The cold response of *CBF* genes in barley is regulated by distinct signaling mechanisms

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A B S T R A C T

Cold acclimation ability is crucial in the winter survival of cereals. In this process *CBF* transcription factors play key role, therefore understanding the regulation of these genes might provide useful knowledge for molecular breeding. In the present study the signal transduction pathways leading to the cold induction of different *CBF* genes were investigated in barley cv. Nure using pharmacological approach. Our results showed that the cold induced expression of *CBF9* and *CBF14* transcription factors is regulated by phospholipase C, phospholipase D pathways and calcium. On the contrary, these pathways have negative effect on the cold induction of *CBF12* that is regulated by a different, as yet unidentified pathway. The diversity in the regulation of these transcription factors corresponds to their sequence based phylogenetic relationships suggesting that their evolutionary separation happened on structural, functional and regulational levels as well. On the *CBF* effector gene level, the signaling regulation is more complex, resultant effect of multiple pathways.

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Introduction

Cold acclimation is a complex trait in cereals involving multiple genetic regulatory networks. The most studied transcription factor (TF) family responsible for cold hardening and frost tolerance is the multigenic CBF/DREB1 family. These TFs belong to the AP2/ERF (APETALA2/ethylene-responsive factor) superfamily and they are able to bind to the CRT/DRE (C-repeat/Dehydration Responsive Elements) in the promoter of several cold induced genes (Skinner et al., 2005). These transcription factors rapidly respond to the cold showing a transient expression for a couple of hours (Stockinger et al., 1997; Gilmour et al., 1998). The expression of *CBF* genes is a very important factor for the strong induction of the cold responsive (COR) genes, like *COR14b* and *DHN5* in barley (Choi et al., 2002; Dal Bosco et al., 2003). This cascade is the initiating event of the cold acclimation of the plants where the whole metabolism is reprogramed and at the end of this process the plants show increased frost tolerance (Thomashow, 1999). The COR genes receive inputs from the *CBF* genes, but *CBF* genes alone are not sufficient for the maximal induction of *COR* genes, they need inputs from the

light sensing machinery like phytochromes and probably from the chloroplast as well (Knight et al., 1991; Crosatti et al., 1995; Crosatti, 1999; Campoli et al., 2009; Kurepin et al., 2013; Vashegyi et al., 2013). The induction of the *CBF* genes is mediated through the constitutively expressed ICE proteins which become activated by cold and able to induce *CBF* expression (Chinnusamy et al., 2003; Badawi et al., 2008). Three *CBF* genes were found in Arabidopsis, they are probably the result of gene duplication and a subsequent divergence (Gilmour et al., 1998). Although there are many data about their function and interaction in dicot model little is known about them in cereals. This question is particularly interesting when it is taken into account that in barley more than 15 *CBF* genes were found (Knox et al., 2010) without having much information about their individual role. What is known from recent overexpression studies performed in barley is that *CBF*2A and wheat *CBF*14 and *CBF*15 were directly proven to be involved in the cold acclimation causing a significant increase in frost tolerance (Soltész et al., 2013; Jeknić et al., 2014). It is also described that these genes are organized in clusters on the long arm of the chromosomes of the homoeologous group 5 (Francia et al., 2004; Miller et al., 2006; Pasquariello et al., 2014) and based on their sequence their phylogenetic tree was also calculated (Skinner et al., 2005; Miller et al., 2006; Badawi et al., 2007; Campoli et al., 2009; Tondelli et al., 2011). In cereals different members of the *CBF* family show different response to cold; there are highly responsive *CBF*s, but there exist unresponsive ones as well (Stockinger et al., 2007; Knox et al., 2008; Campoli

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et al., 2009). These observations suggest that their regulation must occur via different pathways. One experimental approach to investigate this question is to block certain signaling components and investigate subsequent changes in the cold induced expression of the *CBF* genes.

In plants, cold perception and cold signaling is studied mostly in Arabidopsis (Tähtiharju et al., 1997; Xiong et al., 2002; Testerink and Munnik, 2005). According to the most recent results, the cold perception in plants might occur through multiple ways (Knight and Knight, 2012). The fluidity change of the cell membranes or microtubules caused by the decreasing temperature activates signaling cascades leading to elevated intracellular calcium concentration which is decoded by calcium responsive proteins and then the signal ends up with altered expression of cold responsive genes (Örvar et al., 2000; Sangwan et al., 2002; Chinnusamy et al., 2007; Knight and Knight, 2012; Nick, 2013). This complex system involves the activation of phospholipase C (PLC), phospholipase D (PLD) and as a second messenger the lipid mediated cold specific calcium signature (Ruelland et al., 2002; Knight and Knight, 2012). The activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 3-phosphate (IP3). The DAG is converted to phosphatidic acid (PA) by diacylglycerol kinase (DGK), while the IP3 by itself or as a precursor of inositol hexaphosphate or phytic acid (IP6) is responsible for a rapid calcium influx (Lemtiri-Chlieh et al., 2003). On the contrary, PLD pathway directly leads to the production of PA by cleaving phospholipids after the phosphate and releasing PA and alcohol. Although their regulation is different, both pathways lead to the production of PA. Phosphatidic acid is a central signaling molecule participating in many biotic and abiotic processes by activating protein kinases and phosphatases. The whole process is extensively reviewed by Munnik and Testerink (Munnik and Testerink, 2009). The pharmacological modification of these processes is widely used in animal model systems, but there is an increasing number of data using this approach in plant systems as well (Tähtiharju et al., 1997; Vergnolle et al., 2005; Krinke et al., 2007; Xue et al., 2009; Delage et al., 2012). It is well documented, that the PLC pathway can be effectively blocked by U73122, a specific inhibitor of PLC, while the PLD pathway can be inhibited by the PLD antagonist 1-butanol (Vergnolle et al., 2005; Krinke et al., 2007). The increase of the intracellular calcium level can occur from the extracellular space through the plasma membrane channels and from the vacuolar calcium stores through the tonoplast (Knight et al., 1996). Each of them can be inhibited by lanthanum or ruthenium red, respectively (Pottosin et al., 1999; Ruelland et al., 2002; Sangwan et al., 2002).

Our aim was to study the cold induced expression of different *CBF* genes following the inhibition of various signaling steps in barley in order to determine the shared and distinct components in their regulation.

Materials and methods

Plant materials and growth media for gene expression studies

Hordeum vulgare cv. Nure (frost tolerant type) seeds were germinated 5 days on sterile filter paper soaked in MilliQ water in Petri dish. For an inhibitor treatment, the young barley seedlings (developmental phase was Z10 according to Zadoks scale and F1 according to Feekes scale; *Suppl.* Fig. 1) were cultivated in sterile 6 well plates containing 2–2 ml inhibitor solutions (1 mM lanthanum chloride, 10 mM EGTA, 1 mM neomycin, 0.2 v/v % butanol, 50 μM ruthenium red, 10 μM U73122) for 16 h in normal growth conditions (20 °C/20 °C; 16 h/8 h day/night; 75% humidity, 260 μmol/m^2/s white fluorescent light) and then they were placed on inhibitor-free ½ Hoagland-solution. After inhibitor

pretreatments, one part of the seedlings were grown in cold condition for cold treatment (4 °C/4 °C; 16 h/8 h day/night; 75% humidity, 260 μmol/m^2/s white fluorescent light), while the other set of seedlings were left in control condition (20 °C/20 °C; 16 h/8 h day/night; 75% humidity, 260 μmol/m^2/s white fluorescent light).

Sampling and preparation of tissues for RNA analysis for gene expression studies

Samples were taken at the start of cold hardening (0 h) and after 6 h, 12 h and 24 h of hardening and then they were quickly frozen in liquid nitrogen, and stored at −80 °C until their use for RNA preparations. Leaves were harvested from all plants from the crown and each sample contains three seedlings. The whole experiment was repeated three times.

RNA extraction

Samples were taken for RNA extraction from the crown of the seedlings at different time points: 0, 6, 12 and 24 h after the start of cold treatment. Total RNA was extracted from control and cold treated samples by two-step purification protocol. TRIzol extraction was followed by a further purification step using RNeasy Plant Mini Kit (Qiagen) and these RNAs were treated by DNase set (Qiagen). RNA concentration was measured by NanoDrop.

cDNA synthesis

First strand cDNA synthesis was performed using a Molony Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega) and oligo dT 15 primer (Promega) to prime cDNA synthesis. 1 μg of total RNA was mixed with 0.5 μg oligo dT primer to a total of 11 μl, incubated at 75 °C for 5 min, then cooled quickly on ice for minimum 5 min. The reaction was performed following the manufacturer description. The cDNA was diluted in sterile ddH$_2$O to a final volume of 40 μl and the concentration was set to 25 ng/μl. These cDNAs were used as templates in real time RT-PCR reactions.

qRT-PCR

Real-time PCR primers were either taken from the literature or designed from cDNA sequences corresponding to each *CBF* genes, *DHN5* and *COR14b* gene (Table 1). The real time PCR reaction was performed on Bio Rad CFX96 (C1000 Touch Thermal Cycler) real time PCR equipment and KAPA Sybr Fast Universal qPCR reagent (Kapa Biosystems) was used for detection. PCR reactions of 10 μl total volume containing 1 μl cDNA (25 ng/μl), 0.5-0.5 μl of primers (from 10 mM stock), 5 μl Power Sybr Fast Master Mix and nuclease free water to a final volume of 10 μl. For normalization, *HvActin* reference gene (GenBank accession#: AK362208.1) was used for each sample. The PCR program had two stages: first stage was 95 °C 3 min and the second stage consist of 95 °C 5 s and 60 °C 30 s for 40 cycles, than PCR program was finished by melting curve analysis to check the reaction specificity. The data analysis was calculated by relative quantification (dCt method) using beta-actin as internal standard (Livak and Schmittgen, 2001). Data were presented in the form of expression fold changes of each treatment with respect to that of normalized control. The columns and error bars represent 'mean ± standard deviation'.

Statistical analysis

According to the assumption test results, statistical analysis involved unpaired *t*-test with Welch's correction and Kruskal–Wallis nonparametric method followed by