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**Freezing tolerance of winter wheat as influenced by extended growth at low temperature
and exposure to freeze–thaw cycles**

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Abstract

As the seasons progress, autumn-planted winter wheat plants (*Triticum aestivum* L.) first gain, then progressively lose freezing tolerance. Exposing the plants to freeze-thaw cycles of -3/3°C results in increased ability to tolerate subsequent freezing to potentially damaging temperatures. This study was conducted to determine to what extent the length of time grown at low temperature influenced the effectiveness of this freeze-thaw enhancement of freezing tolerance. Plants from six winter wheat lines were grown at 4°C for 1-18 weeks, exposed to 0-2 cycles of freezing to -3°C for 24 h, then thawing for 24 h at 3°C, then tested for their ability to tolerate freezing to -10 to -17°C. The freeze-thaw treatments resulted in increased freezing tolerance after 6 to 12 weeks of growth at low temperature, but had no significant effect before or after that time period. Two cycles of -3/3°C freeze-thaw was consistently more effective than one cycle. Variation in the extent and timing of the effectiveness of the freeze-thaw treatments was found among the wheat lines, suggesting genetic variation that may be useful for prolonging freezing tolerance further into the winter months could be found in winter wheat.

Keywords: wheat (winter), cold, freezing tolerance, freeze-thaw

Running head: Response of winter wheat to freeze-thaw cycles during cold acclimation

Introduction

Winter wheat is planted in the autumn and harvested the following summer, and therefore must survive the winter months in the field. Winter wheat plants simultaneously vernalize and increase freezing tolerance (acclimate to cold) when grown at low, positive temperatures. The effects of the interaction of temperature and photoperiod on vernalization response are complex (Brooking and Jamieson 2002; Allard et al. 2012), and the level of freezing tolerance of the plants declines as saturation of the vernalization requirement is approached when the plants are grown at low, positive temperatures (Fowler et al. 1996a, b; Prasil et al. 2004, 2005; Sarhadi et al. 2010). Recently, a “direct link” between vernalization status and low temperature tolerance was reported, based on the observation that the protein product of one of the major vernalization genes was shown to interact with as many as 500 other genes, including many involved in cold acclimation (Deng et al. 2015). However, the interaction of vernalization and freezing tolerance in plants maintained at sub-freezing temperatures is much more complex. Gusta et al. (1997) investigated the ability of wheat plants to survive being held at temperatures ranging from -4 to -15°C for periods of time ranging from days to months. The freezing tolerance of some of the wheat cultivars remained virtually unchanged for at least 3 months when held at -4°C (Gusta et al. 1997), long after the vernalization requirement was expected to have been met. The authors observed that determinations of freezing tolerance of field-grown plants in the autumn or early winter may not effectively predict over-wintering ability and concluded that a “different mechanism of freezing tolerance exists during a prolonged exposure vs. a short exposure to sub-zero temperatures” (Gusta et al. 1997). Multiple mechanisms contribute to freezing tolerance in

plants, impacting numerous physiological, morphological, and biochemical characteristics of the plant (Levitt 1980). The responses of these numerous characteristics are closely tied to environmental cues, resulting in complex genotype X environment interactions that are not well understood (Fowler et al. 2014). One of these environmental cues is mild freeze-thaw events that occur as autumn transitions to winter and the temperature falls below freezing at night, but then rises above freezing the following day. Over the past 50 years, the date of the first damaging freeze in the autumn has gotten progressively later in the year [see, for example, Betts, (2011)]. Thus, autumn-sown wheat may be exposed to increasing periods of time at low, above-freezing temperatures, and more mild, freeze-thaw events may occur before the onset of potentially damaging, sub-freezing temperatures. We previously demonstrated that the level of freezing tolerance in wheat plants that had been grown at low, above-freezing temperatures for 5 weeks was significantly increased if the plants were exposed to a mild freeze-thaw cycle of -3°C for 24 h, followed by 3°C for 24 h, prior to freezing to a potentially damaging temperature (Skinner and Bellinger 2010). Transcriptome analysis of the crown tissue during this mild freeze-thaw event revealed that multiple metabolic pathways were sequentially activated during the response, and that the numbers of responding genes with a given function increased as the plants continued to grow at 3°C following the freeze (Skinner 2015). This result indicated that the freeze at -3°C activated processes that were amplified and diversified after the freeze stress was removed. We further showed that the $-3/3^{\circ}\text{C}$ freeze-thaw treatment resulted in extensive changes to cellular carbohydrates and lipids (Skinner et al. 2014), indicating that the transcriptome changes are accompanied by structural changes at the cellular level. By understanding the extent into the growth cycle that the freeze-thaw enhancement of freezing tolerance may be effective, and by characterizing differences in the response among cultivars, it

may be possible to develop plant lines that more effectively make use of this phenomenon. Accordingly, the objective of this study was to assess the response to mild freeze-thaw cycles among wheat cultivars grown under low, positive temperatures for 1 to 18 weeks.

Materials and methods

Plant material and freezing survival tests

Five winter wheat cultivars and a winter wheat germplasm line were included in this study. The cultivars were Eltan (Peterson et al, 1991), CDC Kestrel (Fowler, 1997), Norstar (Grant, 1980), Tiber (Kisha et al., 1992), and Froid, a hard red winter wheat developed in Montana, USA, and released in 1968 (<https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?1067585>). Also included was germplasm line Oregon Feed Wheat #5 (ORFW), developed by Bolton (1968) from crosses of winter and spring wheats and further described by Skinner and Garland-Campbell (2008a). The LT_{50} values, the temperatures predicted to be fatal to 50% of the plants, of these wheat lines were previously reported (Skinner and Garland-Campbell 2008a, b) to range from -9.5 to -19.5°C (Table 1). Twenty seeds of each wheat line were planted into a cell (approximate volume = 100ml) of 6-cell horticultural 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).

Seeds were germinated and seedlings grown at 22 °C in a growth chamber (Model E15, Conviron, Pembina, ND) under cool, white fluorescent lights (approximately 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at the soil surface) with a 16-hour photoperiod until the seedlings reached the three-leaf stage. Relative humidity was not controlled.

The plants were then transferred to 4 °C with a 12-hour photoperiod (approximately 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at mid-plant height) for 1 to 18 weeks prior to freezing survival tests. Plants were irrigated weekly with nutrient solution containing macro and micronutrients (Peters Professional, Scotts Co., Camarillo, CA, USA). 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). Snowmax® 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 freezing tests were conducted with target temperatures of -12.5, -14.5, and -16.5°C. The temperature of the plant growth medium in each container near the crowns of the plants was monitored using food piercing temperature probes and an internet-enabled temperature monitor (Model E-16, Sensatronics, Bow, NH, USA). The temperature was recorded every 2 min using a data capture script running on a remote computer. The lowest recorded temperatures naturally varied among the individual containers within a test and therefore the minimum temperature was treated as a continuous variable.

The plants were frozen to potentially damaging temperatures after one of four treatments: (1) no subzero pre-freezing treatment; (2) a 16 hour pre-freezing period at -3°C followed by freezing to potentially damaging temperatures; (3) a freeze/thaw cycle of -3°C for 24 hours followed by 3°C for 24 hours, followed by 16 hours at -3°C, prior to freezing to potentially damaging temperatures; and (4) two cycles of freezing and thawing as in (3), prior to freezing to potentially damaging temperatures. These four treatments were designated NPF (no prefreeze), PF (prefreeze), FTP (freeze-thaw plus prefreeze), and 2FTP (two cycles of freeze-thaw plus

prefreeze), respectively. Freezing to potentially damaging temperatures was effected by programming the freezer to reduce the temperature at a rate of $-2^{\circ}\text{C h}^{-1}$ until the target temperature was reached. For the entire study, the target temperatures ranged from -10°C to -17°C ; the time at the minimum temperature was 1h. Each freezing test contained plants that had been exposed to each of the pretreatments and one length of low temperature growth (1-18 weeks). Each combination of wheat line and pretreatment was represented twice in each freezing test. The value of the response variable (proportion surviving) was determined from the mean of those two representations. Following freezing, the plants were held at 4°C for 24 h with 12 h photoperiod, and then were moved to 22°C with 18h photoperiod. Survival was scored as the proportion of plants that had regrown after 5 weeks. The entire experiment was repeated six times over three years. Cells that had fewer than 10 plants growing prior to the freezing test were included in the test to maintain uniformity of the mass of soil in the freezer, but the data from those individual cells were discarded. Ultimately, the data set comprised 5,025 observations based on 145,078 plants.

Data analysis

The data were analyzed to evaluate the significance of the impact of the prefreezing treatments (NPF, PF, FTP, 2FTP), and length of time of growth at low temperature on survival. For data analysis purposes, the response (survival) was expressed as the arcsine of the square root of the proportion of plants that survived. Survival is expressed in the original scale in this report. The temperature record for each freezing episode for each container was parsed with a computer script (written by the first author) to determine the actual minimum temperature reached and the time the plants remained at those minima. Because of the physical constraints of the system, these values naturally varied from those that were programmed into the freezer and

therefore formed continuous variables. All statistical analyses were carried out using JMP version 12 (<http://www.jmp.com/>). Analysis of variance and means separations were conducted using the “Fit Model” function and regression of survival versus weeks of growth at low temperature was illustrated using the cubic spline smoothing function available in the “Graph Builder” function of JMP version 12. The cubic spline method enables the smoothing of one-dimensional data by varying a penalty applied to local curvature of a regression line fit to the data (Hastie et al., 2009). The function in JMP allows the penalty (represented by the quantity “lambda”) to be varied dynamically and selected empirically through a graphical user interface. In this study, a lambda value of 6.5 resulted in the smoothed curves shown in Figure 1.

Results

Overall mean survival proportions (number of plants that survived / initial number of plants) of the wheat lines were: Eltan, 0.30; Froid, 0.51; CDC Kestrel, 0.40; Norstar, 0.50 and ORFW, 0.04; Tiber, 0.36. This result of about 50% survival of the most freezing tolerant cultivars in the study (Norstar and Froid, Table 1), showed the test conditions were sufficient to distinguish differences in freezing tolerance potential of all of the wheat lines tested. An analysis of variance revealed that each of the sources of variation were statistically significant with the main effects accounting for 62% of the variation (Table 2).

Over the 18 weeks of growth at 4°C, the average proportions of the plants to survive each of the freezing treatments and their statistical separations were: NPF, 0.13a; PF, 0.40b; FTP, 0.42c and 2FTP, 0.45c, where means followed by the same letter were not significantly different ($P=0.05$). In all weeks, survival in the NPF treatment was significantly less ($P=0.05$) than the other treatments, except for the 18th week, where all treatments resulted in similar survival rates (means separation not shown). Freezing survival increased over the first 7 weeks

of growth at 4°C, then declined over 8-18 weeks (Fig. 1). A comparison of plant survival as a function of the freezing treatments and weeks of growth at low temperature revealed that the 2FTP treatment resulted in significantly greater survival than NPF, PF, or FTP during the 6th through 13th week of growth at low temperature (shaded area in Fig. 1). This improvement was no longer significant after 13 weeks; in fact, survival after 2FTP was significantly less than survival after FTP by 17 weeks of growth at low temperature (indicated by the arrow in Fig. 1). However, the effectiveness of improvement of freezing tolerance by the 2FTP treatment varied widely among the individual wheat lines, as shown by comparison of survival following the 2FTP versus PF treatments over weeks 6-13, where significant differences were found (Fig. 2). ORFW, which has very little freezing tolerance (Table 1) responded slightly to the 2FTP treatment during the first 4 weeks of the week 6-13 period, but not after that (Fig. 2). Survival of CDC Kestrel was essentially constantly improved by about 10% by the 2FTP treatment throughout the 6-13 week period (Fig. 2). Eltan and Tiber responded to the 2FTP treatment during 6-11 weeks of growth at 4°C, with peak responses at about 9 and 10 weeks, respectively (Fig. 2). Froid and Norstar, the most freezing tolerant lines in the study (Table 1), responded to the 2FTP treatment later than the other lines in the study, with peak responses occurring at about 11 and 12 weeks of low-temperature growth, respectively (Fig. 2).

Discussion

Winter wheat plants growing in the field lose freezing tolerance as the winter season progresses, but many factors affect the rate and severity of this loss (Fowler et al. 2014). The fulfillment of vernalization requirements often has been cited as the main reason for this loss of freezing tolerance (reviewed in Fowler et al. 2014). However, vernalization of winter wheat usually is completed after about 6 weeks of growth at low temperature (Fowler and Limin,

2004), and the average survival of the wheat lines investigated here was significantly enhanced by exposure to $-3^{\circ}/3^{\circ}\text{C}$ freeze-thaw cycle(s) long after after six weeks of growth (Fig. 1), suggesting this effect was not strictly gated by the vernalization status of the plants. However, the response to the freeze-thaw cycles was essentially lost after about 17 weeks of growth at low temperature, so much so that survival after 2FTP was significantly less than after FTP. We suppose that this significant reduction in survival was because the levels of freezing tolerance were so low that repeated exposure to -3°C damaged the plants, resulting in decreased survival after additional freezing.

The mechanisms responsible for this response to freeze-thaw cycles are unknown, but it previously was shown that the expression levels of hundreds of genes involved in dozens of molecular functions were significantly up- or down-regulated while wheat plants were held at -3°C (Herman et al. 2006; Skinner 2015), or lower temperatures (Skinner 2009). We further demonstrated that both the numbers of significantly regulated genes with similar function, and the number of genes with additional functions increased as the plants subsequently thawed at 3°C following 24 h at -3°C (Skinner 2015). These transcriptomic changes were accompanied by restructuring of carbohydrate and lipid components of the cells (Skinner et al. 2014). The result of this transcriptome and cellular component re-engineering appears to be a significant increase in the ability of the plants to withstand subsequent freezing to potentially damaging temperatures. Genotypic differences controlling the manner in which individual wheat lines expressed this mechanism were found. For example, while freezing tolerance of most of the wheat lines was improved by the exposure to 2FTP for only a portion of the 6-13 weeks of growth at low temperature, CDC Kestrel responded essentially uniformly throughout that time period (Fig. 2). Tiber appeared to show a bimodal response while the other lines did not (Fig. 2).

It previously was shown that segregation of freezing tolerance in the progeny of the cross of CDC Kestrel x Tiber was significantly different from the segregation in the progeny from crosses of CDC Kestrel to Eltan, Froid, or ORFW (Skinner and Garland-Campbell, 2008a). The results presented here, considered along with the previous results (Skinner and Garland-Campbell, 2008a) suggest the differences detectable in the ability to respond to freeze-thaw cycles provide another means to identify genetic variation for freezing tolerance. It may be possible to exploit this ability as a means to extend the length of time the plants remain sufficiently freezing tolerant for a given region, and thereby improve winterhardiness.

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Figure Captions

Figure 1. Survival of wheat lines after 1-18 weeks of growth at low temperature and one of four prefreezing treatments prior to freezing to -10 to -17° . Wheat lines were cultivars Eltan, Froid, CDC Kestrel, Norstar and Tiber, and germplasm line Oregon Feed Wheat #5. The prefreezing treatments were NPF (no prefreeze), PF (prefreeze to -3°C for 16 h), FTP (freeze-thaw plus prefreeze, freeze to -3°C for 24 h then thaw at $+3^{\circ}\text{C}$ for 24 h), and 2FTP (two cycles of FTP). The shaded area indicates survival after 2FTP was significantly greater than after the other treatments. The arrow at 17 weeks indicates the point at which survival after 2FTP became significantly less than survival after FTP.

Figure 2. The improvement in freezing tolerance of six wheat lines after prefreezing treatment 2FTP compared to PF. PF indicates prefreeze to -3°C for 16 h; 2FTP indicates 2 cycles of freeze-thaw plus prefreeze, i.e. freezing to -3°C for 24 h then thawing at $+3^{\circ}\text{C}$ for 24 h, prior to PF then freezing to a challenge temperature of -10 to -17°C . Plants were grown at 4°C for 6 to 13 weeks, the period of time during which the differences in survival following 2FTP compared to PF were statistically significant.

Table 1. Cultivar names, origins, market classes and freezing tolerance potential of winter wheat lines evaluated in this study

Cultivar	Origin	Market Class ^a	Freezing tolerance potential ^b
Eltan	Washington, USA	SWWW	-15.1
Froid	Montana, USA	HRWW	-15.7
CDC Kestrel	Saskatchewan, Canada	HRWW	-14.6
Norstar	Alberta, Canada	HRWW	-19.5
Tiber	Montana, USA	HRWW	-13.5
ORFW ^c	Montana, USA	SWWW	-9.5

^aHRWW= Hard red winter wheat, SWWW=Soft white winter wheat.

^bLT₅₀ values (°C) reported in Skinner and Garland-Campbell, 2008b.

^cORFW “Oregon Feed Wheat #5” is an unreleased germplasm line with very little freezing tolerance, described in Skinner and Garland-Campbell, 2008a.

Table 2. Analysis of variance of freezing tolerance of winter wheat (*Triticum aestivum* L.) lines after 1-18 weeks of growth at 4°C and one of four prefreezing treatments

Source	DF	Sum of Squares	F Ratio	% of variation due to source
Wheat lines ^a	5	252.1	434.9**	17.6
Prefreezing Treatments within weeks of growth ^b	54	257.3	41.1**	17.9
Weeks of growth ^c	17	228.1	115.7**	15.9
Minimum temperature ^d	1	149.0	1284.9**	10.4
Replications	5	8.5	14.6	0.6
Total	5024	1436.3		

^aWheat lines were cultivars Eltan, Froid, CDC Kestrel, Norstar and Tiber, and germplasm line Oregon Feed Wheat #5.

^bPrefreezing treatments were no prefreeze (NPF), prefreeze to -3°C for 16 h (PF), freeze-thaw, prefreeze (FTP), i.e. freeze to -3°C for 24 h, then thaw at 3°C for 24 h, then prefreeze to -3°C for 16 h, and two cycles of FTP (2FTP) prior to freezing to a potentially damaging temperature.

^cWeeks of growth at 4°C and 12 h photoperiod prior to prefreezing and freeze tolerance treatments.

^dThe measured minimum temperature reached during the freezing tolerance testing, ranging from -10 to -17°C, treated as a continuous variable.

**Significant at P<0.001

Fig. 1
98x118mm (300 x 300 DPI)

Fig. 2
135x246mm (300 x 300 DPI)