

Cbf gene regulation in response to varying cold acclimation induction temperatures

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INTRODUCTION

Low temperature (LT) adaptability has been studied intensely in the Triticeae, which includes economically important cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.). Phenotypic studies have shown that the cold induced protective mechanisms in cereals are developmentally regulated and involve acclimation processes that can be stopped, reversed, and restarted (Fowler et al. 1999).

In wheat, *Cbf* (C-repeat binding factor) genes are considered master switches regulating response to cold and are candidate genes for LT tolerance QTL localized at Fr-A2 (Vágújfalvi et al. 2003.; Francia et al. 2004; Båga et al. 2007). The *Cbfs* belong to the AP2/EREBP transcription factor family and interact with the CRT motif present in the promoter region of *Cold*-regulated (*Cor*) genes to induce *Cor* gene expression (Skinner et al. 2005). In the Triticeae, there is a large *Cbf* family consisting of at least 20 members (Badawi et al. 2007) but it is currently not known which of the *CBF* gene(s) are responsible for the phenotypic differences in LT tolerance. Recent fine mapping experiments suggest that molecular variation in *TmCbf12* and linked genes *TmCbf14*, *TmCbf15* are associated with variation in *T. monococcum* (Knox et al. 2008).

In wheat, *Cbf* expression is generally stronger in winter habit or more LT-tolerant genotypes compared to spring habit or less LT-tolerant genotypes (Skinner et al. 2005; Badawi et al. 2007). *Cor* gene expression levels are up-regulated in genotypes with superior freezing tolerance (Limin et al. 1997) and accumulate at warmer temperatures in winter genotypes (Vágújfalvi et al. 2003). Taken together, these results suggest that genotypes with higher threshold induction temperatures can result in earlier initiation of the cold acclimation process and thus better resistance to subsequent freezing temperatures. However, most *Cbf* expression studies to date have focused on characterizing *Cbf* expression induced after cold exposure and have not quantified differences in expression associated with the rate of response to LT exposure. The objectives of this study were to determine if *Cbf* and *Cor* gene expression correlates with induction temperature in wheat. Orthologous *Cbf* and *Cor* genes were also studied in winter barley “Kold” and cold-hardy winter rye “Puma” to allow cross-species comparisons.

MATERIALS AND METHODS

Genetic Material and LT tolerance – Norstar winter wheat, Puma winter rye, and Kold winter barley were selected for detailed evaluation in these studies. Plants for gene expression were grown hydroponically. Imbibed seeds were held in the dark for 2-d at 4°C and then incubated at 25°C for 1-d. Actively germinating seeds were transferred, embryos down, into white light-blocking plastic trays with holes backed by a 1.6-mm mesh screen. Trays were incubated at 25°C for 2-d to allow for further root growth. The seedlings were then placed in hydroponics tanks filled with continuously aerated one-half strength modified Hoagland’s solution in a controlled environment chamber at 20°C with a 16-h light and a photosynthetically active radiation (PAR) of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 10^d. Once, seedlings had developed two to three fully expanded leaves and visible crowns, trays were transferred to the LT-acclimation chambers set at the designated temperatures with 16-hr light at a PAR of 230 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Temperatures measured at crown level were 6, 10, 15, and 18°C. Samples (whole plant excluding the roots) were collected before (0-h = control sample) and after 2, 4, 8, and 48-hr of LT exposure. The 0 and 48-h tissue samples were collected within minutes after the lights came on at the start of the 16-h d. Samples were immediately frozen in liquid nitrogen and stored at -80°C until required for molecular analyses. The experimental design for this experiment was a 5 cultivar x 4 acclimation temperature x 5 acclimation time factorial in a 3 replicate randomized complete block. The procedure outlined by Fowler (2008) was used to determine the threshold induction temperature and LT₅₀ of each cultivar acclimated at 6°C for 0, 2 and 49-d.

Gene Expression – For each plant sample collected, total RNA was extracted from 100 mg of tissue using TRIZOL® reagent. First strand cDNA synthesis was performed using 200 U of SuperScript™ II RT (Invitrogen) and 500 ng of poly-T primer, 5 μg of total RNA. RT-PCR were performed on cDNA samples using gene specific primers (genes examined are presented in Figure 1). Actin was used as a reference gene. Gene expression analyses were performed using 1 μl of cDNA, 1 U of Taq DNA polymerase (Invitrogen) 0.2 mM dNTP, 1.5 mM MgCl₂, 0.15 μM each primer (5% DMSO only for *cbf* fragment amplification) using 18 to 34 cycles (94°C for 30 sec, annealing for 30 sec, 72°C for 30 sec) and a final extension at 72°C for 10

min. Amplification products were separated at 150 V for 30-min on 2% agarose gels.

RESULTS AND DISCUSSION

Once acclimation started, the differences in genetic potentials among wheat, barley, and rye were quickly magnified (Table 1) with cultivars with warmer threshold temperatures showing the most rapid responses to LT after 2-d acclimation. These observations indicate that there is important variability in the mechanisms by which the cultivars considered in this study monitor and respond to temperature. The large differences in threshold induction temperatures, the rapid initial increase in LT tolerance, and the strong relationship between LT_{50} after 2 and 49-d acclimation at 6°C also supports the notion that the cold sensing mechanism and responses in the early stages of acclimation play a critical role in determining plant cold acclimation potential (Fowler, 2008).

Table 1. Threshold induction temperature and LT_{50} after 0, 2, and 49-d acclimation at 6°C for Puma winter rye, Norstar winter wheat, and Kold winter barley.

Cultivar	Threshold	Acclimation Time (days)		
	°C	0	2	49
Norstar	14.7b	-2.7a	-9.3b	-21.7d
Kold	10.5a [†]	-2.0a	-5.3a	-11.7b
Puma	16.9c	-3.3a	-13.3c	-25.0e

[†] a to e - Within columns, means followed by the same letter are not different ($P < 0.05$).

In *T. monococcum*, *Cbf12* and *Cbf14* have been strongly associated with variation in cold tolerance (Knox et al. 2008) and we examined expression of these genes in response to varying induction temperature in winter common wheat, barley, and rye. Regardless of species, the expression patterns of these *Cbfs* were variable, with a complex gene x time x induction temperature interactions (Fig. 1). In Norstar, *Cbf12* was expressed after 2, 4, and 8-hr exposure to 6°C and decreased dramatically at 10°C. In barley, the *Cbf12* orthologue was highly expressed at 2 and 4-hr after treatment at 6°C with little expression at after 48-hr at 15°C. In contrast, except for the 48-h sampling time, the rye *Cbf12* orthologue was expressed at 15°C. *Cbf14* was expressed at 18°C in all three species. These observed differences in expression levels could be due to different upstream regulatory factors that monitor temperature or mutations within a common regulatory factor controlling gene response.

For those *Cbfs* expressed at higher induction temperatures (15°C and 18°C), expression was strongly repressed at 48 hours, just as plants came out of the dark.

These results suggest a light regulated circadian rhythm of *Cbf* expression at higher induction temperatures. This is similar to *Arabidopsis* where endogenous circadian rhythms of *Cbf* transcripts have been reported at higher temperatures (Edwards et al 2006). In contrast to this study, *Arabidopsis* induction of *Cbf* genes by low-temperature treatment also appears to be gated by the circadian clock (Fowler et al. 2005).

Cor gene expression was variable amongst species, with *Wcor14b* and *Wcor410* orthologues being expressed at 15 and 18°C only in Puma. At colder temperatures, *Cor* gene expression was delayed relative to *Cbf12* and *Cbf14* expression. However, at 18°C maximum *Wcor410* gene expression occurred after 8-hr treatment at 15°C and 2-hr at 18°C. *Wcs120* expression in wheat is highly correlated with LT_{50} (Limin et al., 1997), but despite strong expression of *Cbf14* in Norstar at 15°C and 18°C, *Wcs120* was only weakly expressed at warmer temperatures in the present study.

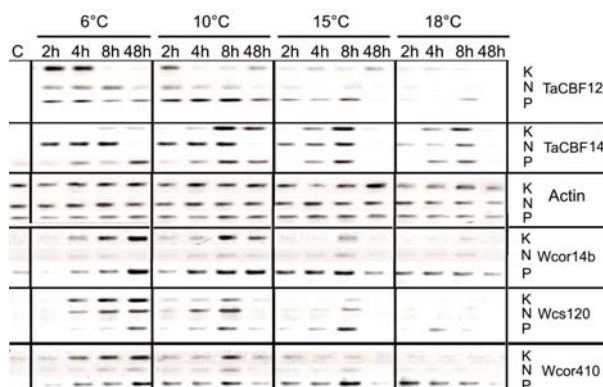


Figure 1. Expression profiles of Norstar (N) wheat, Kold (K) barley, and Puma (P) rye *Cbf12*, *Cbf14*, and *Cor* genes in response to acclimation temperatures ranging from 6 to 18°C.

In Norstar, *Wcs120* expression more closely followed *Cbf12* expression. *Wcor14b* expression was evident only in Puma at 18°C, but expression was not correlated to *Cbf12* or *Cbf14*, especially at 2°C. These results suggest that at higher temperatures, other *Cbf* gene(s) and/or regulatory factors maybe regulating *Cor* gene expression and LT tolerance in rye, and may explain the superior cold tolerance observed in Puma compared to Norstar and Kold (Table 1).

CONCLUSIONS

Large species by threshold induction temperature by time by LT induced gene interactions were noted in this study. This indicated that expression of *Cbf* and *Cor* genes was staged at different temperatures and times during the day and made it difficult to determine cause-and-effect relationships. *Cbf* expression in cereals was regulated not only by induction temperature, but also by

light determined circadian rhythm, particularly at higher temperatures. These observations strongly suggest that sample timing, induction temperature, and light related factors must be considered in future studies involving functional characterization of LT induced genes in cereals.

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