiness in wheat acquired by exposure to -3° C is associated with extensive physiological, morphological, and molecular changes. J Exp Bot 57:3601-3618

Skinner, D.Z., Mackey, B.E. 2009. Freezing tolerance of winter wheat plants frozen in saturated soil. Field Crops Research 113:335-341

Skinner, D.Z., Bellinger, B.S. 2010. Exposure to subfreezing temperature and a freeze-thaw cycle affect freezing tolerance of winter wheat in saturated soil. Plant and Soil. 332(1):289 -297

Skirvin RM, Kohler E, Steiner H, Ayers D, Laughnan A, Norton MA, Warmund M (2000) The use of genetically engineered bacteria to control frost on strawberries and potatoes. Whatever happened to all of that research? Scientia Horticulturae 84:179-189