Quantitative expression analysis of selected COR genes reveals their differential expression in leaf and crown tissues of wheat (Triticum aestivum L.) during an extended low temperature acclimation regimen

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Abstract

A number of COR genes (COld-Regulated genes) have been implicated in the acquisition of low temperature (LT) tolerance in wheat (Triticum aestivum L.). This study compared the relative expression patterns of selected COR genes in leaf and crown tissues of wheat near-isogenic lines to increase understanding of the molecular mechanisms underlying LT acclimation. Reciprocal near-isogenic lines were generated such that the dominant Vrn-A1 and recessive vrn-A1 loci were interchanged in a spring cv. Manitou and a winter cv. Norstar. Phenological development, acquisition of LT tolerance, and WCS120 polypeptide accumulation in these genotypes proceeded at rates similar to those previously reported for 6°C acclimation from 0 to 98 d. However, a differential accumulation of WCS120 polypeptide and expression of the COR genes Wcs120, Wcor410, and Wcor14 was observed in the leaf and crown tissues. COR gene transcript levels peaked at 2 d of the acclimation period in both tissues and differences among genotypes were most evident at this time. COR gene expression was highest for the LT-tolerant and lowest for the tender genotypes. However, expression rates were divergent enough in genotypes with intermediate hardiness that comparisons among tissues and/or times during acclimation often resulted in variable interpretations of the relative expression of the COR genes in the determination of LT tolerance. These observations emphasize the need to pay close attention to experimental conditions, sampling times, and genotype and tissue selection in experiments designed to identify the critical genetic components that interact to determine LT acclimation.

Key words: Dehydrins, low temperature tolerance, near-isogenic lines, real-time PCR, winter cereals.

Introduction

Wheat (Triticum aestivum L.) is one of the most important cereal crops with wide adaptation. The success of wheat in diverse environmental conditions can be attributed to the evolution of types with spring, intermediate (facultative), and winter habit, which differ in their response to temperature and day-length. Winter wheat is generally planted in the autumn, overwinters the cold months in the seedling stage, heads in late spring, and is harvested earlier in summer than spring wheat. Winter wheat requires a period of cold exposure (vernalization) before it will head normally. A vernalization requirement slows the rate of phenological development in the autumn, which increases the plants ability to overwinter and delays flowering until growth resumes in the spring. However, low temperature (LT) damage during high stress winters can lead to substantial reductions in plant stands and lower grain yields. Management practices are important to maximize winter wheat yield and minimize the risk of LT damage (Entz and Fowler, 1991), but there are limitations to the extent these strategies can be employed. Therefore, it is important to gain a better understanding of the
molecular mechanisms underlying LT acclimation to design strategies to increase the LT tolerance genetic potential of the germplasm available to breeding programmes.

Low temperature acclimation is a process by which cool season plants, such as winter wheat, acquire freezing tolerance prior to being subjected to sub-zero conditions. Once initiated, LT acclimation is cumulative and there is an inverse relationship between temperature and degree of acclimation (Fowler et al., 1999). The most rapid changes in LT tolerance, as measured by LT$_{50}$, occur during the initial stages of LT acclimation, but plants cannot fully acclimate until temperatures drop well below the threshold induction level (Fowler, 2008). After the vernalization requirement has been met, there is a transition from vegetative to reproductive stage and the plants’ ability to cold acclimate is limited (Fowler et al., 1996a). A developmental model encompassing these physiological responses (Fowler et al., 1999) suggests that genes determining the length of the vegetative stage act as switches controlling the duration of LT gene expression (Limin and Fowler, 2006; Fowler et al., 1996a, b) and a rate component decides the degree of expression of LT-induced genes (Fowler and Limin, 2004).

A number of LT-induced genes, referred to as COR genes (Cold-Regulated genes) have been identified and characterized in plants (for a review see Thomashow, 1999). Accumulation of several COR gene transcripts has been associated with desiccation and ABA-induction (Allagulova et al., 2003; Borovskii et al., 2002). This is to be expected since many of the physiological changes associated with late embryogenesis, such as ABA production, and salinity and low and high temperature responses, involve osmotic stress. The dehydrins, which belong to the late-embryogenesis abundant (LEA) class of proteins (Close, 1996; Thomashow, 1998; Perras and Sarhan, 1989; Welin et al., 1994), are among the COR genes that have been extensively studied (Close, 1996; Borovskii et al., 2002; Allagulova et al., 2003) and shown to have major roles in conferring LT tolerance. In Arabidopsis, serial analysis of gene expression has revealed higher expression of COR genes in more cold-tolerant leaves than in cold-sensitive pollen (Lee and Lee, 2003). The well-characterized wheat WCS120 family of proteins, induced upon LT exposure, were thought to have a significant role in frost tolerance due to their higher induction in winter hardy than in tender spring wheat plants (Houde et al., 1992a). Because of this relationship, it has been suggested that the WCS120 protein could serve as a molecular marker for frost tolerance in the gramineae (Houde et al., 1992b).

To confound the understanding of LT acclimation further, the spring habit and winter habit in wheat, conferred by the dominant Vrn-A1 locus and the recessive vrn-A1 locus, respectively, have been shown to influence the level of LT tolerance (Sutka et al., 1999; Sutka, 2001) or to be tightly linked to frost tolerance genes (Sutka and Snape, 1989). A MADS-box gene of the API type potentially representing the Vrn-I genes in wheat has been implicated in the determination of the vegetative/reproductive transition phase (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003). In this scheme, the spring habit genotypes are due to constitutive expression of the API alleles while the vernalization-mediated regulation of these alleles occurs in winter genotypes. To determine the genetic and biochemical effect of Vrn-A1 locus on wheat LT tolerance, a set of reciprocal near-isogenic lines (NILs) for the Vrn-A1 locus from spring habit cv. Manitou (Vrn-A1) and winter habit cv. Norstar (vrn-A1) were developed (Limin and Fowler, 2002). Using these NILs, (Danyluk et al., 2003) reported that constitutive expression of TaVRT-1, the putative transcription factor for vegetative/reproductive transition in cereals, was associated with the dominant Vrn-A1 allele and that down-regulation of TaVRT-1 at 4 °C in the winter habit genotypes allowed up-regulation of COR genes such as Wcs120 and Wcs19 in leaf tissues.

Generally, reports on gene expression patterns during LT acclimation have been conducted on RNA extracted from leaves (Danyluk et al., 1994; Ohno et al., 2001; Kobayashi et al., 2004) or crown and leaves combined (Monroy et al., 2007). To our knowledge there have been no comprehensive studies comparing patterns of COR gene expression in leaves and crowns during LT acclimation. Consequently, the objective of this study was to assess the relative expression patterns of selected cold-regulated genes, Wcs120, Wcor410, and Wcor14, in leaf and crown tissues of the above-mentioned reciprocal near-isogenic lines (NILs) of wheat to develop a better understanding of the molecular mechanisms underlying LT acclimation in leaf and crown tissues of the wheat plant. The COR genes were selected based on the localization of their encoded proteins in the cellular compartments. The WCS120 protein family of dehydrins is present in the cytoplasm and nucleus. The 50 kDa member of the WCS120 family is the most abundant (Houde et al., 1995) and was selected for studying its accumulation in leaf and crown tissues of the wheat genotypes. For quantitative real-time PCR (QPCR) analysis primers were designed such that only the Wcs120 transcript coding for the 50 kDa polypeptide was amplified, therefore avoiding the confounding amplification of transcripts from members of the other WCS120 family. The Wcor410 gene was selected as a representative of a gene coding for a plasma membrane localized dehydrin (Danyluk et al., 1994, 1998) and Wcor14 as a representative of a gene coding for a chloroplast targeted protein (Tsvetanov et al., 2000; Vágújfalvi et al., 2000). The accumulation of the WCS120 polypeptides in the leaf and crown tissues of
these genotypes under the same acclimation regimen was also studied.

Materials and methods

Genetic stocks

The genotypes used in this study have previously been characterized (Limin and Fowler, 2002). Briefly, near-isogenic lines (NILs) of winter wheat cultivar Norstar and spring wheat cultivar Manitou were developed such that the winter habit of Norstar was transferred to spring Manitou to produce a winter habit Manitou and the spring habit of spring Manitou was transferred to winter Norstar to produce a spring habit Norstar. Thus the Vrn-A1 locus was substituted, with winter Manitou having the recessive vrn-A1 allele conferring winter habit and the spring Norstar having the dominant Vrn-A1 allele conferring spring habit.

Plant growth, cold acclimation and LT_{so} and FLN determination

The experimental design of the cold acclimation studies was a 4 genotype × 16 acclimation period factorial in a 3 biological replicate randomized complete block design. Plants were grown, maintained and tested for freezing tolerance as previously described (Limin and Fowler, 2002; Fowler and Limin, 2004). Briefly, imbibed seeds were kept in the dark for 2 d at 4 °C and germinated for 3 d in an incubator at 22 °C. Seedlings were then grown hydroponically with continuously aerated half-strength modified Hoagland’s solution for 10 d (Brule-Babel and Fowler, 1988) at 20 °C in 16 h days at 280 μmol m^{-2} s^{-1} PPFD, wherein plants reached the 2–3 leaf stage with visible crowns. These plants were used for the cold acclimation regimens and freeze-tests for LT_{so} determination at the respective time points during the LT acclimation period.

LT_{so} (lethal temperature at which 50% of the plants are killed) was determined as described in Limin and Fowler (1988). At day 0 the plants were transferred to a 6 °C low-temperature growth cabinet for cold acclimation and vernalization under a 16 h photoperiod and 250 μmol m^{-2} s^{-1} PPFD, wherein plants reached the 2–3 leaf stage with visible crowns. These plants were used for molecular analyses were less than a 1 cm long section at the base of the crown) using a modified Trizol™ (Invitrogen, Inc., Burlington, Ontario, Canada) method. Briefly, about 0.5 g of tissue was ground in liquid nitrogen. The ground powder was transferred to 5 ml of Trizol™ reagent in a RNase-free Petri dish and mixed thoroughly. The slurry was transferred to a 15 ml RNase-free tube and mixed with an equal volume of chloroform. The mixture was centrifuged at 2700 g at 2 °C for 10 min, the supernatant transferred to a new tube and the chloroform extraction repeated once more. Total RNA was precipitated using isopropanol. The RNA pellet was briefly dried and resuspended in 600 μl of RNase-free water. The total RNA was then quantified on a spectrophotometer and cleaned using the Purelink Micro-to-Midi RNA clean-up kit (Invitrogen, Inc., Burlington, Ontario, Canada) according to the manufacturer’s instructions.

RNA extraction and cDNA synthesis

Total RNA was extracted from leaves and crown tissues (samples for molecular analyses were less than a 1 cm long section at the base of the crown) using a modified Trizol™ (Invitrogen, Inc., Burlington, Ontario, Canada) method. Briefly, about 0.5 g of tissue was ground in liquid nitrogen. The ground powder was transferred to 5 ml of Trizol™ reagent in a RNase-free Petri dish and mixed thoroughly. The slurry was transferred to a 15 ml RNase-free tube and mixed with an equal volume of chloroform. The mixture was centrifuged at 2700 g at 2 °C for 10 min, the supernatant transferred to a new tube and the chloroform extraction repeated once more. Total RNA was precipitated using isopropanol. The RNA pellet was briefly dried and resuspended in 600 μl of RNase-free water. The total RNA was then quantified on a spectrophotometer and cleaned using the Purelink Micro-to-Midi RNA clean-up kit (Invitrogen, Inc., Burlington, Ontario, Canada) according to the manufacturer’s instructions. The RNA quality was assessed on a Bioanalyzer 2100 (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada) prior to reverse-transcription. For cDNA synthesis, 5 μg of cleaned total RNA was treated with TurboDNA-free DNase (Ambion, Inc., Woodward, Austin, Texas, USA) to remove any genomic DNA contamination. One microgram of the DNase-treated RNA was used for first-strand cDNA synthesis using 500 ng of oligo(dT)_{12-18} (Invitrogen, Inc, Burlington, Ontario, Canada), 900 ng of random primer (Invitrogen, Inc., Burlington, Ontario, Canada), and 200 U of Superscript™ III (Invitrogen, Inc., Burlington, Ontario, Canada) according to the manufacturer’s instructions.

Real-time PCR

For real-time PCR, 1 μl of a 1/5 dilution of the cDNA was used as template. The PCR reaction consisted of the respective primers (Table 1), 1× Quantitect® SYBR® Green PCR Master Mix and 30 nM ROX dye in a 25 μl reaction volume (Qiagen, Inc, Mississauga, Ontario, Canada). Real-time PCR was performed in a MX3000P machine (Stratagene, Cedar Creek, Texas, USA) under conditions optimized for efficient amplification of the respective genes and reference gene. For Wcs120 and Wcor14, amplification was carried out as follows: after the initial activation step at 95 °C for 15 min, 40 cycles each of denaturation at 94 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min were performed. For Wcor410 the amplification conditions were similar, except that the extension time was for 30 s. The wheat Ubiquitin gene was used as reference gene of all the genes of interest (GOI). A dissociation curve was also set up at the end of the 40 cycles in order to ensure that only one product was amplified for each GOI. PCR amplifications for the respective GOI and reference gene were sub-cloned and sequenced to ascertain that the correct GOI were indeed amplified (see Supplementary Table S1 at JXB online). The real-time PCR experiments were conducted on the three biological replicates, with two technical replicates for each sample.

Table 1. Sequences of primers used for real-time PCR amplification and the resulting product size

| Gene   | Forward (F) and reverse (R) primers | Primer sequence 5’⋯3’ | Amplicon size (bp) |
|--------|------------------------------------|----------------------|-------------------|
| Wcs120 | F/w 120b, R/w 120b                 | TTCACGGACACACAGTGTT | 108               |
| Wcor410| F/R 410                            | CTGCCCTTGCTCTTGGATAAG| 110               |
| Wcor14 | F/R 14ba                           | CCTGAGCAGGAAAGAGC   | 124               |
| Ubiquitin | F/R UBI                          | CCTTGCGGACTACACACT   | 164               |
**Protein extraction and western blotting**

Proteins were extracted by grinding frozen leaves in liquid nitrogen, followed by resuspension in buffer containing 0.1 M TRIS-HCl, pH 8.5 and Complete™ EDTA-free protease inhibitor cocktail (Roche Applied Science, Laval, Quebec, Canada), as described by Vitormas et al. (2007). Since the WCS120 proteins are stable upon boiling, their enrichment was possible by boiling the protein sample for 15 min. The protein concentration was determined according to a protein–dye binding method (Bradford, 1976).

WCS120 polypeptides were compared among genotypes and acclimation days using SDS–PAGE on 10% (w/v) gels according to Laemmli et al. (1970). The protein equivalent from 0.3 mg FW of leaves was loaded into each well. BenchMark™ Protein Ladder (Invitrogen, Inc., Burlington, Ontario, Canada) and Kaleidoscope Prestained broad range standards (Bio-Rad Laboratories, Mississauga, Ontario, Canada) were used for molecular weight estimates. Proteins were electrophoretically transferred to nitrocellulose membrane (0.45 μm, GE Healthcare, Quebec, Canada). The membrane was incubated with a 1:5000 dilution of anti-dehydrin antibody (StressGen, Ann Arbor, Michigan, USA) for 2 h after blocking with non-fat dry milk (3%) in TBS. After washing with TBS containing 0.05% Tween-20 and 0.2% Triton X-100 (Sigma-Aldrich, St Louis, Missouri, USA), alkaline phosphatase labelled goat anti-rabbit IgG (KPL, Inc., Maryland, USA) was applied at 1:50 000 dilution. The protein:antibody complex was visualized by PhosphaGlo Reserve AP Chemiluminescent Substrate (KPL, Inc., Maryland, USA). Chemiluminescent emitted from the membrane was detected using a Chemi-Doc XRS (Bio-Rad Laboratories, Mississauga, Ontario, Canada) image capture system. The Quantity One (version 4.6.3.) software (Bio-Rad Laboratories, Mississauga, Ontario, Canada) was used for densitometric quantification of the 50 kDa WCS120 polypeptide.

In order to convert the densitometric values (Intensity mm⁻²) to percentage expression values, the highest value was set at 100% and all other samples were considered expressing at a percentage relative to this sample. In this study, the Norstar leaf sample at 56 d was expressing at highest level and was set at 100%. Two biological replicates were used for densitometric analyses. Western blots were repeated with the third biological replicate and exhibited similar tendencies.

**Data analysis**

Statistical analyses for all data generated in this study were analysed using the Minitab software (Version 14) (Minitab, Inc., Pennsylvania, USA). For the FLN and LT₅₀ data, significant effects of genotype, days of acclimation, and genotype×days of acclimation interactions were determined. For QPCR data, relative expression for the GOI was determined using the ΔΔCt method (Livak and Schmittgen 2001), wherein the expression of the GOI was relative to a control plant sample which was not exposed to low temperature and in this study the spring wheat, Manitou at d 0 was chosen and termed the calibrator. Relative expression was therefore computed as follows:

\[
\Delta C_{t}^{(\text{sample})} = C_{t}^{(\text{GOI} \text{ sample})} - C_{t}^{(\text{Reference gene sample})}
\]

\[
\Delta \Delta C_{t} = \Delta C_{t}^{(\text{sample})} - \Delta C_{t}^{(\text{calibrator})}
\]

\[
\text{Relative expression} = 2^{-\Delta \Delta C_{t}}
\]

In order to determine if there were significant effects due to genotype, days of acclimation, and genotype×days of acclimation on the expression patterns of the GOI, analysis of variance (ANOVA) was conducted. Furthermore, the ANOVA outputs were also observed for significance of the biological and technical effects. The non-significance of the P-values of biological effects indicated that the differences observed were indeed biological in nature. Similarly, the non-significance of the P-values for the technical replicates indicated that the sample to sample variation due to handling and pipetting were not contributing to differences observed in this study.

**Results and discussion**

**Cold acclimation and FLN**

Analysis of variance for final leaf number (FLN) and LT₅₀ showed that genotype, acclimation period, and the genotype×acclimation period interaction were highly significant (P <0.001) for both variables indicating that there were important genotypic differences in the magnitude and patterns of the LT responses. The changes in FLN (Fig. 1) were similar to those reported in previous studies (Limin and Fowler, 2002; Fowler and Limin, 2004). Both spring Norstar and Manitou reached their minimum FLN without being exposed to acclimating conditions confirming that they do not have a vernalization requirement. Forty-nine days of vernalization at 6 °C reduced the FLN of Norstar from 24 to fewer than 13. Similar effects of temperature were observed for winter Manitou where the mean FLN was reduced from 19 to fewer than 10 after 42 d of vernalization. Also, as reported in previous studies (Limin and Fowler, 2002; Fowler and Limin, 2004), both Norstar and spring Norstar had a higher minimum FLN than Manitou and winter Manitou. Increased minimum FLN is another strategy cereals have evolved to delay the vegetative/reproductive transition that is independent of the vernalization response.

The LT₅₀ values (Fig. 2) exhibited a typical curvilinear relationship with days of acclimation at 6 °C (Fowler et al., 1996b). While the spring habit Norstar was less hardy than winter Norstar, winter Manitou gained LT tolerance comparable to spring Norstar. The lowest LT₅₀ was –23 °C at 49 d for Norstar and –13.3 °C at 42 d for winter Manitou. These were also the point of vernalization saturation for these two genotypes as indicated by FLN measurements (Fig. 1). A rapid response to LT during the first week was followed by relatively minor changes in the LT₅₀ of both spring habit genotypes. These observations are in agreement with previously published reports (Fowler et al., 1996b; Limin and Fowler, 2002; Fowler and Limin, 2004), where genotypes with a Norstar background also had an early rapid rate of cold acclimation. Interestingly, while the winter Manitou gained 5–6 °C in additional LT tolerance over Manitou as a result of the vrn-AI allele, spring Norstar lost 10 °C in LT₅₀ compared with winter Norstar due to substitution with the Vrn-A1 allele. The difference in LT₅₀ between Norstar and Manitou was 15 °C. This indicates that gain of the
recessive vrn-A1 allele had a greater effect on the LT tolerance of winter Manitou than the effect of the dominant Vrn-A1 allele from Manitou on spring Norstar. The duration of time in the early developmental stages has been shown to underlie full expression of genetic LT tolerance potential (Limin and Fowler, 2006). The vernalization requirement increased the length of time that the plant is in the vegetative stage with the result that the LT tolerance genes were up-regulated longer thus allowing for more complete expression of cold hardness potential in the winter habit genotypes (Fowler and Limin, 2004).

**WCS120 peptide accumulation**

Immunoblot analyses were performed on proteins extracted from leaves and crowns so that the pattern of WCS120 polypeptide accumulation could be related to plant LT tolerance. The accumulation of the WCS120 polypeptides, as detected by over-exposed chemiluminescent western blots, indicated the presence of four bands corresponding to the WCS180, WCS66, WCS120, and WCS40 polypeptides of the WCS120 family (Figs 3, 4). However, quantitative densiometric determination was only conducted on the 50 kDa WCS120 polypeptide (Fig. 5). Analyses of variance for WCS120 accumulation in crown and leaf tissues were highly significant ($P < 0.001$) for genotype, days of acclimation, and genotype×days of acclimation interaction was also highly significant ($P < 0.001$) in a combined analysis of variance indicating that WCS120 polypeptide accumulated differently in leaf and crown tissues (Fig. 5A, B). In leaf tissues, Norstar was able to sustain a high level of WCS120 accumulation, reaching a maximum by 56 d (Fig. 5A). Winter Manitou and spring Norstar reached a maximum at 21 d and Manitou at 14 d. The leaf WCS120 polypeptide levels in Norstar, spring Norstar and winter Manitou were generally comparable in the first 21 d of LT exposure. In the crown tissues, the highest level of WCS120 was again observed in Norstar, but at 42 d. However, unlike in the leaf tissues, there were distinct differences between the WCS120 levels in the crowns of Norstar compared with the other three genotypes (Fig. 5B). As with leaf tissues, the winter Manitou and spring Norstar WCS120 polypeptide levels were similar for the first 21 d in the crown tissues. The accumulation of WCS120 protein was consistent with published reports (Limin et al., 1995; Fowler et al., 1996a; Vitamvas et al., 2007), in that the most LT-tolerant genotype accumulated the highest levels of the WCS120 polypeptide. However, this study also indicates that there were large differences in the accumulation patterns in the leaves and crowns.

**Accumulation of COR transcripts**

Three different COR genes were selected based on the targeted expression of their encoded proteins. Although Wcs120 and Wcor410 are dehydrins, the former is basic and its protein accumulates in the nucleus and cytoplasm and the latter is acidic with its protein being targeted to the plasma membrane (Houde et al., 1992b; Danyluk et al., 1998). Wcor14 encodes a chloroplast-targeted protein and besides being induced by LT, it is also influenced by light (Crosatti et al., 1995; Takumi et al., 2003). Furthermore, Wcs120 and Wcor14 have C-repeat binding factors (CBF) in their promoter regions and are activated by the CBF transcription factors (Crosatti et al., 2003; Shen et al., 2003; Kobayashi et al., 2005).

Analyses of variance for Wcs120, Wcor410, and Wcor14 transcript accumulation in the leaf and crown tissues showed highly significant ($P < 0.001$) effects of genotype, days of acclimation and genotype×days of acclimation. Similarly, combined analyses of variance showed that tissue×genotype×days of acclimation interaction was highly significant ($P < 0.001$) for genotype, days of acclimation, and tissue×genotype×days of acclimation interaction.
interaction was highly significant ($P < 0.001$) for each of the three genes, indicating that the patterns of \textit{Wcs120}, \textit{Wcor410}, and \textit{Wcor14} transcript accumulation in leaf and crown tissues were different. There was a sharp increase in \textit{Wcs120} transcripts in both the leaves and crowns of all four genotypes after two days of LT acclimation (Figs 6A, 7A). The increase in expression was highest in Norstar and lowest in Manitou in both tissues. Similar differences in expression have been reported for Norstar and spring habit Glenlea cold-acclimated at 4°C (Fowler et al., 1996a). After the sharp increase at 2 d, there was an almost 50% reduction in transcript in the leaves of each genotype by 7 d. Thereafter, Norstar sustained a moderate level of \textit{Wcs120} expression from about 14 d to 42 d. Winter Manitou showed a similar trend, albeit at a lower level than Norstar, with the sustained transcript accumulation spanning 14–28 d.

A different pattern of \textit{Wcs120} transcript accumulation was found in the crown tissue. In winter Norstar after an initial burst at 2 d there was a high and sustained level of transcript accumulation until 63 d. Over most of this period, the expression level for Norstar was at least 50% higher than in the other three genotypes. Also of note was the change in the relative \textit{Wcs120} expression of winter Manitou and spring Norstar in leaf and crown tissue after 2 d acclimation. The expression level of winter Manitou was higher than spring Norstar after 2 d acclimation in the leaves while the reverse was observed in the crown tissue.

\textit{Wcor410} codes for an acidic dehydrin which acts as a cryoprotectant that accumulates in the periphery of plasma membranes in roots, leaves and crowns upon LT exposure of wheat plants (Danyluk et al., 1998, 1994). Observations made in the present study indicate that the temporal expression for \textit{Wcor410} is different in leaf and crown tissue. The expression patterns of \textit{Wcor410} were more comparable to those of \textit{Wcs120} in the leaves than in the crowns (Figs 6, 7). As with \textit{Wcs120}, the \textit{Wcor410} sharply increased in expression at 2 d for all four genotypes in both tissues and Norstar had a higher level of expression than Manitou (Figs 6B, 7B). While there were large differences in leaf tissue, the expression levels in the crown tissues were similar for Norstar and spring Norstar over most of the acclimation period (Figs 6B, 7B). This was strikingly different from the expression in the leaves, where the levels in spring Norstar were 50% or less than in Norstar over the 98 d acclimation regime. A higher \textit{Wcor410} transcript accumulation in leaves of winter Manitou and Norstar in the first 7 d of acclimation could be interpreted as being due to the presence of the recessive \textit{vrn-A1} locus. In contrast, the \textit{Wcor410} expression levels and patterns were similar in Manitou and winter Manitou and in Norstar and spring Norstar (Fig. 7B) indicating that genetic background and not the interchange of the Vrn-A1 locus determined \textit{Wcor410} expression in the crown tissues.

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Fig. 3. Chemiluminescent western blot detection of dehydrins accumulated in leaf tissues of wheat during LT exposure (0–70 d). (A) Winter Norstar (No) and spring Manitou (Ma). (B) Spring Norstar (SN) and winter Manitou (WM). The four \textit{WCS120} polypeptides detected, namely, WCS180, WCS66, WCS120, and WCS40 are indicated and their sizes correspond to 180, 66, 50, and 40 kDa, respectively. (C) Western blot showing comparison of each genotype in relation to each of four specific time points.

Fig. 4. Chemiluminescent western blot detection of WCS120 polypeptide accumulation in crown and leaf tissues during LT exposure (0–70 d). (A) Winter Norstar. (B) Spring Norstar. (C) Spring Manitou. (D) Winter Manitou. Arrowheads indicate 50 kDa \textit{WCS120} polypeptide used for densitometric quantification. L56 is the Norstar leaf sample exposed to LT for 56 d and served as a control for quantification. The asterisk in (C) indicates a 3-fold lower amount of L56 total protein loaded onto the gel. In order to detect the polypeptide in Manitou, a 3-fold higher amount of protein was loaded onto the gel. After densitometric quantification, the values were accordingly adjusted by reducing them 3-fold for Manitou.
Wcor14 had the same dramatic accumulation of transcripts in leaf tissue at 2 d and a decline thereafter (Fig. 6C) that was observed in Wcs120 and Wcor410. Norstar showed the highest Wcor14 expression level at 2 d followed by a gradual decline to 42 d, a drop at 49 d, and low steady-state accumulation to 98 d (Fig. 6C). In agreement with our QPCR data for the hardy cultivar Norstar, Tsvetanov et al. (2000) reported an increase from 1 d to 3 d and then a decline to 20 d using northern blot analysis of Wcor14 transcripts in a winter wheat variety Mironovskaya 808 acclimated at 4 °C. The gradual decline in Wcor14 observed for Norstar also occurred in spring Norstar, but only up to 21 d. After a sharp drop at 42 d, spring Norstar transcript levels remained steady at amounts similar to Norstar. Transcript levels decreased drastically from 2 d to 14 d in winter Manitou, followed by a sudden increase at 21 d to the same level as in Norstar. Thereafter, there was a gradual decline to 42 d followed by steady-state levels to 98 d. Expression in Manitou was about 70% lower than in Norstar at 2 d followed by a steady decline to 35 d and a constant low expression from 42–98 d. These observations are in contrast to the absence of differences in Wcor14 transcript accumulation between Chinese Spring and a winter wheat cultivar Mironovskaya 808 when exposed to 4 °C for 0–10 d (Kobayashi et al., 2004).

Wcor14 is specifically up-regulated by LT (Tsvetanov et al., 2000; Crosatti et al., 2003) and encodes a chloroplast-targeted polypeptide (Crosatti et al., 2003). Therefore, Wcor14 transcript accumulation in the crown tissues is noteworthy because this plant section has few chloroplasts. The cereal crown is composed of the shoot apical meristems, leaf and tiller initials, bases of the unemerged leaves, growing leaf-sheath and root initials, and emerging roots (Pearce et al., 1998). In the present study, the crown tissue used for total RNA extraction was deprived of the roots. Furthermore Miyamura et al. (1990) reported that, in wheat, the number of plastids per cell can range from about 100 (10 mm from the base of the leaf) to about 150 (towards the leaf blade tip). The study also reported that the number of plastids per cell in the crown ranged from about 70 at the shoot apex to about 125 in the slightly chlorophyllous tissues of the crown. Very faint 16S and 23S ribosomal RNA bands were observed in total RNA preparations from crown tissues (see Supplementary Fig. S1 at JXB online) and it is unclear why transcripts deemed to be targeted to chloroplasts were expressed in tissues with very few chloroplasts. Norstar and spring
Norstar had higher Wcor14 expression levels than Manitou and winter Manitou in the first 3 weeks of cold acclimation (Fig. 7C). Norstar showed the highest Wcor14 expression level at 2 d followed by a sharp decline to 7 d and a sustained intermediate level of expression until 63 d. Down-regulation of Wcor14 in spring Norstar started after 2 d and reached levels comparable to those of winter Manitou by 28 d. Regulatory loci at the Vrn-A1/Fr1 loci on chromosome 5A have been shown to control expression of Wcor14 (Vähäjärvi et al., 2000; Kobayashi et al., 2005), more specifically regions associated with cbf-like sequences (Crosatti et al., 2003; Kobayashi et al., 2005). Furthermore, Kume et al. (2005) demonstrated that a cbf homologue, Wcbf2, when activated by LT led to the induction and increase in accumulation of Cor/Lea genes such as Wcor14 and Wcor15. Thus the discrepancy in expression between Norstar and spring Norstar could be due to the influence of the Vrn-A1 locus. Further studies that include measurements of the WCOR14 protein accumulation in crown and leaf tissues need to be conducted to clarify these observations.

**COR gene expression and LT tolerance**

The LT acclimation patterns observed were consistent with those reported earlier for the genotypes and environmental conditions used in this study (Fowler and Limin, 2004). Once acclimation was initiated, both LT50 and WCS120 protein levels were cumulative until the vegetative/reproductive transition was reached. The most rapid changes in LT50 occurred during the initial stages of acclimation. This initial response was accompanied by a burst in Wcs120, Wcor410, and Wcor14 expression that decreased with time. As a result, genotypic differences in levels of COR gene expression were most obvious after 2 d of LT acclimation. These early responses were also indicative of the rate at which genotypes acclimate while factors that determine the length of the vegetative stage, such as vernalization and photoperiod responses, determine the accumulation of LT tolerance (Fowler et al., 1999). Large genotypic differences, like those observed between Norstar and Manitou, were clearly reflected in both COR gene transcript accumulation and protein concentrations in both the leaves and crowns throughout most of the extended LT acclimation regime used in this study. Smaller but important differences in transcript accumulation between genotypes with similar LT tolerance potential, like spring Norstar and winter Manitou, were most evident in the initial stages of LT acclimation. The consistent maximum COR gene expression levels and the magnification of genotypic differences in LT tolerance genetic potential at 2 d both point to the need for further investigation of the gene responses in the different plant tissues and genotypes during the initial stages of LT exposure, preferably during the first few minutes or hours of acclimation.

While the general LT responses were similar, there were major differences in the patterns of COR gene transcript and protein accumulation in leaf and crown tissues. QPCR data showed the highest accumulation of COR transcripts after 2 d of LT exposure in both leaf and crown tissues. However, subsequent studies have suggested that LT tolerance is influenced by other interacting regulatory circuitries (Chinnusamy et al., 2007), besides low temperature, genetic potential, and the factors determining the length of the vegetative stage, such as vernalization and photoperiod (Mahfoozi et al., 2001). Studies in Arabidopsis have shown that light quality (Franklin and Whitelam, 2007) and the circadian clock (Michael et al., 2003) influence LT responses. Takumi et al. (2003) showed that the gene, Wcor15, which encodes a chloroplast-targeted protein, is induced by LT as well as light, similar to its parologue, Wcs19 (Chauvin et al., 1993) and the orthologue, Wcor14 (Takumi et al., 2003). Therefore, the differential expression observed between leaf and crown tissues in this study could have been influenced by differences in the perception of temperature (Limin and Fowler, 1985), day length,
Expression of selected COR genes in wheat leaf and crown tissues

and light quality and quantity due to the relative positioning of the different plant parts. The leaf canopy has a direct effect on the light reaching the crown tissues and, as a result, could be one of the factors responsible for the differences observed between LT responses by leaf and crown tissue. The variable trends among the NILs and between the two tissues also suggest that post-transcriptional/translational regulation and/or mRNA/protein half-life could also play an important role in COR gene transcript accumulation. As a consequence, differences in expression were often divergent enough that comparisons among tissues and/or times during acclimation could result in very different interpretations of the relative roles played by the COR genes in the determination of LT tolerance. These observations emphasize the need for additional research aimed at achieving a more complete understanding of the genetic cascade that determines LT tolerance in wheat. They also identify the need to pay close attention to experimental conditions and genotype and tissue selection in experiments designed to identify the critical genetic components of the highly integrated systems for LT adaptation that are regulated by environmentally-induced complex pathways (Fowler and Limin, 2007).

Supplementary data

Supplementary data can be found at JXB online.

Table S1. Sequences of amplified COR transcripts.

Fig. S1. Bioanalyzer 2100 (Agilent) data for total RNA extracted from three wheat leaf samples and three crown samples.

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