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Quantitative expression analysis of selected low temperature-induced genes in autumn-seeded wheat (*Triticum aestivum* L.) reflects changes in soil temperature

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ABSTRACT

Exposure of winter wheat (*Triticum aestivum* L.) to low autumn temperatures allows it to cold acclimate and withstand sub-zero winter temperatures. Winter damage/kill can however occur. Studies on low temperature (LT)-induced genes have focused on growth chamber-grown plants at constant LT. Ideally a field experiment would be more representative and provide a better understanding of the role of LT-induced genes during cold acclimation. Therefore, the objective of this study was to investigate the expression patterns of selected LT-induced genes, *TaCBF20*, *Wcs120*, *Wcor410* and *Wcor14*, in field-grown wheat (winter-hardy Norstar, tender spring Manitou and the near-isogenic lines, spring Norstar and winter Manitou) over 3 years at Saskatoon, Saskatchewan, Canada. Leaf samples were collected at different intervals during the autumn. Generally, cold acclimation of the four genotypes over the 3 years was in agreement with growth chamber studies. However, variability in LT-induced transcript accumulation in the field-grown plants was observed over the 3 years and it could be attributed to the soil temperature fluctuations. Trends in accumulation in early sampling dates indicated that differences among the genotypes could be delineated, depending on the year and LT-induced genes. This study highlights the importance of integrating field and growth chamber studies to obtain a more complete understanding of the critical roles of LT-induced genes in conferring LT tolerance.

1. Introduction

The low temperatures (LT) experienced during the autumn season enable winter wheat (*Triticum aestivum* L.) to cold acclimate, thereby preparing it to withstand freezing winter temperatures. During the process of cold acclimation, a plant undergoes a series of biochemical and physiological changes (Fowler et al., 1999; Hekneby et al., 2006), which are parts of interacting networks and pathways (Chinnusamy et al., 2007; Dubcovsky et al., 2006; Fowler et al., 2001; Fowler and Limin, 2004; Henderson et al., 2003; Kosova et al., 2008; Limin and Fowler, 2006; Mahfoozi et al., 2000, 2001b). A better understanding of the expression of key genes can help to characterize components of the interacting networks participating in LT tolerance. This knowledge can be used to enhance LT tolerance of winter wheat to reduce the freezing injury/kill and improve winter survival in the field.

Several cold regulated (*COR*) genes are induced in plants exposed to low non-freezing temperatures (Thomashow, 1999, 2001). Studies conducted under controlled LT exposure have consistently shown the rapid up-regulation of genes coding for the CBF transcription factors (Galiba et al., 2009; Thomashow et al., 2001; Van Buskirk and Thomashow, 2006) and the subsequent induction of other down-stream *COR* genes. Winter-hardy wheat genotypes have been shown to accumulate higher levels of *COR* transcripts and polypeptides than LT susceptible genotypes. The WCS120 family of dehydrin polypeptides accumulate at higher levels in LT tolerant genotypes than in the spring genotypes (Houde et al., 1992a). It has also been suggested that the WCS120 polypeptide could be used as a molecular marker for LT tolerance in the gramineae (Houde et al., 1992b).

Besides the requirement for low non-freezing autumn temperatures for cold acclimation, winter wheat also requires the low autumn temperatures to meet its vernalization requirement to switch from the vegetative to the reproductive phase and flower normally in the spring. The determinants of growth habit (*Vrn1*) in wheat have been located on the group 5 chromosomes (Snape et al., 2001). Furthermore, there is a close linkage between the vernalization and LT tolerance genes on the group 5 homoeologues (Baga et al., 2007; Galiba et al., 1995; Sutka, 2001; Toth et al., 2003). Reciprocal near-isogenic lines (NILs) for the Vrn-A1 locus from the spring wheat cv. Manitou (*Vrn-A1*) and the winter wheat cv. Norstar (*vrn-A1*) have been developed to better understand the influence of the Vrn1 locus on LT tolerance potential (Limin and Fowler, 2002). Thus, expression of maximum LT tolerance depends on developmental

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processes such as delayed flowering due to vernalization and photoperiod requirements, both of which can maintain the plant in the vegetative phase thereby allowing LT tolerance accumulation, a consequence of expression of LT-induced genes over a longer period (Fowler et al., 1996a,b; Fowler and Limin, 2004; Mahfoozi et al., 2000, 2001b).

Keeping in mind the interactions between different developmental pathways, the LT acclimation/tolerance process and the complex nature of the hexaploid wheat genome, understanding of LT tolerance has been attempted through controlled environment studies by following the expression patterns of specific LT-induced genes (Ganeshan et al., 2008). Limited studies have attempted to relate growth chamber and field experiments with regard to LT-induced genes. Fowler et al. (1999) have developed a winter survival model based on the known LT response of cereals, including components of acclimation, de-acclimation, LT tolerance and damage, soil temperature, phenological development and genetic coefficients, and validated it with field data from over-wintered cereals in Saskatchewan, Canada. The controlled environment studies, in conjunction with the proposed model, show that in the field the components of vegetative stage maintenance control the duration of expression of LT-induced genes (Fowler et al., 1996a,b; Limin and Fowler, 2006), while rate of LT exposure determines the degree of expression of LT-induced genes (Fowler and Limin, 2004). Furthermore, it has been reported that during initial LT exposure there are rapid changes in LT tolerance (Fowler, 2008; Ganeshan et al., 2008), in addition to genotype-dependent threshold induction temperature requirements for activation of the LT acclimation mechanisms of winter wheat (Fowler, 2008).

In field conditions where environmental fluctuations are unpredictable and uncontrollable, there is likely to be a completely different LT acclimation and LT-induced gene expression profile than in experiments conducted in controlled environment conditions. Studies conducted on field-grown frost tolerant and frost susceptible barley genotypes indicated that the accumulation of *COR* transcripts and corresponding proteins were higher in winter cultivars than in spring cultivars (Giorni et al., 1999). In a recent field study it was shown that there was a higher accumulation of COR14b protein in barley in early winter than in late winter and also in earlier sown plants, reflecting the cumulative acclimation process as the temperature decreases during the autumn (Crosatti et al., 2008). Field-grown winter wheat accumulated high to medium molecular weight dehydrins during autumn and winter and low-molecular weight dehydrins in winter, suggesting differential accumulation of dehydrins during LT acclimation (Stupnikova et al., 2004). Most of the studies have been based on 1-year observation and to our knowledge there are no comprehensive, multi-year, field experiments to assess the expression patterns of *COR* genes and LT acclimation in wheat. Therefore, the objective of this study was to evaluate the expression patterns of selected *COR* genes, *Wcs120*, *Wcor410* and *Wcor14*, and the LT-induced C-repeat binding factor gene, *TaCBF20*, in winter Norstar, spring Manitou and the two NILs, spring Norstar and winter Manitou in a 3-year field test. The patterns of expression of the three selected *COR* genes were recently studied in these wheat genotypes grown at 6 °C for 98 days (Ganeshan et al., 2008). *TaCBF20* (Badawi et al., 2007), which was previously characterized and given the nomenclature *TaCBF1*, has been shown to be induced within 15 min of exposure to 4 °C temperature (Jaglo et al., 2001). Only transcript accumulation was considered in the present field study, since we recently demonstrated that the accumulation of a selected dehydrin polypeptide, WCS120, mirrored accumulation of its corresponding *Wcs120* transcripts in growth chamber experiments with the same genotypes used in this study (Ganeshan et al., 2008).

Table 1
Sampling intervals for field-grown wheat plants in each of 3 years. Sampling dates were September 07, 01, and 01 in 2004, 2005 and 2006, respectively.

Sampling	Sampling intervals from seeding date (days)		
	2004	2005	2006
1st	23	29	26
2nd	36	41	40
3rd	50	55	49
4th	–	70	59
5th	–	84	–

2. Materials and methods

2.1. Plant material and field design

The wheat (*Triticum aestivum* L.) genotypes used in this study consisted of a winter habit cv. Norstar, a spring habit cv. Manitou, a spring habit Norstar and a winter habit Manitou. The latter two are near-isogenic lines (NILs) derived from reciprocal crosses of Norstar and Manitou, such that the *Vrn-A1* locus was the only difference between the two NILs (Limin and Fowler, 2002). The experimental design for the field trial was a four-genotype × two-replicate randomized complete block design for each of 3 years, namely, 2004, 2005 and 2006. Seeding was done on 7th September in 2004 and on 1st September in 2005 and 2006. Each year samples of 20–30 plants from each replicate were collected at various time intervals, measured in days after seeding (Table 1). Plants were sampled three times in 2004, five times in 2005 and four times in 2006. Only three sampling dates up to 50 days were possible in 2004 due to low soil temperatures. The first sampling date after planting was 23 days in 2004, 29 days in 2005 and 26 days in 2006. Leaves were frozen in liquid N_2 and stored at −80 °C, till needed for RNA extraction. Crown tissues were processed for freeze tests as described in the next section. Soil temperature at crown depth was also measured during the growth period using two probes at different locations in the planted area (Fowler et al., 1999).

2.2. Freeze tests

Crown tissues collected from the field were used for freeze tests to determine the LT_{50} (temperature at which 50% of the plants were killed) for each genotype on each sampling date. Freeze tests were performed as previously reported (Limin and Fowler, 1988). Briefly, 5–6 crowns of each genotype were grouped together and placed in a freezer at −3 °C for 12 h and the temperature was decreased after 12 h at a rate of 2 °C h^{-1} until −17 °C, followed by cooling at a rate of 8 °C h^{-1}. Groups of crowns were removed at each of five pre-selected temperatures (−7 °C, −9 °C, −11 °C, −13 °C and −15 °C) during the decrease in temperature and stored at 4 °C overnight. The crowns were then grown in a controlled environment at 20 °C and 16 h photoperiod at 280 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). After 3 weeks, the number of plants showing roots and green leaves were counted to determine the LT_{50} values.

2.3. Real-time PCR

Total RNA was extracted from leaves using a modified Trizol™ (Invitrogen Life Technologies, Inc., Burlington, Ont., Canada) method (Ganeshan et al., 2008). Five micrograms of total RNA was treated with the Turbo DNA-free™ DNAse kit (Ambion Inc., Austin, TX, USA) and 1 μg of DNAse-treated RNA was reverse-transcribed using Superscript III (Invitrogen Life Technologies, Inc., Burlington, Ont., Canada) and oligod$T_{(12–18)}$ (Invitrogen Life Technologies, Inc., Burlington, Ont., Canada). Three microlitres of a 1/15 dilution of the cDNA was used for real-time PCR in a 25 μL volume. For