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C-Repeat Binding Factor and Dehydrin Genes are Induced Co-Ordinately in Drought Tolerance Response of Wheat Cultivars

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Abstract

Four bread wheat genotypes with contrasting drought stress tolerance were studied. Expression levels of dehydrin (*Wdhn13*) and C-repeat binding factor (*Cbf14*, *Cbf15*) genes were investigated in leaves of two drought tolerant (Plainsman V, Mv Emese) and two sensitive (GK Élet, Cappelle Desprez) cultivars by semi-quantitative RT-PCR during drought treatment at anthesis. Coordinate induction of *Cbf14*, *Cbf15* and *Wdhn13* genes occurred at a late stage of stress treatment in all cultivars except the most sensitive Cappelle Desprez, where no induction was evident. The most pronounced late induction of genes was observed in the tolerant Mv Emese genotype. *Cbf14*, *Cbf15* and *Wdhn13* showed largely parallel changes of expression in stressed adult plants. The mRNA level of the same set of genes was measured in leaves of non-stressed seedlings with qRT-PCR method. Expression level of *Wdhn13* was high and low in seedlings of tolerant and sensitive cultivars, respectively. *Cbf15* specific transcript was barely detectable in leaves of non-stressed seedlings. In order to shed light on any potential difference in hormone responsiveness, seedlings were subjected to ABA treatment *in vitro*. At low hormone concentrations (10 and 20 μM ABA) consistently weaker ABA induced root growth retardation of GK Élet was found in comparison with the other three cultivars. Results highlight pronounced and late induction of a set of defence genes and low ABA sensitivity as features appearing in drought tolerant and sensitive responses, respectively. Data is discussed in the light of multifactorial determination of the complex phenotype of drought tolerance in wheat.

Keywords: wheat (*Triticum aestivum* L.), drought stress, abscisic acid, dehydrin genes, CBF genes

1. Introduction

C-repeat binding factor (*Cbf*) genes are AP2/ERF transcription factors implicated both in cold stress response (Galiba et al., 2009) and in ABA mediated drought stress tolerance (Mizoi et al., 2012; Knight et al., 2004; Xiao et al., 2006; Kidokoro et al., 2015). *Cbf14* and *Cbf15* genes of wheat were first identified in *Triticum monococcum* L. and mapped to a major locus determining frost tolerance in this species (Miller et al., 2006). *Cbf14* and *Cbf15* of *Triticum aestivum* were functionally tested by transformation of barley, proving the role of these genes in freezing tolerance (Soltész et al., 2013). Along with alleviation of consequences of freezing stress, transcriptional activation of some dehydrin genes was also noted in the transgenic barley lines. Dehydrins are protective proteins against cellular damage in freezing and osmotic stress with multiple ways of functioning (Battaglia et al., 2008; Hara, 2010), complementing other molecular defences (Hegedűs et al., 2004) activated by these stress factors. Fifty-four wheat genes have been selected as candidates for coding dehydrins, most of them induced by dehydration (Wang et al., 2014). *Wdhn13* is a relatively well characterized member of this group. Its ABA inducibility has been suggested by some authors (Kurahashi et al., 2009), but has not been proposed by others (Ohno et al., 2003). This gene was found to exhibit variable expression/inducibiliy in divergent cultivars in response to dehydration (Rampino et al., 2006).

Connection between abscisic acid (ABA) signalling and drought tolerance has been well established (*e.g.* Lee & Luan, 2012; Golldack et al., 2014). ABA sensitivity may be manifested at any of the divergent processes this

hormone regulates, such as germination, growth retardation or other stress responses leading to tolerance. Although these responses probably do not use the same set of signalling components, ABA sensitivity at germination for instance was found to be associated with enhanced drought tolerance in a number of cases (Cutler et al., 1996; Hugouvieux et al., 2001; Papp et al., 2004). Measuring retardation of root elongation at the seedling stage is a relatively simple way to characterize responsiveness to exogenously applied ABA (Thole et al., 2014). This trait was found to be associated with high drought stress tolerance levels in a set of synthetic hexaploid wheat lines derived from *Aegilops tauschii* (Kurahashi et al., 2009).

In this study, expression levels of genes potentially associated with a drought stress response were investigated in four bread wheat genotypes of contrasting drought tolerance. The cultivars were already characterized earlier and were shown to exhibit tolerance (Plainsman V, Mv Emese) or sensitivity (GK Élet, Cappelle Desprez) against drought stress (Guóth et al., 2009; Jäger et al., 2014). We hypothesized that known regulators of water stress tolerance and/or effector genes might contribute to this contrasting behaviour. As candidates, a dehydrin gene and *CBF* transcription factors were characterized under drought conditions at anthesis. The transcription pattern of these genes was also established in non-stressed seedlings of the same cultivars. Further on we set out to compare ABA sensitivity of root elongation at the seedling stage to explore ABA responsiveness of the cultivars.

2. Materials and methods

2.1 Growth of Plant Material, Sampling and Drought Stress Treatment

Seeds of four winter wheat (*Triticum aestivum* L.) cultivars (Plainsman V, Mv Emese, GK Élet, Cappelle Desprez) were imbibed in distilled water for 1 hour at 22 °C. All subsequent steps of the procedure were performed at this temperature. Imbibition was followed by surface sterilization in sodium hypochlorite with 2% available chlorine for 8 min and by rinsing 4 times with sterile distilled water. Imbibed seeds were germinated in glass Petri dishes on a filter paper moistened with distilled water in the darkness. The seedlings were subjected to ABA treatment immediately after the emergence of the radicle.

For water stress treatment plants of the winter wheat varieties were planted in a soil-sand-peat mixture (3:1:1, v/v/v) after 7 weeks of vernalisation at a temperature of 4 °C, and grown in PGV-15 growth chambers (Conviron, Winnipeg, Canada) using the spring climatic program T1 (Tischner et al., 1997). The min/max temperature rose from 12.5/5.5 °C to 21/14 °C until anthesis. Irrigation was carried out regularly in the morning at a rate of 150 ml/pot/day. In order to compensate for the delay in heading and flowering of Cappelle Desprez compared to the other three cultivars (according to preliminary experiments), plants of the former variety were planted 2 weeks earlier to align developmental differences. Drought stress was generated by total water withholding starting at the time of inflorescence emergence (Zadoks' growth stage 53) to complete anthesis (Zadoks' growth stage 67) until the volumetric water content of the soil dropped below 10 % in the pots. Leaf samples were collected from both drought-stressed and control plants. Soil volumetric water content (VWC) of 20 pots per genotype and treatment was measured using an HH2 moisture meter (Delta-T Devices Ltd., Cambridge, UK) at field capacity and during treatments. Relative water content (RWC) of leaves was determined on whole flag leaves of three plants per genotype and treatment at the end of water withholding by measuring fresh weight (FW) at excision, saturated weight (SW) after 24 h rehydration on distilled water at 4 °C in the dark, and dry weight (DW) after oven drying for 48 h at 80 °C. The leaf RWC (%) was calculated using the following equation:

$$\text{RWC}\ (\%) = [(\text{FW} - \text{DW})/(\text{SW} - \text{DW})] \times 100 \quad (1)$$

For sample preparation of non-stressed young seedlings, plants were planted as above and were grown at constant 21 °C temperature under 10/14 hours light/dark illumination cycle. At Zadok's growth stage 13 leaves of seedlings were harvested for RNA preparation (Zadoks et al., 1974).

2.2 RNA Extraction, RT-PCR, qRT-PCR, DNA Fragment Isolation and Sequencing

Leaf samples were grinded in liquid nitrogen and RNA was isolated with Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the instructions of the manufacturer. First strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) was used for reverse transcription of the RNA samples. RT-PCR was performed with GoTaq Flexi DNA Polymerase from Promega (Madison, WI, USA) according to the instructions of the manufacturer, applying a total of 30 cycles. An equal use of cDNA templates was confirmed by RT-PCR with control primers TalKa and TalKb, amplifying sequence Ta2776, as suggested by Paolacci et al. (2009). The gene-specific primers for the dehydrin gene were designed with the Primer Premier software (Premier Biosoft, Palo Alto, CA, USA) and were purchased from Biomi Kft (Gödöllő, Hungary) or Sigma (St Luis, MI, USA). Primers specific for the CBF genes were kindly provided by Gábor Galiba (Agricultural Institute of HAS, Martonvásár, Hungary). All primers used are listed in Table 1. qRT-PCR was performed in a Rotor Gene 6000

instrument (Corbett Research, Australia), by applying conditions recommended by the manufacturer and 60 °C annealing temperature. Quality of products was confirmed with melting curve analysis by heating samples from 66 °C to 99 °C in 0.5 °C increments. Evaluation of data was done with the help of Comparative Quantitation Analysis program (McCurdy et al., 2008). RT-PCR and qRT-PCR were performed at least two times on different biological samples with a minimum of two technical repetitions in each case. RT-PCR products were purified from agarose gels using an Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Uppsala, Sweden). Primary DNA sequences were determined by Biomi Kft (Gödöllő, Hungary). Differences of genes expression levels were tested with one-way ANOVA.

2.3 Measuring Root Growth Retardation of Seedlings in Response to ABA Treatment

Synchronously germinated seedlings were transferred into Petri dishes with filter paper wetted with distilled water or with 10, 20 or 50 μM ABA (Sigma) solution. At least 40 seedlings per treatment and genotype were incubated for further one week in the darkness and the lengths of their primary roots were recorded. The experiment was repeated three times. Differences in root length were tested with one-way ANOVA and a subsequent Dunnett's T3 (T3) test (Dunnett, 1955).

3. Results

3.1 Drought Stress Treatment

As a consequence of water deprivation soil volumetric water content declined below 10% in all cultivars by the end of the treatment (Figure 1). There was no consistent, significant difference in the dynamics of water loss among the cultivars based on repetitions of the experiment. Relative water content of leaves remained significantly higher in the drought tolerant cultivars (Figure 2).

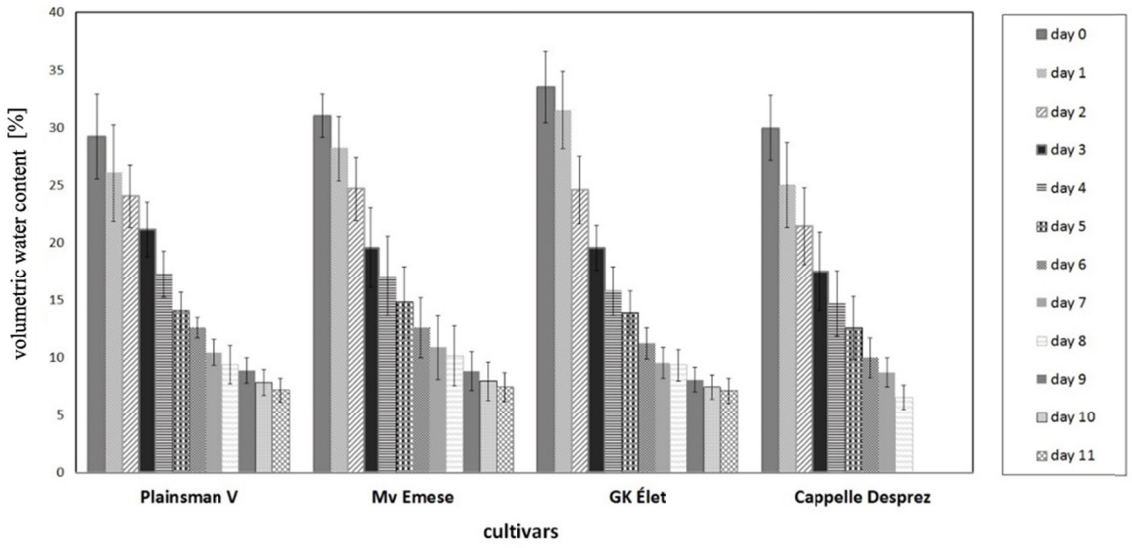

Figure 1. Volumetric water content (VWC) of soil during drought stress. Number of days of the treatment is indicated. Means ± standard deviations are shown

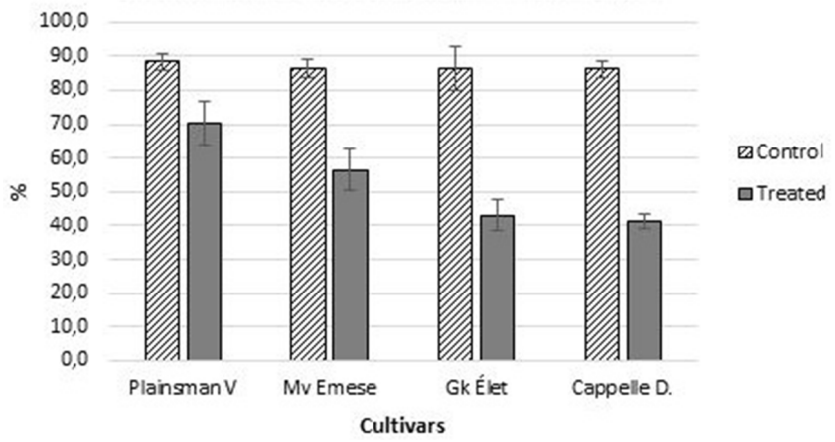

Figure 2. Relative water content (RWC) of leaves at the end of the treatment. Means ± standard deviations are shown

3.2 Transcriptome Analysis in Adult Plants under Water Deprivation

Gene specific RT-PCR product from *Wdhn13* was most induced in Mv Emese at 6th-10th days of water deprivation (Figure 3). mRNA of this gene was barely detectable in the other three genotypes, with low level of induction in Plainsman V and Gk Élet. Declining signal in semi quantitative RT-PCRs of all studied genes was followed by a wave of induction around the 8th day of treatment, except for the most sensitive Cappelle Desprez, where this induction did not occur. Parallel changes in expression of *Cbf14*, *Cbf15* and *Wdhn13* genes were noted, but this effect was not stringent. In order to confirm identity of the PCR products representative RT-PCR fragments of *Cbf14*, *Cbf15* and *Wdhn13* were extracted from agarose gels and sequenced directly.

Table 1. Gene specific and control primers used in RT-PCRs and qRT-PCR

Primer name	Primer sequence
Cbf14a	5'-CCAAACCAGTGTCATTCAA-3'
Cbf14b	5'-TTGTCTCAACTTCGCCACT-3'
Cbf15a	5'-GTGTCTCAACTTCGCCGACT-3'
Cbf15b	5'-ATGTGTCCAGGTCCATTTTCC-3'
Wdhn13a	5'-ATTCTGCAAAGTAGCGGGTC-3'
Wdhn13b	5'-AGAACCAGTGTCAGATTTCCCT-3'
TalKa	5'-GTAGCATTATGTTTGTGCCTTG-3'
TalKb	5'-GGAGAGCCAGTCAAGACCCTCG-3'

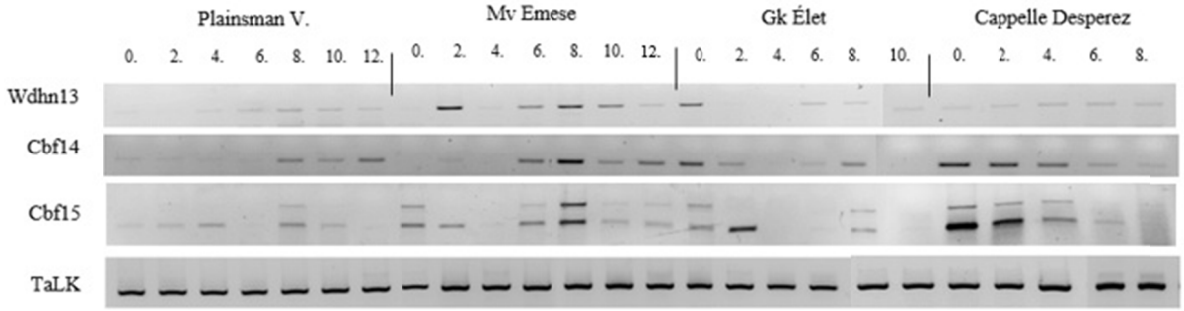

Figure 3. RT-PCR products for the studied genes in the four genotypes during drought stress treatment. Numbers indicate days of treatment

3.3 Transcript Abundance of Cbf14, Cbf15 and Wdhn13 Genes in Non-Stressed Seedlings

Quantitative RT-PCR was performed on cDNA samples prepared from unstressed seedlings of the cultivars studied. Transcript abundance of the genes was investigated in leaves of 3 week old non-stressed plants. Data show high expression of *Wdhn13* occurring exclusively in the tolerant cultivars (Plainsman V and Emese) (Figure 4). mRNA levels of the *CBF14* gene were generally modest, highest in the cultivar Plainsman V. mRNA of the *Cbf15* gene however was not detected at all under these conditions, or its level was extremely low.

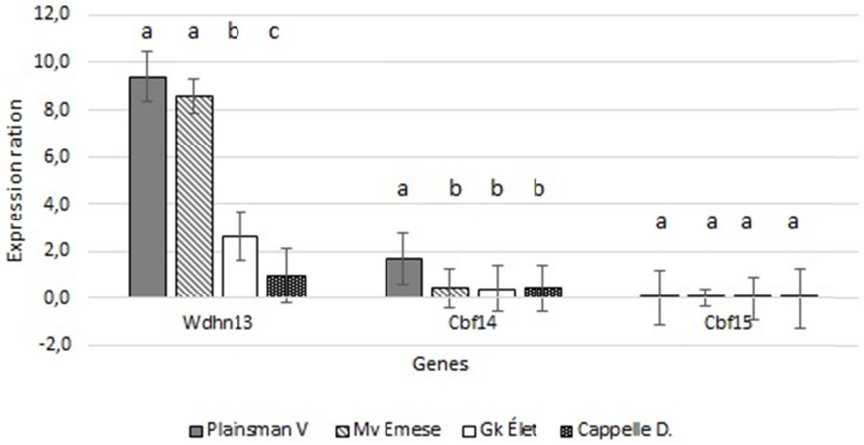

Figure 4. Expression levels of the genes, measured by qRT-PCR, in unstressed seedlings. One-way ANOVA with post-hoc Tukey HSD test was used to test for statistical differences. Means ± standard deviations are shown. Different letters indicate differences among the cultivars for each gene separately at $p < 0.05$ of probability

3.4 ABA Sensitivity of Seedlings

Exogenous application of ABA at low concentrations retarded root elongation to a different degree. Cultivar GK Élet showed significantly the weakest response to 10 and 20 µM ABA applications (Figure 5).

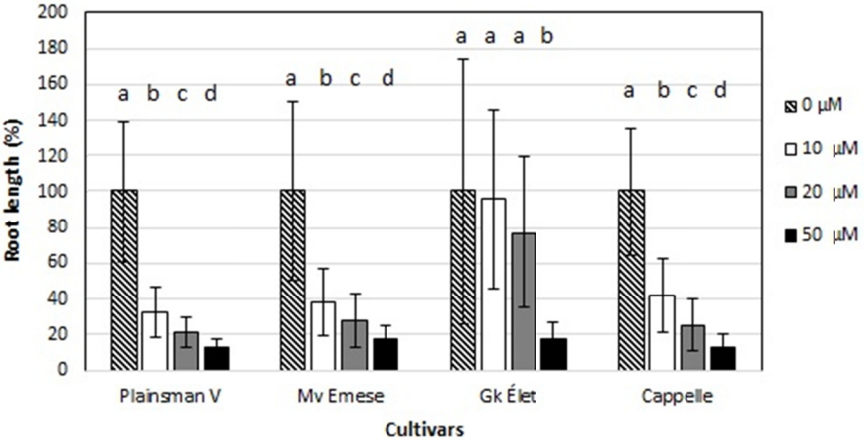

Figure 5. Relative root lengths of seedlings after one week growth at the indicated ABA concentrations (10, 20 and 50 µM) were compared to untreated controls. Welch's ANOVA test ($F^{(3, 125,9)} = 158,5$; $p = 0,000$) was used to test for statistically significant differences among data sets of genotypes and hormone concentrations. Means ± standard deviations are shown. Different letters indicate differences at $p < 0.05$ of probability

4. Discussion

Drought tolerance is a complex phenotype with multi factorial determination (Ashraf, 2010). Studies presented here focused on expression of potentially stress related genes as well as ABA sensitivity in wheat genotypes with

contrasting ability to tolerate water stress. The investigated attributes may indicate differences in stress response mechanisms with potential contribution to drought hardiness.

This work is an extension of earlier experiments (Jäger et al., 2014) where several traits potentially contributing to different drought tolerance level of four selected bread wheat cultivars have already been identified. Smaller leaf area and lower stomatal number pro leaves were found in the tolerant varieties, while a thin and water permeable cuticle was described in the sensitive cv. Cappelle Desprez. Besides morphological traits, stress induced morpho-physiological changes also differentiated the cultivars especially after repeated exposures to water deficit. Loss of cellular integrity (indicated by increased electrolyte leakage), more severe ultrastructural damage to the chloroplasts and faster decline of relative water content of leaves were characteristic to the sensitive cultivars during repeated drought cycles (Jäger et al., 2014). Apparently the two tolerant genotypes exhibited stronger defencesagainst oxidative damage at the cellular level. This coincided with higher glutathione synthase and glutathione peroxidase activity in cv. Plainsman V as was established by Gallé and co-workers (2009). Therefore, several facets of the more effective drought tolerance response of Plainsman V and Mv Emese have been already uncovered. Different responses to water shortage were confirmed in our present experiments, as tolerant cultivars exhibited significantly higher relative water content values in leaves already at the end of one water deprivation period applied (Figure 2).

Given the high complexity of the drought tolerant phenotype we hypothesized that further relevant physiological differences could still be established among these cultivars. In a search for further clues explaining differences, expression levels of potential stress tolerance related genes were studied.

Dehydrin genes are well appreciated targets of *Cbf* genes when responding to cold. Parallel expression of *DHN* and *Cbf* genes has been found in cold stress (*e.g.* Kume et al., 2005). *Cbf* and other cold responsive genes were found activated by ABA independent and ABA dependent pathways as well (Knight et al., 2004). More recently *Cbf* gene expression has been also linked to induction of a dehydrin gene during the drought tolerance response of *Brachypodium distachyon* (Ryu et al., 2014). *Wdhn13* is probably subject to regulation by multiple transcription factors, including *Cbf* genes. The *Wcbf2* wheat transcription factor has been shown to activate the promoter of *Wdhn13* in tobacco (Takumi et al., 2008). Transcriptional regulation of *Wdhn13* involving *Cbf* genes under drought conditions fits well with our data. In this study dehydrin and *Cbf* gene specific RT-PCR reactions were performed on leaf samples from drought stressed plants of the four genotypes around anthesis. Data indicated that *Wdhn13*, *Cbf14* and *Cbf15* genes showed coordinate induction in Mv Emese during a late stage of the water limitation period. This induction of dehydrin and *CBF* genes occurred at approximately one week after total water deprivation and was most pronounced in Mv Emese, a genotype with high tolerance and yield under drought stress conditions. Based on this expression pattern in drought stressed adult plants *Wdhn13* (and probably other dehydrins) may be regulated by both *Cbf* genes. In order to confirm association between drought, stress-induced *CBF*/dehydrin gene expression at anthesis and drought tolerance, a larger number of genotypes ought to be examined. Transcript profiles of *Wdreb2* and other potential regulators as well as more dehydrin genes should be tested in this respect.

We presumed that a clearer picture could be obtained about interactions among dehydrin and *Cbf* genes by examining plants exposed to as low a level of stress as possible. To this end the same set of genes was investigated in leaves of non-stressed young seedlings. According to observations of Rorat and co-workers (2004) in five Solanaceae species and barley, leaves of young, developing seedlings exhibited high dehydrin protein levels even in the absence of stress. Similarly, light induced expression of a dehydrin-encoding gene, *HaDhn1*, was found in sunflower seedlings in the absence of environmental stresses (Natali et al., 2007). This made us speculate that wheat seedlings may also express at least some *DHN* and/or *Cbf* genes spontaneously (*i.e.* probably as a result of the unavoidable, very low level of stress received during cultivation). In order to minimize stress factors as much as possible, wheat seedlings were well watered and a constant temperature was applied to exclude cold induced gene expression. Target gene expression was investigated by quantitative qRT-PCR. Cultivar specific expression of the genes was found, which didn't match with that found around anthesis (day 0 of drought treatment). This confirms that transcription of *Cbf* and dehydrin genes may be subject to development specific regulation. In seedlings of some cultivars *Cbf14* and *Cbf15* transcripts were found expressed at moderate and at very low levels, respectively. This contrasted with parallel expression of *Cbf14* and *Cbf15* in adult stage plants under drought stress implying that the two genes may be regulated by different mechanisms. Independent regulation of the two genes was also shown earlier by Knox et al. (2008) in cold stress.

The dehydrin gene *Wdhn13* was found highly expressed in unstressed seedlings of the two tolerant varieties but at a lower level in the sensitive cultivars. A potential connection between dehydrin expression early in plant

development and drought tolerance of adult plants should be further investigated on a larger set of genes and cultivars. It is worth noting however that high levels of a desiccation induced dehydrin protein at the seedling stage werealso correlated with adult stage drought tolerance in wheat (Lopez et al., 2001).

ABA sensitivity of root growth has been associated with drought tolerance in wheat (Kurahashi et al., 2009). As ABA is also known to activate expression of some dehydrin genes, we performed an in vitro experiment to this end. In our study ABA induced root growth retardation of GK Élet was found to be consistently weaker at low external hormone concentrations (10 and 20 μM ABA applied) in comparison with the other three cultivars. This data suggest that ABA insensitivity may contribute to the low level of drought tolerance of GK Élet, and confirm that ABA responsiveness may be a factor of drought sensitivity in some wheat cultivars. No direct correlation was found however between inducibility of the genes studied and ABA sensitivity of the cultivars. Stress induced modification of root growth in cvs Mv Emese and GK Élet has been also characterized by Tari et al. (2010). Smaller osmotically (PEG) induced reduction of root growth rate was found in GK Élet, which corresponds well with our findings. In the experiment of Tari et al. (2010) endogenous ABA content of the root tips remained unchanged in GK Élet, but decreased in Mv Emese. Our data about ABA insensitivity can explain why the relatively higher ABA content could still not have led to efficient growth retardation in Gk Élet root tips. ABA responsiveness of root elongation can be measured at the seedling stage, making this method especially feasible for early pre-screening drought sensitivity of breeding lines.

We conclude that pronounced and coordinate induction of *Cbf* and dehydrin genes (*Cbf14*, *Cbf15* and *Wdhn13*) in a late stage of water stress in a tolerant genotype (Mv Emese) at anthesis may represent a module in the multi facet phenotype of drought hardiness. We propose that the other tolerant cultivar involved in our studies (Plainsman V) probably uses alternative strategies to avoid detrimental effects of drought. Morphological traits may support lower level of water loss in this cultivar (Jäger et al., 2014). Evading water shortage by accelerated development of the root system may also be a part of an acclimation process in Plainsman V. This aspect of the water stress response however, was not investigated in our studies. A further basic factor of drought tolerance is ABA responsiveness, which is apparently weak in the cultivar Gk Élet, probably contributing to its sensitivity. Our experiments also uncovered expression of the *Wdhn13* dehydrin gene in leaves of unstressed seedlings in the tolerant cultivars. Whether this implies differences in sensitivity of signalling steps leading to defencegene expression needs further investigations.

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