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The regulatory role of vernalization in the expression of low-temperature-induced genes in wheat and rye

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Abstract Low temperature is one of the primary stresses limiting the growth and productivity of wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.). Winter cereals low-temperature-acclimate when exposed to temperatures colder than 10°C. However, they gradually lose their ability to tolerate below-freezing temperatures when they are maintained for long periods of time in the optimum range for low-temperature acclimation. The overwinter decline in low-temperature response has been attributed to an inability of cereals to maintain low-temperature-tolerance genes in an up-regulated state once vernalization saturation has been achieved. In the present study, the low-temperature-induced *Wcs120* gene family was used to investigate the relationship between low-temperature gene expression and vernalization response at the molecular level in wheat and rye. The level and duration of gene expression determined the degree of low-temperature tolerance, and the vernalization genes were identified as the key factor responsible for the duration of expression of low-temperature-induced genes. Spring-habit cultivars that did not have a vernalization response were unable to maintain low-temperature-induced genes in an up-regulated condition when exposed to 4°C. Consequently, they were unable to achieve the same levels of low-temperature tolerance as winter-habit cultivars. A close association between the point of vernalization saturation and the start of a decline in the *Wcs120* gene-family mRNA level and protein accumulation in plants maintained at 4°C indicated that vernalization genes have a regulatory influence over low-temperature gene expression in winter cereals.

Key words Low-temperature tolerance · Vernalization · *Wcs120* gene family · Gene regulation · Gene expression

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Introduction

Vernalization response and low-temperature acclimation are two important mechanisms that cereals have evolved to cope with low-temperature stress. Vernalization response reduces the risk of winter cereals entering the extremely cold-sensitive reproductive growth stage until the danger of low-temperature damage has passed. Low-temperature acclimation allows winter cereals to protect critical cell structures and physiological processes during periods of freezing temperatures. Vernalization and low-temperature responses are regulated through complex genotypic and environmental interactions that induce a large number of physical and biochemical changes in the plant. Both responses have similar optimum temperature ranges for induction (Olein 1967; Ritchie 1991) and they are regulated by genetic systems that are interrelated (Brule-Babel and Fowler 1988; Sutka and Snape 1989; Roberts 1990). In fact the vernalization gene *vrn1*, which is recessive to spring growth-habit, may be pleiotropic, affecting both growth-habit and low-temperature tolerance in wheat (Brule-Babel and Fowler 1988).

Winter cereals gradually lose their ability to tolerate below-freezing temperatures when they are maintained for long periods of time in the optimum range for low-temperature acclimation (Andrews 1960a; Olein 1967; Roberts and Grant 1968; Tsenov 1973; Tumanov et al. 1976; Roberts 1979). Satisfaction of vernalization requirements has often been suggested as the primary reason for the decline in low-temperature tolerance of overwintering cereals (Andrews 1960b; Marshall 1969; Koch 1973; Vincent 1973; George 1982; Roberts 1990). Recent studies have shown that there is a close relationship between the time to vernalization saturation and the start of a decline in the low-temperature tolerance of wheat and rye cultivars grown in the optimum range for low-temperature acclimation

(Fowler et al. 1996). These studies indicate that cereals have evolved at least two mechanisms to regulate responses to low-temperature stress: (1) one to deal with short-term exposure to below-freezing temperatures during the growing season, and (2) one to cope with the long-term below-freezing stresses of winter. According to this hypothesis, low-temperature tolerance is a function of the degree and duration of low-temperature gene expression and the vernalization genes are primarily responsible for the duration of low-temperature gene expression (Fowler et al. 1996).

Recent developments in molecular biology have provided an opportunity for the investigation of low-temperature gene expression at the RNA and protein levels. Expression of the low-temperature-induced *Wcs120* gene family of wheat has been shown to be correlated with freezing tolerance in Gramineae species (Houde et al. 1992a; 1992b; 1995). The WCS120 family consists of five proteins with molecular masses of 200, 180, 66, 50, and 40 kDa. Sequence analysis and molecular characterization of four members of this family has revealed repeated glycine- and lysine-rich domains (Houde et al. 1992a; Ouellet et al. 1993; Chauvin et al. 1994) that may explain their common antigenicity. These proteins are up-regulated specifically by low temperature and are expressed at high levels in freezing-tolerant Gramineae species (Houde et al. 1992b). In the present study, *Wcs120* cDNA and an anti-WCS120 antibody were used to investigate the relationship between low-temperature gene expression and vernalization response in wheat and rye.

Materials and methods

Plant material and experimental design

Two wheat ('Glenlea' spring wheat and 'Norstar' winter wheat) and two rye ('Gazelle' spring rye and 'Puma' winter rye) cultivars and eight vernalization/low-temperature periods (0, 2, 7, 21, 49, 63, 84, and 98 days) were evaluated. The wheat and rye species were *Triticum aestivum* L. and *Secale cereale* L., respectively. The experimental design was a two-level three-replicate factorial. Analyses of variance were conducted to determine the significance of treatment differences.

LT_{50} and vernalization determination

Imbibed seeds were held for 2 days at 5°C and then transferred to an incubator and held for 3 days at 22°C. The seedlings were then grown at 17°C with a 16-h day for 12.5 days before being exposed to conditions for low-temperature acclimation and vernalization. Plants were transferred to a 4°C chamber with a 16-h photoperiod and a light intensity of 325 μmol m^{-s} s^{-1} for vernalization/low-temperature acclimation. The plants were grown hydroponically in half-strength Hoagland's solution, which was changed weekly.

The procedure outlined by Limin and Fowler (1988) was used to determine the LT_{50} (the temperature at which 50% of the plants are killed by low-temperature stress) of each cultivar at the end of each vernalization/low-temperature acclimation period. Plants were also transferred to a 20°C chamber with a 16-h photoperiod and a light intensity of 350 μmol m^{-2} s^{-1} at the end of each vernalization/low-temperature acclimation period. These plants were grown until the flag leaf had emerged and the final number of leaves on the main shoot could be determined for each cultivar (Wang et al. 1995). Vernalization saturation was considered complete for each cultivar once the cold treatment no longer reduced its final leaf number.

Protein extraction, separation and immunoblot analysis

Soluble proteins were extracted from leaf tissues by grinding in a pre-cooled mortar with Tris buffer [0.1 M Tris, pH 9.5 containing 1 mM phenylmethylsulphonyl (PMSF)]. The extract was immediately centrifuged for 5 min at 12 000 *g* and the supernatant was adjusted to the final buffer concentration with 2× SDS electrophoresis sample buffer (Laemmli 1970). Equal amounts (5 mg) of proteins were separated on 10% SDS-PAGE and transferred electrophoretically to nitrocellulose (Hybond C-extra, Amersham). After blocking with powdered milk (2%) in PBS (phosphate-buffered saline) containing 0.2% Tween-20 (Blotto), the membrane was incubated with a 1:10 000 dilution of the purified WCS120 antibody (Houde et al. 1992b). After washing with PBS-Tween, the proteins recognized by the primary antibody were revealed with peroxidase coupled to anti-rabbit IgG (Jackson, Immunoresearch Inc.) as a secondary antibody (1:25 000 dilution). The complex was revealed using the ECL chemiluminescence detection kit (Amersham).

Quantitation of WCS120 proteins was performed using densitometry scanning as previously described (Houde et al. 1995). Densitometry readings were normalized by setting the maximum protein accumulation of the winter cultivars in each species to 100%.

Northern-blot analysis

Total RNA (10 μg) samples (Danyluk and Sarhan 1990) were mixed with ethidium bromide before electrophoresis on formaldehyde-agarose gels as suggested by Rosen and Villa-Komaroff (1990). After electrophoresis, RNA was transferred to nitrocellulose membranes (Hybond C-extra, Amersham) in 20 × SSC (saline sodium citrate). The filters were air-dried and then baked for 1 h at 80°C prior to hybridization with the ^{32}P-labelled *pWcs120* insert (Houde et al. 1992a). Filters were washed at 55°C with several buffer changes of decreasing SSC concentration (5–0.1×) and then autoradiographed on Kodak XRP films with intensifying screens (DuPont, Cronex Lightning plus) at −80°C. A control clone, p 2.1, and a wheat tubulin gene that did not display differential hybridization during cold acclimation were used in addition to ethidium bromide staining to verify the equal loading and the quality of mRNA.

Relative levels of *Wcs120* mRNA transcripts were determined by densitometry scanning of the Northern blots. Densitometry readings were normalized by setting the maximum mRNA accumulation of the winter cultivars in each species to 100%.

Results and discussion

The final leaf numbers of the rye and wheat cultivars considered in this study (Fig. 1) followed the typical vernalization response patterns reported for spring- and winter-habit cultivars (Fowler et al. 1996). The final leaf numbers of Glenlea and Gazelle were not influenced by exposure to a vernalization temperature of 4°C. Therefore, any vernalization requirement that these spring habit cultivars may have had was met during the 17.5-day establishment period prior to the start of the vernalization treatment (Jedal et al. 1986). Seven days at 4°C did not reduce the final leaf number of either Norstar winter wheat or Puma winter rye. However, there was a rapid decline in the final leaf number of the winter cultivars between vernalization-days 7

and 49 and both reached their final leaf number (indicating that vernalization saturation had been achieved) by day 49 at 4°C.

The rye and wheat cultivars considered in this study followed typical patterns of low-temperature acclimation (Fig. 2) reported for spring- and winter-habit cultivars (Fowler et al. 1996). All cultivars began low-temperature acclimation at a rapid rate following exposure to a constant temperature of 4°C. The rate of low-temperature acclimation then gradually slowed and eventually started to decline producing a curvilinear relationship between the LT_{50} and the stage of acclimation. Glenlea spring wheat and Gazelle spring rye achieved maximum low-temperature tolerance by 21 days at 4°C. In contrast, Norstar winter wheat and Puma winter rye required approximately 49 days of acclimation to reach their coldest LT_{50}. A longer low-temperature acclimation response allowed the winter wheat and rye cultivars to achieve minimum LT_{50}s which were 14.6 and 17.5°C, respectively, colder than the minimum survival temperatures of their spring counterparts. Consequently, maintenance of the low-temperature-acclimation response over a longer period of time was the primary factor responsible for the superior low-temperature tolerance of the winter-compared to the spring-habit cultivars.

A gradual loss of low-temperature tolerance (Fig. 2) started at approximately the same time as vernalization saturation was complete (Fig. 1) for the winter cereals considered in this study. This inability of winter cereals to maintain low-temperature tolerance when stored for long periods of time at temperatures in the vernalization/acclimation range has been attributed to a down-regulation of the low-temperature-tolerance genetic system once vernalization saturation has been achieved (Fowler et al. 1996). Northern- and Western-blot analyses performed with *Wcs120* cDNA clones and antibodies directed against the WCS120 protein family provided the opportunity to investigate the relationship between the stage of vernalization and low-temperature-induced gene expression at the molecular level.

Low-temperature-induced gene expression, as measured by the accumulation of WCS120 proteins, closely followed the changes in LT_{50} observed for both spring and winter wheat cultivars (Figs. 2 and 3). This confirms earlier observations that the antibodies raised against WCS120 proteins can be used as molecular markers to assess the development of low-temperature tolerance in cereals (Houde et al. 1992b).

The level of *Wcs120* mRNA transcripts (Fig. 4) peaked before the WCS120 proteins (Fig. 3) reached their maximum level of expression in the winter- and spring-habit cultivars of both wheat and rye. Compared to the spring wheat and rye cultivars, the winter habit cultivars had a higher *Wcs120* mRNA level that was maintained for a longer period of low-temperature exposure (Fig. 4). Both the spring- and winter-habit cultivars produced very low levels of *Wcs120* mRNA at 17°C. Exposure to 4°C resulted in a very rapid accumulation of *Wcs120* mRNA transcripts in all four cultivars. However, this initial burst was followed by a rapid decline in *Wcs120* mRNA level in both

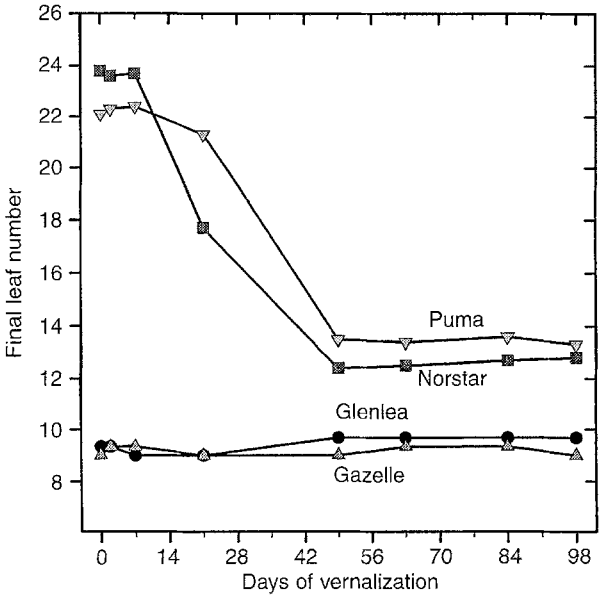

Fig. 1 Final leaf number (SE=0.52) of Glenlea spring wheat, Gazelle spring rye, Norstar winter wheat, and Puma winter rye grown at 4°C for 0 to 98 days

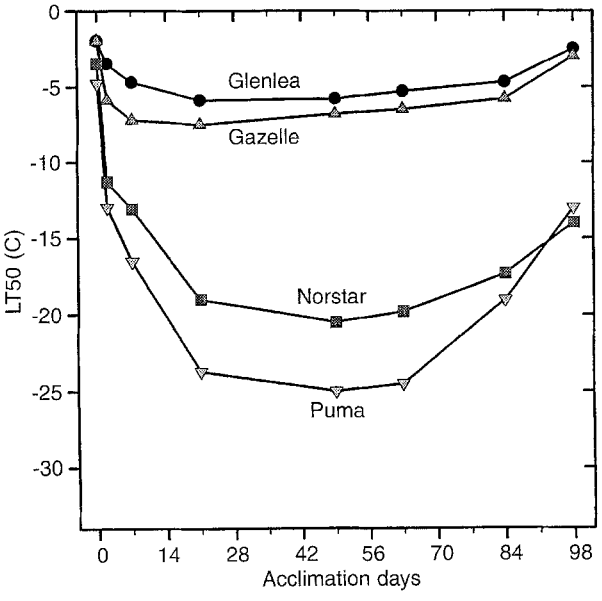

Fig. 2 Low-temperature tolerance (LT_{50}, SE=0.53) of Glenlea spring wheat, Gazelle spring rye, Norstar winter wheat, and Puma winter rye grown at 4°C for 0 to 98 days

Glenlea spring wheat and Gazelle spring rye. In contrast, high levels of *Wcs120* mRNA transcripts were maintained for approximately 49 days when Norstar winter wheat and Puma winter rye were held at a constant 4°C. *Wcs120* mRNA levels of the winter cultivars decreased rapidly after 49 days of acclimation, but their levels at 84 and 98 days remained in the same range as the maximum expression observed for the spring-habit cultivars after 2 days at

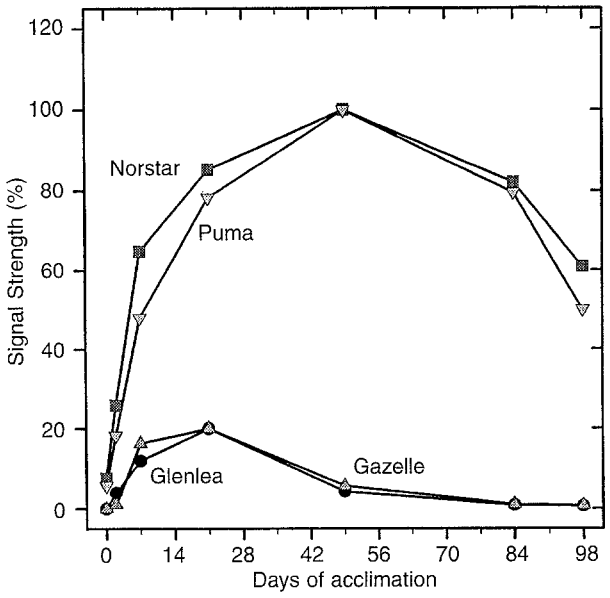

Fig. 3 Relative levels of WCS120 protein accumulation (SE=1.82) for Glenlea spring wheat, Gazelle spring rye, Norstar winter wheat, and Puma winter rye grown at 4°C for 0 to 98 days. Signal strengths were normalized by setting maximum densitometer scans of Western blots of the winter cultivars in each species at 100%

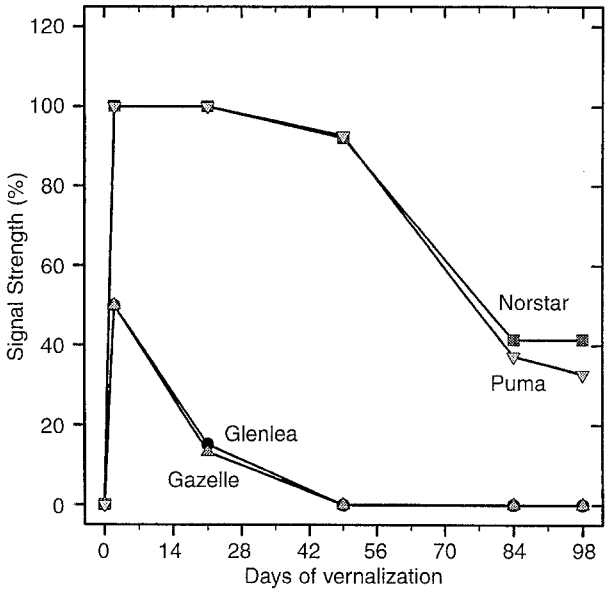

Fig. 4 Relative levels of *Wcs120* mRNA transcripts (SE=0.32) for Glenlea spring wheat, Gazelle spring rye, Norstar winter wheat, and Puma winter rye grown at 4°C for 0 to 98 days. Signal strengths were normalized by setting maximum densitometer scans of Northern blots of the winter cultivars in each species at 100%

4°C. These observations suggest that the poor low-temperature tolerance of spring compared to winter-habit cultivars is the result of an inability of spring types to maintain low-temperature-tolerance genes in an up-regulated state. They also indicate that the maintenance of a high level of

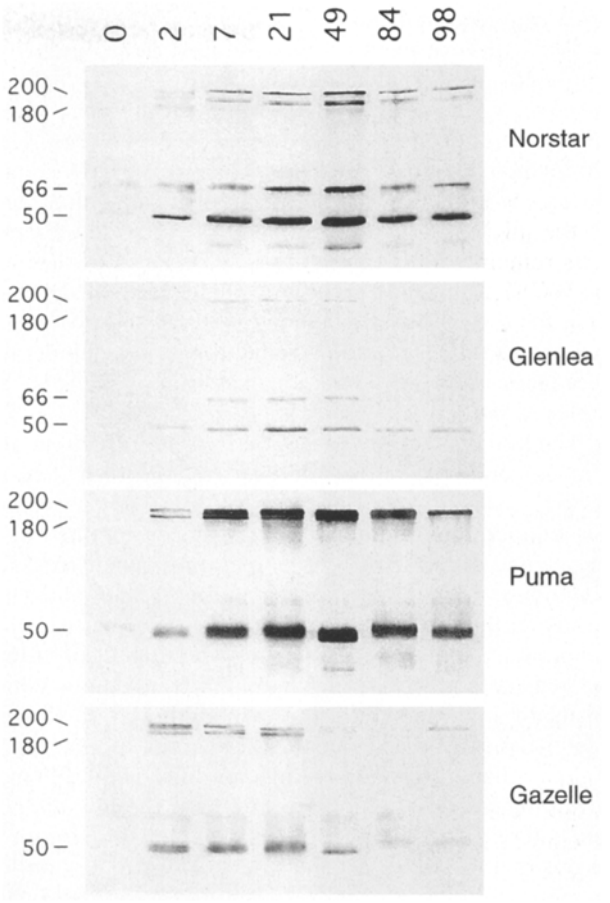

Fig. 5 Kinetic analyses of proteins identified by the anti-WCS120 antibody in Glenlea spring wheat, Gazelle spring rye, Norstar winter wheat, and Puma winter rye after 0, 2, 7, 21, 49, 84, and 98 days of acclimation at 4°C

the low-temperature-tolerance-gene mRNA signal through long periods of low-temperature exposure is the primary factor that differentiates hardy from non-hardy genotypes (Limin et al. 1995).

Molecular analyses showed that similar WCS120 proteins were expressed by spring- and winter-habit cultivars of the same species (Fig. 5). Protein levels were very low in non-acclimated plants and then increased rapidly in all four cultivars following exposure to 4°C (Fig. 3). The pattern of protein accumulation reflected the changes in plant LT_{50}, which produced peaks at 21 and 49 days of acclimation for spring- and winter-habit cultivars, respectively. The fact that the WCS120 proteins increased and decreased in unison throughout the entire 98 day period at 4°C suggests that low-temperature response is a function of the degree and duration of gene expression and is not due to the activation of different sets of low-temperature genes. This is in agreement with earlier observations that hardy and non-hardy genotypes possess similar WCS120 protein products that are coordinately regulated by low temperature (Houde et al. 1992b).

The *Wcs120* gene family mRNA level (Fig. 4) and the protein accumulation patterns (Fig. 3) observed in this

study provided molecular evidence consistent with the hypothesis that low-temperature-induced genes are down-regulated once vernalization saturation (Fig. 1) is achieved in winter wheat and rye. The continued presence of high levels of protein (Fig. 3) after the mRNA level (Fig. 4) had diminished demonstrates that the products of low-temperature gene induction can be maintained for some time after the mRNA signal has dropped off provided temperatures remain cool (Limin et al. 1995). The slow decay of the WCS120 protein curve (Fig. 3) mirrored the decline in LT_{50} for the cultivars evaluated (Fig. 2) and provides an explanation for the gradual reduction in low-temperature tolerance after vernalization saturation (Fig. 1) was achieved in the winter cereals.

The kinetic analyses of WCS120 protein accumulation (Fig. 3) and the close inverse relationship between WCS120 protein levels and LT_{50} (Fig. 2) indicate that the low-temperature tolerance of cereals is determined by the degree and duration of low-temperature gene expression. The low-temperature response pattern of the *Wcs120* gene family further indicates that an inability to maintain low-temperature-induced genes in an up-regulated condition is the main reason why spring-habit cultivars do not achieve levels of low-temperature-tolerance similar to those of winter-habit cultivars. These observations support the hypothesis that the vernalization genes play a key role in determining the duration of expression of the low-temperature-tolerance genes and provide an explanation for the apparent pleiotropic effect that the *Vrn1* gene has on low-temperature tolerance and vernalization response in wheat (Brule-Babel and Fowler 1988; Sutka and Snape 1989; Roberts 1990). They also suggest that any factor that delays the transition from vegetative to reproductive stages, such as a vernalization or photoperiod requirement, should be expected to increase the level of expression of low-temperature tolerance genes in cereals exposed to acclimating temperatures.

There is genetic evidence suggesting that *Vrn1* is homoeologous to other spring-habit genes in wheat and related species. This group includes the vernalization genes *Vrn1, Vrn4* and *Vrn3,* on chromosomes 5A, 5B and 5D of wheat, *Sh2* on chromosome 7 of barley, and *Sp1* on chromosome 5 of rye (Plaschke et al. 1993; Pan et al. 1994; Galiba et al. 1995; Laurie et al. 1995). A close linkage has been reported between several of these vernalization loci and low-temperature-tolerance genes (Roberts 1990; Hayes et al. 1993; Pan et al. 1994) suggesting that at least some of the genes conditioning low-temperature response in cereals exist in clusters (Fowler et al. 1993). While the results of the present study provide strong support for the hypothesis that the vernalization genes are pleiotropic, affecting both growth habit and low -temperature tolerance in wheat and rye, the likelihood of a close genetic linkage of genes conditioning plant low-temperature response is not ruled out. In fact, the role that vernalization genes play in determining plant low-temperature tolerance may simply be restricted to a regulatory influence on the duration of expression of low-temperature-induced genes.

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