The role of genes encoding wheat HD-Zip I transcription factors in response to drought and frost in transgenic wheat

Yunfei Yang

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Faculty of Sciences

School of Agriculture, Food and Wine

The University of Adelaide

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Abstract

Drought and frost are common abiotic stresses that negatively affect plant development, growth and yield worldwide. To survive, plants have their own defence mechanisms to adapt to harsh environmental conditions. Transcription factors (TFs), as regulators of gene expression, compose an important part of the plant defence system. TFs from homeodomain leucine zipper class I (HD-Zip I) are suggested to participate in regulatory networks of gene expression to abiotic stresses in plants. However, little information is known about the roles of HD-Zip I TFs in response to abiotic stresses in wheat. Hence, the roles of three wheat genes *TdHDZI-3*, *TdHDZipI-4* and *TaHDZipI-5*, encoding γ-clade TFs of HD-Zip I were characterised in transgenic wheat.

In this study, the *TaHDZipI-5* gene product showed its transcriptional activating activity and its activation domain located at the C-terminus using an in-yeast trans-activation assay. The TaHDZipI-5 TF was suggested to have homo-dimerization ability and may hetero-dimerize with TaHDZipI-3. The differences between DNA interactions in homo- and hetero-dimers were analysed using 3D models, and the hetero-dimerisation was indicated to be more stable than the homo-dimerisation form.

The overexpression of *TaHDZipI-5* transgene driven by the constitutive promoter *Ubiquitin*, conferred drought and cold tolerance to transgenic wheat. However, a series of undesired phenotypic features, such as reduced plant size and biomass, delayed flowering and decreased grain yields, occurred in transgenic plants with constitutive overexpression of the *TaHDZipI-5* transgene. Two stress-inducible promoters, *OsWRKY71* and *TdCor39*, which were previously employed to avoid the negative influences of *TaDREB3* gene expression on phenotype and yield in transgenic barley, were then used for relieving the negative effects made by the overexpression of the *TaHDZipI-5* transgene. The attempt to improve the phenotypes of transgenic wheat was not as successful as intended. A possible reason might be that the selected promoters may not meet strict spatial requirements for the overexpression of the *TaHDZipI-5*. However, the inducible overexpression of *TaHDZipI-5* driven by each of the promoters conferred frost tolerance to transgenic wheat.

In the second part of the study, promoters of genes encoding HD-Zip I TFs TdHDZipI-3 (HDZI-3) and TdHDZipI-4 (HDZI-4), were characterised under drought and cold stresses using transgenic wheat. A novel CBF/DREB transcription factor protein TaCBF5L was isolated from

roots of drought-stressed wheat, using Y1H method with DRE cis-element as a bait. The *TaCBF5L* gene was transformed into wheat plants for the characterisation of the *HDZI-3* and *HDZI-4* promoters. Based on the phenotypic results, the *TaCBF5L* transgene driven by *HDZI-4* significantly improved grain yield, seed number and biomass under severe drought. In addition, both *HDZI-3* and *HDZI-4* promoters improved cold tolerance of transgenic wheat by increasing the plant survival rates, though no improvement was observed in plant yield phenotypes under well-watered conditions or moderate drought.

To better understand the activity of the two promoters in transgenic wheat under different stresses, we tried to find downstream genes of TaCBF5L. Several stress-related genes encoding LEA/DHN/COR were found to be downstream regulated by TaCBF5L under severe drought and cold conditions. It is notable that these potential downstream genes showed different expression under different abiotic stresses. It is probably because TaCBF5L co-operates with other TFs under drought and frost conditions, which could result in divergent expression of downstream genes.

In summary, the functions of the three γ-clade HD-Zip I wheat genes were characterised under drought and cold conditions, which could further explain how wheat plants give response to stress. Besides, these genes can be used as potential tools in bio-engineering to improve the plant stress-tolerance and grain yields under hostile environment. However, the interactions of the genes and corresponding TFs comprise a complicated transcriptional network and the mechanisms still need to be further explored in the future.

Thesis declaration

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- Yang Y., Luang S., Harris J., Riboni M., Li Y., Bazanova N., Hrmova M., Haefele S., Kovalchuk N. and Lopato S. (2017). Overexpression of the class I homeodomain transcription factor TaHDZipI-5 increases drought and frost tolerance in transgenic wheat. *Plant biotechnology Journal*, DOI: 10.1111/pbi.12865, 1–14

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Abbreviation

ABA	Abscisic acid
ACPFG	The Australian Centre for Plant Functional Genomics
AD	Activation domain
AP2/ERF	APETALA2/ ethylene responsive factor
At	*Arabidopsis thaliana*
Bd	*Brachypodium distachyon*
BD	Binding domain
BLAST	The Basic Local Alignment Search Tool
bp	Base pair
CDS	Coding DNA sequence
CRT	C-repeat-binding factor
CPX	Centrophenoxine
DRE	Dehydration-responsive element
DREB	Dehydration-responsive element binding protein
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
ERF	Ethylene responsive factor
EST	Expressed sequence tag
GAPdH	*Glyceraldehyde-3-Phosphate Dehydrogenase*
GFP	Green fluorescent protein
HD	Homeodomain
HD-Zip	Homeodomain leucine zipper
HD-Zip I	Homeodomain-leucine zipper class I

HD-Zip II	Homeodomain-leucine zipper class II
HD-Zip IV	Homeodomain-leucine zipper class IV
Hv	*Hordeum vulgare*
IWGSC	International Wheat Genome Sequencing Consortium
LB	Lysogeny broth
LEA	Late embryogenesis abundant
MESA	MOPS-EDTA-Sodium Acetate
MOPS	3-(N-morpholino) propane sulfonic acid
MQW	Milli-Q water
MYB	Myeloblastosis
NCBI	The National Centre for Biotechnology Information
Os	*Oryza sativum*
PCR	Polymerase chain reaction
PLACE	Plant *cis*-acting regulatory elements
Q-PCR	Quantitative real-time PCR
RT-PCR	Reverse transcription PCR
SSC	Saline-sodium citrate
Sb	*Sorghum bicolor*
Ta	*Triticum aestivum*
Td	*Triticum durum*
TF(s)	Transcription factor(s)
WT	Wild type
WW	Well-watered
Y1H	Yeast one-hybrid
Y2H	Yeast two-hybrid
Zm	*Zea mays*

Publications arising from this thesis

1. Yang, Y., Sornaraj, P., Borisjuk, N., Kovalchuk, N. and Haefele, S.M. (2016) Transcriptional network involved in drought response and adaptation in cereals. In: *Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives* (Shanker, A.K. and Shanker, C. eds), pp 3–29. Rijeka: InTech.

2. Yang Y., Luang S., Harris J., Riboni M., Li Y., Bazanova N., Hrmova M., Haefele S., Kovalchuk N. and Lopato S. (2017). Overexpression of the class I homeodomain transcription factor TaHDZipI-5 increases drought and frost tolerance in transgenic wheat. *Plant biotechnology Journal*, DOI: 10.1111/pbi.12865, 1–14

3. Yang Y, Riboni M, Li Y, Ismagul A, Bazanova N, Chirkova L, Hussain S, Hrmova M, Haefele S, Lopato S, Kovalchuk N. Characterisation of drought-responsive HD-Zip I promoters in transgenic wheat plants. Manuscript in preparation.

Chapter 1 Introduction

1.1 General introduction

Drought and frost, as major abiotic stresses, are posing a threat to crops' growth and yields globally. Traditional breeding can enhance the stress tolerance of crop plants, but it has its limitations and the process of plant selection is usually much slower than transgenic technology (Ruan et al., 2012). A particular target in transgenic technology is transcription factors (TFs). TFs are major regulators of gene expression and metabolic pathway involved in various plant functions. Stress-related TFs temporally and spatially modulate transcription of numerous target genes to provide an efficient way for plants to respond to environmental challenges such as heat, drought and frost.

1.2 Function of HD-Zip I genes in different plant species

Homeodomain leucine zipper (HD-Zip) proteins are a group of TFs which participate in plant growth and development processes. They were classified into four subgroups according to their structural and functional differences. The subgroup I members of HD-Zip TFs were identified to be specifically involved in abiotic stress responses. HD-Zip class I TFs have been reported to be isolated from many species, such as *Arabidopsis* (Ariel et al., 2007), sunflowers (Cabello et al., 2012) and wheat (Lopato, 2006), and some of these TF genes were found to be inducible by environmental stresses such as drought, low temperature and salt.

1.2.1 Role of HD-Zip I TFs from *Arabidopsis* in environmental stresses endurance

The HD-Zip I subfamily in *Arabidopsis* comprises 17 members, and some of the members might play important roles in controlling abscisic acid (ABA) synthesis (Nakamura et al., 2006). Among them are *ATHB1/HAT5, ATHB3/HAT7, ATHB5-7, ATHB12, ATHB13, ATHB16, ATHB20-23, ATHB40,* and *ATHB5I-54* (Ariel et al., 2007). Existing data show that increased ABA concentrations and drought affect the transcript level of ATHB6, -7, and -12 (Lee and Chun, 1998, Söderman et al., 1996). According to Henriksson et al. (2005), factors like ABA, NaCl, low temperature and different light intensity can regulate the transcript level of each member of the *Arabidopsis* HD-Zip subfamily I, and at least some *HD-Zip I* genes can control changes in plant development under environmental stimuli and stresses.

1.2.2 Sunflower HD-Zip I TFs involved in response to abiotic stresses

HaHB1 and HaHB4 are members of the HD-Zip I subfamily which have been isolated from *Helianthus annuus* (common sunflower). According to Cabello et al. (2012), HaHB1 plays a significant role in response to cold temperature. *GUS* reporter gene expression driven by the

HaHB1 promoter has been observed in meristems and siliques of transgenic *Arabidopsis* plants under frost (Cabello et al., 2012). The injury evaluation results and other phenotypical data show that the expression of *HaHB1* enhances the cold tolerance in transgenic sunflower by stabilizing the cellular membrane and reducing formation of extracellular ice crystals (Cabello et al., 2012). HaHB4, is another TF from the sunflower HD-Zip I subfamily. Its involvement in desiccation response in transgenic *Arabidopsis* has been demonstrated (Dezar et al., 2005). *GUS* gene expression controlled by the *HaHB4* promoter has been detected in hypocotyls, stems and leaves of transgenic lines, but not in reproductive tissues (Dezar et al., 2005). In short, both HaHB1 and HaHB4 TFs play roles in environmental stresses. However, HaHB1 is involved in low temperature response, whilst HaHB4 is responsive to drought.

1.2.3 Wheat HD-Zip I TFs participated in abiotic stresses acclimation

Although numerous HD-Zip class I genes in *Arabidopsis* (Ariel et al., 2007), rice (Agalou et al., 2008) and sunflower (Dezar et al., 2005) have been isolated and their involvement in environmental stresses has been demonstrated (Lee and Chun, 1998, Harris et al., 2011, Dezar et al., 2005), there is quite limited information about wheat genes coding for HD-Zip class I TFs. Only isolation of TaHDZipI-1 and TaHDZipI-2, which belong to the wheat HD-Zip subfamily I, has been reported so far (Lopato et al., 2006). According to the research of Lopato et al. (2006), the expression levels of *TaHDZipI-1* and *TaHDZipI-2* genes in flowers and grain were almost the same, but fairly different in the rest of tissues. The gene *TaHDZipI-1* expression was detected in seedlings and mature tissues. It was highly expressed in stems, whilst *TaHDZipI-2* was predominantly expressed in shoots of seedlings and grain, with no expression detected in mature tissues (Lopato et al., 2006).

Three members of the γ-clade HD-Zip subfamily I from *Triticum aestivum* L. cv. RAC875, TaHDZipI-3, -4 and -5, have been isolated by Harris et al. (2016). TaHDZipI-4 and TaHDZipI-5 were isolated from a wheat cDNA library, prepared from spikes and leaves subjected to drought and heat stresses by using an Y2H screen, with TaHDZipI-3 protein as a bait (Harris et al., 2016). Preliminary characterisation demonstrated that the inducibility of the native genes encoded all the three members by elevated concentrations of ABA, cold and drought except for *TaHDZipI-3* (Harris et al., 2016). Besides, the endogenous *TaHDZipI-5* gene showed the highest expression level compared with the other two genes, hence was considered the best candidate out of the three for improving drought and cold stress tolerance in wheat. Therefore, to further characterise its role in stress-response of transgenic wheat has become one part of my project.

Besides, the functions the *TaHDZipI-3*, *TaHDZipI-4* and *TaHDZipI-5* promoters were also intended to be investigate. However, their sequences were not available in the BAC library, hence, their homologous sequences of *TdHDZipI-3*, *TdHDZipI-4* and *TdHDZipI-5* promoters from Durum wheat, were alternatively used for the investigation. In the former work, the TdHDZipI-5 promoter had not been investigated. It was decided this promoter required further investigation as part of this research project. The *TdHDZipI-3* and *TdHDZipI-4* promoters were formerly analysed by Harris (2014). *TdHDZipI-4* has been proven to be inducible by ABA though *TdHDZipI-3* showed no activity when exposed to different stresses. Hence, only *TdHDZipI-4* was further characterised. In Harris' work, it was found that *TdHDZipI-4* contained two *cis*-elements, DPBFCOREDCDC3 (ACACNNG) and MYCCONSENSUSAT (CANNTG). The MYCCONSENSUSAT element was found to be responsive to cold and ABA signaling (Chinnusamy et al., 2003), though limited information about the DPBFCOREDCDC3 element was found through the references. In order to understand how exactly the role the *TdHDZipI-3* and *TdHDZipI-4* promoters play and to find decent promoter candidate for gene overexpression optimization in wheat plants under different abiotic stresses, characterisation of these two promoters in *TaCBF5L* transgenic wheat under drought and frost has become one part of my project.

1.3 Constitutive and stress-inducible promoters for gene expression in transgenic wheat

1.3.1 Constitutive promoters

Maize polyubiquitin and cauliflower mosaic virus (CaMV) 35S are two promoters which are constitutively and ectopically active in most tested plants (Christensen and Quail, 1996). Two polyubiquitin genes, *Ubi 1* and *Ubi 2*, have been isolated from maize by Christensen et al. (1992). Both genes are constitutively expressed at room temperature and are strongly induced by heat (Cornejo et al., 1993). CaMV 35S is another constitutive promoter of viral origin, which is also used in transgenic monocots (Christensen and Quail, 1996). However, it was demonstrated that 35S is less active in monocot than dicot cells (Bruce et al., 1989, McElroy and Brettell, 1994). Because maize polyubiquitin has stronger activity than 35S promoter in monocots, it became a useful alternative of the 35S promoter in plant genetic engineering (Christensen and Quail, 1996).

It has been reported that maize polyubiquitin (Egawa et al., 2006, Zhang et al., 2010, Lopato and Langridge, 2011, Morran et al., 2011) and 35S (Yamaguchi-Shinozaki and Shinozaki, 2001,

Kasuga et al., 2004, Cabello et al., 2012) promoters have been used for constitutive expression of DREB/CBF and HD-Zip TFs in crop plants. Use of these promoters in combination with stress-responsive genes enhanced stress tolerance in transgenic plants (Yamaguchi-Shinozaki and Shinozaki, 2006, Harris et al., 2011), however, it led to growth retardation and pleiotropic phenotypes (Lopato and Langridge, 2011, Morran et al., 2011, Saint Pierre et al., 2012). Stress-inducible promoters such as *Cor39* (Kovalchuk et al., 2013) and *OsHOX24* (Agalou et al., 2008) only worked on the overexpression of target genes under specific stresses, which can reduce the negative influence of the transgene on plant development. Hence, they were used as alternative promoter candidates.

1.3.2 Stress-inducible promoters of *LEA/DHN/Cor/Rab* genes

A number of stress responsive-promoters of late embryogenesis/dehydrin/cold responsive/ responsive to ABA (*LEA*/*DHN*/*Cor*/*Rab*) genes have been reported (Skriver and Mundy, 1990, Ohno et al., 2001, Babu et al., 2004, Xiao et al., 2007, Brini et al., 2007, Amar et al., 2013). These promoters usually have relatively moderate basal levels (constitutive level) of promoter activity and were shown to be activated by several abiotic stresses. The information about *Cor39*, which belongs to the *LEA*/*DHN*/*Cor*/*Rab* genes, is summarized below.

1.3.2.1 The Cor39 promoter from durum wheat

The *Cor39* gene from *Triticum aestivum* L. cv Winoka can be activated to the maximum level after several hours of cold treatment (Guo et al., 1992). In *Vitro* transcription or translation experiment and DNA sequence analysis showed that the *Cor39* gene expression product is a 39 kD hydrophilic polypeptide with two sequence repeats (Guo et al., 1992). One sequence repeat is rich in lysine and was found in other proteins (e.g. Cor47 and group II LEA proteins); the second one is rich in glycine and can also be allocated in some members of group II LEA proteins (Guo et al., 1992).

The *TdCor39* promoter was isolated from the BAC library prepared from *Triticum durum* cv. Langdon (Kovalchuk et al., 2013). It is sensitive to low temperatures and can be activated by cold in stems, leaves and developing spikes of transgenic barley and rice (Kovalchuk et al., 2013). In leaves, the activity of the *TdCor39* gene is strongly induced by cold, and moderately induced by drought and mechanical wounding. Conversely, the *TdCor39* gene expression level is low in embryos, coleoptiles and leaves in the absence of stress. According to Kovalchuk et al. (2013), a reduction in detrimental phenotypes has been observed if transgenic barley was transformed with the pTdCor39-*TaDREB3* construct, instead of the construct for constitutive

expression of the same transgene (Kovalchuk et al., 2013). In contrast to *TaDREB3*, the *TaHDZipI-5* gene expression is induced by ABA. It was expected that the combination of the *TdCor39* promoter (ABA inducible) with the *TaHDZipI-5* gene (ABA inducible) would produce better results than the *TaDREB3* gene, and this became a section of my project.

1.3.2.2 The OsWRKY71 promoter from rice

The *OsWRKY71* gene originates from rice (*Oryza sativa* L.). The expression of this promoter is ABA independent. It is moderately induced by cold and weakly induced by drought and high salinity (Mare et al., 2004). The *OsWRKY71* promoter can be activated under low temperature in stems, leaves and developing spikes of transgenic barley and rice (Kovalchuk et al., 2013). According to the results obtained by Kovalchuk et al. (2013), the pOsWRKY71-TaDREB3 promoter-gene combination provides better phenotypes of transgenic barley plants than the pTdCor39-TaDREB3 construct, and hence the *OsWRKY71* promoter seems to be a promising tool for the optimization of phenotypes and improvement of frost and drought tolerance of transgenic wheat plants. In this thesis, the *OsWRKY71* promoter has been evaluated in transgenic wheat using drought and frost tolerance tests.

1.4 The aim of the project

The current project is aiming to:

- Assess the functions of stress-related *TaHDZipI-5* transgene using constitutive and stress-inducible promoters in wheat stress response;
- Determine the *cis*-elements in the *TaHDZipI-5* homologous gene promoter which contributes to ABA activation
- Identify the activation domain of TaHDZipI-5 TF; and
- Further characterise the wheat HD-Zip I gene promoters *TdHDZipI-3* and *TdHDZipI-4* in transgenic wheat under drought and cold stresses.

Chapter 2 Literature review

This chapter is derived entirely from the published work:

Yang, Y., Sornaraj, P., Borisjuk, N., Kovalchuk, N. and Haefele, S.M. (2016) Transcriptional network involved in drought response and adaptation in cereals. In: *Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives* (Shanker, A.K. and Shanker, C. eds), pp 3–29. Rijeka: InTech.

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Author contribution statements

Yunfei Y (candidate)

Reviewed the literature and composed 83% of the draft and generated all figures and chapters.

I accept the accuracy of this contribution statement and the inclusion of this paper in the thesis.

Signed.... *Date* 18/03/2016

Sornaraj P

Reviewed the literature and composed 17% of the draft.

I accept the accuracy of this contribution statement and the inclusion of this paper in the thesis.

Signed..... *Date* 19/03/16

Borisjuk N

Improved the structure of the draft, supervised the generation of figures and performed editing of the manuscript.

I accept the accuracy of this contribution statement and the inclusion of this paper in the thesis.

Signed.. *Date* 22-3-16

Kovalchuk N

Inspired the initial planning of the manuscript.

I accept the accuracy of this contribution statement and the inclusion of this paper in the thesis.

Signed..... *Date* 23.03.16

Haefele S

Investigated and inspired the initial planning of the manuscript. Performed substantial editing of the manuscript, acted as the corresponding author and submitted the manuscript.

I accept the accuracy of this contribution statement and the inclusion of this paper in the thesis.

Signe *Date* 18.03.2016

Chapter 1

Transcriptional Network Involved in Drought Response and Adaptation in Cereals

Yunfei Yang, Pradeep Sornaraj, Nikolai Borisjuk, Nataliya Kovalchuk and Stephan M. Haefele

Additional information is available at the end of the chapter

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Abstract

Drought is the major abiotic stress in many wheat environments, decreasing grain yields and farmer's income. Finding ways to improve drought tolerance in wheat is therefore a global effort. Transcription factors (TFs) play important roles in drought tolerance by stimulating plant's protective genome activities in response to heat and water limitation. TFs are specialized proteins which can bind to specific DNA elements in gene promoters and modulate gene expression in response to various external and internal stimuli. Thus TFs is a crucial part of plant signal transduction pathway mediated by signal receptors, phytohormones and other regulatory compounds. The activities of TFs are closely related to their structure, and their binding specificity is determined by the homo-/hetero-dimerization of TFs. The expression of downstream genes may produce a subset of TFs or regulate other functional proteins involved in physiological drought adaptation. Thus, the hierarchic regulations of TF activities, downstream gene expression and protein–protein interaction comprise a complex regulatory network, which participates in drought response and adaptation in cereal crops. Basic mechanisms of this regulatory network have been described, but more insight is needed to find new tools for enhancing cereals' adaptation to drought stress.

Keywords: Abiotic stress, cereals, drought, regulatory networks, transcription factors

1. Introduction

Drought is the major environmental factor that limits crop growth and yield globally. Improving crop performance under water limiting conditions is, therefore, an important research focus of plant scientists around the world. Limited water availability evokes adaptive physiological responses regulated by changes in expression of numerous stress-responsible genes.

Transcription factors (TFs) are groups of proteins that bind to specific regulatory DNA elements located in gene promoters upstream of transcription initiation sites, repressing or activating target gene expression. Intensive research in recent years has shown that temporal and spatial modulation of stress-related TFs provides an efficient way for plants to deal with unfavourable growth conditions. TFs involved in drought response were identified and characterized in various cereal species, including all major food crops such as in rice [1–4], wheat [5–8], barley [9, 10], and maize [11, 12]. Overexpression of several target genes encoding stress-responsive TFs led to improved survival rate under water limitation in transgenic plants [4, 10, 12–14].

The activities of TFs are closely related to their structure. TFs usually contain a DNA-binding domain (DBD) and a transcriptional activation domain (TAD) [15]. The DBD enables TFs to bind with specific promoter elements of target genes, and TAD mediates regulation of the downstream gene either directly or in cooperation with other proteins. TAD usually represents a low-complexity sequence that prevents protein self-folding and facilitates protein–protein interactions (PPI). The transcriptional response to drought in cereals is controlled by a large number of TFs, which have been grouped into several different families based on their structure and binding specificity. The main TFs discussed in this article belong to the following families: the DRE-binding protein/C-repeat binding factors (DREB/CBF) [1, 13, 16, 17], the NAM/ATAF1/CUC2 (NAC) factors [18, 19], the MYB family [20, 21], the WRKY family [9, 22], the basic leucine zipper family (bZIP) [14, 23–25], and the homeodomain-leucine-zipper (HD-Zip) family [26–28].

When plants suffer water deficiency, receptors from the cell membrane/cell wall sense the extracellular stress signals and convert them into intracellular secondary messengers such as Ca^{2+} and inositol phosphate [29]. How exactly the signal is transmitted toward gene activation is still poorly understood and is a subject of intensive multidisciplinary investigations. However, it is well agreed that plant hormones, especially abscisic acid (ABA), play significant role in drought stress–related transcription, in many cases through modulating phosphorylation status of transcription factors and other regulatory proteins.

The objective of this article is to review the involvement of TFs in drought response and adaptation in cereals and to illuminate the complexity of the factors and processes involved. The article is subdivided into four sections, which will (1) give examples of drought-related hierarchy in TF interactions regulated by plant hormones, (2) provide an overview of major families of cereal TFs involved in drought response, (3) overview existing data on TF target gene networks activated in response to drought, and (4) describe the homo- and hetero-dimerization in relation to TF's activities.

2. Plant hormone crosstalk in drought relevant regulatory pathways

Phytohormones play critical roles in linking the stress-responsive signaling cascades. ABA is a key plant hormone that functions as a link between environmental stress reception and adaptive transcriptional programs such as the regulation of cellular mechanisms, carbohydrate

and lipid metabolism. Similar to ABA, gibberellic acid (GA) and jasmonic acid (JA) play important functions in cellular stress network signaling. Different receptors have been reported to recognize and bind with these plant growth regulators in order to activate or modulate downstream responses [30].

Drought and ABA-mediated signals are perceived through three main pathways by different receptors (see Figure 1). The first receptor PYR/PYL/ RCARs (PYRABACTIN RESISTANCE / PYRABACTIN RESISTANCE-LIKE / REGULATORY COMPONENT OF ABA RECEPTOR-SPYR) binds ABA and inactivates the type 2C protein phosphatases (PP2Cs), which leads to the accumulation of SNF1-RELATED PROTEIN KINASES (SnRK2s) [30]. SnRK2s activate ABA-responsive TFs such as *Arabidopsis* AREB1, AREB2, and AREB3. These TFs regulate ABA-dependent gene expression involved in several physiological processes such as the movement of stomatal guard cells, thereby increasing the tolerance to drought [30]. AtMYB44 is one of the TFs that negatively regulate the target genes coding PP2Cs, which leads to stomatal closure and reduced transpiration losses [31]. Protein-coupled receptor-type G proteins (GTGs) such as GTG1/GTG2 are involved in the second ABA reception pathway, which was first reported in *Arabidopsis* [30]. GTG1/GTG2 proteins are membrane-localized receptors with functions in seedling and pollen tube growth and development, acting through voltage-dependent anion channels. The third ABA receptor is the H subunit of Mg-chelatase (CHLH/ABAR), which regulates the lipid metabolism linked to drought tolerance in plants [30].

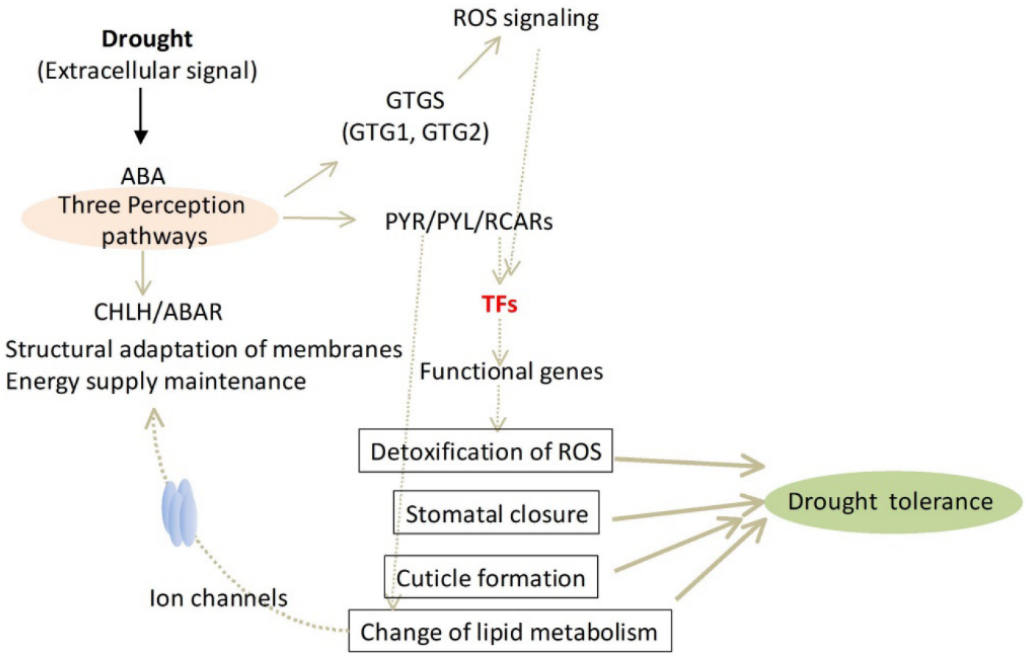

Figure 1. A schematic model of the signal chain from drought stress perception to physiological responses and drought tolerance.

It was also demonstrated that ABA can increase the transcription level of reactive oxygen species (ROS) network genes [30]. ROS are reactive oxygen–based molecules such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which not only toxically damage cells through membrane peroxidation and de-esterification under environmental stresses but also trigger stress endurance in plants [30]. For example, ABA has been shown to trigger the activity of cytosolic aldehyde oxidase and xanthine dehydrogenase, which separately produce H_2O_2 and O_2^- in drought [32].

The GA receptor GA INSENSITIVE DWARF1 (GID1) was reported from rice and is a homolog of the *Arabidopsis* GID1a/b/c [30]. GA-responsive TFs GRAS (GA insensitive [GAI], REPRESSOR of *ga1-3* [RGA], and SCARECROW [SCR]) are GA signaling repressors involved in GA-controlled plant development [30]. Subgroup of GRAS, called DELLA proteins, can interact with GID1 and lead to DELLA protein degradation. The downstream gene of DELLA TFs encoding a RING-H2 zinc finger factor XERICO is involved in ABA and GA transduction pathways under abiotic stresses [30]. Further, the DELLA protein RGL3 can be responsive to JA and interact with the JA regulator OsJAZ (jasmonic acid ZIM-domain protein) under drought [30]. Thus, DELLA proteins can be considered as the interface of ABA, GA, and JA signaling pathways in response to water deficiency [30].

The regulation network of TFs plays an important role in stress-relevant hierarchic regulatory pathways. OsNAC10, a NAC TF, can up-regulate the downstream genes encoding AP2 and WRKY TFs involved in ROS detoxification and scavenging for drought response through the ABA synthesis pathway. The mechanisms of plant response to drought include cell wall development and cuticle formation [30]. The promoter region of the gene *OsNAC6* contains various recognition sites such as ABREs, MYBRS, MYCRS, W-boxes, and GCC boxes, which can be separately recognized by TFs AREB/ABF, MYB, MYC, WRKY, and ERF [33]. These TFs are likely to bind to the corresponding *cis*-elements and co-regulate the expression of *OsNAC6* that participate in the ABA induction pathway and abiotic stress response in plants. In the bZIP family, the gene encoding OsbZIP12 was also found to have MYBRS, MYCRS, and W-box motifs in its promoter region, which can be recognized by TFs MYB, MYC, and WRKY, respectively [34]. Besides, OsNAC5 and OsbZip23 might co-regulate the expression of the downstream gene *OsLEA3* since both of them enhance the transcription level of *OsLEA3* [35]. OsDREB1F might interact directly/indirectly with some bZIP family members in the ABA-dependent pathway that activate transcription of the ABA responsive genes *rd29B* and *RAB18* [3]. However, more in-depth studies are needed to identify these events and to explain the underlying mechanism.

3. Major families of cereal TFs involved in drought response

Transcription factors are classified into several family groups mainly based on characteristic amino acid sequences of its conserved DBDs [36, 37]. Of these, the families DREB/CBF, NAC, MYB, WRKY, bZIP, and HD-Zip are the main TFs involved in drought. Their structural features, classification, and representative family members in cereals are summarized in Table 1 and Figure 2.

TF	Species	TFs name	Cis-element recognition	Downstream genes	Accession/or locus number	Reference	ABA (D/I)	Transgenic plants	Stress inducible/tolerance
DREB	Rice	OsDREB1A	DRE/CRT (G/ACCGAC)	e.g., *cor15A, rd29A, rd17*	AP001168	[1, 129]	D	Rice, *Arabidopsis*	Drought, salt, cold
		OsDREB1E	DRE/CRT (G/ACCGAC)	U	AY785896	[44]	I	Rice	Drought
		OsDREB1F	DRE/CRT (G/ACCGAC)	*rd29A, COR15a, rd29B, RAB18*	AY345234	[3]	D/I	Rice, *Arabidopsis*	Drought, salt, cold
		OsDREB1G	DRE/CRT (G/ACCGAC)	U	XM_483622	[44]	I	Rice	Drought
		OsDREB2A	DRE/CRT (G/ACCGAC)	U	AF300971	[45]	I	Rice	Drought
		OsDREB2B	DRE/CRT (G/ACCGAC)	U	AK099221	[44]	I	Rice	Drought
	Wheat	TaDREB1	DRE/CRT (TACCGACAT)	*rd29A*	AF303376	[41]	I	Rice, *Arabidopsis*	Drought, salt, cold
		TaDREB2	DRE/CRT (G/ACCGAC)	e.g., *TaRAB16.5, TaWZY2, TaWIt10*	GU785008	[13]	I	Barley, wheat	Frost, drought
		TaDREB3	DRE/CRT (G/ACCGAC)	e.g., *TaRAB16.5, TaWZY2, TaWIt10*	GU785009	[13]	I	Barley, wheat	Frost, drought
	Barley	HvDREB1	DRE/CRT (G/ACCGAC)	*rd29A*	DQ012941	[47]	D/I	*Arabidopsis*	Drought, salt, cold
		HvCBF4	DRE/CRT (G/ATCGAC)	e.g., *Atase, LRR, CytP450*	AF298230	[48]	I	Rice	Drought, salt, cold
	Maize	ZmDREB1A	DRE/CRT (G/ACCGAC)	e.g., *COR15A, KIN1, KIN2*	AF450481	[11]	I	*Arabidopsis*	Drought, cold, salt
		ZmDREB2A	DRE/CRT (G/ACCGAC)	*rd29A, rd29B*	AY108198	[110]	I	*Arabidopsis*	Drought, heat
		ZmDREB2.7	DRE/CRT (G/ACCGAC)	U	GRMZM2G028386	[46]	I	*Arabidopsis*	Drought
NAC	Rice	OsNAC5	NACRS (CACG)	*OsLEA3, Os06g0681200*	AK102475	[35, 57, 130]	D	Rice	Drought, salt , cold,
		OsNAC6	NACRS (CACG)	*AK104277, AK110725*	B028185.1	[33, 35, 58]	D	Rice	Drought, cold, salt
		OsNAC9	NACRS	e.g., *NCED, Ca^{2+}ATPase, CCR*	GSE31855	[59]	U	Rice	Drought
		OsNAC10	U	U	U	[60]	D	Rice	Drought, salinity
		OsNAC52	NACRS	e.g., *rd29A, rd29B, RD22*	AAT44250	[61]	D	*Arabidopsis*	Drought
		ONAC045	NACRS	*OsLEA3-1, OsPM1*	CT829509	[19]	D	Rice	Drought, salt
		SNAC1	U	U	AK067690	[18]	D	Rice	Drought, salt
	Wheat	TaNAC-2	NACRS	e.g., *rd29A, rd29B, RD22*	U	[52]	D/I	*Arabidopsis*	Drought
		TaNAC67	NACRS	*DREB2A, COR15, ABI1, ABI2*	KF646593	[54]	D	*Arabidopsis*	Drought, salt, cold
		TaNAC69	NACRS	*Chitinase, ZIM, glyoxalase I*	U	[53]	U	Wheat	Drought
		TaNAC69-1	U	U	AY625682	[131]	U	N	Drought, salinity, heat
	Maize	ZmSNAC1	U	U	U	[132]	D	Sorghum	Drought, cold, salt
		ZmSNAC052	U	U	KM987612	[55]	U	N	Drought, cold
		Zma000584	U	U	KP283536	[55]	U	N	Drought, cold
		Zma006493	U	U	KM670443	[55]	U	N	Drought
		Zma001259	U	U	KM670444	[55]	U	N	Drought, cold, salt
MYBs	Rice	OsMYB3R-2	MYBRS	*Dehydration-responsive element-binding protein 2A, COR15A, RCI2A*	BAD81765	[20]	U	*Arabidopsis*	Drought
		OsMYB4	U	U	Y11414	[71]	U	Apple	Drought, cold
		OsMYB48-1	MYBRS	e.g., *OsPP2C68, RAB21, OsNCED4*	Os01g74410.2	[72]	D	Rice	Drought, salinity
	Wheat	TaMYB3R1	U	U	HQ236494	[73]	D	N	Drought, salt, cold
		TaMYB30-B	MYBRS	*rd29A, ERD1*	U	[7]	I	*Arabidopsis*	Drought
	Maize	ZmMYB-R1	U	U	JQ337942	[70]	D	N	Drought, salt, heat, cold
WRKY	Rice	OsWRKY3	U	U	Os03g55080	[81]	D	N	Drought
		OsWRKY4	U	U	Os06g44010	[81]	U	N	Drought, cold, flood
		OsWRKY8	U	U	Os11g02480	[81]	U	N	Drought, cold
		OsWRKY11	U	U	AK108745	[22]	U	Rice	Drought, heat
		OsWRKY18	U	U	Os02g08440	[81]	U	N	Drought, cold, flood
		OsWRKY22	U	U	Os01g61080	[81]	U	N	Drought, cold, flood
		OsWRKY24	U	U	Os01g43650	[81, 82]	D	N	Drought, cold

TF	Species	TFs name	Cis-element recognition	Downstream genes	Accession/or locus number	Reference	ABA (D/I)	Transgenic plants	Stress inducible/tolerance
WRKY	Rice	OsWRKY42	U	U	Os04g21950	[81]	U	N	Drought, cold
		OsWRKY45	U	U	Os05g14370	[78]	D	*Arabidopsis*	Drought, disease
		OsWRKY50	U	U	Os02g26430	[81]	U	N	Drought, cold, flood
		OsWRKY53	U	U	Os08g29660	[81]	U	N	Drought, cold, flood
		OsWRKY78	U	U	Os11g29870	[81]	U	N	Drought, cold, flood
		OsWRKY84	U	U	Os01g54600	[81]	U	N	Drought, cold, flood
		OsWRKY96	U	U	Os01g14440	[81]	U	N	Drought, cold, flood
		OsWRKY100	U	U	Os09g16510	[81]	U	N	Drought, cold, flood
	Wheat	TaWRKY2	U	*STZ*	EU665425	[82]	D	*Arabidopsis*	Drought, salt
		TaWRKY10	U	U	HQ700327	[83]	U	Tobacco	Drought, salinity
		TaWRKY19	U	*Cor6.6, rd28A, rd29B*	EU665430	[82]	D	*Arabidopsis*	Drought, salt, freezing stress
		TaWRKY44	(TTGACC/ TTAACC)	e.g., *NtSOD, NtAPX, NtCAT*	KR827395	[111]	U	Tobacco	Drought, salt, osmotic stresses
	Barley	Hv-WRKY38	U	U	CAD60651	[133]	U	N	Drought
bZIP	Rice	OsbZip23	ABRE	*OsLEA3-1*, et al.	AK072062	[23, 92]	D	Rice	Drought, salt
		OsbZip12	ABRE	*LEA3, Rab16*	U	[34]	D	Rice	Drought
		OsbZip16	ABRE	*LEA3-1, RAB16C*	Os02g09830	[91]	D	Rice	Drought
		OsbZip45	ABRE	U	Os05g0569300	[92]	D	Rice	Drought
		OsbZip46	ABRE	e.g., *RAB21*	AK103188	[98]	D	Rice	Drought, heat, hydrogen peroxide
		OsbZIP52/ RISBZ5	G-box	*OsLEA3, OsTPP1*, RAB25	Os06g45140	[24]	I	Rice	Drought, cold
		OsbZip71	ABRE or DRE	*OsCAT, OsNHX1, OsMY*	Os09g13570	[25]	D	Rice	Drought, salt
		OsbZip72	ABRE	*LEA3, Rab16*	Os09g28310	[25, 93]	D	Rice	Drought
	Wheat	TabZip60	ABRE	e.g., *Atrd29A, AIRD20, Atrd29B*	KJ562868, KJ806555-KJ806560	[84]	D	*Arabidopsis*	Drought, salt, freezing stress
		TaABP1	U	U	HQ166718	[134]	D	Tobacco	Drought, salt, cold
	Barley	HvbZip13	U	U	U	[86]	D	N	Drought
		HvbZip15	U	U	AK365526.1	[86]	D	N	Drought
		HvbZip18	U	U	AK251589.1	[86]	D	N	Drought
		HvbZip20	U	U	AK359622.1	[86]	D	N	Drought
		HvbZip23	U	U	AK374525.1	[86]	D	N	Drought
		HvbZip29	U	U	AK359391.1	[86]	D	N	Drought, cold
		HvbZip34	U	U	AK365082.1	[86]	D	N	Drought
		HvbZip40	U	U	AK249686.1	[86]	D	N	Drought
		HvbZip42	U	U	AK368116.1	[86]	D	N	Drought
		HvbZip49	U	U	AK369418.1	[86]	D	N	Drought
		HvbZip52	U	U	AK372616.1	[86]	D	N	Drought
		HvbZip53	U	U	AK359129.1	[86]	D	N	Drought
		HvbZip77	U	U	U	[86]	D	N	Drought
	Maize	ZmbZip17	U	U	NM_001158672	[89]	D	N	Drought, heat, salt
		ZmbZip37	U	U	GRMZM5C858197	[88]	U	N	Drought
		ZmbZip72	ABRE	e.g., *rd29B, RAB18, HIS1-3*	HQ328839	[90]	D	*Arabidopsis*	Drought, salt, osmotic stress
		ZmbZip74	U	U	*GRMZM2G448607*	[88]	U	N	Drought
		ZmbZip112	U	U	*GRMZM2G103647*	[88]	U	N	Drought
HD-Zip	Rice	Oshox22	CAAT (G/C) ATTG	U	AY224440	[27]	D	Rice	Drought, salt
		OsHox4	U	U	AF145728	[104]	U	Rice	Drought
	Maize	Zmhdz10	CAATAATTG	U	JX514832	[108]	D	Rice, *Arabidopsis*	Drought, salt

U, Unknown; D, ABA-dependent; I, ABA-independent; N, No transgenic.

Table 1. Overview on the main cereal transcription factor family members involved in drought.

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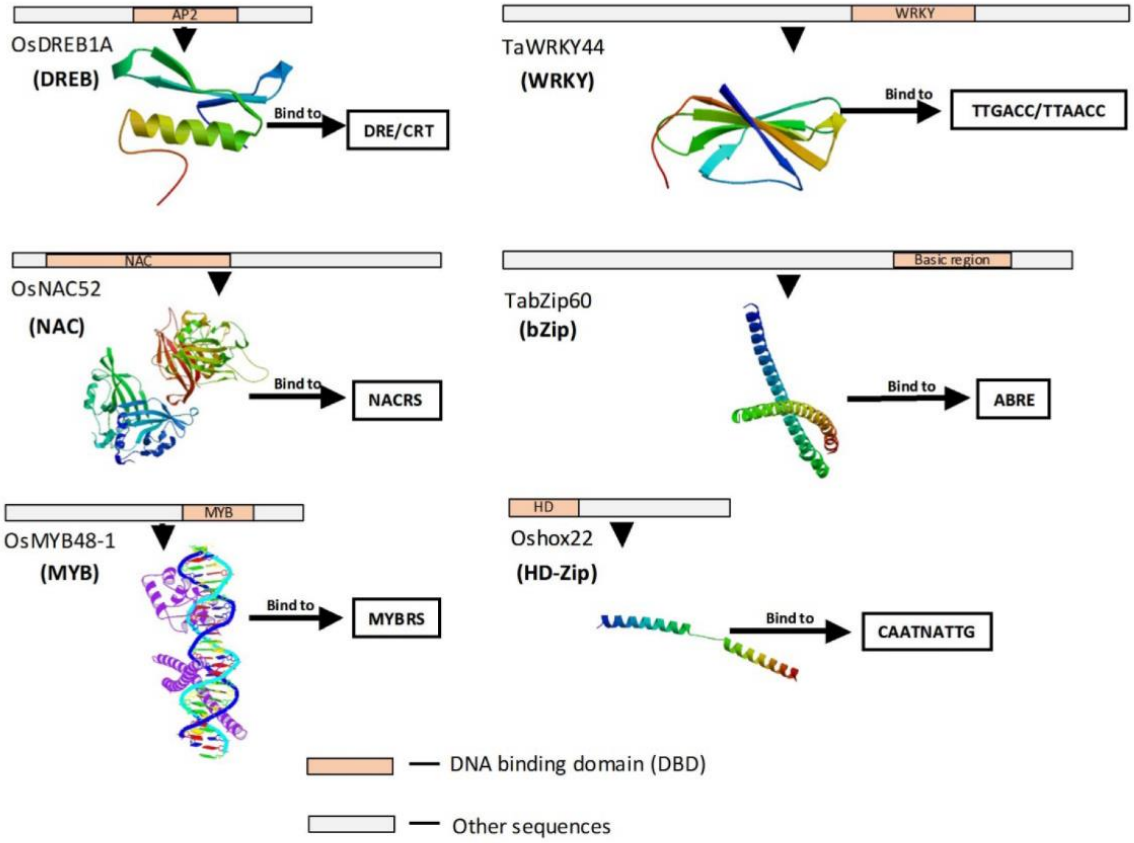

Figure 2. Schematic representation of domain compositions, secondary structures, and recognition sites of major drought-related TF families. The secondary structures were predicted using SWISS-MODEL (http://swissmodel.expasy.org/).

3.1. DREB/CBF family

The DREB/CBF family is a member of the AP2/EREBF superfamily of TFs, responsive to several stresses including drought [3, 8]. A cDNA encoding the first identified DREB/CBF family member CBF1 was isolated from *Arabidopsis thaliana* and characterized by Stockinger et al. [38]. DREB/CBF TFs possess about 60 amino acid long AP2 DBD which specifically recognizes a dehydration-responsive C-repeat (DRE/CRT) *cis*-element. The AP2 is a highly conserved domain of DREB family members. It contains two conserved motifs: the YRG and RAYD motifs. The YRG motif is considered to determine DNA binding and the RAYD motif, which forms an α-helix on the C-terminus, is supposed to play a role in PPI [39]. Drought responsive DREB TFs were also found in other plant species such as *Brassica napus* [40], *Triticum aestivum* [41], *Atriplex hortensis* [17], and *Oryza sativa* [42].

Many reported drought-inducible cereal DREBs were shown to be regulators improving stress endurance. In wheat, the gene *TaDREB1* [41] was induced by drought, salt, and cold. The transgenic barely containing *TaDREB2* and *TaDREB3* [13] showed improved tolerance in drought and low temperature conditions. In rice, 13 transcriptional factors including seven

DREB1 types (OsDREB1A, 1B, 1C, 1D, 1E, 1F, and 1G) and six DREB2 types (OsDREB2A, 2B, 2C, 2D, 2E, and OsAB14) [43] were isolated and analyzed. The overexpression of OsDREB1A [1] and OsDREB1F [3] resulted in transgenic *Arabidopsis* and rice plants with higher tolerance to salt, drought, and low temperature. OsDREB1G, 2A, and 2B were identified to be strong candidates in drought responsive pathways, while OsDREB1E could slightly improve the drought survival rate in transgenic rice [44, 45]. In different wheat cultivars, TaDREB1 was demonstrated to be inducible by drought, salt, low temperature, and ABA [41]. TaDREB2 and TaDREB3 significantly improved frost and drought tolerance in transgenic barley and wheat [13]. In maize, ZmDREB1A [11], –2A [94], and ZmDREB2.7 [46] contributed to drought tolerance. In barley, the gene *HvDREB1* [47] was induced by drought, salt, and low temperature, while the constitutive expression of HvCBF4 [48] increased the survival rate of transgenic rice under drought.

3.2. NAC family

The NAM/ATAF/CUC (NAC) TFs contain a unique feature, a conserved N-terminus DBD and a dissimilar C-terminus regulatory domain, and they are spread across the plant kingdom [37]. The name NAC is an abbreviation of three genes designated as no apical meristem (*NAM*), *Arabidopsis* transcription activation factor (*ATAF*), and cup-shaped cotyledon (*CUC*), which encode proteins containing homologous sequences as the NAC domain [49, 50]. *NAM* isolated from petunia by Souer et al. [49] was the first gene demonstrated to encode a NAC protein, followed by the gene *CUC2* from *Arabidopsis* [50]. Mutation of both genes resulted in the absence of apical shoot meristems [51] and led to floral abnormalities such as the alteration of petal primordia positions during the development stage. These evidences show that the TFs NAM and CUC play important roles in shoot apical meristem formation and determine the organ primordia positions in the floral meristem [49, 50]. But in relation to abiotic stress, the wheat genes *TaNAC2* [52] and *TaNAC69* [53] were strongly expressed under water deficiency and salinity. TaNAC67 was found to decrease the cell membrane instability, preventing water loss and enhancing other physiological processes that were considered to be responsive to drought, low temperature, and salt stress [54]. In maize, ZmNAC052, Zma000584, Zma006493, Zma001259z [55], and ZmSNAC1 showed increased transcription levels under water deficiency, indicating their potential role in drought tolerance regulation. In rice, SNAC1 [56], OsNAC5 [57], –6 [58], –9 [59], and –10 [60] altered the root structure for plant adaptation during drought. Further, the overexpression of *OsNAC045* [19] and *OsNAC52* [61] induced ABA sensitivity and conferred drought resistance in transgenic rice and transgenic *Arabidopsis*, respectively.

3.3. MYBs family

MYB is a group of ancient TFs found in viruses [62] and eukaryotes such as plants, animals, and fungi [63]. The first gene (c1) identified to encode MYB in plants was from *Zea Mays* [64]. MYB TFs contain a conserved DBD called MYB domain characterized by one to three imperfect repeated amino acid sequences (R1, R2, and R3). Each repeat sequence has around 50–53 amino acids which form three α-helixes [65]. These three α-helices form a helix-turn-helix structure

when interacting with DNA. The MYB TFs are mostly classified into three classes according to the number of the MYB domain repeats: R1-MYB, R2R3-MYB [66], and R1R2R3-MYB [67]. These TFs participate in responses to dehydration, salt, cold, and drought [20, 68, 69]. In maize, the ZmMYB-R1 was induced by ABA, drought, low temperature, high salt, and heat [70]. The overexpression of rice genes *OsMYB3R-2* [20], *OsMYB4* [71], and OsMYB48-1 [72] improved the adaptive response to drought and other stresses in transgenic plants. In wheat, TaMYB3R1 [73] and TaMYB30-B [7] were found to be potentially involved in drought adaptation.

3.4. WRKY family

WRKY proteins belong to the superfamily WRKY-GCM1 of zinc finger TFs [74]. They exist in numerous plant species [75] and were reported to be involved in several biotic and abiotic stress responses and developmental processes such as embryogenesis and leaf senescence [75]. WRKY family members contain a highly conserved WRKY domain with 60 amino acids comprised of two motifs. One is the conserved WRKYGQK motif on the N-terminus and the other one is a zinc-finger-like motif on the C-terminus [76]. There are three main groups in the WRKY family according to the different number of WRKY domains and the variable structure of the zinc-finger-like motif [76]. Group I has two WRKY domains, whereas groups II and III have one WRKY domain. The zinc finger motifs of the WRKY domain in groups I and II are the same, but different in group III [75]. The group II has been divided into five subgroups by Eulgem et al. [76], designated as IIa, IIb, IIc, IId, and IIe, according to the conserved motifs outside the WRKY domain. Within these five subgroups, Zhang et al. [77] distinguished another three new groups (2_a+2_b, 2_c, 2_d+2_e). Several drought-related WRKY TFs were found in rice, wheat, and barley [78–80]. In rice, *OsWRKY3, –4, –8, –18, –22, –24, –42, –50, –53, –78, –84, –96,* and *–100* were found to be co-expressed in drought and cold stress, and some of them were even expressed in different organs of flooded plants [81]. OsWRKY11 was identified to be involved in drought and heat response [22]. OsWRKY45 was found to be sensitive to ABA and considered to play a role in stomatal closure to improve drought and salt tolerance [78]. In barely, HvWRKY38 was shown to have a function in drought and cold response [9]. In wheat, the overexpression of TaWRKY2, –19 [82], and –10 [83] led to improved drought and salt adaptation in transgenic plants.

3.5. bZIP family

The basic leucine zipper (bZIP) family is another big group of TFs involved in diverse functions such as hormone and sugar signaling and organ development [84]. bZIP proteins commonly have a basic region for DNA binding and a conserved leucine zipper motif [85]. These TFs specifically bind to a DNA sequence with a core *cis*-element ACGT-like TACGTA (A-box), GACGTC (C-box), CACGTG (G-box), and an ABA-responsive element (ABRE) [84]. Some bZIP members were identified to participate in transducing ABA-dependent stress signals and were named as ABRE binding proteins (AREBs) or ABRE binding factors (ABFs) [86]. Numerous bZIP proteins were demonstrated or predicted to be involved in abiotic stress response in cereal plants, e.g., 89 in rice [87], 171 in maize [88], and 141 in barley [86]. They were classified into 11 groups A, B, C, D, E, F, G, H, I, S, and U according to the phylogenetic trees and DNA

binding motif [86] in maize, rice, and barely. The overexpression of several cereal bZIP TFs was identified to be in response to drought stress. In barley, 11 HvbZIP members were identified to be down- or up-regulated by drought [86]. In maize, ZmbZIP37, -17, and -112 showed high expression levels in drought stress conditions [88, 89] and the overexpression of ZmbZIP72 enhanced the drought tolerance in transgenic *Arabidopsis* [90]. In rice, OsbZIP12 [34], -16 [91], -23, -45 [92], -71 [25], and -72 [93] play a positive role in drought tolerance through ABA signal, while OsbZIP52 [24] and -46 [94] were suggested to be a negative regulator in water deficiency. In wheat, the gene encoding *TabZIP60* was highly induced by salt, cold, and ABA, and the overexpression of *TabZIP60* enhanced the drought and frost tolerance in transgenic *Arabidopsis* [84].

3.6. HD-Zip family

The homeodomain leucine zipper (HD-Zip) family is a group of proteins that are unique to the plant kingdom. All members of the HD-Zip family contain the combination of homeodomain (HD) [6] with a following leucine zipper (Zip or LZ). HD is a conserved protein domain containing a 60 amino acids sequence, which is present in all eukaryotic species [95]. HD is a folded structure with three helices, which are responsible for the specific protein-DNA interactions [95]. HD-Zip family proteins have been classified into HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV [96–100] according to different domain structure and functions. According to Chan et al. [101], HD-Zip I TFs have less conserved motifs than HD-Zip II, and the sequences of HD-Zip I outside the HD-Zip domain are quite different, whereas HD-Zip II TFs have several common sequences outside the HD-Zip domain. The HD-Zip III TFs have four additional amino acids on the conjunction of HD and LZ compared with other three subfamilies [102]. HD-Zip I TFs contain no lipid/sterol-binding domain, designated StAR-related lipid transfer (START) domain, which was found in HD-Zip III and HD-Zip IV TFs [102]. Hence, the special structural feature of HD-Zip I TFs is the presence of HD and leucine zipper and the absence of common sequences outside the HD-Zip domain [103].

In rice, the *HD-Zip I* subfamily has 14 members: *Oshox4-6*, *Oshox*8, *Oshox*12-14, *Oshox*16, and *Oshox*20-25 [104]. Three of them, *OsHOX6*, *OsHOX22* and *OsHOX24*, are homologs of the ABA and abiotic stress-inducible genes *AtHB7* and *AtHB12 in Arabidopsis* [105]. *OsHOX22* and *OsHOX24* have been identified to be involved in drought, cold, and ABA response. *OsHOX22* is strongly activated by high salinity and ABA, but it is weakly induced by frost [27]. Zhang et al. [27] found that the insertion of T-DNA into the *OsHOX22* promoter region led to a decreased gene expression level of *OsHOX22* and reduced ABA content, but improved drought and salt endurance of rice seedlings. The authors believe that *OsHOX22* is a negative regulator for stress response by regulating an ABA-mediated signal transduction pathway and ABA biosynthesis [27]. The *OsHOX24* promoter has shown strong activation by water deficiency and high salinity [106]. According to the results of Agalou et al. [104], *OsHOX22* and *OsHOX24* can be induced by drought in drought-sensitive and drought-resistant cultivars, whereas *OsHOX6* can only be induced in drought sensitive cultivars. Although the role of OsHOX6 and OsHOX24 TFs is still not clear, the homologs of these TFs, AtHB7 and AtHB12, have been found to be involved in ABA modulation by regulating the protein phosphatase 2C

activation and an ABA receptor gene activity [107]. In short, TFs Oshox6, –22, and –24 are responsive to dry conditions, similarly as the *ATHB7* and *ATHB12*, and their involvement in drought response might have relevance for ABA synthesis regulation [104].

In maize, the HD-Zip TF Zmhdz10 was found to play an important role in drought response [108], and in wheat, only two HD-Zip TFs, TaHDZipI-1 and TaHDZipI-2, were reported so far [109]. However, there is no information about the function of wheat HD-Zip proteins in drought response.

4. TFs target gene network activated in response to drought

TFs are involved in target gene network regulation through their DBD interaction with different gene promoter *cis*-elements mediated by ABA-dependent or ABA-independent signal transduction pathway (see Figure 3).

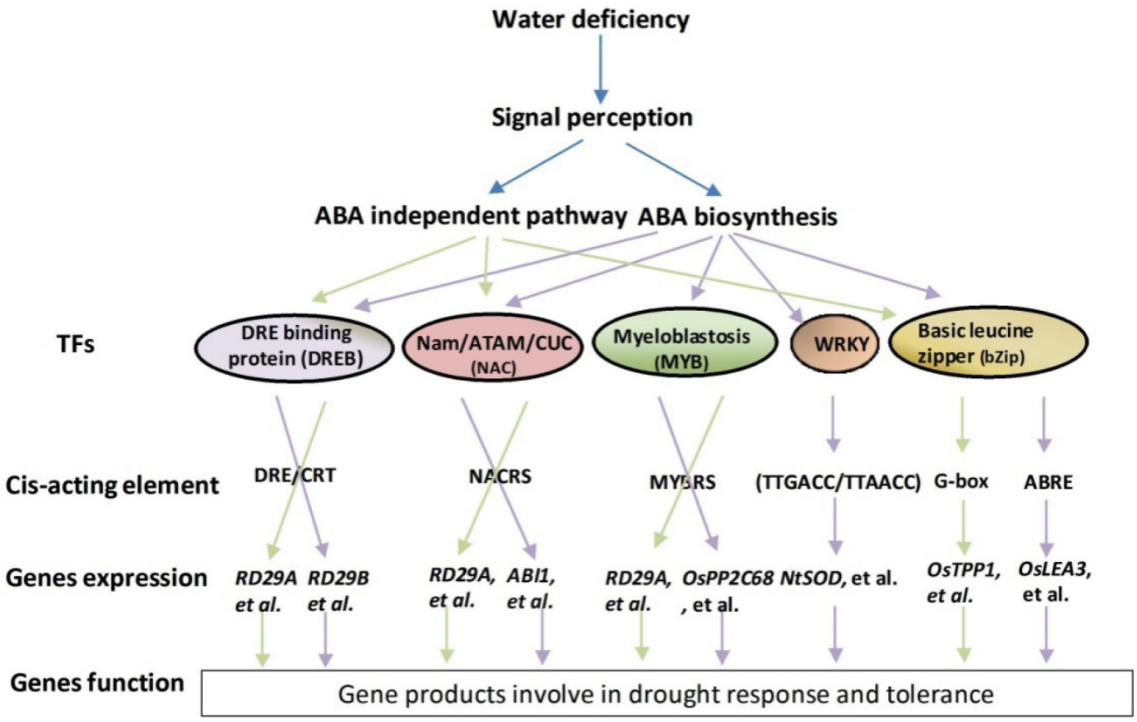

Figure 3. Cereal TFs target gene networks in response to drought through ABA-dependent and -independent pathways. The green arrows show the ABA-independent pathway and the purple arrows show the ABA-dependent pathway.

Most DREB family members such as OsDREB1A [1] and TaDREB1 [41] were found to be activated in ABA-independent pathways. They can enhance the stress tolerance by activating the expression of downstream genes such as late embryogenesis abundant (*LEA*) genes driven

by DRE/CRT *cis*-element. *LEA* genes, such as *COR15A* and *rd29A* (*Cor78*), are also designated as dehydrins (DHNs) or cold-responsive (COR) genes. Their expression products participate in cells protection from stresses by enhancing the membrane stability and correction of protein folding [13]. However, recent studies showed that some members of DREB also participate in ABA-dependent pathways. Wang et al. [3] found that the overexpression of *OsDREB1F* in transgenic rice results in the expression of ABA-induced genes *rd29B* and *RAB18*, whose promoters only contain an AREB element that cannot be recognized by DREB TFs. So far, there is no explanation on illuminating the way for OsDREB1F activating the expression of these two genes. The hypothesis is that OsDREB1F might interact with bZIP family members that can bind with an AREB element and modulate the transcription of *rd29B* and *RAB18* [3]. The same is true for ZmDREB2A [110] and the mechanism is still not clear. Besides, ZmDREB2A was identified to activate some downstream genes encoding detoxification enzymes that can protect cells from ROS. However, these genes do not contain a DRE/CRT element. Hence, it is possible that ZmDREB2A indirectly affects these genes' expression [110]. Besides, DREBs in different species might have different preference in regulating the expression of downstream genes with different core elements in their promoters. The rice OsDREB1A, e.g., prefers to interact with the CRT/DRE core element GCCGAC of genes such as *cor15A*, *rd29A*, and *rd17* instead of core element ACCGAC, while the *Arabidopsis* DREB1A and maize ZmDREB1A have equal competition for recognizing core element GCCGAC and core element ACCGAC in the downstream genes [1, 11].

In the WRKY family, more than 10 TFs isolated from rice were found to co-express under drought and cold stresses, but the downstream genes were still not determined [81]. TaWRKY44 from wheat was identified to recognize the core element (TTGACC/TTAACC) in the promoter region of downstream genes and up-regulate genes encoding antioxidant enzymes such as NtSOD, stress-defensive proteins such as NtERD10C, and lipid-transfer proteins such as NtLTP1 to increase plants survival rate in drought. Thus, TF TaWRKY44 participates in regulating antioxidant enzyme activity and decreasing the ROS levels in order to prevent oxidative damage in plant cells [111].

Members of the MYB family regulate the expression level of different target genes involved in the ABA-dependent and independent pathways. The overexpression of OsMYB48-1 was found to regulate genes such as *OsPP2C68*, *RAB21*, and *OsNCED4*, respectively, involving in ABA early signaling, late response, and the ABA synthesis pathway, contributing to increased drought tolerance under water deficiency [72]. In transgenic *Arabidopsis*, the overexpression of *OsMYB3R-2* increased the expression level of the downstream genes *DREB2A*, *COR15A*, and *RCI2A* and enhanced the plants adaptation to abiotic stresses [20]. Besides, TaMYB30-B was found to induce the expression of stress inducible genes *rd29A* and *ERD1*, involved in the ABA-independent pathway [7].

Members of the bZIP family were also found to regulate downstream gene transcription through the ABA-dependent and independent pathway. OsbZIP52 was suggested to bind to G-box *cis*-elements and down-regulate genes such as *OsLEA3* and *OsTPP* that can improve drought or cold tolerance in rice via the ABA-independent pathway [24]. The expression of

downstream genes *LEA3* and *Rab16* was activated by the transcription factor OsbZIP12 under water deficiency mediated by the ABA synthesis pathway [34].

Most TFs in the NAC family participate in the ABA synthesis pathway. OsNAC5 and OsNAC6 can recognize a core sequence (CACG) of the downstream gene *OsLEA3* and regulate the gene expression that changed root structure and resulted in higher drought tolerance through the ABA signaling pathway [35]. OsNAC6 also participates in up-regulating the transcription of genes encoding peroxidase, which can catalyze a series of oxidative reactions [33]. Some TFs even participate in both ABA-dependent and independent pathways. For example, TaNAC67 was found to up-regulate 10 abiotic stress responsive genes such as *rd29A* and *rd29B*, which were separately related to ABA-independent and -dependent pathways and four ABA synthesis/responsive genes such as *ABI1* [54], thereby improving stress tolerance in plants.

5. Homo- and hetero-dimerization of TFs

Homo- and hetero-dimerization of TFs plays an important role in certain cases and is considered as a pre-requisite for binding of DNA *cis*-elements. Formation of homo- and hetero-dimers plays a further function in modulating the DNA-binding specificity of TFs. Inability to form a dimeric complex may absolutely abolish the DNA binding ability of certain classes of TFs. The high complexity in the selection of hetero-dimerization partners and inability of some TFs to homo-dimerize but hetero-dimerize suggests that homo- and hetero-dimerization of TFs are not random processes, but that specific interactions between monomeric TFs forms are preferred. Hence, dimerization is likely to fulfill specific functions in gene regulation.

The dimerization ability of NAC proteins has been localized to the NAC domain [112, 113]. The residues in the highly conserved NAC domain are involved in the dimer contact and consist of hydrophobic interactions, a twisted anti parallel β-sheet sandwiched between two helices and two prominent salt bridges formed by the conserved arginine and glutamate [114, 115]. Experimental data suggest that NAC TFs are capable of forming both homo- and hetero-dimers. The NAC domain of NAC1 [116] and ANACO19 [113, 114] were shown to form homo-dimers. The NAC domains of OsNAC5 were shown to interact with the NAC domains of OsNAC5, OsNAC6, and SNAC1, generating both homo- and hetero-dimeric complexes. BnNAC14, a *Brassica napus* NAC protein, was shown to form hetero-dimers with BnNAC5-8, BnNAC485, and BnNAC3, but not homo-dimers. Mutational and deletion studies suggested that conserved NAC domains, in particular, the amino acids in close proximity to both the amino and carboxy-terminals, are necessary for mediating the formation of homo- or hetero-dimers [106, 112, 117].

Dimerization of the bZIP class of TFs is mediated by leucine zipper motifs, i.e., non-canonical repeats of leucine or other hydrophobic amino acid residues creating an amphipathic α-helix. The electrostatic attraction and repulsion of the polar residues situated next to the hydrophobic residues enables the formation and stabilization of dimers [118, 119]. Homo-dimeric rice OsbZIP71 is capable of exchanging its subunit to form hetero-dimers with members of the Group-C, in particular, with OsbZIP15, OsbZIP20, OsbZIP33, and OsbZIP88, suggesting a

possible role of hetero-dimerization in efficient binding to *cis*-elements on promoters of target genes [25]. A member of Group-A, the G-box-binding factor AtGBF4, interacts with the Group-G AtGBF1 and the Group-H AtGBF2. Similarly, the members of *Arabidopsis* Group-E, bZIP34 and bZIP61, form hetero-dimers with bZIP51 of Group-I and bZIP43 of Group-S, but none of these TFs belonging to Groups E and I can form homo-dimers due to electrostatic violations in the leucine zipper regions [15, 120, 121]. These data suggest that dimerization between members of within and between groups of bZIP TFs is highly specific and acts as a crucial mechanism to modulate the affinity for *cis*-elements and function of TFs.

In HD-Zips, the leucine zipper that is immediately downstream of the helical domain enables dimerization of HD-Zip TFs, which is a pre-requisite for DNA binding. The HD-Zip leucine zipper is a canonical repeat of leucine amino acid at every seventh residue creating an amphipathic α-helix, which forms a coiled coil structure during dimerization. Formation of hydrophobic interface and complementary charge interactions by the residues present in the coiled coil structure permit or inhibit the formation of dimers from monomeric HD-Zip TFs [105]. *In-vitro* studies have shown that dimerization of HD-Zip is a pre-requisite for DNA binding, and it is assumed that members of HD-Zip Class I and Class II families form hetero-dimers exclusively with other members of their own family [105, 122–124].

Though there is clear evidence for homo- and hetero-dimerization of WRKY proteins, the extent to which they form a functional dimer is unknown and yet to be determined. Of the seven WRKY subclasses, interaction between members of four WRKY class TFs have been experimentally demonstrated. In Group IIa WRKY TFs, dimerization is mediated by a canonical leucine zipper sequence, whereas in members belonging to other Group II and Group III subclasses, presence of leucine/isoleucine/valine residues at approximate seven-residue intervals at their N-termini form an amphipathic alpha helices similar to the secondary structure of a basic leucine zipper and mediate dimerization [125]. It is suggested that these potential leucine zipper sequences might mediate the formation of homo- and hetero-dimers within and between members of different subclasses of WRKY TFs. For example, *Arabidopsis* WRKY TFs belonging to the Group IIa, AtWRKY 18, AtWRKY40, and AtWRKY60, form homo- and hetero-dimers [126]. Similarly AtWRKY30 interacted with AtWRKY53, AtWRKY54, and AtWRKY70 and formed hetero-dimers through leucine zipper motifs present at the N-termini of the subclass of WRKY TFs [127]. Interaction between different subclasses was observed in rice. OsWRKY71, a Group IIa WRKY TF, interacted not only with itself, but also with a Group IId WRKY protein, OsWRKY51 [128]. Formation of homo- and hetero-dimer complexes between different WRKY TFs can have positive or negative effects on their DNA binding activities.

Formation of homo- and hetero-dimers offers an additional large combinatorial flexibility in the regulation of transcription. Performing an accurate analysis and developing a deeper understanding of roles of TFs in various biological processes will require the knowledge of other interacting partners, downstream genes, and location of expression in plant organs along with mechanism of homo- and hetero-dimerization of particular TFs. Thus, it may prove difficult to attempt to make informative conclusions about the roles of specific TFs on the basis of their singular overexpression without this level of knowledge.

6. Conclusions

TFs play a vital role in regulating gene transcription through different signal pathways to enable plants to adapt to harsh environments and abiotic stresses such as drought. Those TFs can recognize and interact with specific *cis*-elements of target genes via DBDs. Some TFs can up-/down-regulate downstream gene transcription, which encodes a subset of TFs integrated in plant hormone signaling pathways, forming a complex hierarchic regulatory network. ABA, JA, and GA, the main plant hormones, act as key regulators in balancing plant growth and abiotic stress response. TFs, as the node of the cellular stress network and growth process, function as the interface of different phytohormone signal transduction pathways. A further layer of complexity is the formation of homo- and hetero-dimers, playing an important role in regulating DNA-binding specificity of TFs. These networks of signal pathways are regulating the activity of stress response TFs and other stress-relative genes, which in turn modulate physiological functions, such as stomatal movement, cuticle formation, and carbohydrate and lipid metabolism, to limit water loss and adapt to drought conditions. However, the hierarchy of TF interactions, the downstream genes' network, the interaction mechanism of the signal transduction pathways, and the protein–protein dimerization are not fully explored and still need more effort to be understood. More knowledge about plant protection system in hostile environments will help to find new tools for enhancing the plants to adaptat to abiotic stresses.

Author details

Yunfei Yang, Pradeep Sornaraj, Nikolai Borisjuk, Nataliya Kovalchuk and Stephan M. Haefele*

*Address all correspondence to: stephan.haefele@acpfg.com.au

Australian Centre for Plant Functional Genomics (ACPFG), University of Adelaide, Adelaide, Australia

References

[1] Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, et al. OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought, high salt and cold responsive gene expression. The Plant Journal. 2003;33(4):751-763. DOI: 10.1046/j.1365-313X.2003.01661.x

[2] Chen JQ, Meng XP, Zhang Y, Xia M, Wang XP. Over-expression of OsDREB genes lead to enhanced drought tolerance in rice. Biotechnology Letters. 2008;30(12): 2191-2198.DOI: 10.1007/s10529-008-9811-5

[3] Wang Q, Guan Y, Wu Y, Chen H, Chen F, Chu C. Overexpression of a rice OsDREB1F gene increases salt, drought, and low temperature tolerance in both Arabidopsis and rice. Plant Molecular Biology. 2008;67(6):589-602. DOI: 10.1007/s11103-008-9340-6

[4] Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, et al. Genome-wide analysis of NAC transcription factor family in rice. Gene. 2010;465(1):30-44. DOI: 10.1016/j.gene.2010.06.008

[5] Liu H, Zhou X, Dong N, Liu X, Zhang H, Zhang Z. Expression of a wheat MYB gene in transgenic tobacco enhances resistance to Ralstonia solanacearum, and to drought and salt stresses. Functional & Integrative Genomics. 2011;11(3):431-443. DOI: 10.1007/s10142-011-0228-1

[6] Rahaie M, Xue GP, Naghavi MR, Alizadeh H, Schenk PM. A MYB gene from wheat (Triticum aestivum L.) is up-regulated during salt and drought stresses and differentially regulated between salt-tolerant and sensitive genotypes. Plant Cell Reports. 2010;29(8):835-844. DOI: 10.1007/s00299-010-0868-y

[7] Zhang L, Zhao G, Xia C, Jia J, Liu X, Kong X. A wheat R2R3-MYB gene, TaMYB30-B, improves drought stress tolerance in transgenic Arabidopsis. Journal of Experimental Botany. 2012;63(16):5873-5885. DOI: 10.1093/jxb/ers237

[8] Kovalchuk N, Jia W, Eini O, Morran S, Pyvovarenko T, Fletcher S, et al. Optimization of TaDREB3 gene expression in transgenic barley using cold-inducible promoters. Plant Biotechnology Journal. 2013;11(6):659-670. DOI: 10.1111/pbi.12056

[9] Mare C, Mazzucotelli E, Crosatti C, Francia E, Cattivelli L. Hv-WRKY38: A new transcription factor involved in cold-and drought-response in barley. Plant Molecular Biology. 2004;55(3):399-416. DOI: 10.1007/s11103-004-0906-7

[10] Oh SJ, Kwon CW, Choi DW, Song SI, Kim JK. Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. Plant Biotechnology Journal. 2007;5(5):646-656. DOI: 10.1111/j.1467-7652.2007.00272.x

[11] Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K, et al. Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in Zea mays L. Plant and Cell Physiology. 2004;45(8):1042-1052. DOI: 10.1093/pcp/pch118

[12] Wang CT, Dong YM. Overexpression of maize ZmDBP3 enhances tolerance to drought and cold stress in transgenic Arabidopsis plants. Biologia. 2009;64(6):1108-1114. DOI: 10.2478/s11756-009-0198-0

[13] Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, et al. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnology Journal. 2011;9(2):230-249. DOI: 10.1111/j.1467-7652.2010.00547.x

[14] Kobayashi F, Maeta E, Terashima A, Kawaura K, Ogihara Y, Takumi S. Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat. Journal of Experimental Botany. 2008;59(4):891-905. DOI: 10.1093/jxb/ern014

[15] Veerabagu M, Kirchler T, Elgass K, Stadelhofer B, Stahl M, Harter K, et al. The interaction of the arabidopsis response regulator arr18 with bzip63 mediates the regulation of PROLINE DEHYDROGENASE expression. Molecular Plant. 2014;7(10): 1560-1577. DOI: 10.1093/mp/ssu074

[16] Zhao L, Hu Y, Chong K, Wang T. ARAG1, an ABA-responsive DREB gene, plays a role in seed germination and drought tolerance of rice. Annals of Botany. 2010;105(3): 402-409. DOI: 10.1093/aob/mcp303

[17] Shen YG, Zhang WK, Yan DQ, Du BX, Zhang JS, Liu Q, et al. Characterization of a DRE-binding transcription factor from a halophyte Atriplex hortensis. Theoretical and Applied Genetics. 2003;107(1):155-161. DOI: 10.1007/s00122-003-1226-z

[18] Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, et al. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(35):12987-12992. DOI: 10.1073/pnas.0604882103

[19] Zheng X, Chen B, Lu G, Han B. Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. Biochemical and Biophysical Research Communications. 2009;379(4):985-989. DOI: 10.1016/j.bbrc.2008.12.163

[20] Dai X, Xu Y, Ma Q, Xu W, Wang T, Xue Y, et al. Overexpression of an R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic Arabidopsis. Plant Physiology. 2007;143(4):1739-1751. DOI: 10.1104/pp. 106.094532

[21] Qin Y, Wang M, Tian Y, He W, Han L, Xia G. Over-expression of TaMYB33 encoding a novel wheat MYB transcription factor increases salt and drought tolerance in Arabidopsis. Molecular Biology Reports. 2012;39(6):7183-7192. DOI: 10.1007/ s11033-012-1550-y

[22] Wu X, Shiroto Y, Kishitani S, Ito Y, Toriyama K. Enhanced heat and drought tolerance in transgenic rice seedlings overexpressing OsWRKY11 under the control of HSP101 promoter. Plant Cell Reports. 2009;28(1):21-30. DOI: 10.1007/ s00299-008-0614-x

[23] Xiang Y, Tang N, Du H, Ye H, Xiong L. Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. Plant Physiology. 2008;148(4): 1938-1952. DOI: 10.1104/pp.108.128199

[24] Liu C, Wu Y, Wang X. bZIP transcription factor OsbZIP52/RISBZ5: A potential negative regulator of cold and drought stress response in rice. Planta. 2012;235(6): 1157-1169. DOI: 10.1007/s00425-011-1564-z

[25] Liu C, Mao B, Ou S, Wang W, Liu L, Wu Y, et al. OsbZIP71, a bZIP transcription factor, confers salinity and drought tolerance in rice. Plant Molecular Biology. 2014;84(1-2):19-36. DOI: 10.1007/s11103-013-0115-3

[26] Shen H, Liu C, Zhang Y, Meng X, Zhou X, Chu C, et al. OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice. Plant Molecular Biology. 2012;80(3): 241-253. DOI: 10.1007/s11103-012-9941-y

[27] Zhang S, Haider I, Kohlen W, Jiang L, Bouwmeester H, Meijer AH, et al. Function of the HD-Zip I gene Oshox22 in ABA-mediated drought and salt tolerances in rice. Plant Molecular Biology. 2012;80(6):571-585. DOI: 10.1007/s11103-012-9967-1

[28] Zhao Y, Ma Q, Jin X, Peng X, Liu J, Deng L, et al. A novel maize homeodomain-leucine zipper (HD-Zip) I gene, Zmhdz10, positively regulates drought and salt tolerance in both rice and Arabidopsis. Plant and Cell Physiology. 2014;55(6):1142-1156. DOI: 10.1093/pcp/pcu054

[29] Wohlbach DJ, Quirino BF, Sussman MR. Analysis of the Arabidopsis histidine kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. Plant Cell Online. 2008;20(4):1101-1117. DOI: 10.1105/tpc.107.055871

[30] Golldack D, Li C, Mohan H, Probst N. Tolerance to drought and salt stress in plants: Unraveling the signaling networks. Frontiers in Plant Science. 2014;5. DOI: 10.3389/fpls.2014.00151

[31] Cominelli E, Galbiati M, Tonelli C. Transcription factors controlling stomatal movements and drought tolerance. Transcription. 2010;1(1):41-45. DOI: 10.4161/trns.1.1.12064

[32] Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell & Environment. 2010;33(4):453-467. DOI: 10.1111/j.1365-3040.2009.02041.x

[33] Nakashima K, Tran L-SP, Van Nguyen D, Fujita M, Maruyama K, Todaka D, et al. Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. The Plant Journal. 2007;51(4): 617-630. DOI: 10.1111/j.1365-313X.2007.03168.x

[34] Joo J, Lee YH, Song SI. Overexpression of the rice basic leucine zipper transcription factor OsbZIP12 confers drought tolerance to rice and makes seedlings hypersensitive to ABA. Plant Biotechnology Reports. 2014;8(6):431-441. DOI: 10.1007/s11816-014-0335-2

[35] Takasaki H, Maruyama K, Kidokoro S, Ito Y, Fujita Y, Shinozaki K, et al. The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible

genes and stress tolerance in rice. Molecular Genetics and Genomics. 2010;284(3): 173-183. DOI: 10.1007/s00438-010-0557-0

[36] Kerppola TK, Curran T. Transcription factor interactions: basics on zippers. Current Opinion in Structural Biology. 1991;1(1):71-79. DOI: 10.1016/0959-440X(91)90014-K

[37] Wang ZY, Dane F. NAC (NAM/ATAF/CUC) transcription factors in different stresses and their signaling pathway. Acta Physiologiae Plantarum. 2013;35(5):1397-1408. DOI: 10.1007/s11738-012-1195-4

[38] Stockinger EJ, Gilmour SJ, Thomashow MF. Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(3):1035-1040. DOI: 10.1073/pnas.94.3.1035

[39] Kizis D, Lumbreras V, Pagès M. Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. FEBS Letters. 2001;498(2-3):187-189. DOI: 10.1016/S0014-5793(01)02460-7

[40] Gao MJ, Allard G, Byass L, Flanagan AM, Singh J. Regulation and characterization of four CBF transcription factors from Brassica napus. Plant Molecular Biology. 2002;49(5):459-471. DOI: 10.1023/A:1015570308704

[41] Shen YG, Zhang WK, He SJ, Zhang JS, Liu Q, Chen SY. An EREBP/AP2-type protein in Triticum aestivum was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theoretical and Applied Genetics. 2003;106(5):923-930. DOI: 10.1007/s00122-002-1131-x

[42] Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, et al. OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt-and cold-responsive gene expression. The Plant Journal. 2003;33(4):751-763. DOI: DOI 10.1046/j.1365-313X.2003.01661.x

[43] Pandey B, Sharma P, Saini M, Pandey DM, Sharma I. Isolation and characterization of dehydration-responsive element-binding factor 2 (DREB2) from Indian wheat (Triticum aestivum L.) cultivars. Australian Journal of CropScience. 2014;8(1):165-167.

[44] Chen JQ, Meng XP, Zhang Y, Xia M, Wang XP. Over-expression of OsDREB genes lead to enhanced drought tolerance in rice. Biotechnology Letters. 2008;30(12): 2191-2198. DOI: 10.1007/s10529-008-9811-5

[45] Cui M, Zhang W, Zhang Q, Xu Z, Zhu Z, Duan F, et al. Induced over-expression of the transcription factor OsDREB2A improves drought tolerance in rice. Plant Physiology and Biochemistry. 2011;49(12):1384-1391. DOI: 10.1016/j.plaphy.2011.09.012

[46] Liu S, Wang X, Wang H, Xin H, Yang X, Yan J, et al. Genome-wide analysis of ZmDREB genes and their association with natural variation in drought tolerance at

seedling stage of Zea mays L.. PLOS Genetics. 2013;9(9):e1003790. DOI: 10.1371/journal.pgen.1003790

[47] Xu ZS, Ni ZY, Li ZY, Li LC, Chen M, Gao D-Y, et al. Isolation and functional characterization of HvDREB1—a gene encoding a dehydration-responsive element binding protein in Hordeum vulgare. Journal of Plant Research. 2009;122(1):121-130. DOI: 10.1007/s10265-008-0195-3

[48] Lourenço T, Saibo N, Batista R, Ricardo CP, Oliveira MM. Inducible and constitutive expression of HvCBF4 in rice leads to differential gene expression and drought tolerance. Biologia Plantarum. 2011;55(4):653-663. DOI: 10.1007/s10535-011-0164-x

[49] Souer E, van Houwelingen A, Kloos D, Mol J, Koes R. The no apical meristem gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell. 1996;85(2):159-170. DOI: Doi 10.1016/S0092-8674(00)81093-4

[50] Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. Genes involved in organ separation in Arabidopsis: An analysis of the cup-shaped cotyledon mutant. The plant cell. 1997;9(6):841-857. DOI: 10.1105/tpc.9.6.841

[51] Brini F, Hanin M, Lumbreras V, Irar S, Pages M, Masmoudi K. Functional characterization of DHN-5, a dehydrin showing a differential phosphorylation pattern in two Tunisian durum wheat (*Triticum durum* Desf.) varieties with marked differences in salt and drought tolerance. Plant Science. 2007;172(1):20-28. DOI: 10.1016/j.plantsci.2006.07.011

[52] Mao X, Zhang H, Qian X, Li A, Zhao G, Jing R. TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. Journal of Experimental Botany. 2012;63(8):2933-2946. DOI: 10.1093/jxb/err462

[53] Xue GP, Way HM, Richardson T, Drenth J, Joyce PA, McIntyre CL. Overexpression of TaNAC69 leads to enhanced transcript levels of stress up-regulated genes and dehydration tolerance in bread wheat. Molecular Plant. 2011;4(4):697-712. DOI: 10.1093/mp/ssr013

[54] Mao X, Chen S, Li A, Zhai C, Jing R. Novel NAC transcription factor TaNAC67 confers enhanced multi-abiotic stress tolerances in Arabidopsis. PloS One. 2014;9(1):e84359. DOI: 10.1371/journal.pone.0084359

[55] Lu M, Sun Q, Zhang D, Wang T, Pan J. Identification of 7 stress-related NAC transcription factor members in maize (Zea mays L.) and characterization of the expression pattern of these genes. Biochemical and Biophysical Research Communications. 2015;46(2):144-150. DOI: 10.1016/j.bbrc.2015.04.113

[56] Liu G, Li X, Jin S, Liu X, Zhu L, Nie Y, et al. Overexpression of rice NAC gene SNAC1 improves drought and salt tolerance by enhancing root development and re-

ducing transpiration rate in transgenic cotton. PloS One. 2014;9(1). DOI: 10.1371/journal.pone.0086895

[57] Jeong JS, Kim YS, Redillas MC, Jang G, Jung H, Bang SW, et al. OsNAC5 overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field. Plant Biotechnology Journal. 2013;11(1):101-114. DOI: 10.1111/pbi.12011

[58] Rachmat A, Nugroho S, Sukma D, Aswidinnoor H. Overexpression of OsNAC6 transcription factor from Indonesia rice cultivar enhances drought and salt tolerance. Emirates Journal of Food and Agriculture. 2014;26(6):519-527. DOI: 10.9755/ejfa.v26i6.17672

[59] Redillas MC, Jeong JS, Kim YS, Jung H, Bang SW, Choi YD, et al. The overexpression of OsNAC9 alters the root architecture of rice plants enhancing drought resistance and grain yield under field conditions. Plant Biotechnology Journal. 2012;10(7):792-805. DOI: 10.1111/j.1467-7652.2012.00697.x

[60] Jeong JS, Kim YS, Baek KH, Jung H, Ha S-H, Do Choi Y, et al. Root-specific expression of OsNAC10 improves drought tolerance and grain yield in rice under field drought conditions. Plant Physiology. 2010;153(1):185-197. DOI: 10.1104/pp.110.154773

[61] Gao F, Xiong A, Peng R, Jin X, Xu J, Zhu B, et al. OsNAC52, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. The Plant Cell, Tissue and Organ Culture (PCTOC). 2010;100(3):255-262. DOI: 10.1007/s11240-009-9640-9

[62] Klempnauer K-H, Gonda TJ, Bishop JM. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: The architecture of a transduced oncogene. Cell. 1982;31(2):453-463. DOI: 10.1016/0092-8674(82)90138-6

[63] Lipsick JS. One billion years of Myb. Oncogene. 1996;13(2):223-235.

[64] Paz-Ares J, Ghosal D, Wienand U, Peterson P, Saedler H. The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO Journal. 1987;6(12):3553.

[65] Du H, Zhang L, Liu L, Tang X, Yang W, Wu Y, et al. Biochemical and molecular characterization of plant MYB transcription factor family. Biochemistry (Moscow). 2009;74(1):1-11. DOI: 10.1134/S0006297909010015

[66] Stracke R, Werber M, Weisshaar B. The R2R3-MYB gene family in Arabidopsis thaliana. Current Opinion in Plant Biology. 2001;4(5):447-456. DOI: 10.1016/S1369-5266(00)00199-0

[67] Braun EL, Grotewold E. Newly discovered plant c-myb-like genes rewrite the evolution of the plant myb gene family. Plant Physiology. 1999;121(1):21-24. DOI:http://dx.doi.org/10.1104/pp.121.1.21

[68] Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K. An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. The Plant Cell. 1993;5(11):1529-1539. DOI: 10.1105/tpc.5.11.1529

[69] Shinozaki K, Yamaguchi-Shinozaki K, Seki M. Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology. 2003;6(5):410-417. DOI: 10.1016/S1369-5266(03)00092-X

[70] Liu L, Hao Z, Weng J, Li M, Zhang D, Bai L, et al. Identification of drought-responsive genes by cDNA-amplified fragment length polymorphism in maize. Annals of Applied Biology. 2012;161(3):203-213. DOI: 10.1111/j.1744-7348.2012.00565.x

[71] Pasquali G, Biricolti S, Locatelli F, Baldoni E, Mattana M. Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples. The Plant Cell Reports. 2008;27(10):1677-1686. DOI: 10.1007/s00299-008-0587-9

[72] Xiong H, Li J, Liu P, Duan J, Zhao Y, Guo X, et al. Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. PloS One. 2014;9(3):e92913. DOI: 10.1371/journal.pone.0092913

[73] Cai H, Tian S, Liu C, Dong H. Identification of a MYB3R gene involved in drought, salt and cold stress in wheat (Triticum aestivum L.). Gene. 2011;485(2):146-152. DOI: 10.1016/j.gene.2011.06.026

[74] Babu MM, Iyer LM, Balaji S, Aravind L. The natural history of the WRKY–GCM1 zinc fingers and the relationship between transcription factors and transposons. Nucleic Acids Research. 2006;34(22):6505-6520. DOI: 10.1093/nar/gkl888

[75] Chen L, Song Y, Li S, Zhang L, Zou C, Yu D. The role of WRKY transcription factors in plant abiotic stresses. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 2012;1819(2):120-128. DOI: 10.1016/j.bbagrm.2011.09.002

[76] Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. Trends in Plant Science. 2000;5(5):199-206. DOI: 10.1016/S1360-1385(00)01600-9

[77] Zhang Y, Wang L. The WRKY transcription factor superfamily: Its origin in eukaryotes and expansion in plants. BMC Evolutionary Biology. 2005;5(1):1. DOI: 10.1186/1471-2148-5-1

[78] Qiu Y, Yu D. Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in Arabidopsis. Environmental and Experimental Botany. 2009;65(1):35-47. DOI: 10.1016/j.envexpbot.2008.07.002

[79] Xu Q, Feng W, Peng H, Ni Z, Sun Q. TaWRKY71, a WRKY transcription factor from wheat, enhances tolerance to abiotic stress in transgenic Arabidopsis thaliana. Cereal

Research Communications. 2013;42(1):47-57. DOI: http://dx.doi.org/10.1556/CRC.2013.0051

[80] Marè C, Mazzucotelli E, Crosatti C, Francia E, Cattivelli L. Hv-WRKY38: A new transcription factor involved in cold-and drought-response in barley. Plant Molecular Biology. 2004;55(3):399-416. DOI: 10.1007/s11103-004-0906-7

[81] Berri S, Abbruscato P, Faivre-Rampant O, Brasileiro AC, Fumasoni I, Satoh K, et al. Characterization of WRKY co-regulatory networks in rice and Arabidopsis. BMC Plant Biology. 2009;9(120). DOI: 10.1186/1471-2229-9-120

[82] Niu CF, Wei W, Zhou QY, Tian AG, Hao YJ, Zhang WK, et al. Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. Plant, Cell & Environment. 2012;35(6):1156-1170. DOI: 10.1111/j.1365-3040.2012.02480.x

[83] Wang C, Deng P, Chen L, Wang X, Ma H, Hu W, et al. A wheat WRKY transcription factor TaWRKY10 confers tolerance to multiple abiotic stresses in transgenic tobacco. PloS One. 2013;8(6):e65120. DOI: 10.1371/journal.pone.0065120

[84] Zhang L, Zhang L, Xia C, Zhao G, Liu J, Jia J, et al. A novel wheat bZIP transcription factor, TabZIP60, confers multiple abiotic stress tolerances in transgenic Arabidopsis. Physiologia Plantarum. 2015;153(4):538-554. DOI: 10.1111/ppl.12261

[85] Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, et al. bZIP transcription factors in Arabidopsis. Trends in Plant Science. 2002;7(3):106-111. DOI: 10.1016/S1360-1385(01)02223-3

[86] Pourabed E, Golmohamadi FG, Monfared PS, Razavi SM, Shobbar Z-S. Basic leucine zipper family in barley: Genome-wide characterization of members and expression analysis. Molecular Biotechnology. 2015;57(1):12-26. DOI: 10.1007/s12033-014-9797-2

[87] Nijhawan A, Jain M, Tyagi AK, Khurana JP. Genomic survey and gene expression analysis of the basic leucine zipper transcription factor family in rice. Plant Physiology. 2008;146(2):333-350. DOI: 10.1104/pp.107.112821

[88] Wei K, Chen J, Wang Y, Chen Y, Chen S, Lin Y, et al. Genome-wide analysis of bZIP-encoding genes in maize. DNA Research. 2012;19(6):463-476. DOI: 10.1093/dnares/dss026

[89] Jia Z, Lian Y, Zhu Y, He J, Cao Z, Wang G. Cloning and characterization of a putative transcription factor induced by abiotic stress in Zea mays. African Journal of Biotechnology. 2009;8(24):6764-6771. DOI: 10.5897/AJB09.1280

[90] Ying S, Zhang D, Fu J, Shi Y, Song Y, Wang T, et al. Cloning and characterization of a maize bZIP transcription factor, ZmbZIP72, confers drought and salt tolerance in transgenic Arabidopsis. Planta. 2012;235(2):253-266. DOI: 10.1007/s00425-011-1496-7

[91] Chen H, Chen W, Zhou J, He H, Chen L, Chen H, et al. Basic leucine zipper transcription factor OsbZIP16 positively regulates drought resistance in rice. Plant Science. 2012;193-194:8-17. DOI: 10.1016/j.plantsci.2012.05.003

[92] Park SH, Jeong JS, Lee KH, Kim YS, Do Choi Y, Kim J-K. OsbZIP23 and OsbZIP45, members of the rice basic leucine zipper transcription factor family, are involved in drought tolerance. Plant Biotechnology Reports. 2015;9(2):89-96. DOI: 10.1007/s11816-015-0346-7

[93] Lu G, Gao C, Zheng X, Han B. Identification of OsbZIP72 as a positive regulator of ABA response and drought tolerance in rice. Planta. 2009;229(3):605-615. DOI: 10.1007/s00425-008-0857-3

[94] Tang N, Zhang H, Li X, Xiao J, Xiong L. Constitutive activation of transcription factor OsbZIP46 improves drought tolerance in rice. Plant Physiology. 2012;158(4):1755-1768. DOI: 10.1104/pp.111.190389

[95] Ariel FD, Manavella PA, Dezar CA, Chan RL. The true story of the HD-Zip family. Trends in Plant Science. 2007;12(9):419-426. DOI: 10.1016/j.tplants.2007.08.003

[96] Byrne ME. Shoot meristem function and leaf polarity: The role of class III HD–ZIP genes. PLoS Genetics. 2006;2(6):e89. DOI: 10.1371/journal.pgen.0020089

[97] Nakamura M, Katsumata H, Abe M, Yabe N, Komeda Y, Yamamoto KT, et al. Characterization of the class IV homeodomain-leucine zipper gene family in Arabidopsis. Plant Physiology. 2006;141(4):1363-1375. DOI: 10.1104/pp.106.077388

[98] Zhu W, Zhang L, Zhang N, Xing Y, Jiang B. The clone of wheat dehydrin-like gene wzy2 and its functional analysis in Pichia pastoris. African Journal of Biotechnology. 2012;11(40). PubMed PMID: CABI:20123203761. DOI: 10.5897/AJB11.3470

[99] Hu R, Chi X, Chai G, Kong Y, He G, Wang X, et al. Genome-wide identification, evolutionary expansion, and expression profile of homeodomain-leucine zipper gene family in poplar (Populus trichocarpa). PloS One. 2012;7(2):e31149. DOI: 10.1371/journal.pone.0031149

[100] Reinhart BJ, Liu T, Newell NR, Magnani E, Huang T, Kerstetter R, et al. Establishing a framework for the ad/abaxial regulatory network of Arabidopsis: Ascertaining targets of class III homeodomain leucine zipper and KANADI regulation. The Plant Cell Online. 2013;25(9):3228-3149. DOI: 10.1105/tpc.113.111518

[101] Chan RL, Gago GM, Palena CM, Gonzalez DH. Homeoboxes in plant development. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression. 1998;1442(1):1-19. DOI: 10.1016/S0167-4781(98)00119-5

[102] Mukherjee K, Bürglin TR. MEKHLA, a novel domain with similarity to PAS domains, is fused to plant homeodomain-leucine zipper III proteins. Plant Physiology. 2006;140(4):1142-1150. DOI: 10.1104/pp.105.073833

[103] Arce AL, Raineri J, Capella M, Cabello JV, Chan RL. Uncharacterized conserved motifs outside the HD-Zip domain in HD-Zip subfamily I transcription factors; a potential source of functional diversity. BMC Plant Biology. 2011;11(1):42. DOI: 10.1186/1471-2229-11-42

[104] Agalou A, Purwantomo S, Övernäs E, Johannesson H, Zhu X, Estiati A, et al. A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. Plant Molecular Biology. 2008;66(1-2):87-103. DOI: 10.1007/s11103-007-9255-7

[105] Harris JC, Hrmova M, Lopato S, Langridge P. Modulation of plant growth by HD-Zip class I and II transcription factors in response to environmental stimuli. New Phytologist. 2011;190(4):823-837. DOI: 10.1111/j.1469-8137.2011.03733.x

[106] Nakashima K, Jan A, Todaka D, Maruyama K, Goto S, Shinozaki K, et al. Comparative functional analysis of six drought-responsive promoters in transgenic rice. Planta. 2013;239(1):47-60. DOI: 10.1007/s00425-013-1960-7

[107] Olsson A, Engström P, Söderman E. The homeobox genes ATHB12 and ATHB7encode potential regulators of growth in response to water deficit in Arabidopsis. Plant Molecular Biology. 2004;55(5):663-677. DOI: 10.1007/s11103-004-1581-4

[108] Zhao Y, Ma Q, Jin X, Peng X, Liu J, Deng L, et al. A novel maize homeodomain–leucine zipper (HD-Zip) I gene, Zmhdz10, positively regulates drought and salt tolerance in both rice and Arabidopsis. Plant and Cell Physiology. 2014;55(6):1142-1156. DOI: 10.1093/pcp/pcu054

[109] Lopato S, Bazanova N, Morran S, Milligan AS, Shirley N, Langridge P. Isolation of plant transcription factors using a modified yeast one-hybrid system. Plant Methods. 2006;2(1):3. DOI: 10.1186/1746-4811-2-3

[110] Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Tran LSP, et al. Regulation and functional analysis of ZmDREB2A in response to drought and heat stresses in Zea mays L. The Plant Journal. 2007;50(1):54-69. DOI: 10.1111/j.1365-313X.2007.03034.x

[111] Wang X, Zeng J, Li Y, Rong X, Sun J, Sun T, et al. Expression of TaWRKY44, a wheat WRKY gene, in transgenic tobacco confers multiple abiotic stress tolerances. Frontiers in Plant Science. 2015;6(615):1-14. DOI: 10.3389/fpls.2015.00615

[112] Xie Q, Frugis G, Colgan D, Chua N-H. Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes & Development. 2000;14(23):3024-3036. DOI: 10.1101/gad.852200

[113] Olsen AN, Ernst HA, Lo Leggio L, Johansson E, Larsen S, Skriver K. Preliminary crystallographic analysis of the NAC domain of ANAC, a member of the plant-specific NAC transcription factor family. Acta Crystallographica. Section D: Biological Crystallography. 2003;60(1):112-115. DOI: 10.1107/S0907444903022029

[114] Ernst HA, Nina Olsen A, Skriver K, Larsen S, Lo Leggio L. Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. EMBO Reports. 2004;5(3):297-303. DOI: 10.1016/j.tplants.2004.12.010

[115] Olsen AN, Ernst HA, Leggio LL, Skriver K. NAC transcription factors: Structurally distinct, functionally diverse. Trends in Plant Science. 2005;10(2):79-87. DOI: 10.1016/j.tplants.2004.12.010

[116] Takada S, Hibara K-i, Ishida T, Tasaka M. The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development. 2001;128(7):1127-1135.

[117] Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, et al. Molecular characterization of Brassica napus NAC domain transcriptional activators induced in response to biotic and abiotic stress. Plant Molecular Biology. 2003;53(3):383-397. DOI: 10.1023/B:PLAN.0000006944.61384.11

[118] Ehlert A, Weltmeier F, Wang X, Mayer CS, Smeekens S, Vicente-Carbajosa J, et al. Two-hybrid protein–protein interaction analysis in Arabidopsis protoplasts: Establishment of a heterodimerization map of group C and group S bZIP transcription factors. The Plant Journal. 2006;46(5):890-900. DOI: 10.1111/j.1365-313X.2006.02731.x

[119] Shen H, Cao K, Wang X. AtbZIP16 and AtbZIP68, two new members of GBFs, can interact with other G group bZIPs in Arabidopsis thaliana. BMB Reports. 2008;41(2):132-138. DOI: 10.5483/BMBRep.2008.41.2.132

[120] Weltmeier F, Ehlert A, Mayer CS, Dietrich K, Wang X, Schütze K, et al. Combinatorial control of Arabidopsis proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors. The EMBO Journal. 2006;25(13):3133-3143. DOI: 10.1038/sj.emboj.7601206

[121] Shen H, Cao K, Wang X. A conserved proline residue in the leucine zipper region of AtbZIP34 and AtbZIP61 in Arabidopsis thaliana interferes with the formation of homodimer. Biochemical and Biophysical Research Communications. 2007;362(2):425-430. DOI: 10.1016/j.bbrc.2007.08.026

[122] Sessa G, Morelli G, Ruberti I. The Athb-1 and-2 HD-Zip domains homodimerize forming complexes of different DNA binding specificities. EMBO Journal. 1993;12(9):3507.

[123] Meijer A, De Kam R, d'Erfurth I, Shen W, Hoge J. HD-Zip proteins of families I and II from rice: Interactions and functional properties. Molecular and General Genetics MGG. 2000;263(1):12-21. DOI: 10.1007/PL00008671.

[124] Johannesson H, Wang Y, Engström P. DNA-binding and dimerization preferences of Arabidopsis homeodomain-leucine zipper transcription factors in vitro. Plant Molecular Biology. 2001;45(1):63-73. DOI: 10.1023/A:1006423324025

[125] Chi Y, Yang Y, Zhou Y, Zhou J, Fan B, Yu J-Q, et al. Protein–protein interactions in the regulation of WRKY transcription factors. Molecular Plant. 2013;6(2):287-300. DOI: 10.1093/mp/sst026

[126] Xu X, Chen C, Fan B, Chen Z. Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. The Plant Cell. 2006;18(5):1310-1326. DOI: 10.1105/tpc.105.037523

[127] Besseau S, Li J, Palva ET. WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in Arabidopsis thaliana. Journal of Experimental Botany. 2012;63(7): 2667-2679. DOI: 10.1093/jxb/err450

[128] Xie Z, Zhang ZL, Zou X, Yang G, Komatsu S, Shen QJ. Interactions of two abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurone cells. The Plant Journal. 2006;46(2):231-242. DOI: 10.1111/j.1365-313X.2006.02694.x

[129] Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, et al. Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant and Cell Physiology. 2006;47(1):141-153. DOI: 10.1093/pcp/pci230

[130] Song S-Y, Chen Y, Chen J, Dai X-Y, Zhang W-H. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta. 2011;234(2): 331-345. DOI: 10.1007/s00425-011-1403-2

[131] Baloglu MC, Oz MT, Oktem HA, Yucel M. Expression analysis of TaNAC69-1 and TtNAMB-2, wheat NAC family transcription factor genes under abiotic stress conditions in durum wheat (Triticum turgidum). Plant Molecular Biology Reporter. 2012;30(5):1246-1252. DOI: 10.1007/s11105-012-0445-3

[132] Lu M, Ying S, Zhang D-F, Shi Y-S, Song Y-C, Wang T-Y, et al. A maize stress-responsive NAC transcription factor, ZmSNAC1, confers enhanced tolerance to dehydration in transgenic Arabidopsis. Plant Cell Reports. 2012;31(9):1701-1711. DOI: 10.1007/s00299-012-1284-2

[133] Xiong X, James VA, Zhang H, Altpeter F. Constitutive expression of the barley HvWRKY38 transcription factor enhances drought tolerance in turf and forage grass (Paspalum notatum Flugge). Molecular Breeding. 2010;25(3):419-432. DOI: 10.1007/s11032-009-9341-4

[134] Cao X-y, Ming C, Xu Z-s, Chen Y-f, Li L-c, Yu Y-h, et al. Isolation and functional analysis of the bZIP transcription factor gene TaABP1 from a Chinese wheat landrace. Journal of Integrative Agriculture. 2012;11(10):1580-1591. DOI: 10.1016/S2095-3119(12)60161-0

Chapter 3 General materials and methods

3.1 Introduction

This chapter provides the details of the methods that were only generally mentioned in Chapters 4 and 5. The plasmid constructions, plant transformations, plant growth and stress treatments are described in Chapters 4 and 5 and the detailed descriptions were already given in each of the two chapters, respectively. Hence, these parts will not be repeated in this chapter.

3.2 Plant materials

Bread wheat (*Triticum aestivum* L. cv. Gladius) was used as the donor plants for transformation experiments. The homozygous sublines of transgenic wheat used in this research are listed in Table 3.1.

Table 3.1 Transgenic wheat sublines

Construct	ID of transgenic wheat	Generations	Number of independent homozygous lines
pUbi-TaHDZipI-5	GL167	$T_2/T_3/T_4$	3
pWRKY71-TaHDZipI-5	GL245	T_3/T_4	3
pCor39-TaHDZipI-5	GL246	T_2/T_3	3
pHDZI-3-TaCBF5L	GL180	$T_2/T_3/T_4$	3
pHDZI-4- TaCBF5L	GL181	$T_2/T_3/T_4$	4

3.3 Genomic DNA extraction

Fresh leaf tissue of each plant was collected in Collection Microtube (Qiagen), freeze-dried overnight, ground using a Qiagen grinder (Retsch mill, Type MM 300) at frequency of 25 oscillations per second for 1 minute and stored at -20 ^{o}C. Six hundred microliters of extraction buffer (100 ml 1.0 M Tris-HCl with pH 7.5, 100 ml 0.5 M ethylenediamine tetra-acetic acid (EDTA) with pH 8.0, 125 ml 10 % (v/v) sodium dodecyl sulfate (SDS) and 675 ml H_2O, total volume 1 L) was added into each tube, incubated at 65 ^{o}C for half an hour, and then kept in a 4 ^{o}C refrigerator for 15 minutes. When the tubes were cooled down to the room temperature, 300 µl of 6 M ammonium acetate (4 ^{o}C) was added into each tube, mixed vigorously with a vortex, and then kept at 4 ^{o}C for 15 minutes. Each tube was centrifuged at 4000 rpm for 15 minutes. After that, the supernatant was moved into new Collection Microtube and mixed with 360 µl 100 % (v/v) iso-propanol. These tubes were kept at room temperature for 15 minutes to precipitate DNA, and then centrifuged at 4000 rpm for 20 minutes. The supernatant was removed and the DNA pellet was washed with 400 µl of 70 % (v/v) ethanol. Afterwards, the

tube was placed upside down for 15 minutes until the DNA pellet dried, and then added with 200 µl milli-Q water for the DNA to dissolve at 4 °C overnight. Each tube was centrifuged at 4000 rpm for 20 minutes. Finally, the supernatants were transferred from these tubes into 96 micro-well plates and stored under -20 °C.

3.4 Polymerase chain reaction method

One Taq Quick-Load 2× Master Mix with Standard Buffer (New England Biolabs, Australia) was used in the polymerase chain reaction (PCR). The whole reaction was conducted in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad) with specific primers (Chapter 4 Table 1, Chapter 5 Table 1). The cycling parameters for the PCR are listed in Table 3.2. The PCR amplification product size was confirmed by separating by electrophoresis on a 1–1.5 % (w/v) agarose gel alongside a HyperLadder™ 1kb/ 50bp (Bioline, London, United Kingdom) as a DNA fragment size marker.

Table 3.2 PCR routine

Step	Temperature (°C)	Length of time
1. Denaturation	95	4 minutes
2. Denaturation	95	30 seconds
3. Annealing	55	30 seconds
4. Extension	72	48 seconds
Cycling 39 times from step 2 to 4		
5. Final extension	72	1 minute
6. Cooling	15	Unlimited time

3.5 Homozygous sublines selection in transgenic wheat

3.5.1 T_0 generation transgenic wheat growth, sampling and harvest

Leaves of T_0 plantlets transformed with *TaHDZipI-5/TaCBF5L* were collected at the three/four-leaf-stage in 2-ml tubes and stored at -80 °C. Total RNA of each leaf sample was isolated using Direct-zol™ RNA Mini Prep or TRIZOL. The copy number of each sample was checked by quantitative real-time PCR (Q-PCR). Plants were grown in PC2 rooms of the greenhouse until maturation. Their T_1 progeny seeds were harvested and dried at 37 °C for one week, and then stored at 4 °C in darkness.

3.5.2 T_1 generation transformed sublines selection, growth, sampling and harvest

T_1 progeny seeds from T_0 transgenic line with good germination rate were selected and germinated in Petri dishes for two or three days. These seedlings were then transferred into 12

cm green square pots in the greenhouse. Leaf samples were collected from these plants under the three/four-leaf-stage for DNA/RNA isolation. The copy number of each sample was identified using Q-PCR. Their T_2 progeny seeds were harvested and dried at 37 ºC for one week, and then stored at 4 ºC in darkness.

3.5.3 T_2/T_3 homozygous sublines selection

T_2/T_3 progeny seeds from three or more T_1/T_2 independent sublines with single copy number were selected. Twelve seeds from each subline were germinated in Petri dishes for 2 or 3 days, and then grown in bins/pots in the greenhouse. Leaf samples were collected for genomic DNA/RNA isolation. PCR was used for primary homozygous sublines selection. Northern blot hybridization/Q-PCR was used for gene expression analysis. Sublines were assumed homozygous if all the twelve samples had transgene expression.

3.6 Isolation of RNA

50–100 mg frozen leaf samples (collected from two/three-week-old plants) grinded in a 2-ml tube with two 1.2 mm stainless steel balls by Geno/Grinder®. 500 ml TRIzol® Reagent (Life Technologies Corporation, Grand Island, NY, USA) were added into each tube, vortexed for 15 minutes and centrifuged at 16000×g for 7 minutes. The supernatant was transferred into a new tube (1.5 ml) and mixed with the same volume of absolute ethanol. The mixture was sharply vortexed and then transferred into a Zymo-Spin ™ IIC column with a clean collection tube. The collection tube was replaced by a new one after centrifuging at 12000×g for 1 minute. Each column was washed with 400 µl RNA wash buffer by centrifuging at 12000×g for 1 minute and the supernatant was discarded. 80 µl of DNase I mix (5 µl DNaseI (1 U/µl) + 8 µl 10 × DNase I Reaction buffer + 3 µl DNase/RNase-Free Water + 64 µl RNA wash buffer) was added to each column and the columns were incubated at 25–37 ºC for 15 minutes and then centrifuged at 12000×g for 30 seconds. 400 µl Direct-zol™ RNA prewash was added into the column and centrifuged at 12000×g for 1 minute and the supernatant was removed. 700 µl RNA Wash Buffer was added into the column and centrifuged at 12000×g for 1 minute to remove supernatant. The column was dried at 12000×g centrifuge for 2 minutes. DNase/RNase-Free Water was added into the column matrix and centrifuged at 16000×g for 1 minute, then the eluted RNA solution was re-centrifuged at the same speed for 1 minute, and stored at -80 ºC.

3.7 Northern blot hybridization

Northern blot hybridization was used for checking *TaCBF5L* transgene expression in transformed wheat under promoter HDZI-3/HDZI-4.

3.7.1 Preparation of 10× MOPS-EDTA-Sodium Acetate buffer

MOPS-EDTA-Sodium Acetate (MESA) buffer was a commonly used buffer for running RNA electrophoresis in Northern blot hybridization methods. 42 g 3-(N-morpholino) propane sulfonic acid (MOPS) and 10.88 g $NaOAc{\cdot}3H_2O$ / 6.5 g anhydrous NaOAc were dissolved in 0.9 L Milli-Q water (MQW). 20 ml of 0.5 M / 3.72 g EDTA was add into the mixture and the pH was adjusted into 7 using 1 M NaOH. The total volume was 1 L.

3.7.2 Gel loading buffer (10 ml) preparation

Formaldehyde and formamide were considered as the denaturing agent for RNA samples to decrease second structure. 6 ml formamide and 2.1 ml Formaldehyde (36 %) were mixed with 1.2 ml 10× MOPS/EDTA, 80 µl Ethidium-Bromide and 600 Brom Phenol Blue 10 × sucrose.

3.7.3 Agarose gel (1.76 %) preparation

3 g Agarose (Ultra-Pure, Invitrogen, Cat No. 16500-500) was added into a 200 ml triangular flask with 20 ml 10× MOPS/EDTA and 144 autoclaved MQW. The flask was microwaved until the agarose mixture dissolved and then moved out at room temperature for cooling down. 6 ml of 37 % formaldehyde was added into the gel when the gel solution temperature reduced to 55–60 ^{o}C. After that, the agarose gel was gently poured into the pre-wetted electrophoresis apparatus (owl tank) with comb thick side down.

3.7.4 RNA Samples preparation and agarose gel running

Eppendorf-type polypropylene tubes (1.5 ml) were marked and placed on the 80-well micro-tube rack (Heathrow Scientific, Nottingham, United Kingdom) in order. The rack was then kept on ice. 18 µl of loading buffer was added into each tube, and then mixed with aliquot volume of RNA reached to around 1600 ng/µl. After that, the MQW was added into the mixture until the total volume was 28 µl. Each tube was spin quickly and then heated at 65 ^{o}C for 10–15 minutes. After that, the samples were put on ice for 10 minutes. Each RNA sample mixture was downloaded onto the gel as well as 3 µl RNA ladder (λ marker, Promega, USA), positive controls and negative controls. The electrophoresis was at the voltage of 40 V for 20 minutes, then at 60 V for 2.5 hours. Pictures for electrophoresis results under UV illumination were taken and recorded with date and time. The lanes of the gel were marked and the gel was gently wetted with some 20× saline-sodium citrate (SSC) buffer (3 M sodium chloride with 300 mM trisodium citrate, pH 7.0). A blotting tank was filled with 10× SSC buffer with a large rectangular glass over. Two Whatman papers were pre-wetted by 10× SSC and placed on the

middle of the glass with ends in the buffer. The gel was then faced down on the pre-wet Whatman papers.

3.7.5 Northern blotting

Hybond N^+ membrane was soaked in 4× SSC for 1 minute, and then placed on the top of the gel with caution to prevent formation of bubbles. Parafilm was placed on each side of the gel to make sure that buffer transferred the membrane. After that, the membrane was covered with two wet sheets of gel-size Whatman papers, two dry sheets of the same size Whatman papers and a stack of dry paper towels and a glass plate in order. A weight (0.2–0.75 kg) was then placed on the top of the glass plate. The RNA transfer was lasted over the night. After the transferring, the weight, glass plate, paper towels and Whatman papers were removed. The gel was disposed and the membrane was rinsed in 4× SSC to eliminate gel remains and then air-dried on a sheet of paper towel.

3.7.6 Probe preparation

The DNA template of the*TaCBF5L* transgene was amplified by PCR with primers of a whole length of the *TaCBF5L* cDNA (Chapter 5 Table 1). After that, 3 µl of random primer was mixed with 5 µl of DNA template (30–50 ng) and boiled for 5 minutes to denature the DNA probe, and then moved back on ice for at least 3 minutes. The mixture was then added with 12.5 µl 2× oligo labelling buffer and 1.5 µl Klenow's polymerase. After that, the mixture was added with the $dCT^{32}P$ at room temperature and then placed on ice at a workstation behind a safety shield. A Pasteur pipette was plugged with Miracloth inside as the column and added with the Sephadex G-100 using P1000 pipette. When the Sephadex G-100 reached to the neck of the Pasteur pipette, 1× TE was added into the column until full. The column neck was then sealed with parafilm. Three micro-centrifuge tubes were prepared and marked as No.1–No.3. They were put on a rack under the column. A Geiger counter was used to check the radioactivity. After radiolabelling accomplished, the parafilm was removed from the column and all the DNA probe was added on the top of the column. The dropping solution was collected by the No.1 micro-centrifuge tube until the radioactivity reached into 50–100 cps. Then the drops were collected by the No.2 tube until the radioactivity of the drops reached to the peak (around 1 kps) and decreased into 500 cps or from 2 kps into 700–800 cps. The No.3 tube was used to collect the rest of the drops. Then the No.1 and No.3 tubes were thrown into the radioactive waste bins. The No.2 tube with DNA probe was boiled for 5 minutes and kept on ice for another 5 minutes.

3.7.7 Pre-hybridization

Membrane was soaked into 5× SSC for 5 minutes and rolled into a cylinder with RNA side back to the wall. 30 ml of 5× SSC was poured into a hybridization tube and then placed with the rolled membrane inside. After that, the tube was added with 20 ml of pre-hybridization solution, and then rotated and pre-hybridized at 65 ^{o}C in the rotisserie oven overnight. The pre-hybridization solution was removed out of the tube.

3.7.8 Hybridization

The hybridization solution was incubated at 65 ^{o}C for 5 minutes and added into a hybridization tube with 5–10 ml. The tube was then added with the DNA probe and kept at 42 ^{o}C overnight. The following day, the hybridization solution was removed from the tube. The tube was washed down with 15 L water, and then added with 30 ml 2× SSC and 0.1 % SDS and then incubated at 42 ^{o}C for 20 minutes. The bottle was washed down again with water for around 10 minutes and added with 1× SSC and 0.1 % SDS, and then rewarmed at 65 ^{o}C for 20 minutes; this step was repeated twice. After that, all the solution was removed from the tube and the tube was washed down by water for around 10 minutes. The membrane was taken out from the hybridization bottle and dried on the paper towel for 10 minutes and then marked with name and date and stored in a plastic bag.

3.8 Quantitative real-time PCR

3.8.1 cDNA synthesis

2 µg RNA were added into a 0.2-ml PCR tube (on ice) and adjusted with Milli-Q water up to total volume of 8 µl. The tube was then added with 0.4 µl Oligo $(dT)_{18}$ primer, 1 µl $dNTP_s$ and 3.6 µl RNase free water. The mixture was kept at 65 ^{o}C for 5 minutes, and then immediately transferred to ice for at least 1 minute. After that, 4 µl first stand buffer (5×), 1 µl 0.1 M DTT, 0.5 µl RNase OUT, 0.35 µl Super Script ^{TM} III RT (200 U/µl) and 1.15 µl RNase free water were added into each tube. The mixture was at 50 ^{o}C for 1 hour for cDNA synthesis reaction, and the incubation was terminated at 70 ^{o}C for 15 minutes. Each cDNA sample was diluted for 20 times and stored at -20 ^{o}C.

3.8.2 cDNA quality identification assessment by using RT-PCR with primers designed based on housekeeping genes' primers

The quality of cDNA was evaluated by reverse transcription PCR (RT-PCR), which was performed using primers derived from a wheat housekeeping gene, calreticulin (*TaCRT)*. The

primers (Table 3.3) were designed on two exons with intron inside in order to distinguish genomic DNA from cDNA. Taq DNA Polymerase, cycling parameters, PCR components, thermal cycler, agarose gel concentration and DNA weight marker were the same as described in Chapter 3.4. The cDNA samples free of genomic DNA contamination were used for Q-PCR.

Table 3.3 Primers designing for housekeeping genes in RT-PCR

House-keeping gene name	**Primer type**	**Primer forward (5'-3')**	**PCR product length without introns (bp)**	**PCR product length with introns (bp)**
TaCRT	Primer forward	GGCGGAAGGATCAG GTGCATGCAG	184	296
	Primer reverse	GTTGTGCTCACGGAA GACGAAGCC		

3.8.3 Determination of transgene expression level using Q-PCR

To determine the expression level of target genes, Q-PCR was performed with specific primers (Chapter 4 Table 1, Chapter 5 Table 1). The Q-PCR reaction components are listed in Table 3.4. Reactions were performed in QuantStudio6 (Life Technologies): 20 seconds at 95 °C followed by 40 cycles of 1 second at 95 °C, 20 seconds at 60 °C, and fluorescent acquisition at 60°C. Followed by melt curve analysis: 15 seconds at 95 °C, 1 minute at 60 °C then increased temperature from 60 °C to 95 °C with fluorescence readings acquired at 0.05 °C/s increments. Three 20 µL PCR reaction mixtures with the same set of primers were combined and purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany). The purified product was quantified using QUBIT fluorometer and then sequenced by AGRF. After that, the PCR product was diluted into 10^9 copies per microliter according to dilute 20 / (([Concentration (ng/µL)] / 10^9) / ([Size (bp)] × 660) × 6.022 × 10^{23}) × 10^{10} µL of the product in TE buffer to a final of 200 µl stock solution. A dilution series covering six orders of magnitude was prepared. The 10^9 stock solution was diluted to produce a dilution series with six orders of magnitude from 10^7 to 10^2 copies per microliter. Three replicates of each of the six standard concentrations as well as no-template controls were included with every Q-PCR experiment for plotting the standard curve. Q-PCR was also performed for each unknown cDNA sample with three replications. Three optimised primers out of four control genes

(Chapter 4 Table 1, Chapter 5 Table 1) were used for normalising the copy number of target genes in unknown cDNA samples.

Table 3.4 Q-PCR component

Name of Q-PCR component	**volume（μl）**
Primer forward（5 μM）	0.4
Primer reverse（5 μM）	0.4
Kapa Sybr Fast Universal 2X qPCR Master Mix (Geneworks)	5
50X ROX Low (Geneworks)	0.2
H2O	2
cDNA sample	2
Total volume	10

3.9. Identification of ABA responsive cis-elements in the *TdHDZipI-5A* promoter

3.9.1 Primers designing and PCR for truncated *TdHDZipI-5A* promoter sequences amplification

A 2354-bp-long fragment of the *TdHDZipI-5A* promoter sequence was isolated from *T. durum* BAC library and used for promoter analysis. The likely ABA responsive cis-elements of upstream sequences of *TdHDZipI-5A* promoter was predicted by using PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/). Four pairs of primers (Chapter 4, Table 1) were designed between potential cis-elements of each promoter sequence and four promoter truncations of TdHDZipI-5b were obtained by PCR.

3.9.2 Truncated *TdHDZipI-5A* sequences cloning using pCR™8/GW/TOPO® TA Cloning® Kit

pCR™8/GW/TOPO® TA Cloning® Kit (Invitrogen, Melbourne, Victoria, Australia) was used for cloning of truncated *TdHDZipI-5A* sequences. 1 μl of PCR product was mixed with 1 μl salt solution, 0.5 μl pCR^{TM}8 TOPO vector as well as 3.5 μl Milli-Q water. The mixture was incubated at room temperature for 5 minutes. 2 μl mixtures were added into a 2 ml tube with 50 μl *Escherichia coli* (*E. coli*) strain DH5α and kept on ice for 30 minutes. After that, the tube

with mixture was incubated at 42 ºC for 30 seconds and then added with 250 µl SOC media (0.5 % yeast extract, 2 % tryptone, 10 mM $MgCl_2$, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$ and 20 mM glucose). The tubes were incubated in Orbital mixer incubator (Ratek, Australia) in 37 ºC with 200 rounds per minute for 2 hours. Then they were centrifuged at 1000 rounds per minute for 1 minute. Most supernatant was removed and the remains were mixed with the precipitated cells and the slurry was spread over Lysogeny broth (LB) solid media (100 µg/ml Spectinomycin) with sterile disposable spreader bars (Techno Plas Pty Ltd, St. Marys, South Australia, Australia). The transformed *E.coli* were incubated at 37 ºC overnight (12–16 hours) and then stored at 4 ºC in refrigerator. 2 ml of LB liquid media containing 100 µg/ml Spectinomycin was added into a 15 ml cultural tube. Single positive *E.coli* colony was picked from the LB solid media and used for inoculation of 2 ml LB liquid media with small (0.2–10 µl) pipette tip. The *E.coli* cells were incubated in Orbital mixer incubator (Ratek) at 37 ºC with rotation 200 rounds per minute overnight (12–16 hours), the plasmids were isolated using ISOLATEII Plasmid Mini kit (Cat No. BIO-52057, BIOLINE) and stored at -20 ºC. The isolated plasmids were considered as entry clone for sub-cloning by LR reaction.

3.9.3 Sub-cloning of the truncated *TdHDZipI-5A* sequences upstream GUS reporter gene using LR reaction cloning

Gateway® LR Clonase™ II Enzyme Mix (Invitrogen) was used for LR reaction cloning. 0.5 µl entry clone was mixed with 1 µl destination vector pMDC164 (150 ng/µl) and 6.5 µl TE buffer (pH 8.0). The LR Clonase™ II enzyme mix was thaw on ice for 2 minutes and then added into the mixture. The mixture was incubated at 25 ºC for 1 hour and the enzyme was deactivated by 1 µl protein K solution at 37 ºC for 10 minutes. 2 µl mixtures were added into a 2 ml tube with 50 µl *Escherichia coli* (E. coli) strain DH5α and kept on ice for 30 minutes. After that, the tube with mixture was incubated at 42 ºC for 30 seconds and then added with 250 µl SOC media. The tubes were incubated in Orbital mixer incubator (Ratek, Australia) in 37 ºC with 200 rounds per minute for 2 hours. Then they were centrifuged at 1000 rounds per minute for 1 minute. Most of supernatant was removed and the rest was mixed with the precipitated cells and spread on the solid LB media (100 µg/ml Kanamycin) with inoculating loops/Spreader (TECHNO-PLAS). The transformed *E.coli* were incubated at 37 ºC overnight (12–16 hours) and then stored at 4 ºC in refrigerator. 2 ml of the liquid LB media (100 µg/ml Kanamycin) was added into a 15-ml cultural tube. Single positive *E. coli* colony was slightly picked from the LB solid media into a tube with 2 ml liquid LB media using small size (0.2–10 µl) pipette tip. The *E. coli* cells were incubated in Orbital mixer incubator (Ratek) at 37 ºC overnight (12–16 hours) with

rotation 200 rounds per minute and the plasmids were isolated using ISOLATEII Plasmid Mini kit (Cat No. BIO-52057, BIOLINE) and then stored at -20 ^{o}C. The sub-cloned truncated sequences of the *TdHDZipI-5A* were confirmed by Sanger sequencing in AGRF and the constructs were designated as TdHDZipI-5b D1~TdHDZipI-5b D4 from the longest to the shortest length.

3.9.4 Identification functional cis-elements in the *TdHDZipI-5A* promoter deletions using transient expression assays based on biolistic bombardment of wheat cells

Transient expression assay (TEA) is a technique that enables transgene expression to be analysed in several hours or days (Fromm et al., 1985), which is much faster than expression in stably transformed transgenic lines. TEA has been initially developed by Fromm in 1985, he used electroporation mediated transformation of protoplasts (Fromm et al., 1985). Later, other variants of transient expression assay were developed, which used either Agrobacterium mediated transformation (Barton and Chilton, 1983, Janssen and Gardner, 1990) or biolistic bombardment (Morikawa et al., 1989) for the delivery of foreign genes into cells. In my research, TEA has been used for the identification of functional stress responsive *cis*-acting elements among computer-predicted elements in *TdHDZipI-5A* promoter. The detailed process of TEA is described as follows:

Cell culture from wheat roots were grown in an autoclaved triangular flask with 100 ml autoclaved liquid ½ Murashige-Skoog + 2 CPX + Arg (½-strength Murashige-Skoog (MS) medium with 100 mg casein hygrolysate, 100 mg L-Arginine A5006, 2 mg Centrophenoxine (CPX), pH 5.8, total volume 1 L) in a shaker. The cultivation condition was at 25 ^{o}C in darkness for weekly subculture. Six days after subculture, the flask with cell cultivation was moved from a shaker into a laminar-flow hood. The flask was shaken gently by wrist until the cell cultivation inside was mixed evenly, and 6 µl of the cell suspension was taken from the flask and spread on a piece of filter paper (diameter 3.5 cm) using filtration device Pyrex XX1504700 (Millipore, Eschborn, FRG), and then was placed above the Petri dish with 25 ml OSS media (½-strength Murashige-Skoog (MS) medium with 40 µL $GuSO_4$, 120 g Sucrose, 200 mM ABA, 1 L milli-Q water, pH 5.8, total volume 1 L). It was incubated for 2 hours before biolistic bombardment. For each sample, 5 µl 1000 ng promoter truncation plasmid (pTdHDZipI-5D-*GUS*) was mixed into 25 µl gold suspension for vortex and then covered with 5 µl GM solution. The mixture was vortexed for 20–30 seconds and incubated at room temperature for 20 minutes. Then it was centrifuged (13,000×g) for 5 minutes at 4^{o} C. The supernatant was carefully removed and the pellet was washed with ethanol (75 %, v/v) twice and dried. Then the gold coated DNA pellets

were dissolved into 30 µl absolute ethanol and mixed by metallic rack. These coated micro-carriers for each sample were used for three repetitions. The macro-carriers were soaked in 95 % ethanol for 2 hours and dried, and then each of them was covered with 5.5 µl coated micro-carriers. 900 psi rupture discs were washed by isopropanol and then air dried. The gun apparatus was cleaned with ethanol and the PDS-1000/He ballistic device (Bio-Rad, Munich, Germany) was turned on. Then the rupture disc was placed into the retaining cap, whilst the coated macro-carriers were placed on the launch. After that, the Petri dish with ABA induced cell cultivars was placed on the target plate shelf. The door of the gun apparatus was closed, and the vacuum was started until the vacuum gauge reached 28 hg. The helium was released and hold until the disc was ruptured. Then the vacuum was released and the bombarded Petri-dish was removed from the target shelf. After bombardment, each filter paper covered with transformed cells was moved into a clean Petri-dish and added with 6 ml autoclaved liquid ½ Murashige-Skoog + 2 CPX + Arg (½-strength Murashige-Skoog (MS) medium with 0.5 mM ABA in darkness at room temperature for 24 hours. After that, the solution was removed gently by pipette from each filter paper stained with GUS staining solution for 48 hours, and the GUS activity for each cells suspensions group was determined by number of GUS foci.

Chapter 4 Overexpression of the class I homeodomain TaHDZipI-5 increases drought and frost tolerance in transgenic wheat

This chapter is derived entirely from the published work:

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Principal Author

Name of Principal Author (Candidate)	Yunfei Yang		
Contribution to the Paper	Designed the experiment with supervisors, performed experiments, analysed data, wrote and edited the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	3/2/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.
iv.

Name of Co-Author	Sukanya Luang		
Contribution to the Paper	Constructed 3-D models of TaHDZipI-5 dimerisations with the HDZI cis-element and defined the foundation of the domain.		
Signature		Date	5/2/2018

Name of Co-Author	John Harris		
Contribution to the Paper	Determined an endogene *TaHDZipI-5* expression in a variety of unstressed bread wheat tissues.		
Signature		Date	5/2/2018
Name of Co-Author	Matteo Riboni		
Contribution to the Paper	Performed Q-PCR to determine the transgene expression under different abiotic stresses.		
Signature		Date	5/2/2018
Name of Co-Author	Yuan Li		
Contribution to the Paper	Performed Q-PCR to determine the copy number of the transgene TaHDZipI-5 in T_0/T_1 transgenic plants.		
Signature		Date	5/2/2018
Name of Co-Author	Natalia Bazanova		
Contribution to the Paper	Performed a yeast assay for identification of the TaHDZipI-5 activation domain.		
Signature		Date	5/2/2018
Name of Co-Author	Maria Hrmova		
Contribution to the Paper	Wrote one part of the manuscript, performed 3-D models construction and bioinformatics guidance, edited the manuscript and acted as the corresponding author.		
Signature		Date	5/2/2018
Name of Co-Author	Stephan Haefele		
Contribution to the Paper	Supervised experiments and edited the manuscript.		
Signature		Date	5/2/2018

Name of Co-Author	Nataliya Kovalchuk		
Contribution to the Paper	Supervised experiments and edited the manuscript.		
Signature		Date	5/2/2018
Name of Co-Author	Sergiy Lopato		
Contribution to the Paper	Conceived and designed the experiments, supervised experiments, analysed data and edited the manuscript.		
Signature		Date	5/2/2018

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Overexpression of the class I homeodomain transcription factor TaHDZipI-5 increases drought and frost tolerance in transgenic wheat

Yunfei Yang, Sukanya Luang[†], John Harris[‡], Matteo Riboni, Yuan Li, Natalia Bazanova[§], Maria Hrmova*, Stephan Haefele[¶], Nataliya Kovalchuk and Sergiy Lopato

School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, SA, Australia

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**Correspondence* (Tel +61 8 8313 7160; fax +61 8 8313 7102; email: maria.hrmova@adelaide.edu.au)
[†]Present address: Institute of Molecular Biosciences, Mahidol University, Nakhon-Pathom, Thailand.
[‡]Present address: South Australian Research and Development Institute, GPO Box 397, Adelaide, SA, 5064, Australia
[§]Present address: Commonwealth Scientific and Industrial Research Organisation, Glen Osmond, SA 5064, Australia
[¶]Present address: Rothamsted Research, West Common Harpenden, Hertfordshire, AL5 2JQ, UK
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Summary

Characterization of the function of stress-related genes helps to understand the mechanisms of plant responses to environmental conditions. The findings of this work defined the role of the wheat *TaHDZipI-5* gene, encoding a stress-responsive homeodomain–leucine zipper class I (HD-Zip I) transcription factor, during the development of plant tolerance to frost and drought. Strong induction of *TaHDZipI-5* expression by low temperatures, and the elevated *TaHDZipI-5* levels of expression in flowers and early developing grains in the absence of stress, suggests that *TaHDZipI-5* is involved in the regulation of frost tolerance at flowering. The TaHDZipI-5 protein behaved as an activator in a yeast transactivation assay, and the TaHDZipI-5 activation domain was localized to its C-terminus. The TaHDZipI-5 protein homo- and hetero-dimerizes with related TaHDZipI-3, and differences between DNA interactions in both dimers were specified at 3D molecular levels. The constitutive overexpression of *TaHDZipI-5* in bread wheat significantly enhanced frost and drought tolerance of transgenic wheat lines with the appearance of undesired phenotypic features, which included a reduced plant size and biomass, delayed flowering and a grain yield decrease. An attempt to improve the phenotype of transgenic wheat by the application of stress-inducible promoters with contrasting properties did not lead to the elimination of undesired phenotype, apparently due to strict spatial requirements for *TaHDZipI-5* overexpression.

Introduction

Drought and frost are significant limitations to plant growth and development and substantially decrease crop yields globally, including in Australia. Depending on seasonal conditions, a sudden frost at flowering can be a major cause of wheat and barley grain yield losses. Overnight frost events during flowering will damage the sensitive reproductive tissues, often resulting in a near-total loss of grain. If crops are sown late with the aim to avoid productivity losses due to frost, severe yield losses may occur in hot and dry periods at the end of the growing season. In addition, late planting often leads to reduced grain size, yield and quality. The cost to Australian wheat and barley industries caused by frost is estimated to be around AUS$ 360 million in direct and indirect losses annually (GRDC National Frost Initiative, goo.gl/hKWf34). Thus, identification, characterization and application of candidate genes for the molecular breeding of crop acclimation to both frost and drought are of the utmost importance.

Environmental stresses such as frost or drought trigger specific signal transduction pathways, which activate the expression of stress-responsive genes (Braam *et al.*, 1997; Bray, 1997; Hwang *et al.*, 2002; Tena *et al.*, 2001; Zhu, 2016). Gene expression starts from the modulation of transcription by stress-related transcription factors (TFs), which regulate a number of physiological processes under stress, including cuticular wax biosynthesis (Aharoni *et al.*, 2004; Bi *et al.*, 2016, 2017; Borisjuk *et al.*, 2014; Seo *et al.*, 2011), stomatal closure (Ren *et al.*, 2010; Tan *et al.*, 2017), reactive oxygen species (ROS) detoxification (Jiang and Deyholos, 2009) and structural alterations in plasma membranes (Pearce, 1999). Manipulation using genes encoding stress-related TFs offers the possibility to regulate large groups of genes

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involved in the same physiological processes, and therefore, this intervention draws the attention of plant biotechnologists (Agarwal *et al.*, 2017; Gahlaut *et al.*, 2016; Hrmova and Lopato, 2014).

An attractive target for this approach is the family of homeodomain–leucine zipper (HD-Zip) TFs, which contains proteins regulating plant development after plants are exposed to environmental stimuli and stresses (Brandt *et al.*, 2014; Harris *et al.*, 2011; Perotti *et al.*, 2017). All HD-Zip TFs possess a highly conserved homeodomain (HD) and leucine zipper (Zip or LZ) motifs (Ariel *et al.*, 2007; Harris *et al.*, 2016; Mattsson *et al.*, 1992; Ruberti *et al.*, 1991; Schena and Davis, 1992, 1994). An HD is a folded helix-turn-helix motif, which contains 60 amino acid residues (Gehring *et al.*, 1990; Laughon and Scott, 1984; Otting *et al.*, 1990) and functions during the recognition of specific DNA sequences (Gehring *et al.*, 1990; Shepherd *et al.*, 1984). LZ, adjacent to HD, participates in dimerization of HD-Zip TFs by forming a coiled coil structure (Harris *et al.*, 2016; Ruberti *et al.*, 1991; Szilák *et al.*, 1997). Dimerization could affect the affinity of HD-Zip proteins to specific DNA binding sites and hence potentially regulate the strength of activation of target genes (Chew *et al.*, 2013; Harris *et al.*, 2016; Palena and Gonzalez, 1999; Szilák *et al.*, 1997).

The HD-Zip family of proteins has been classified into four subfamilies, designated HD-Zip classes I to IV, based on unique features in the domain structure and specificity of *cis*-element binding (Ariel *et al.*, 2007). The members of HD-Zip class I differ from the other family members by the absence of common domains and/or motifs besides the HD and Zip domains (Ariel *et al.*, 2007; Chan *et al.*, 1998; Mukherjee and Bürglin, 2006; Ponting and Aravind, 1999; Schrick *et al.*, 2004). The members of the HD-Zip I family recognize a specific 9-bp pseudo-palindromic binding site: CAATNATTG (Meijer *et al.*, 1997; Sessa *et al.*, 1993). No obvious requirements for the central nucleotide of the *cis*-element have been observed for wheat HD-Zip I TFs (Harris *et al.*, 2016; Kovalchuk *et al.*, 2016). However, it is not clear how homo- or hetero-dimerization of HD-Zip I TFs influences DNA binding and the activation of target genes (Chew *et al.*, 2013; Harris *et al.*, 2016; Hrmova and Lopato, 2014).

Homeodomain–leucine zipper class I TFs were isolated from a variety of species such as *Arabidopsis thaliana* (Ariel *et al.*, 2007; Schena and Davis, 1992), resurrection plant *Craterostigma plantagineum* (Deng *et al.*, 2002; Frank *et al.*, 1998), sunflower (Cabello and Chan, 2012; Cabello *et al.*, 2012), rice (Agalou *et al.*, 2008), maize (Zhao *et al.*, 2011) and wheat (Harris *et al.*, 2016; Lopato *et al.*, 2006). Some of these TFs have been reported to respond to various abiotic stresses on transcriptional and/or post-translational levels (Bhattacharjee *et al.*, 2016; Harris *et al.*, 2016; Kovalchuk *et al.*, 2016; Olsson *et al.*, 2004; Wu *et al.*, 2016; Zhao *et al.*, 2014). For instance, transcription of *Athb7* and *Athb12* from *Arabidopsis* was induced by elevated levels of abscisic acid (ABA) and by water deficiency (Olsson *et al.*, 2004; Söderman *et al.*, 1996). Transcription of *Hahb1* from sunflower was responsive to low temperatures (Cabello *et al.*, 2012), while *Hahb4* was activated by desiccation (Dezar *et al.*, 2005a,b). Transcription of wheat *TaHDZipI-2* was not influenced by ABA and was partially suppressed by low temperatures; however, the transactivation activity of the TaHDZipI-2 protein was strongly increased by the addition of exogenous ABA (Kovalchuk *et al.*, 2016). In contrast, *TaHDZipI-4* and *TaHDZipI-5* were activated by ABA on both transcriptional and post-translational levels (Harris *et al.*, 2016).

The effect of overexpression of HD-Zip TFs on the ability of transgenic plants to survive severe stress conditions has also been demonstrated (Bhattacharjee *et al.*, 2016; Cabello *et al.*, 2012; Cabello and Chan, 2012; Kovalchuk *et al.*, 2016; Wu *et al.*, 2016; Zhang *et al.*, 2012). For instance, overexpression of the *Oshox22* and *Oshox24* genes (HD-Zip I γ-clade) in transgenic rice and *Arabidopsis* led to an increased ABA content and increased sensitivity to drought and high salinity (Bhattacharjee *et al.*, 2016, 2017; Zhang *et al.*, 2012). In contrast, overexpression of the similar *ZmHDZ4* gene (HD-Zip I γ-clade) from maize in transgenic rice enhanced plant tolerance to drought, despite an increased sensitivity to ABA. *ZmHDZ4*-expressing transgenic plants had a lower relative electrolyte leakage, lower malondialdehyde levels and increased proline contents under drought compared to wild-type (WT) plants (Wu *et al.*, 2016). All of these changes could potentially contribute to enhanced drought tolerance.

Improvement of cold/frost tolerance was demonstrated only for the representatives of the HD-Zip I α-clade. Constitutive overexpression of *AtHB13* from *Arabidopsis* and *HaHB1* from sunflower had little influence on the growth and yield of transgenic *Arabidopsis*, but stabilized cell membrane integrity under cold, drought and high salinity conditions and increased plant stress tolerance (Cabello and Chan, 2012; Cabello *et al.*, 2012). Frost tolerance enhancement of transgenic barley seedlings was achieved by constitutive overexpression of *TaHDZipI-2*, the wheat orthologue of *AtHB13*. However, it was accompanied by negative changes in the phenotype of transgenic plants and a significant yield loss compared to those of control plants (Kovalchuk *et al.*, 2016).

In our previous projects, five genes encoding the members of HD-Zip subfamily I TFs, designated *TaHDZipI-1* to *TaHDZipI-5*, were isolated from wheat and partially characterized (Harris *et al.*, 2016; Kovalchuk *et al.*, 2016; Lopato *et al.*, 2006). *TaHDZipI-1* (ζ-clade) expression was detected in seedlings and mature vegetative tissues, while *TaHDZipI-2* (α-clade) was predominantly expressed in shoots of seedlings and during early grain development, with no expression detected in mature tissues (Lopato *et al.*, 2006). *TaHDZipI-2* was demonstrated to function as a regulator of plant growth, flowering time and frost tolerance (Kovalchuk *et al.*, 2016). Overexpression of *TaHDZipI-2* in transgenic barley directly or indirectly regulated a number of genes responsible for barley adaptation to cold, vernalization, flowering time and shape of spikes (Kovalchuk *et al.*, 2016). The *TaHDZipI-3* gene (γ-clade) was initially identified as a close homologue of *AtHB7* and *AtHB12* from *Arabidopsis*, and its induction of transcription by drought was demonstrated by Harris *et al.* (2016). In contrast to the homologous genes from *Arabidopsis*, *TaHDZipI-3* was not activated by cold and was not able to function as an activator in yeast or in wheat cells. Therefore, the full-length coding region of this protein provided ideal bait for a yeast 2-hybrid (Y2H) screen. The screen identified two interacting partners, which were identified to be the monocot-specific members of the γ-clade, designated *TaHDZipI-4* and *TaHDZipI-5*. In contrast to *TaHDZipI-3*, transcription of both monocot-specific genes was ABA-dependent and was strongly up-regulated by both cold and drought (Harris *et al.*, 2016).

This study is directed to identify regulatory genes that could be used for the improvement of frost and drought tolerance in economically important plants, such as wheat and barley. *TaHDZipI-5* was selected for further characterization because it was more strongly induced by cold and drought than the two

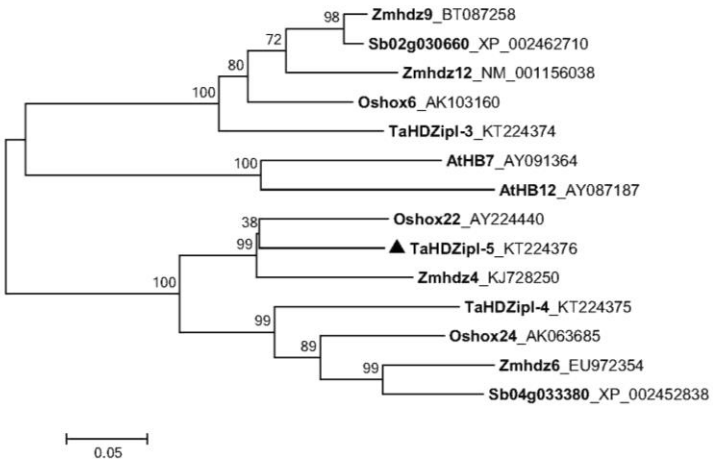


Figure 1 A rectangular phylogenetic tree displaying the evolutionary relationships of HD-Zip I γ-clade TFs from *Arabidopsis* and selected monocots. Abbreviations of species: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Sb, *Sorghum bicolor*; Ta, *Triticum aestivum*; Zm, *Zea mays*.

other genes from the wheat HD-Zip I γ-clade (Harris *et al.*, 2016). In this work, we studied the expression levels of *TaHDZipI-5* in a variety of wheat tissues, analysed the TaHDZipI-5 transactivation properties and revealed the determinants of homo- and heterodimerized TaHDZipI-5 and TaHDZipI-3 in complex with a defined *cis*-element at the 3D molecular level. *TaHDZipI-5* was initially constitutively overexpressed in transgenic wheat, and comparative evaluations of transgenic and WT plants for growth characteristics and yield components, and tolerance to extreme stress conditions were performed. Overexpression of *TaHDZipI-5* significantly improved plant tolerance to both stresses; however, it negatively influenced plant growth and grain yields. Stress-inducible expression of *TaHDZipI-5* was applied in an attempt to reduce the negative influence of the transgene on plant development, the onset of flowering and yield.

Results

A reconstruction of the phylogenetic relationship of HD-Zip I γ-clade proteins

A phylogenetic tree was constructed using sequences of HD-Zip I γ-clade proteins from the dicot model plant *Arabidopsis* and from several monocots including sorghum, rice, maize and wheat. Protein sequences were either derived from a previous study (Henriksson *et al.*, 2005) or were taken from NCBI databases and compared with the translated sequence of *TaHDZipI-5* (Table S2). The phylogenetic tree (Figure 1) shows that TaHDZipI-5 shares a closer evolutionary relationship with Oshox22 from rice (69% sequence identity) and Zmhdz4 from maize (63% sequence identity), than with other entries in the tree.

Endogenous *TaHDZipI-5* expression in a variety of unstressed bread wheat tissues

Expression of the endogenous *TaHDZipI-5* gene was analysed in a variety of tissues of unstressed wheat plants. *TaHDZipI-5* had the highest expression level in endosperm (Figure 2a). Additionally, high expression levels were found in roots and reproductive plant tissues sampled around fertilization. The lowest expression levels of *TaHDZipI-5* were detected in coleoptiles; these were about 30-fold lower than those in the endosperm.

Functional *cis*-elements responsible for ABA-dependent *TdHDZipI-5* promoter activation

Promoter sequences of two homeologous genes, *TdHDZipI-5A* and *TdHDZipI-5B*, were isolated from a durum wheat BAC library (Cenci et al., 2003), because the respective bread wheat sequences were not yet available at the time when this work commenced. The comparison of durum wheat promoter sequences (*TdHDZipI-5A* and *TdHDZipI-5B*) with corresponding sequences from bread wheat, identified in the Whole Genome Reference Assembly Pseudomolecules v1.0 databases of the International Wheat Genome Sequencing Consortium (IWGSC), revealed more than 99% sequence identity in a region containing functional *cis*-elements (Figure S3). Sequences of the promoters (each approximately 1300 bp long) were aligned using LALIGN (Huang and Miller, 1991) to find the best local alignments (Figure S4). Several ABRE and MYB responsive elements were predicted in conserved positions in both *TdHDZipI-5* promoter regions (PLACE software; Higo *et al.*, 1999). *TdHDZipI-5A* promoter deletions were generated based on putative *cis*-acting elements at −1055, −366, −336 and −175 bp positions, and these were named D1, D2, D3 and D4 (Figures 2b and S4). To define the functional *cis*-elements, 0.5 mM ABA was used to induce the activation of the *GUS* reporter gene by four promoter deletions in a transient expression assay performed in cultured wheat cells. Transformation with D1, D2 and D3 led to step-by-step decreasing numbers of GUS foci, while D4 could not activate *GUS* gene expression (Figure 2b). Therefore, the putative *cis*-element responsible for the ABA-dependent activation of *TdHDZipI-5A* is the MYB responsive (MYBR) element GGATA, which is located in the 161-bp region between D3 (−336 np) and D4 (−175 np), upstream of the transcription initiation site (Figure 2b). Two upstream ABA-responsive elements (ABREs) and/or one MYBR element enhanced the ABA-inducible promoter activation.

Identification of the TaHDZipI-5 activation domain using an in-yeast activation assay

Results of the in-yeast activation assay showed that the yeast strains carrying pGBKT7-TaHDZipI-5 grew well on SD/-Trp

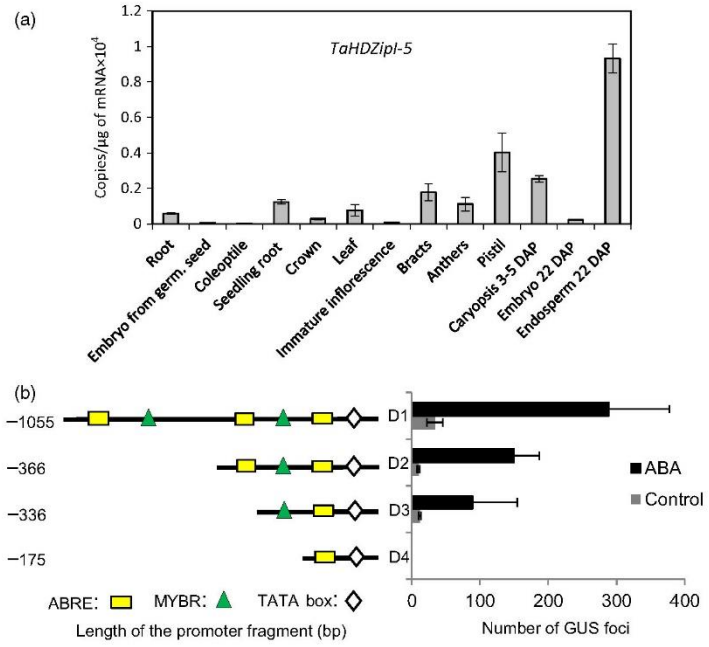
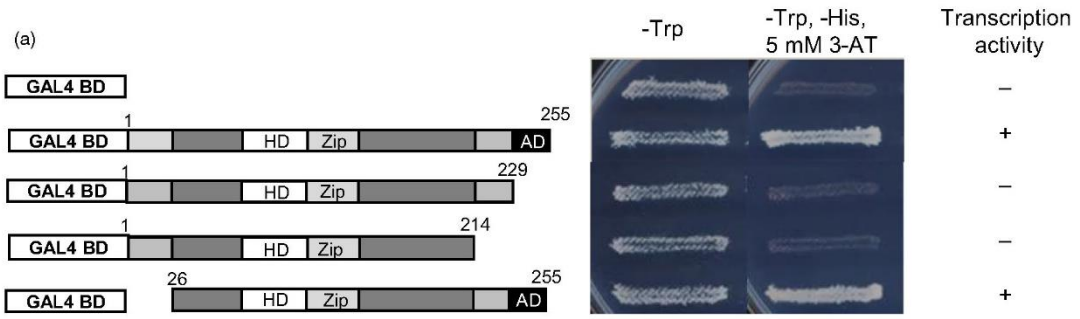


Figure 2 Characterization of the *TaHDZipI-5* gene. (a) Transcript numbers of the *TaHDZipI-5* gene in wheat tissues were estimated by Q-PCR. (b) Mapping of *cis*-elements responsible for the abscisic acid (ABA)-dependent activation of the *TdHDZipI-5A* promoter, using a transient expression assay in wheat cell culture. Depicted is a schematic representation of ABA-responsive element (ABRE) and MYB responsive (MYBR) *cis*-elements in four promoter deletions (D1–D4) of the *TdHDZipI-5A* promoter, and the graph shows activation of GUS expression by the deletions detected in a transient expression assay, in the presence (black bars) or absence (control; grey bars) of 0.5 mM ABA in the culture medium. Error bars were calculated from three technical replicates.



(b)

TaHDZipI-5	**FPESFCATPELWEP-WPWPPVEWNAVA**
Oshox22	**FPESFCATPELWEP-WPL--VEWNAVA**
Zmhdz4	**FPES**Y**CA**M**PELWEP-WPL--VEWNAVA**
Sb06g024000	**FPES**Y**CA**M**PELWEP-WPL--VEWNAVA**
TaHDZipI-4	LQ**E**P**FCATPELWE**TS**WPL--VEWNAVA**
Oshox24	L**P**DP**FC**GM**P**D**LWE**T-**WP**M--**VEWNAVA**
Sb04g033380	LHDPL**CATP**D**LW**DT-**WPL**--L**EWNAVA**

Figure 3 Identification of the TaHDZipI-5 activation domain (AD) using an in-yeast activation assay. (a) Transcription activity determined through the in-yeast activation assay, where homeodomain (HD), Zip and AD designate putative homeodomain, leucine zipper and AD, respectively. Amino acid residues at the beginning and end of each truncated protein are indicated with numbers. (b) Conserved sequences of the identified AD in TaHDZipI-5 and close homologues from other monocots. Ta, *Triticum aestivum*; Zm, *Zea mays*; Os, *Oryza sativa*; Sb, *Sorghum bicolor*. Amino acid residues, which are the same in more than the half of the investigated sequences, are in bold.

medium (confirming transformation) and the SD/-Trp/-His medium containing 5 mM 3-AT (confirming the yeast *HIS3* reporter gene activation by a plant activation domain-AD) (Figure 3a). To locate the position of the TaHDZipI-5 AD, different truncated variants of the TaHDZipI-5 protein were tested with the in-yeast activation assay. The empty pGBKT7 vector was transformed into

yeast and used as a negative control. After 4 days of cultivation, the yeast carrying the 26–255 residue fragment grew well on SD/-Trp/-His medium, while the yeast carrying the 1–229 aa and 1–214 residue fragments were not able to grow on the selective medium, suggesting that the putative AD localizes to the C-terminal part of the protein (amino acid residues 229–255) (Figure 3a). The identified AD region is represented by a C-terminal sequence that is conserved in TaHDZipI-5 homologues from other monocot plants (Figure 3b).

Based on molecular model predictions of TaHDZipI-5, homo-dimerization and hetero-dimerization influence the DNA binding specificity

Harris *et al.* (2016) have recently shown that the expression levels of *TaHDZipI-4* and *TaHDZipI-5* increased under cyclic drought conditions, while those of *TaHDZipI-3* remained low. These authors proposed a model explaining how TaHDZipI-3 binds to DNA *cis*-elements in a homo-dimeric form and that a hetero-

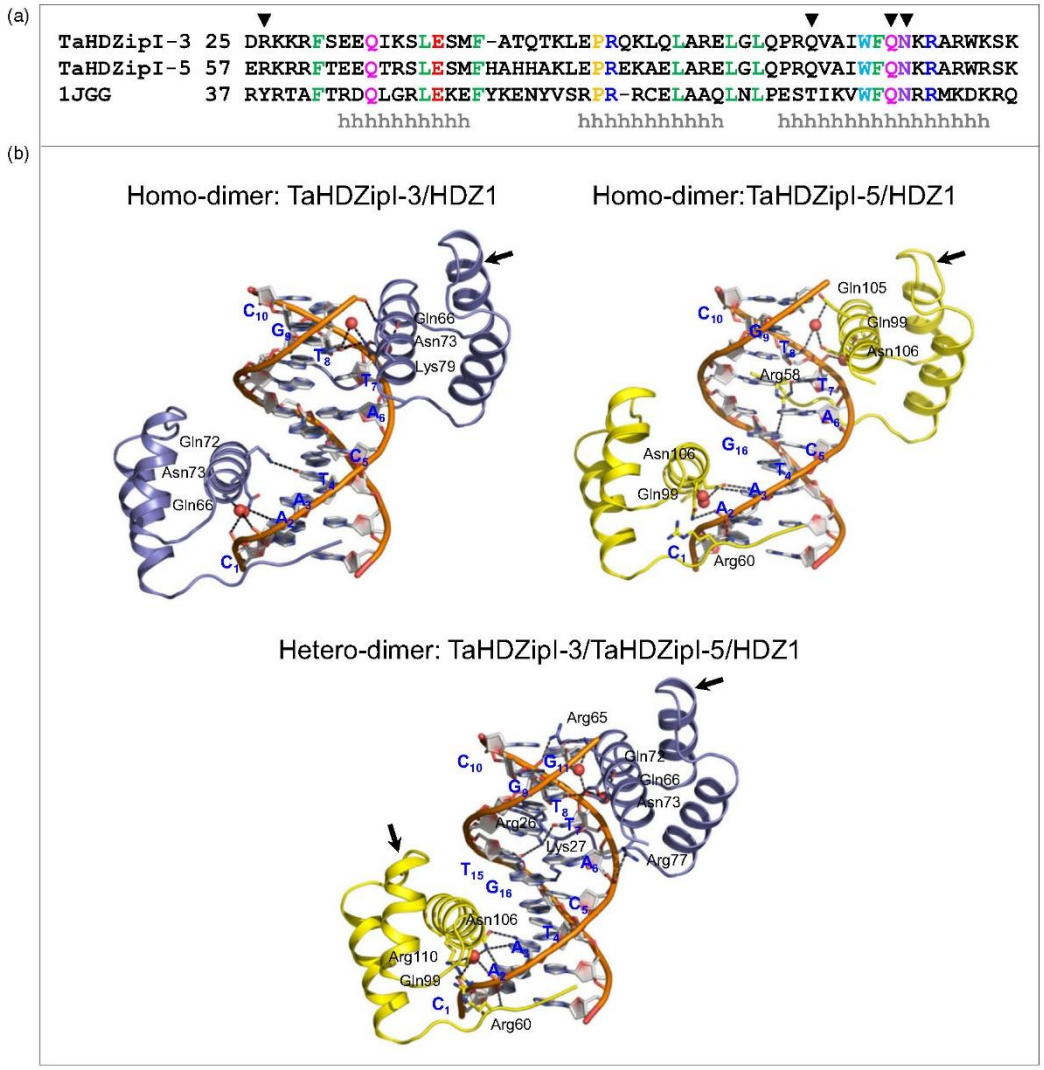


Figure 4 Molecular features of homeodomains (HDs) of TaHDZipI-3 and TaHDZipI-5 in homo- and hetero-dimeric forms in complex with the HDZ1 *cis*-element. (a) A sequence alignment of TaHDZipI-3 and TaHDZipI-5 HDs and of even-skipped HD from *Drosophila melanogaster* (PDB: 1JGG). Identical amino acid residues are coloured based on their properties. α-Helical secondary structural elements are indicated with 'h' below the sequences. HD residues that interact with DNA *cis*-elements are indicated by inverted triangles (▼). (b) Ribbon representations of homo-dimeric TaHDZipI-3 and TaHDZipI-5, and hetero-dimeric TaHDZipI-3/TaHDZipI-5 models in complex with the HDZ1 *cis*-element; blue descriptions and atomic colour representations are used for HDZ1. The ribbons of TaHDZipI-3 and TaHDZipI-5 are coloured in blue and yellow, respectively. DNA-interacting residues are shown in sticks, and DNA sugar-phosphate backbones are coloured in cpk-orange. Water molecules are shown as red spheres. Interactions (less than 3.5 Å; Table S3) between residues and HDZ1 are shown in black dashed lines. Arrows point to differences in folding of α-helices between TaHDZipI-3 and TaHDZipI-5.

dimeric form of TaHDZipI-4 and TaHDZipI-5 (also members of the class I γ-clade HDs) would initiate a stress response. In the current work, we compared 3D models of homo-dimeric TaHDZipI-3 and TaHDZipI-5, and of hetero-dimeric TaHDZipI-3/TaHDZipI-5, in complex with a HDZ1 *cis*-element to seek whether DNA interactions differed between homo- and hetero-dimeric structural models.

Structural bioinformatic comparisons of wheat TaHDZipI-3 and TaHDZipI-5 proteins showed the presence of HD domains with well-defined boundaries. An alignment of the template (PDB: 1JGG) used for structural modelling and TaHDZipI-3 and TaHDZipI-5 indicated that there was not a high level of sequence identity between the investigated proteins (Figure 4, top panel). HDs of TaHDZipI-3 shared respective 26% identity and 53% similarity to the template (PDB: 1JGG), while TaHDZipI-3 and TaHDZipI-5 between themselves shared 31% identity and 52% similarity. The positions of 15 identical residues between the template and target sequences of HDs (Figure 4a) indicated which residues might participate in DNA binding. Secondary structure element predictions in TaHDZipI-3 and TaHDZipI-5 indicated the presence of three α-helices (marked as 'h' in Figure 4, top panel) that carried most of these identical residues.

Homo-dimeric (TaHDZipI-3/TaHDZipI-3 or TaHDZipI-5/TaHDZipI-5) and hetero-dimeric (TaHDZipI-3/TaHDZipI-5) HD models in complex with HDZ1 (5′-CAATCATTGC-3′/5′-GCAATGATTG-3′; interacting nucleobases are underlined) (Sali and Blundell, 1993) were constructed to understand the differences in binding of DNA. The structural models of HDs of TaHDZipI-5 showed the presence of three α-helices, interconnected with loops similarly to TaHDZipI-3 (Figure 4, bottom panel); however, minor structural differences were observed between TaHDZipI-3 and TaHDZipI-5 (*cf.* arrows in Figure 4, bottom panel). Data from structural modelling indicated that differences in DNA binding to *cis*-elements resulted from differences in the presence of charged and polar residues at the N-terminus and at the third α-helix of each HD monomer that contacted DNA at a major groove (Figure 4, bottom panel; Table S3).

More specifically, our molecular analysis showed that Arg58 in TaHDZipI-5 at the N-terminus of the first HD monomer bound directly to two nucleobases T7 and G16, while Gln105 and Asn106 contacted the T8 nucleobase indirectly through a water molecule (Figure 4 bottom panel). In addition, Asn106 in TaHDZipI-5, which corresponds to Asn73 in TaHDZipI-3, bound to the nucleobase A3. Further, the analyses of HDZ1-binding modes in TaHDZipI-3/TaHDZipI-5 showed that two positively charged residues of the TaHDZipI-3 monomer at the N-terminus bound to the nucleobases T7 and T15 *via* Arg26 and to the nucleobase G16 through Lys27. Additionally, Asn73 at the major groove α-helix bound to the nucleobase T8, while Gln72 contacted T8 *via* a water molecule and could also form a hydrogen bond to the nucleobase G11. On the other hand, the interactions of the TaHDZipI-5 HD monomer with HDZ1 were similar to those of the second HD monomer in the homo-dimeric form.

A detailed analysis of hydrogen bond patterns in homo-dimeric (TaHDZipI-5/TaHDZipI-5) and hetero-dimeric (TaHDZipI-3/TaHDZipI-5) DNA complexes (Table S3) showed that three nucleobases (A2, T4 and T8) were bound to Asn73 (participating through both monomers) and Gln72 (Harris *et al.*, 2016). Besides, four nucleobases (A3, T7, T8 and G16) were bound to Arg58, Gln105 and Asn106 (participating through both monomers) in homo-dimeric TaHDZipI-5. The analysis of interactions in hetero-dimeric TaHDZipI-3/TaHDZipI-5 showed the participation of seven nucleobases (A2, A3, T7, T8, G11, T15 and G16) that were contacted through Arg26, Lys27, Gln72, Asn73 of TaHDZipI-3 and through Asn106 of TaHDZipI-5; Arg26 and Gln72 residues formed bidentate interactions with DNA. Free energies of homo-dimeric TaHDZipI-5 (290 kcal/mol) and hetero-dimeric TaHDZipI-3/TaHDZipI-5 (244 kcal/mol), calculated through FoldX (Schymkowitz *et al.*, 2005), indicated that hetero-dimeric TaHDZipI-3/TaHDZipI-5 was more stable than its TaHDZipI-5 homo-dimeric form.

Evaluation of transgenic wheat plants constitutively expressing *TaHDZipI-5*

Initially, transgenic wheat plants (cv. Gladius) were generated using a construct where expression of the transgene *TaHDZipI-5* was driven by a constitutive maize polyubiquitin promoter. Three independent transgenic T_1 lines, L1, L2 and L4 containing a single copy of the transgene (Figure S5), were used for characterization of plant phenotypes and yield components under well-watered conditions. Two of three transgenic lines showed a very similar phenotype to that of WT plants (Figure S6). However, L1 plants were significantly shorter than WT plants; they had a significantly lower seed number per spike and a lower grain yield than WT plants (Figure S6). All transgenic lines showed delay in flowering time compared to that of WT plants (Figure S6).

The T_3 progeny of transgenic and WT plants were grown in two large containers with different watering regimes (Figure S1; Table S4). Using pilot experiments described in the Materials and Methods, all three starting T_2 lines, L1-3-9, L2-7-9 and L4-8-9 (pUbi-TaHDZipI-5), were identified to be homozygous for the transgene. The phenotypic data of transgenic wheat lines were compared to those of WT plants (Figure 5). The data obtained from the well-watered bin correlated well with those obtained for T_1 transgenic plants grown in pots. The progeny of L1-3-9 had reduced plant height, less dry biomass, fewer tillers, spikes and seeds, and about 90% lower grain yield (seed weight per plant) than WT plants. The other two lines showed up to a 25%–30% decrease in all parameters compared with those of WT plants, except for flowering time where differences did not exceed 2 days (Figure 5). In contrast to the data obtained under well-watered conditions, the differences in growth characteristics and yield parameters between progenies of these two lines (L2-7-9 and L4-8-9) and WT plants were small under drought (Figure 5).

Drought tolerance (survival) of transgenic wheat seedlings was significantly higher than that of WT plants

Three-week-old transgenic wheat seedlings (T_2 progenies of sublines L2-7 and L4-8 transformed with pUbi-TaHDZipI-5, and WT plants, ten plants for each class) were used in three independent drought tolerance experiments. We only examined lines with minimal phenotypic differences compared to those of WT plants. Two control plants and two transgenic plants from each line were planted in the same pot with the aim to minimize influence of differences in seedling sizes and respective differences in water consumption on water availability in the soil. Only 10% of WT plants survived the applied drought conditions and recovered after rewatering (Figure 6a). In contrast, both tested transgenic lines showed a significantly stronger ability to recover than WT plants, with over half of all tested plants surviving (Figure 6a).

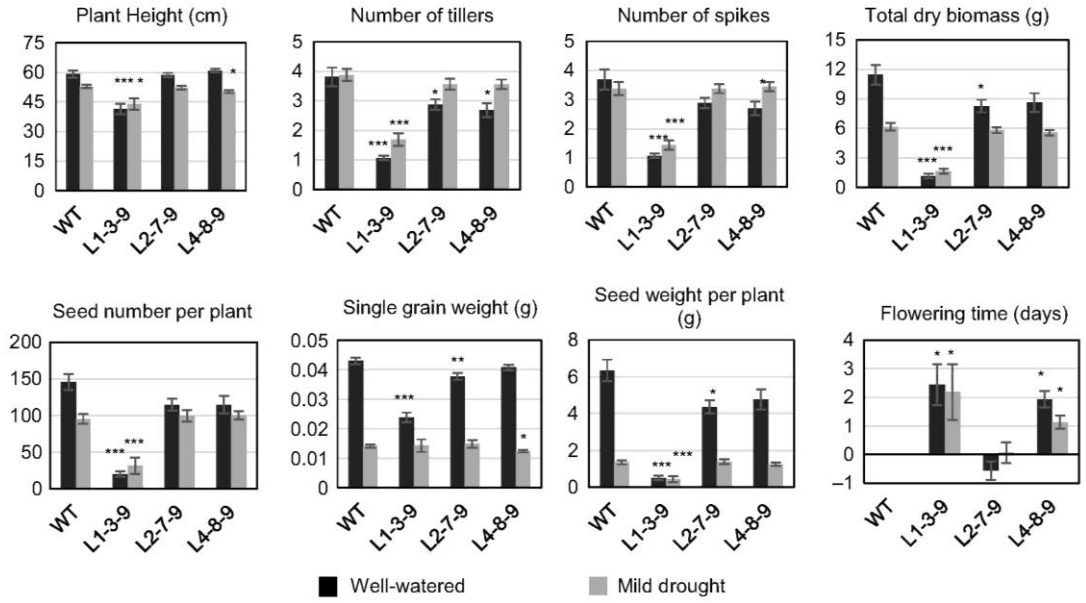


Figure 5 Growth characteristics and yield components of control wild-type (WT) (*Triticum aestivum* cv. Gladius) and T_3 transgenic wheat transformed with pUbi-TaHDZipI-5 under well-watered (black boxes) and under drought conditions (grey boxes). Flowering time of transgenic plants was compared to the average flowering time of 16 control WT plants, which is represented as day 0. Differences between transgenic lines and WT plants in each of the well-watered and drought conditions were tested using unpaired Student's *t*-tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Transgenic wheat seedlings tolerate frost better than WT plants

Three-week-old T_1 (Figure 6b) and T_3 (Figure 6c) generations of transgenic seedlings were used in frost tolerance tests. Twelve plants of each transgenic line and twelve WT plants were treated in a semi-automated cold cabinet, using an updated program (Figure S2) based on the earlier established protocol for barley (Kovalchuk *et al.*, 2013). This included 6.5-h exposure to −7 °C for the T_1 generation and the same exposure time to −8 °C for T_3 plants; both generations were tested in three independent experiments. All tested transgenic lines showed a higher survival rate than those of WT plants in both experiments (Figure 6b,c). Levels of transgene expression were determined by Q-PCR using RNA isolated from unstressed wheat leaves collected from transgenic and control plants before frost tolerance tests were conducted (Figure 6d).

Evaluation of transgenic wheat plants with stress-inducible expression of *TaHDZipI-5*

With the aim to avoid or minimize the influence of *TaHDZipI-5* overexpression on the developmental phenotype of transgenic wheat, we replaced the *ZmUbiquitin* constitutive promoter with one of the stress-inducible promoters from *OsWRKY71* and *TdCor39* genes. These stress-inducible promoters were previously employed to avoid the negative influences of *TaDREB3* gene expression on phenotype and yield in transgenic barley (Kovalchuk *et al.*, 2013). Generation of transgenic plants, selection of homozygous lines, assessment of yield components and evaluation of frost tolerance were performed similarly as for transgenic wheat plants transformed with the pUbi-TaHDZipI-5 construct (Figures 7 and 8). Surprisingly, cold-inducible expression of the transgene led to a reduced difference between flowering time of transgenic and WT plants only for the plants with the *pWRKY71* promoter, whereas both stress-inducible promoters, *pWRKY71* and *pCor39*, stabilized the single grain weight in transgenics (Figures 7a and 8a). Although both promoters showed the low levels of basal promoter activity in unstressed transgenic wheat plants (Figures 7b and 8b), many of the negative phenotype and yield characteristics failed to improve, compared to transgenic plants with constitutive transgene expression. Nevertheless, both transgenics demonstrated the substantial enhancement of frost tolerance during the vegetative developmental stage (Figures 7c and 8c).

Discussion

The *TaHDZipI-5* cDNA was isolated in a Y2H screen using *TaHDZipI-3* as bait, from a cDNA library prepared from flag leaves and spikes of *Triticum aestivum* L. genotype RAC875, subjected to drought and heat stresses. Subsequently, the gene and gene product were characterized at the molecular level (Harris *et al.*, 2016). *TaHDZipI-5* expression was induced by drought, frost and ABA treatment. Based on a close evolutionary relationship with the *Arabidopsis* γ-clade of the HD-Zip I subfamily and conserved intron/exon structure, the *TaHDZipI-5* gene was identified as a monocot-specific member of the γ-clade (Harris *et al.*, 2016). The closest homologues of *TaHDZipI-5* from rice and maize, *Oshox22* and *Zmhdz4*, participate in ABA-mediated drought response, and expression of these genes is up-regulated by water deficiency (Wu *et al.*, 2016; Zhang *et al.*, 2012).

The analysis of transgenic rice and *Arabidopsis* plants revealed that constitutive overexpression of *Oshox22* and another monocot-specific γ-clade member from rice, *Oshox24*, leads to

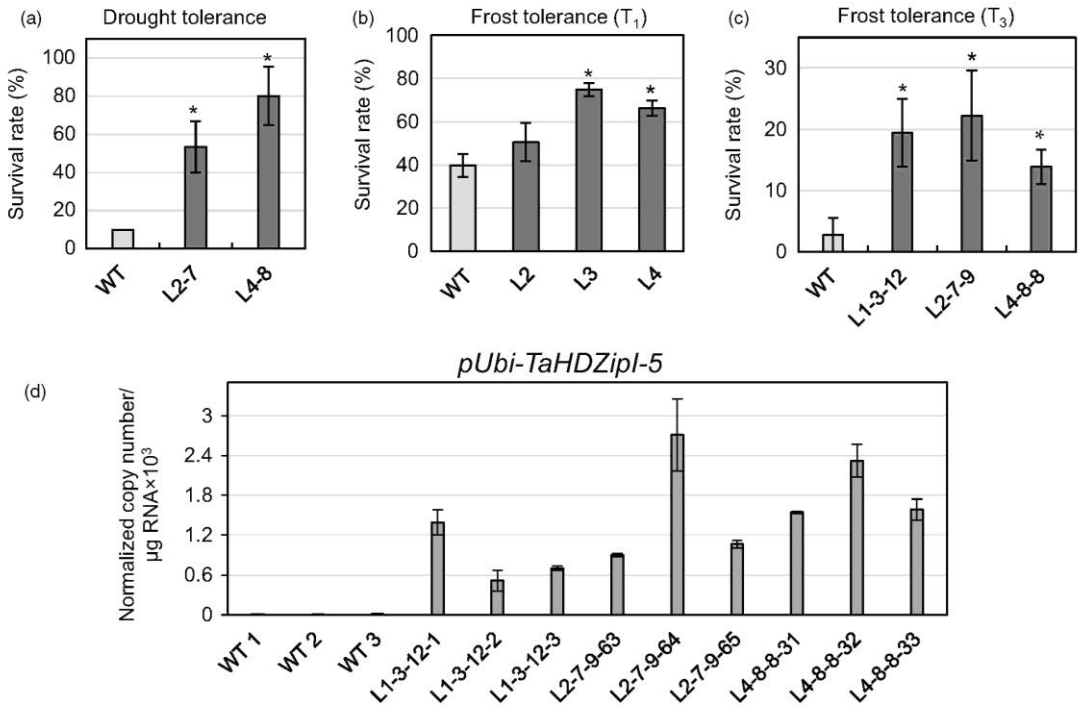


Figure 6 Comparison of drought and frost tolerance of wild-type (WT) (*Triticum aestivum* cv. Gladius) and transgenic wheat transformed with pUbi-TaHDZipI-5. (a) Drought tolerance of two independent transgenic lines (T_2 progeny), sizes of which were similar to those of the control WT plants', is shown as the survival rate of plants recovered after the terminal drought stress, followed by rewatering. (b, c) Survival rates of plants recovered after seedling-stage frost: (b) T_1 progeny and (c) T_3 progeny of transgenic plants. Error bars represent ± SD of three independent experiments. Values represent means ± SE (*n* varies for each column and is shown in each case directly on the graphs). Significant differences between transgenic lines and WT plants were tested using an unpaired Student's *t*-test (*$P < 0.05$). (d) The expression levels of the *TaHDZipI-5* transgene in leaves of T_3 plants in unstressed control WT and transgenic plants, sampled prior to frost treatment. Error bars represent ± SD of three technical replicates.

negative regulation of response to drought/dehydration and, hence, to increased sensitivity of transgenic plants to drought (Bhattacharjee *et al.*, 2016, 2017; Zhang *et al.*, 2012). In contrast, overexpression of the very similar gene from maize, *Zmhdz4,* positively regulated plant responses to stress in transgenic *Arabidopsis* and conferred tolerance to drought in transgenic rice (Wu *et al.*, 2016).

Regulation of HD-Zip I gene expression by low temperatures was demonstrated for *Arabidopsis* (Cabello *et al.*, 2012), tomato (Zhang *et al.*, 2014), paper mulberry (Peng *et al.*, 2015), sunflower (Cabello *et al.*, 2012), rice (Zhang *et al.*, 2012) and wheat (Harris *et al.*, 2016). The overexpression of α-clade HD-Zip class I TFs conferred cold/frost tolerance to transgenic *Arabidopsis* and barley (Cabello *et al.*, 2012; Kovalchuk *et al.*, 2016); however, the influence on the overexpression of monocot-specific γ-clade members on cold or frost tolerance of transgenic plants remains to be determined.

The molecular characterization of the *TaHDZipI-5* gene and its protein product by Harris *et al.* (2016) and in this work demonstrated its role in wheat tolerance to drought and frost. These studies were conducted through overexpression of the *TaHDZipI-5* gene in transgenic wheat and the evaluation of transgenic plants. We compared phenotypes and yield components of transgenic and control WT wheat plants under optimal growth conditions and under a slowly increasing drought. The ultimate aims of this work were to optimize transgene performance, decrease negative influences of the transgene on plant development using stress-inducible promoters and select the optimal homozygous lines for field trials in Australian agricultural regions that are prone to seasonal frosts and long periods of drought (annual and monthly potential frost days; http://www.bom.gov.au/jsp/ncc/climate_averages/frost/index.jsp).

The analysis of *TaHDZipI-5* expression in a variety of wheat tissues revealed that the level of this gene was elevated in flowers, developing grain and particularly in the endosperm, a plant tissue that contains increased ABA. In contrast, the number of *TaHDZipI-5* transcripts in vegetative tissues was low (Figure 2a). Expression of *TaHDZipI-5* in flowers shortly before fertilization, and during the early stages of grain development, and the strong induction of *TaHDZipI-5* expression by low temperatures possibly suggest the involvement of this gene in the protection of wheat tissues that are most vulnerable to night frosts.

To understand the function of the *TaHDZipI-5* gene, we isolated gene promoters and revealed DNA-specific *cis*-elements responsible for the ABA-dependent promoter activation. As the gene/promoter sequences of *TaHDZipI-5* were not available in databases, we analysed the *TdHDZipI-5A* and *TdHDZipI-5B*

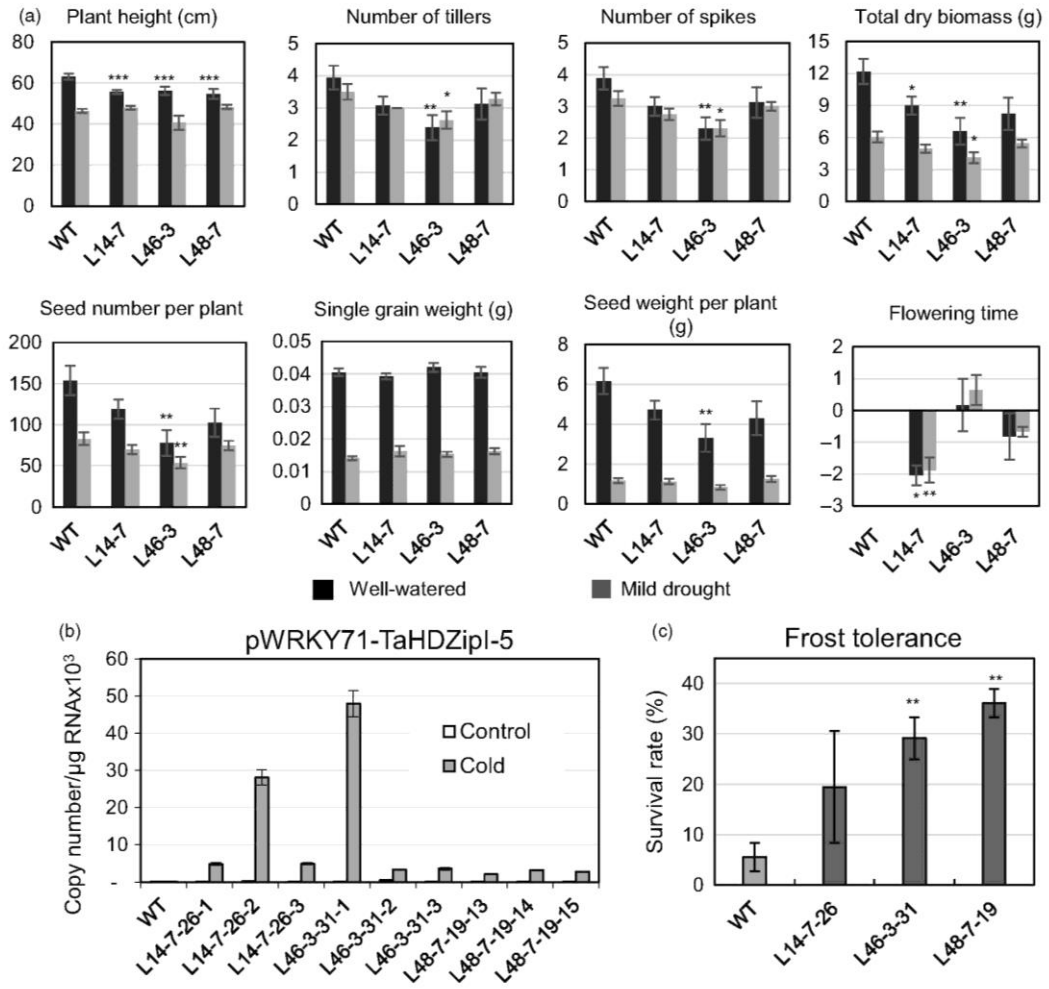


Figure 7 Characteristics of transgenic wheat transformed with pWRKY71-TaHDZipI-5. (a) Comparisons of plant growth and yield characteristics of wild-type (WT) and transgenic T_2 plants under well-watered conditions (black boxes) and mild drought (grey boxes). (b) Transgene expression levels in control WT and transgenic T_4 plants at 23 °C (control) and 4 °C (cold). (c) Frost tolerances of WT and transgenic wheat transformed with pWRKY71-TaHDZipI-5 are shown as the survival rate of plants recovered after the terminal frost treatment. Error bars represent ± SD for three independent experiments. Differences between transgenic lines and WT plants were tested in the unpaired Student's *t*-test (*$P < 0.05$, **$P < 0.01$, *** for $P < 0.001$).

promoters of homeologous genes from durum wheat. Firstly, several concentrations of ABA were tested to select the minimal endogenous concentration (0.5 mM) leading to a strong promoter activation (data not shown). Secondly, we analysed the 1055-bp sequence of the *TaHDZipI-5A* promoter (including 5′UTR), which could activate ABA-dependent TFs. Mapping revealed that the promoter was activated through the proximal MYBR element GGATA (−310 bp from the translation start), which was identified by Baranowskij *et al.* (1994) as the binding site for MYBSt1, a MYB-like protein with an endosperm-related function (Mercy *et al.*, 2003). Additionally, the activity of the *TdHDZipI-5A* promoter was enhanced by two ABREs and/or one MYBR elements, situated upstream of the proximal MYBR element (Figures 2b and S3). All mapped *cis*-elements were found in the promoters of both homeologous durum genes in conserved positions. Predicted ABRE situated in the D4 fragment of the promoter close to the TATA box was not involved in promoter activation by ABA.

Using a transient expression assay in wheat cells and a reporter construct with synthetic promoter, Harris *et al.* (2016) demonstrated that TaHDZipI-5 acted as a transcriptional activator. In this work, the transactivation TaHDZipI-5 domain was defined in an in-yeast activation assay. A series of CDS deletions encoding truncated variants of TaHDZipI-5 were generated, and the constructs were transformed in yeast cells to detect transactivation activity. Similar to Zmhdz4 (Wu *et al.*, 2016) and Oshox22 (Zhang *et al.*, 2012), TaHDZipI-5 was found to contain an AD at the C-terminal region of the protein (Figure 3a), which is a highly

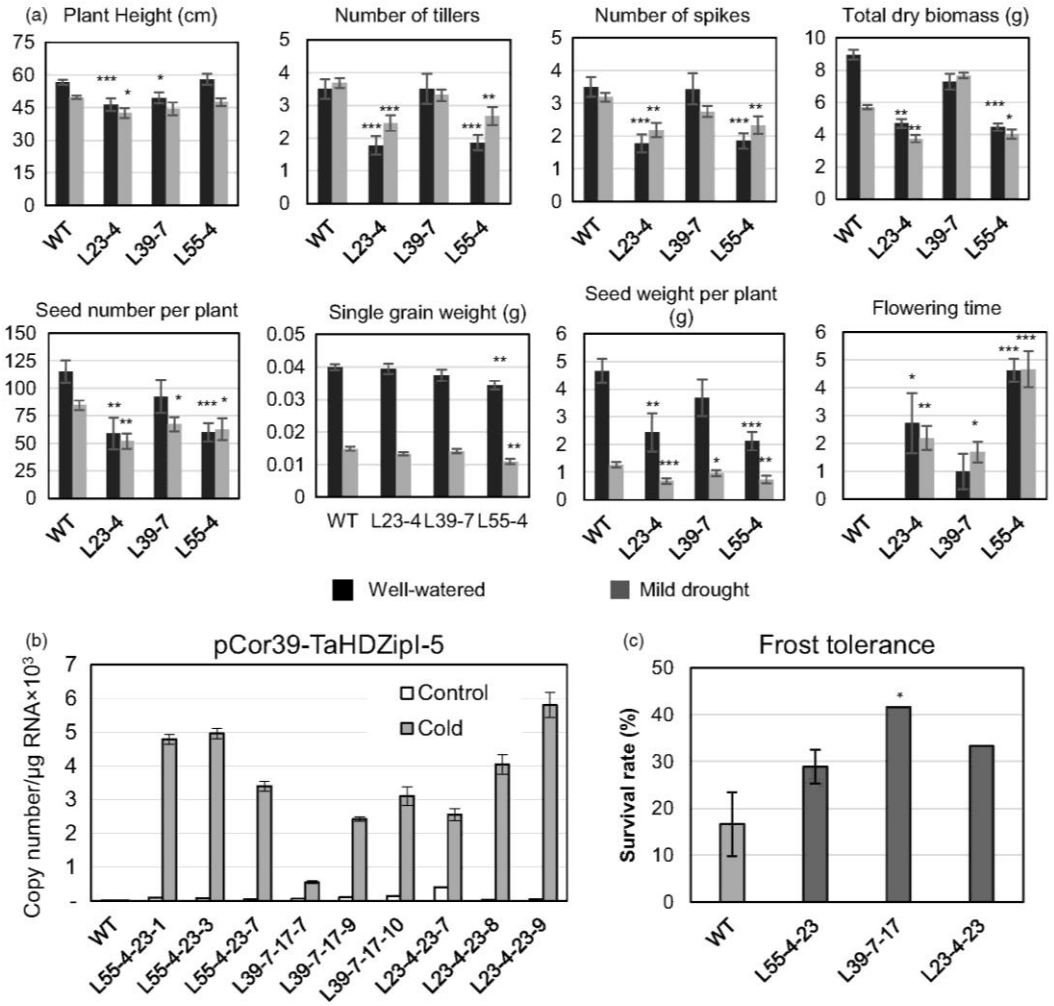


Figure 8 Characteristics of transgenic wheat transformed with pCor39-TaHDZipI-5. (a) Comparison of plant growth and yield characteristics of wild-type (WT) and transgenic T_2 plants, under well-watered conditions (black boxes) and mild drought (grey boxes). (b) Transgene expression levels in WT and transgenic T_4 plants at 23 °C (control) and 4 °C (cold). (c) Frost tolerance of WT and transgenic wheat transformed with pCor39-TaHDZipI-5 is shown as the survival rate of plants recovered after the terminal frost treatment. Error bars represent ± SD for three independent experiments. Differences between transgenic lines and WT plants were tested in the unpaired Student's *t*-test (*$P < 0.05$, **$P < 0.01$, *** for $P < 0.001$).

conserved sequence in homologous proteins from various grasses (Figure 3b).

According to our previous study (Harris *et al.*, 2016), all wheat HD-Zip I γ-clade members homo-dimerize and also interact with other members through hetero-dimerization. We revealed that TaHDZipI-5 displayed an equal propensity to form homo-dimeric TaHDZipI-5 and hetero-dimeric TaHDZipI-3/TaHDZipI-5 complexes. However, the DNA interaction differences between TaHDZipI-5 homo- and hetero-dimers remained unclear. Hence, we constructed the 3D models of homo-dimers of TaHDZipI-5 or TaHDZipI-3 and hetero-dimeric TaHDZipI-3/TaHDZipI-5, in complex with HDZ1 *cis*-elements, to explore the differences in DNA binding between homo- and hetero-dimeric structures. 3D models showed that DNA interactions in the TaHDZipI-3/TaHDZipI-5 hetero-dimer were more stable than those in the TaHDZipI-5 homo-dimer. These models suggested that the TaHDZipI-3/TaHDZipI-5 hetero-dimer is more efficient in binding DNA. This may indicate that the TaHDZipI-3/TaHDZipI-5 hetero-dimer could be more efficient also *in vivo*, during the activation of target promoters, than the TaHDZipI-5 homo-dimer.

Initially, we overexpressed the *TaHDZipI-5* gene in transgenic wheat plants using a constitutive polyubiquitin promoter from maize. Homozygous T_1 or T_2 sublines were selected in a pilot experiment using T_2 and T_3 generations, and seeds of selected sublines were used for the analyses of growth characteristics and yield components under sufficient and limited watering (Figure 5). This experiment was performed in large containers to better reflect interplant competition for water, light and nutrients that might occur in the field. We observed that the *TaHDZipI-5* transgene negatively influenced plant phenotypes by

decreasing the numbers of tillers and spikes per plant and consequently decreasing the total plant biomass and seed number compared to those of WT plants. Under well-watered conditions, the differences in all characteristics were significantly higher than under drought (Figure 5). Differences in flowering time resulted in 1- to 3-day delays in T_3 sublines compared to the average flowering times of control plants. The differences in flowering times of selected T_1 plants amounted up to 2 weeks (Figure S6), but these differences decreased in two subsequent generations of transgenic lines. In contrast, the remainder of growth and yield characteristics in the T_1 generation of transgenic plants diverged less, most probably because they represented a mixture of homo- and heterozygous plants, including those of null segregants, which were not excluded from this analysis.

Under well-watered conditions, lines L2 and L4 had more similar phenotypes compared to those of WT plants and L1 in T_1 and T_3 generations. Thus, these two lines were selected for drought tolerance evaluation, which could be defined as plant's ability to survive severe drought at the vegetative developmental stage. Seedlings of transgenic and control plants were grown and assessed in the same pot, to accommodate differences in seedling sizes. These data, using T_2 plants, suggested approximately fivefold to eightfold enhancement of drought tolerance in both transgenic lines compared to control plants (Figure 6a). This observation is in contradiction with that obtained for *Oshox22*, overexpressed in transgenic rice (Zhang *et al.*, 2012), but correlates with the data obtained for *Zmhdz4*, overexpressed in transgenic *Arabidopsis* and rice (Wu *et al.*, 2016). For these discrepancies, we offer the following explanation. Both *Zmhdz4* and *TaHDZipI-5* originate from plants which have different physiological responses to drought compared to rice. Therefore, *Zmhdz4* and *TaHDZipI-5* may have different biological roles to *Oshox22*, which might be connected to small, but functionally important differences in protein structure or to differences in spatial or temporal patterns of gene expression. These hypotheses require further investigation.

Frost tolerance experiments were performed once with T_1 transgenic plants (Figure 6b) with null segregants identified and excluded from the analysis and twice using T_3 homozygous transgenic lines (Figure 6c,d). Both experiments were performed similarly; however, in the second experiment (using T_3 homozygous transgenic lines), the minimal incubation temperature was 1 °C lower than that in the first experiment (using T_1 plants). This resulted in a lower survival rate of WT plants in the second experiment. An enhancement of frost tolerance was observed in all tested lines in both experiments, confirming that *TaHDZipI-5* is a promising candidate gene for improvement of wheat frost tolerance. The analysis of potential downstream stress-inducible LEA (late embryogenesis abundant)/COR (cold-responsive)/DHN (dehydrin) genes in control WT and transgenic lines with the constitutive overexpression of the *TaHDZipI-5* transgene revealed the up-regulation of *TaCOR14B* (GenBank: AF207546) and *TaRAB15* (GenBank: X59133) transcripts in several transgenic lines (Figure S7). However, this up-regulation did not correlate with the levels of *TaHDZipI-5* transgene transcripts (Figure 6d).

Constitutive overexpression of *TaHDZipI*-5 led to a negative effect of the transgene on the plant phenotype similar to overexpression of *Oshox22* in transgenic rice; this also resulted in a stunted phenotype of transgenic plants, a smaller size of plants and fewer tillers (Zhang *et al.*, 2012). To eliminate or decrease the negative effect of the transgene on the phenotype of transgenic wheat lines, the constitutive promoter was replaced with stress-inducible *pWRKY71* and *pCor39* promoters, which were previously used to optimize the phenotype of transgenic barley (Kovalchuk *et al.*, 2013). Both promoters are active in vegetative and flowering parts of the plant. The first promoter originates from the rice *OsWRKY71* gene, and in barley, it was moderately activated by cold and weakly activated by drought. The activity of the promoter was ABA-independent, and the basal level of activity in transgenic barley was low. In contrast, the second promoter, which originates from the *TdCor39* gene, was strongly activated in barley by frost and drought. *TdCor39* was strongly induced by ABA and had a moderate basal level of activity in barley (Kovalchuk *et al.*, 2013).

Transgenic wheat lines with the *TaHDZipI*-5 transgene driven by *pWRKY71* and *pCor39* promoters showed activation of both promoters under low temperature (Figures 7b and 8b), and low levels of transgene expression in the absence of stress. Inducible transgene expression improved single grain weight compared to when the constitutive polyubiquitin promoter was used. Single grain weights did not decrease in plants with either stress-inducible promoters compared to WT. Differences in flowering times between transgenic and control plants were smaller in plants with the *pWRKY71* promoter, compared to those containing *pUbi* promoter constructs. However, the improvement or elimination of other negative changes in phenotypes of transgenic lines growing under conditions of sufficient watering was not achieved. This disappointing result was somewhat surprising because the levels of *pWRKY71* and *pCor39* promoter activities in the absence of stress (basal levels) were low. One feasible explanation for these results could be that the application of heterologous promoters led to a 'poisonous' effect of the transgene on plant development because of the wrong spatial pattern of *TaHDZipI-5* expression. This problem could be ameliorated by (i) using the native *TaHDZipI-5* promoter boosted by the addition of enhancing elements, so that the spatial activity of the promoter remains unchanged (Chen *et al.*, 2017), (ii) enhancing the efficiency of translation of the native *TaHDZipI-5* gene or (iii) manipulation of native TaHDZipI-5 protein structure to increase the strength of binding to target *cis*-elements or the efficacy of ABA-dependent protein activation. These options could be explored with the recently developed technology of genome editing using engineered nucleases, without the generation of transgenic plants with an extra copy of the target gene.

Experimental procedures

Plasmid construction and plant transformation

The generation of vectors for plant transformation has been described previously (Eini *et al.*, 2013; Kovalchuk *et al.*, 2013; Morran *et al.*, 2011). Briefly, the *2x35S* promoter was excised from the pMDC32 vector (Curtis and Grossniklaus, 2003) and replaced with one of three promoters: the constitutive *ZmUbiquitin* promoter (Eini *et al.*, 2013), or stress-inducible *OsWRKY71* or *TdCor39* promoters (Kovalchuk *et al.*, 2013), resulting in pUbi, pWRKY71 and pCor39 vectors, respectively. A 767-bp fragment of the *TaHDZipI-5* coding sequence (CDS) (GenBank accession KT224376) (Harris *et al.*, 2016) was isolated from *T. aestivum* L. cv. RAC875 and cloned into a pENTR-D-TOPO vector (Invitrogen, Melbourne, Victoria, Australia). The cloned insert was verified by sequencing and subcloned into the pUbi, pWRKY71 and pCor39 vectors, resulting in pUbi-TaHDZipI-5, pWRKY71-TaHDZipI-5 and pCor39-TaHDZipI-5 constructs.

Constructs were transformed in the Australian elite bread wheat cv. Gladius using a biolistic bombardment method (Ismagul *et al.*, 2014; Kovalchuk *et al.*, 2009). Genomic DNA was isolated from leaf tissue using a freeze-drying method described by Shavrukov *et al.* (2010). Transgene integration was confirmed by PCR using a forward primer from the 3′ end of the *TaHDZipI-5* coding region and a reverse primer from the 5′ end of the *nos* terminator (Table S1). Transgene genomic copy number was estimated in the T_0 and/or T_1 progenies of selected transgenic lines by quantitative real-time PCR (Q-PCR), based on the $2^{-\Delta\Delta Ct}$ method (Kovalchuk *et al.*, 2013; Li *et al.*, 2004). *Nos* terminator primers and a specific DNA probe (Yadav *et al.*, 2015) were used for transgene amplification, and endogenous *Puroindoline-b* (*Pin-b*) gene primers and probe (Li *et al.*, 2004; Yadav *et al.*, 2015) were used for template loading normalization (Table S1). T_1 lines with a single copy number were selected for further analysis. The selections of homozygous T_1 lines were conducted in pilot experiments using the T_2 progeny of transgenic T_1 lines with two copies of transgene or T_3 progeny of T_2 lines. In these experiments, transgene integration was assessed by PCR in twelve seedlings of each line. The line was considered as homozygous if the expected PCR product was observed for all twelve plants. Seeds of homozygous T_1 (or T_2) lines from each construct were selected and used for phenotyping and stress tolerance tests.

Appendix S1 contain the descriptions of gene expression by quantitative real-time PCR, cloning of promoters and the identification of ABA-responsive *cis*-elements, analysis of evolutionary relationships, in-yeast activation assay, construction of 3D models, analysis of transgenic plants, drought tolerance tests and survival rates of seedlings under terminal drought, and frost tolerance tests.

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Conflict of interest

The authors declare no conflict of interest.

References

Agalou, A., Purwantomo, S., Overnas, E., Johannesson, H., Zhu, X., Estiati, A., Kam, R.J.D. *et al.* (2008) A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. *Plant Mol. Biol.* **66**, 87–103.

Agarwal, P.K., Gupta, K., Lopato, S. and Agarwal, P. (2017) Dehydration responsive element binding transcription factors and their applications for the engineering of stress tolerance. *J. Exp. Bot.* **68**, 2135–2148.

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., Arkel, G.V. and Pereira, A. (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. *Plant Cell*, **16**, 2463–2480.

Ariel, F.D., Manavella, P.A., Dezar, C.A. and Chan, R.L. (2007) The true story of the HD-Zip family. *Trends Plant Sci.* **12**, 419–426.

Baranowskij, N., Frohberg, C., Prat, S. and Willmitzer, L. (1994) A novel DNA binding protein with homology to Myb oncoproteins containing only one repeat can function as a transcriptional activator. *EMBO J.* **13**, 5383–5392.

Bhattacharjee, A., Khurana, J.P. and Jain, M. (2016) Characterization of rice homeobox genes, *OsHOX22* and *OsHOX24*, and over-expression of *OsHOX24* in transgenic *Arabidopsis* suggest their role in abiotic stress response. *Front. Plant Sci.* **7**, 627.

Bhattacharjee, A., Sharma, R. and Jain, M. (2017) Over-expression of *OsHOX24* confers enhanced susceptibility to abiotic stresses in transgenic rice via modulating stress-responsive gene expression. *Front. Plant Sci.* **8**, 628.

Bi, H., Luang, S., Li, Y., Bazanova, N., Morran, S., Song, Z., Perera, M.A. *et al.* (2016) Identification and characterization of wheat drought-responsive MYB transcription factors involved in the regulation of cuticle biosynthesis. *J. Exp. Bot.* **67**, 5363–5380.

Bi, H., Luang, S., Li, Y., Bazanova, N., Borisjuk, N., Hrmova, M. and Lopato, S. (2017) Wheat drought-responsive WXPL transcription factors regulate cuticle biosynthesis genes. *Plant Mol. Biol.* **94**, 15–32.

Borisjuk, N., Hrmova, M. and Lopato, S. (2014) Transcriptional regulation of cuticle biosynthesis. *Biotechnol. Adv.* **32**, 526–540.

Braam, J., Sistrunk, M.L., Polisensky, D.H., Xu, W., Purugganan, M.M., Antosiewicz, D.M., Campbell, P. *et al.* (1997) Plant responses to environmental stress: regulation and functions of the *Arabidopsis TCH* genes. *Planta*, **203**, S35–S41.

Brandt, R., Cabedo, M., Xie, Y. and Wenkel, S. (2014) Homeodomain leucine-zipper proteins and their role in synchronizing growth and development with the environment. *J. Integr. Plant Biol.* **56**, 518–526.

Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.* **2**, 48–54.

Cabello, J.V. and Chan, R.L. (2012) The homologous homeodomain–leucine zipper transcription factors HaHB1 and AtHB13 confer tolerance to drought and salinity stresses via the induction of proteins that stabilize membranes. *Plant Biotechnol. J.* **10**, 815–825.

Cabello, J.V., Arce, A.L. and Chan, R.L. (2012) The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins. *Plant J.* **69**, 141–153.

Cenci, A., Chantret, N., Kong, X., Gu, Y., Anderson, O.D., Fahima, T., Distelfeld, A. *et al.* (2003) Construction and characterization of a half million clone BAC library of durum wheat (*Triticum turgidum* ssp *durum*). *Theor. Appl. Genet.* **107**, 931–939.

Chan, R.L., Gago, G.M., Palena, C.M. and Gonzalez, D.H. (1998) Homeoboxes in plant development. *Biochim. Biophys. Acta*, **1442**, 1–19.

Chen, J., Fan, X., Qian, K., Zhang, Y., Song, M., Liu, Y., Xu, G. *et al.* (2017) pOsNAR2.1: OsNAR2.1 expression enhances nitrogen uptake efficiency and grain yield in transgenic rice plants. *Plant Biotechnol. J.* **15**, 1273–1283.

Chew, W., Hrmova, M. and Lopato, S. (2013) Role of homeodomain leucine zipper (HD-Zip) IV transcription factors in plant development and plant protection from deleterious environmental factors. *Int. J. Mol. Sci.* **14**, 8122–8147.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133**, 462–469.

Deng, X., Phillips, J., Meijer, A.H., Salamini, F. and Bartels, D. (2002) Characterization of five novel dehydration-responsive homeodomain leucine zipper genes from the resurrection plant *Craterostigma plantagineum*. *Plant Mol. Biol.* **49**, 601–610.

Dezar, C.A., Fedrigo, G.V. and Chan, R.L. (2005a) The promoter of the sunflower HD-Zip protein gene Hahb4 directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA. *Plant Sci.* **169**, 447–456.

Dezar, C.A., Gago, G.M., González, D.H. and Chan, R.L. (2005b) *Hahb-4*, a sunflower homeobox-leucine zipper gene, is a developmental regulator and

confers drought tolerance to *Arabidopsis thaliana* plants. *Transgenic Res.* **14**, 429–440.

Eini, O., Yang, N., Pyvovarenko, T., Pillman, K., Bazanova, N., Tikhomirov, N., Eliby, S. *et al.* (2013) Complex regulation by Apetala2 domain-containing transcription factors revealed through analysis of the stress-responsive *TdCor410b* promoter from durum wheat. *PLoS ONE*, **8**, e58713.

Frank, W., Phillips, J., Salamini, F. and Bartels, D. (1998) Two dehydration-inducible transcripts from the resurrection plant *Craterostigma plantagineum* encode interacting homeodomain-leucine zipper proteins. *Plant J.* **15**, 413–421.

Gahlaut, V., Jaiswal, V., Kumar, A. and Gupta, P.K. (2016) Transcription factors involved in drought tolerance and their possible role in developing drought tolerant cultivars with emphasis on wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* **129**, 2019–2042.

Gehring, W.J., Müller, M., Affolter, M., Percival-Smith, A., Billeter, M., Qian, Y.Q., Otting, G. *et al.* (1990) The structure of the homeodomain and its functional implications. *Trends Genet.* **6**, 323–329.

Harris, J.C., Hrmova, M., Lopato, S. and Langridge, P. (2011) Modulation of plant growth by HD-Zip class I and II transcription factors in response to environmental stimuli. *New Phytol.* **190**, 823–837.

Harris, J.C., Sornaraj, P., Taylor, M., Bazanova, N., Baumann, U., Lovell, B., Langridge, P. *et al.* (2016) Molecular interactions of the γ-clade homeodomain-leucine zipper class I transcription factors during the wheat response to water deficit. *Plant Mol. Biol.* **90**, 435–452.

Henriksson, E., Olsson, A.S., Johannesson, H., Johansson, H., Hanson, J., Engström, P. and Söderman, E. (2005) Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships. *Plant Physiol.* **139**, 509–518.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* **27**, 297–300.

Hrmova, M. and Lopato, S. (2014) Enhancing abiotic stress tolerance in plants by modulating properties of stress responsive transcription factors. In *Genomics of Plant Genetic Resources* (Tuberosa, R., Graner, A. and Frison, E., eds), pp. 291–316. Dordrecht, the Netherlands: Springer.

Huang, X. and Miller, W. (1991) A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math.* **12**, 337–357.

Hwang, I., Chen, H.C. and Sheen, J. (2002) Two-component signal transduction pathways in *Arabidopsis*. *Plant Physiol.* **129**, 500–515.

Ismagul, A., Iskakova, G., Harris, J.C. and Eliby, S. (2014) Biolistic transformation of wheat with centrophenoxine as a synthetic auxin. Biolistic transformation of wheat with centrophenoxine as a synthetic auxin. *Methods Mol. Biol.* **1145**, 191–202.

Jiang, Y. and Deyholos, M.K. (2009) Functional characterization of *Arabidopsis* NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. *Plant Mol. Biol.* **69**, 91–105.

Kovalchuk, N., Smith, J., Pallotta, M., Singh, R., Ismagul, A., Eliby, S., Bazanova, N. *et al.* (2009) Characterization of the wheat endosperm transfer cell-specific protein TaPR60. *Plant Mol. Biol.* **71**, 81–98.

Kovalchuk, N., Jia, W., Eini, O., Morran, S., Pyvovarenko, T., Fletcher, S., Bazanova, N. *et al.* (2013) Optimization of *TaDREB3* gene expression in transgenic barley using cold-inducible promoters. *Plant Biotechnol. J.* **11**, 659–670.

Kovalchuk, N., Chew, W., Sornaraj, P., Borisjuk, N., Yang, N., Singh, R., Bazanova, N. *et al.* (2016) The homeodomain transcription factor TaHDZipI-2 from wheat regulates frost tolerance, flowering time and spike development in transgenic barley. *New Phytol.* **211**, 671–687.

Laughon, A. and Scott, M.P. (1984) Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA-binding proteins. *Nature*, **310**, 25–31.

Li, Z.W., Hansen, J.L., Liu, Y., Zemetra, R.S. and Berger, P.H. (2004) Using real-time PCR to determine transgene copy number in wheat. *Plant Mol. Biol. Rep.* **22**, 179–188.

Lopato, S., Bazanova, N., Morran, S., Milligan, A.S., Shirley, N. and Langridge, P. (2006) Isolation of plant transcription factors using a modified yeast one-hybrid system. *Plant Methods*, **2**, 3–17.

Mattsson, J., Söderman, E., Svenson, M., Borkird, C. and Engström, P. (1992) A new homeobox-leucine zipper gene from *Arabidopsis thaliana*. *Plant Mol. Biol.* **18**, 1019–1022.

Meijer, A.H., Scarpella, E., Dijk, E.L., Qin, L., Taal, A.J., Rueb, S., Harrington, S.E. *et al.* (1997) Transcriptional repression by Oshox1, a novel homeodomain leucine zipper protein from rice. *Plant J.* **11**, 263–276.

Mercy, I.S., Meeley, R.B., Nichols, S.E. and Olsen, O.A. (2003) *Zea mays ZmMybst1* cDNA, encodes a single Myb-repeat protein with the VASHAQKYF motif. *J. Exp. Bot.* **54**, 1117–1119.

Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S. *et al.* (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant Biotechnol. J.* **9**, 230–249.

Mukherjee, K. and Bürglin, T.R. (2006) MEKHLA, a novel domain with similarity to PAS domains, is fused to plant homeodomain-leucine zipper III proteins. *Plant Physiol.* **140**, 1142–1150.

Olsson, A., Engström, P. and Söderman, E. (2004) The homeobox genes *ATHB12* and *ATHB7* encode potential regulators of growth in response to water deficit in *Arabidopsis*. *Plant Mol. Biol.* **55**, 663–677.

Otting, G., Qian, Y.Q., Billeter, M., Müller, M., Affolter, M., Gehring, W.J. and Wüthrich, K. (1990) Protein-DNA contacts in the structure of a homeodomain-DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J.* **9**, 3085–3092.

Palena, C.M. and Gonzalez, D.H. (1999) A monomer–dimer equilibrium modulates the interaction of the sunflower homeodomain leucine-zipper protein Hahb-4 with DNA. *Biochem. J.* **341**, 81–87.

Pearce, R.S. (1999) Molecular analysis of acclimation to cold. *Plant Growth Regul.* **29**, 47–76.

Peng, X., Wu, Q., Teng, L., Tang, F., Pi, Z. and Shen, S. (2015) Transcriptional regulation of the paper mulberry under cold stress as revealed by a comprehensive analysis of transcription factors. *BMC Plant Biol.* **15**, 108.

Perotti, M.F., Ribone, P.A. and Chan, R.L. (2017) Plant transcription factors from the homeodomain-leucine zipper family I. Role in development and stress responses. *IUBMB Life*, **69**, 280–289.

Ponting, C.P. and Aravind, L. (1999) START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. *Trends Biochem. Sci.* **24**, 130–132.

Ren, X., Chen, Z., Liu, Y., Zhang, H., Zhang, M., Liu, Q., Hong, X. *et al.* (2010) ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis. *Plant J.* **63**, 417–429.

Ruberti, I., Sessa, G., Lucchetti, S. and Morelli, G. (1991) A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. *EMBO J.* **10**, 1787–1791.

Sali, A. and Blundell, T. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815.

Schena, M. and Davis, R.W. (1992) HD-Zip proteins: members of an *Arabidopsis* homeodomain protein superfamily. *Proc. Natl Acad. Sci. USA*, **89**, 3894–3898.

Schena, M. and Davis, R.W. (1994) Structure of homeobox-leucine zipper genes suggests a model for the evolution of gene families. *Proc. Natl Acad. Sci. USA*, **91**, 8393–8397.

Schrick, K., Nguyen, D., Karlowski, W.M. and Mayer, K.F. (2004) START lipid/sterol-binding domains are amplified in plants and are predominantly associated with homeodomain transcription factors. *Genome Biol.* **5**, R41.

Schymkowitz, J.W., Rousseau, F., Martins, I.C., Ferkinghoff-Borg, J., Stricher, F. and Serrano, L. (2005) Prediction of water and metal binding sites and their affinities by using the Fold-X force field. *Proc. Natl Acad. Sci. USA*, **102**, 10147–10152.

Seo, P.J., Lee, S.B., Suh, M.C., Park, M.J., Go, Y.S. and Park, C.M. (2011) The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis*. *Plant Cell*, **23**, 1138–1152.

Sessa, G., Morelli, G. and Ruberti, I. (1993) The Athb-1 and-2 HD-Zip domains homodimerize forming complexes of different DNA binding specificities. *EMBO J.* **12**, 3507.

Shavrukov, Y., Gupta, N.K., Miyazaki, J., Baho, M.N., Chalmers, K.J., Tester, M., Langridge, P. *et al.* (2010) HvNax3 – a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Funct. Integr. Genomics*, **10**, 277–291.

Shepherd, J.C., McGinnis, W., Carrasco, A.E., De Robertis, E.M. and Gehring, W.J. (1984) Fly and frog homoeo domains show homologies with yeast mating type regulatory proteins. *Nature*, **310**, 70–71.

Söderman, E., Mattsson, J. and Engström, P. (1996) The *Arabidopsis* homeobox gene *ATHB-7* is induced by water deficit and by abscisic acid. *Plant J.* **10**, 375–381.

Szilák, L., Moitra, J. and Vinson, C. (1997) Design of a leucine zipper coiled coil stabilized 1.4 kcal mol^{-1} by phosphorylation of a serine in the e position. *Protein Sci.* **6**, 1273–1283.

Tan, Y., Li, M., Yang, Y., Sun, X., Wang, N., Liang, B. and Ma, F. (2017) Overexpression of *MpCYS4*, a phytocystatin gene from *Malus prunifolia* (Willd.) Borkh., enhances stomatal closure to confer drought tolerance in transgenic *Arabidopsis* and apple. *Front. Plant Sci.* **8**, 33.

Tena, G., Asai, T., Chiu, W.L. and Sheen, J. (2001) Plant mitogen-activated protein kinase signaling cascades. *Curr. Opin. Plant Biol.* **4**, 392–400.

Wu, J., Zhou, W., Gong, X. and Cheng, B. (2016) Expression of *ZmHDZ4*, a maize homeodomain-leucine zipper I gene, confers tolerance to drought stress in transgenic rice. *Plant Mol. Biol. Rep.* **34**, 845–853.

Yadav, D., Shavrukov, Y., Bazanova, N., Chirkova, L., Borisjuk, N., Kovalchuk, N., Ismagul, A. *et al.* (2015) Constitutive overexpression of the *TaNF-YB4* gene in transgenic wheat significantly improves grain yield. *J. Exp. Bot.* **66**, 6635–6650.

Zhang, S., Haider, I., Kohlen, W., Jiang, L., Bouwmeester, H., Meijer, A.H., Schluepmann, H. *et al.* (2012) Function of the HD-Zip I gene *Oshox22* in ABA-mediated drought and salt tolerances in rice. *Plant Mol. Biol.* **80**, 571–585.

Zhang, Z., Chen, X., Guan, X., Liu, Y., Chen, H., Wang, T., Mouekouba, L.D. *et al.* (2014) A genome-wide survey of homeodomain-leucine zipper genes and analysis of cold-responsive HD-Zip I members' expression in tomato. *Biosci. Biotechnol. Biochem.* **78**, 1337–1349.

Zhao, Y., Zhou, Y., Jiang, H., Li, X., Gan, D., Peng, X., Zhu, S. *et al.* (2011) Systematic analysis of sequences and expression patterns of drought-responsive members of the HD-Zip gene family in maize. *PLoS ONE*, **6**, e28488.

Zhao, Y., Ma, Q., Jin, X., Peng, X., Liu, J., Deng, L., Yan, H. *et al.* (2014) A novel maize homeodomain–leucine zipper (HD-Zip) I gene, *Zmhdz10*, positively regulates drought and salt tolerance in both rice and *Arabidopsis*. *Plant Cell Physiol.* **55**, 1142–1156.

Zhu, J.K. (2016) Abiotic stress signaling and responses in plants. *Cell*, **167**, 313–324.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Soil water tension monitored at 10 and 30 cm depths in large containers used for plant growth under well-watered conditions or increasing drought.

Figure S2 Details of frost tolerance experiments.

Figure S3 Alignments of *TdHDZipI-5A* and *TdHDZipI-5B* promoter sequences and sequences of corresponding genes of *Triticum aestivum* cv. Chinese Spring, identified in the Whole Genome Reference Assembly Pseudomolecules v1.0 databases of the International Wheat Genome Sequencing Consortium, using the BLAST software (Altschul *et al.*, 1997).

Figure S4 Alignment of *TdHDZipI-5B* (5B) and *TdHDZipI-5A* (5A) promoters. LALIGN (Huang and Miller, 1991) was used to find the best local alignments.

Figure S5 (a) Transgene copy numbers in T_1 transgenic plants estimated by Q-PCR. Plants seeds used in analyses are indicated by arrows. (b) Examples of selection of homozygous lines by PCR using transgene-specific primers.

Figure S6 Growth characteristics and yield components of control wild-type (WT) and transgenic T_1 wheat (*Triticum aestivum* cv. Gladius) plants transformed with pUbi-TaHDZipI-5.

Figure S7 Expression levels of three stress-inducible LEA (Late Embryogenesis Abundant)/COR (cold-responsive)/DHN (dehydrin) genes (*TaWZY2*, GenBank: EU395844; *TaCOR14B*, GenBank: AF207546; *TaRab15*, GenBank: X59133) and the TaDREB3 (GenBank: DQ353853) regulatory gene, in leaves of unstressed control WT plants and T_3 sublines of tree independent transgenic lines.

Table S1 List of PCR primers and DNA probes used in this study.

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Table S3 Hydrogen bonds of homo-dimeric TaHDZipI-3 and TaHDZipI-5, and hetero-dimeric TaHDZipI-3/TaHDZipI-5 with HDZ1 (5′-CAATCATTGC-3′/5′-GCAATGATTG-3′).

Table S4 Characteristics of the T_2/T_3 progenies of *TaHDZipI-5* transgenic lines analysed in large containers under well-watered or mild drought condition.

Appendix S1 Materials and methods.

Overexpression of the class I homeodomain transcription factor TaHDZipI-5 increases drought and frost tolerance in transgenic wheat

Yunfei Yang, Sukanya Luang[†], John Harris[††], Matteo Riboni, Yuan Li, Natalia Bazanova[‡], Maria Hrmova*, Stephan Haefele[‡‡], Nataliya Kovalchuk, Sergiy Lopato

University of Adelaide, School of Agriculture, Food and Wine, Glen Osmond, SA 5064, Australia

*Correspondence: maria.hrmova@adelaide.edu.au; Tel: +61 8 8313 7160; Fax: +61 8 8313 7102

Current addresses:

[†]Institute of Molecular Biosciences, Mahidol University, Nakhon-Pathom, Thailand

[††]South Australian Research and Development Institute, GPO Box 397, Adelaide, SA 5064, Australia

[‡]Commonwealth Scientific and Industrial Research Organisation, Glen Osmond, SA 5064, Australia

[‡‡]Rothamsted Research, West Common Harpenden, Hertfordshire, Al5 2JQ, UK

Supplementary data

Supplementary materials and methods

Analysis of gene expression by quantitative real-time PCR (Q-PCR)

Expression of the transgene in unstressed and cold (4°C) treated leaves of transgenic plants was demonstrated in different generations by Q-PCR (Fletcher, 2014). Total RNA was extracted

from tissues using TRIzol® Reagent (Life Technologies Corporation, Grand Island, NY, USA). Complementary DNA (cDNA) was synthesized from RNA as a template *via* reverse transcription (Fletcher, 2014). cDNA quality was assessed by reverse transcription polymerase chain reaction (RT-PCR) with intron-spanning primers of the wheat house-keeping gene *calreticulin* (*TaCRT*) (Table S1). To determinate the transgene expression level, Q-PCR was performed with primers of the target gene *TaHDZipI-5* (Table S1), described by Ferdous *et al.* (2015). Three out of four optimised wheat house-keeping genes, *TaActin*, *TaCyclophilin*, *TaGAPdH* and *TaEFA*, were used for transgene expression normalisation (Table S1). All experiments were carried out with three biological and three technical replicates.

Cloning of TdHDZipI-5A *and* TdHDZipI-5B *promoters and the identification of abscisic acid (ABA)-responsive* cis*-elements*

A fragment of the coding region of *TaHDZipI-5* (GeneBank accession KT224376) was isolated by PCR, using full-length cDNA as a template. It was used as a probe to screen a BAC library prepared from genomic DNA of *Triticum turgidum L. ssp. durum cv. Langdon* (Cenci *et al.*, 2003), as described by Kovalchuk *et al.* (2009). Both durum wheat homeologues of the *TaHDZipI-5* gene were identified by PCR using DNA of selected BAC clones as templates, and primers were derived from the coding region of *TaHDZipI-5* cDNA. Genes of the *T. turgidum* ssp *durum*, orthologues of *TaHDZipI-5*, are designated as *TdHDZipI-5A* and *TdHDZipI-5B*. The promoter sequences were identified through sequencing of BAC clones as described by Kovalchuk *et al.* (2009). Approximately 1300-bp-long fragments of the *TdHDZipI-5* promoter sequences were used for promoter analyses. These were cloned into the pENTR-D-TOPO vector, verified by sequencing and re-cloned into pMDC164 (Curtis and Grossniklaus, 2003) upstream of the *GUS* reporter gene. Sequences of the promoters were aligned using LALIGN version 2.1u09 (Huang and Miller, 1991). Potential ABA responsive

cis-elements of promoters were predicted (PLACE software; Higo *et al.*, 1999). Forward primers (Table S1) were designed to prevent interruptions of potential ABA responsive *cis*-elements; four 5'-deletions of the *TdHDZipI-5A* promoter, which included 5'UTRs, were generated by PCR. PCR products were cloned into the pENTR-D-TOPO vector (Invitrogen, Melbourne, Victoria, Australia), confirmed by sequencing and transferred by recombination into pMDC164; these were designated as *TdHDZipI-5A* D1, (1,055 bp), D2 (366 bp), D3 (336 bp), and D4 (175 bp), respectively. The identification of functional *cis*-elements responsible for ABA-induced activation of the *TdHDZipI-5A* promoter by deletions, was performed using a transient expression assay based on the biolistic bombardment of cultured wheat cells (Eini *et al.*, 2013). After one hour of cell recovery following bombardment, the liquid medium was exchanged for the same medium containing 0.5 mM ABA. Blue GUS foci were numbered after 24 hours of incubation with ABA.

Analysis of evolutionary relationship of selected HD-Zip I γ-clade proteins

HD-Zip I γ-clade protein sequences from *Arabidopsis* (Henriksson *et al.*, 2005) and selected monocots (Agalou *et al.*, 2008; Harris *et al.*, 2016; Hu *et al.*, 2012; Zhao *et al.*, 2011) were derived from the Plant Transcription Factor Database (Jin *et al.*, 2017) and EST database at National Center for Biotechnology Information. Multiple sequence alignments (Table S2) were performed using the MAFFT version 7 algorithm online (Katoh and Standley, 2013). A phylogenetic tree was constructed with the Neighbour Joining (NJ) algorithm, p-distance and the bootstrap method (1000 replicates of bootstrap) in the Molecular Evolutionary Genetics Analysis version 6 (MEGA6) program (Tamura *et al.*, 2013).

In-yeast activation assay

A transactivation assay in yeast was used to identify the activation domain of TaHDZipI-5. The full-length open reading frame (ORF) or various truncated fragments of *TaHDZipI-5* were individually fused in frame with the yeast GAL4 DNA-binding domain in the pGBKT7 vector (Invitrogen, Victoria, Australia). Constructs were transformed into yeast (*Saccharomyces cerevisiae* strain AH109). Transformed yeast cells were examined on synthetic defined (SD) (-Trp) medium and replica-plated to SD (-Trp / -His) medium. Yeast growth on the SD medium reflected the growth of yeast containing the native activation domain in truncated TaHDZipI-5 sequences.

Construction of 3D models of homeodomains (HDs) of TaHDZipI-5 in homo- and hetero-dimeric forms in complex with the HDZ1 cis*-element*

Structural models of homo-dimeric TaHDZipI-5 HDs and hetero-dimeric TaHDZipI-3/TaHDZipI-5 HDs were constructed using the crystal structure of *Drosophila melanogaster* HD (PDB: 1JGG) as a template (Hirsch and Aggarwal, 1995), through the MODELLER program suite v9.16 (Sali and Blundell, 1993). A sequence alignment between TaHDZipI-3, TaHDZipI-5 and the template was performed with MUSCLE (Edgar, 2004) and visualised in Annotator (Gille *et al.*, 2014). The DNA *cis*-element HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3') was constructed from the AT-rich *cis*-element (5'-TAATTGAATT-3'/5'-AATTCAATTA-3') of 1JGG using Coot (Emsley *et al.*, 2010). Fifty models were generated using different random starting coordinates, and models with the lowest score of the Modeller Objective Function (MOF) (Shen and Sali, 2006) and Discrete Optimised Protein Energy (DOPE) (Eswar *et al.*, 2006) were selected. The final protein models were chosen based on conformational energy calculations with ProSa2003 (Sippl, 1993) that were validated by PROCHECK (Laskowski *et al.*, 1993). DOPE/MOF/z-score parameters for TaHDZipI-5/HDZ1 and TaHDZipI-3/TaHDZipI-5/HDZ1 were -9,716/898/-4.45 and -10,253/810/-6.30,

respectively. The construction of structural models of homo-dimeric TaHDZipI-3 HDs was described previously (Harris *et al.*, 2016). The stabilities of 3D models were calculated using FoldX (Schymkowitz *et al.*, 2005).

Analysis of transgenic plants

Three independent T_1 lines of transgenic wheat with a single copy of the transgene were selected for primary phenotypic characterisation and seed multiplication. Twelve seeds of control plants (WT) and twelve T_1 transgenic seeds from each line were sown into 12-cm square pots filled with coco-peat soil, with one plant per pot, and grown under well-watered conditions in a greenhouse with day/night temperatures of 23°C (16 hours) and 19°C (8 hours). Leaves of three-week old control and transgenic seedlings of each line were sampled for genomic DNA isolation. Plant height, tiller and spike number, seed weight, total dry biomass, seed number, flowering time and single grain weight were recorded for each plant. The transgene copy number was determined by Q-PCR (Fig. S1).

Comparative evaluations of growth and yield components of transgenic T_3 lines and control plants grown under well-watered and mild-drought conditions were performed in two large containers filled with a mixture of coco-peat, sand and clay soil (1:1:1) (Shavrukov *et al.*, 2016). Three independent T_3 lines of all four transgenics were used for the evaluation of growth and yield components. Untransformed WT plants were used as control. Transgenic plants were grown in two identical containers, one with well-watered conditions and one with slowly increasing drought. In each container, 16 plants of each transgenic line and the same number of WT plants were randomly grown in rows, with eight plants per row. Leaf samples of each plant were collected for DNA/RNA isolation at the three-leaf stage of seedling development. In the well-watered container, plants were regularly watered until maturity. In the drought-

subjected container, plants were regularly watered until mid-tillering and watering stopped thereafter. Plants showed signs of mild wilting at the beginning of flowering. The soil water potential of each container was automatically monitored and recorded by Magpie-3 (Measuring Engineering Australia) using sensors in two depths (10 cm and 30 cm) below the soil surface (Fig. S1). Growth and yield characteristics of transgenic lines and control plants were monitored in both containers. The data for each measured parameter for each line were statistically analysed using Student *t-tests* (unpaired, two-tails), and null-segregants were excluded from the analyses in cases where lines were heterozygous.

Drought tolerance test or the survival rate of seedlings under terminal drought

Two independent homozygous lines with minimal differences in a seedling size to those of control plants were used in a drought survival test, conducted in a PC2 glasshouse. WT plants were used as control. Seeds were sown in five 6-inch round pots filled with the same amount of coco-peat soil. Before sowing seeds, the soil in each pot was water-saturated by soaking the pot in water overnight in plastic trays. The following day, pots were removed and drained for 24 hours, and each pot was weighed after drainage. The soil moisture weight was calculated as the difference between the soil weight after drainage and the dry soil weight (measured after incubation for a week at 65°C). Two plants of each line and WT plants were grown in each pot in the growth room under 24°C during 16 hours day light and 19°C during 8 hours darkness. Plants in each pot were well-watered for three weeks, after which watering was stopped. During the well-watered stage, each pot was weighted daily and water was added if the soil water content was below 80% of soil moisture weight. After 25 to 28 days of drought, plants were re-watered and survival rates were assessed after a three-week recovery.

Frost tolerance test (survival rate of seedlings subjected to frost)

Three T_3 independent homozygous lines of transgenic plants were used in a frost survival test. Untransformed WT plants were used as control. Seeds were sown in twelve 6-inch round pots filled with coco-peat soil. One plant of each line and WT plants were grown in a pot (Fig. S2A). Plants in each pot were well-watered and kept in a PC2 room (24/16 °C of day/night temperature, 16 hours day length) for three weeks and later placed into a cold cabinet (BINDER, Tuttlingen, Germany). Plants in the cold cabinet were exposed to temperatures decreasing gradually from 18 °C to a minimum temperature of -8 °C with 6.5 hours under the lowest temperature, and then slowly returned back to 18 °C (Fig. S2B). Pots were insulated to protect plant roots from frost-damage. Leaf tissues for RNA isolation were collected before the stress application (Fig. S2B). In addition, leaves of plants with stress-inducible promoters and control plants were collected at 4 °C. The ice nucleating agent SNOMAX® (Sno-Quip Pty Ltd, Mittagong, NSW, Australia) (2 g/L) was used to spray plants to prevent water crystallisation below 0 °C. After the frost treatment, pots were transferred back to the PC2 growth room for recovery. Survival rates were estimated after two weeks of recovery.

Analysis of expression of potential downstream genes in transgenic lines with constitutive overexpression of TaHDZipI-5

The analysis of the downstream gene expression was performed by Q-PCR, as described by Fletcher *et al.* (2014). Gene-specific primers from 3'UTRs (Table S1) were used to analyse the expression levels of *TaWZY2* (GenBank: EU395844), *TaCOR14B* (GenBank: AF207546; Tsvetanov *et al.*, 2000), *TaRAB15* (GenBank: X59133; King *et al.*, 1992) and *TaDREB3* (GenBank: DQ353853; Lopato *et al.*, 2006) genes in three independent control WT plants and three T_3 sublines of each of three independent transgenic lines with the constitutive overexpression of *TaHDZipI-5*. Three technical replicates were used in this experiment.

Legends to supplementary figures

Fig. S1. Soil water tension monitored at 10 cm and 30 cm depths in large containers used for plant growth under well-watered conditions or increasing drought. An arrow (no watering) indicates the point at which watering was withdrawn.

Fig. S2. Details of frost tolerance experiments. (a) Position of seedlings in pots during frost tolerance tests. (b) Temperature and light conditions during frost tolerance experiments in a semiautomatic cold cabinet.

Fig. S3. Alignments of *TdHDZipI-5A* and *TdHDZipI-5B* promoter sequences and sequences of corresponding genes of *Triticum aestivum* cv. Chinese Spring, identified in the Whole Genome Reference Assembly Pseudomolecules v1.0 databases of the International Wheat Genome Sequencing Consortium, using the BLAST software (Altschul *et al.*, 1997).

Fig. S4. Alignment of *TdHDZipI-5B* (5B) and *TdHDZipI-5A* (5A) promoters. LALIGN (Huang and Miller, 1991) was used to find the best local alignments. Primers used for generation of promoter deletions are underlined. Characteristic elements present in sequences of both promoters are indicated with boxes of different colours. MYBR - MYB recognition element; ABRE - abscisic acid responsive element; ATG - translational start.

Fig. S5. (a) Transgene copy numbers in T_1 transgenic plants estimated by Q-PCR. Plants seeds used in analyses are indicated by arrows. (b) Examples of selection of homozygous lines by PCR using transgene-specific primers. Homozygous T_1 sublines of two independent single-transformation-event lines were selected using the analysis of the transgene (*TaHDZipI-5*) presence in the T_2 progeny. H_2O - sample containing no DNA, WT - sample containing DNA

isolated from WT untransformed plant, P - positive control, where 1000-fold diluted plasmid DNA was used as a template.

Fig. S6. Growth characteristics and yield components of control wild-type (WT) and transgenic T_1 wheat (*Triticum aestivum* cv. Gladius) plants transformed with pUbi-TaHDZipI-5. Plants were grown under well-watered conditions. Flowering time of transgenic plants was compared with the average flowering time of 12 control WT plants, which is represented as day 0. Differences between transgenic lines and WT plants were tested in the unpaired Student's t-test (* $P<0.05$, ** $P<0.01$, *** for $P < 0.001$).

Fig. S7. Expression levels of three stress-inducible LEA (Late Embryogenesis Abundant)/COR (Cold-Responsive)/DHN (Dehydrin) genes (*TaWZY2*, GenBank: EU395844; *TaCOR14B*, GenBank: AF207546; *TaRab15*, GenBank: X59133) and the TaDREB3 (GenBank: DQ353853) regulatory gene, in leaves of unstressed control WT plants and T_3 sublines of tree independent transgenic lines. Expression levels of the *TaHDZipI-5* transgene in the same lines are shown in Fig. 6D. No correlation was found between the expression levels of the *TaHDZipI-5* transgene and downstream genes. Error bars represent ± SD of three technical replicates.

Legends to supplementary tables

Table S1. List of PCR primers and DNA probes used in this study.

Table S2. A sequence alignment of 14 entries (with GenBank accession numbers) used to generate a phylogenetic tree displaying the evolutionary relationships of HD-Zip I γ-clade TFs from *Arabidopsis* and selected monocots, shown in Fig. 1. Asterisks (*) indicate positions with a single conserved residue; colons (:) indicate conservation between residues with strongly

similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix); full stops (.) indicate conservation between residues with weakly similar properties (scoring ≤ 0.5 in the Gonnet PAM 250 matrix).

Table S3. Hydrogen bonds of homo-dimeric TaHDZipI-3 and TaHDZipI-5, and hetero-dimeric TaHDZipI-3/TaHDZipI-5 with HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3').

Table S4. Characteristics of the T_2/T_3 progenies of *TaHDZipI-5* transgenic lines analysed in large containers under well-watered or mild drought condition.

Supplementary references

Agalou, A., Purwantomo, S., Overnas, E., Johannesson, H., Zhu, X., Estiati, A., Kam, R.J.D. *et al.* (2008) A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. *Plant Mol. Biol.* **66**, 87–103.

Altschul, S. F., Madden, T. L., Schaeffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Cenci, A., Chantret, N., Kong, X., Gu, Y., Anderson, O.D., Fahima, T., Distelfeld, A. *et al.* (2003). Construction and characterization of a half million clone BAC library of durum wheat (*Triticum turgidum* ssp *durum*). *Theor. Applied Genet.* **107**, 931–939.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133**, 462–469.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

Eini, O., Yang, N., Pyvovarenko, T., Pillman, K., Bazanova, N., Tikhomirov, N., Eliby, S. *et al.* (2013) Complex regulation by Apetala2 domain-containing transcription factors revealed through analysis of the stress-responsive *TdCor410b* promoter from durum wheat. *PloS One* **8**, e58713.

Emsley, P., Lohkamp, B., Scott, W.G. and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr.* **66**, 486–501.

Eswar, N., Webb, B., Marti-Renom, M.A., Madhusudhan, M.S., Eramian, D., Shen, M.-Y., Pieper, U. *et al.* (2006) Comparative protein structure modeling using Modeller. *Curr. Protoc. Bioinform.*: John Wiley & Sons, Inc.

Ferdous, J., Li, Y., Reid, N., Langridge, P., Shi, B.J. and Tricker, P.J. (2015) Identification of reference genes for quantitative expression analysis of microRNAs and mRNAs in barley under various stress conditions. *PloS One* **10**, e0118503.

Fletcher, S.J. (2014) qPCR for quantification of transgene expression and determination of transgene copy number. *Meth. Mol. Biol.* **1145**, 213–237.

Gille, C., Fähling, M., Weyand, B., Wieland, T. and Gille, A. (2014) Alignment-annotator web server: rendering and annotating sequence alignments. *Nucleic Acids Res.* 42, W3-W6.

Harris, J.C., Sornaraj, P., Taylor, M., Bazanova, N., Baumann, U., Lovell, B., Langridge, P. *et al.* (2016) Molecular interactions of the γ-clade homeodomain-leucine zipper class I transcription factors during the wheat response to water deficit. *Plant Mol. Biol.* **90**, 435–452.

Henriksson, E., Olsson, A.S., Johannesson, H., Johansson, H., Hanson, J., Engström, P. and Söderman, E. (2005) Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships. *Plant Physiol.* **139**, 509–518.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* **27**, 297–300.

Hirsch, J.A. and Aggarwal, A.K. (1995) Structure of the even-skipped homeodomain complexed to AT-rich DNA: new perspectives on homeodomain specificity. *EMBO J.* **14**, 6280-6291.

Hu, R., Chi, X., Chai, G., Kong, Y., He, G., Wang, X., Shi, D. *et al.* (2012) Genome-wide identification, evolutionary expansion, and expression profile of homeodomain-leucine zipper gene family in poplar (*Populus trichocarpa*). *PloS One* **7**, e31149.

Huang, X. and Miller, W. (1991) A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math.* **12**, 337–357.

Jin, J., Tian, F., Yang, D.C., Meng, Y.Q., Kong, L., Luo, J. and Gao, G. (2017) PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res.* **45**, D1040–D1045.

Katoh, K. and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780.

King, S.W., Joshi, C. and Nguyen, H.T. (1992) DNA sequence of an ABA-responsive gene (*rab15*) from water-stressed wheat roots. *Plant Mol. Biol.* **18**, 119–121.

Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291.

Lopato, S., Bazanova, N., Morran, S., Milligan, A.S., Shirley, N. and Langridge, P. (2006) Isolation of plant transcription factors using a modified yeast one-hybrid system. *Plant Meth.* **2**, 3–17.

Sali, A. and Blundell, T. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815.

Schymkowitz, J.W., Rousseau, F., Martins, I.C., Ferkinghoff-Borg, J., Stricher, F. and Serrano, L. (2005) Prediction of water and metal binding sites and their affinities by using the Fold-X force field. *Proc. Natl. Acad. Sci. USA* **102**, 10147–10152.

Shavrukov, Y., Baho, M., Lopato, S. and Langridge, P. (2016) The *TaDREB3* transgene transferred by conventional crossings to different genetic backgrounds of bread wheat improves drought tolerance. *Plant Biotechnol. J.* **14**, 313–322.

Shen, M., Sali, A. (2006) Statistical potential for assessment and prediction of protein structures. *Prot. Sci.* **15**, 2507–2524.

Sippl, M.J. (1993) Recognition of errors in three-dimensional structures of proteins. *Proteins: Struct. Funct. Bioinf.* **17**, 355–362.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729.

Tsvetanov, S., Ohno, R., Tsuda, K., Takumi, S., Mori, N., Atanassov, A. and Nakamura, C. (2000) A cold-responsive wheat (*Triticum aestivum* L.) gene *wcor14* identified in a winter-hardy cultivar Mironovska 808. *Genes Genet. Syst.* **75**, 49–57.

Zhao, Y., Zhou, Y., Jiang, H., Li, X., Gan, D., Peng, X., Zhu, S. *et al.* (2011) Systematic analysis of sequences and expression patterns of drought-responsive members of the *HD-Zip* gene family in maize. *PloS One* **6**, e28488.

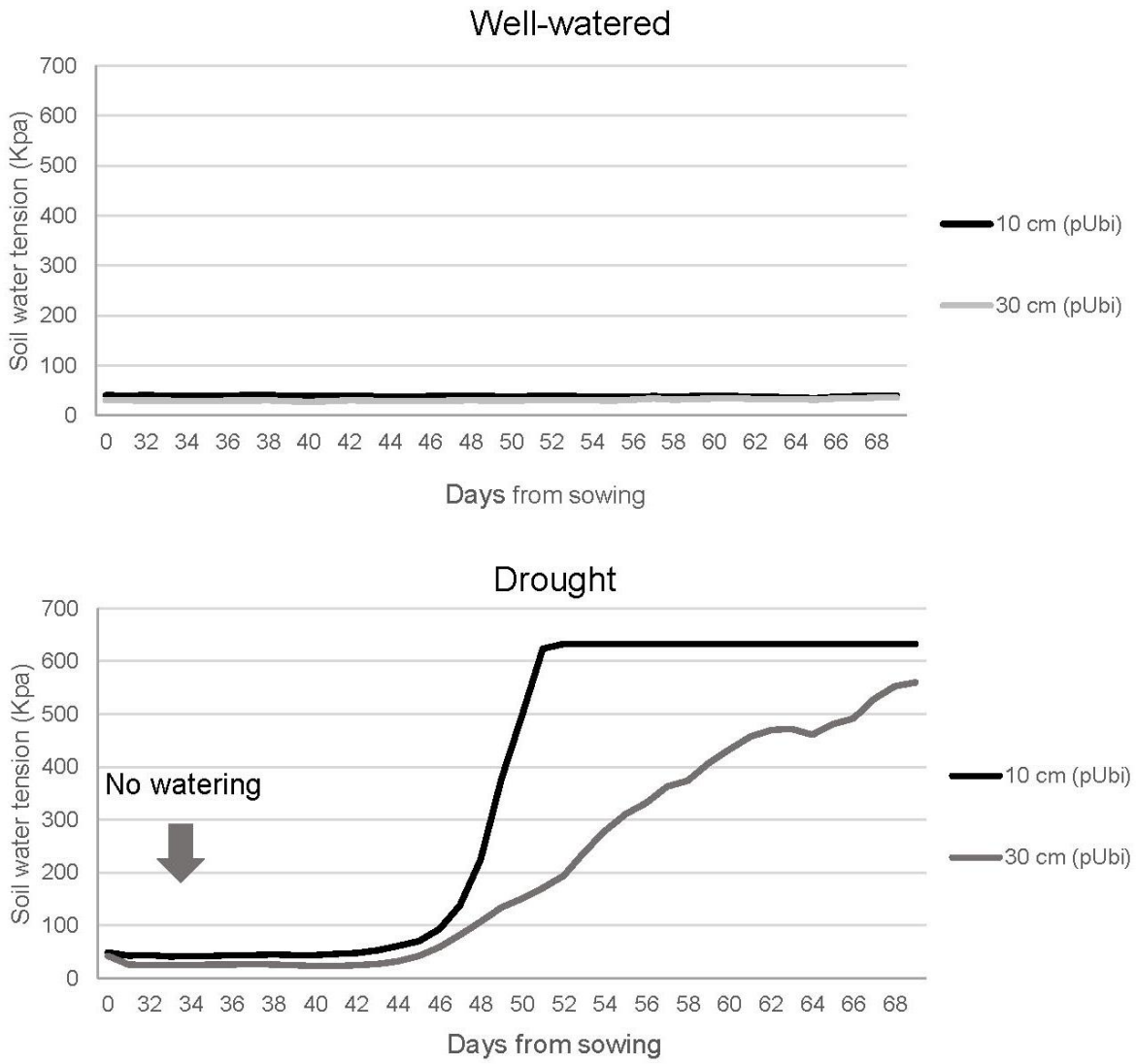

Fig. S1. Soil water tension monitored at 10 cm and 30 cm depths in large containers used for plant growth under well-watered conditions or increasing drought. An arrow (no watering) indicates the point at which watering was withdrawn.

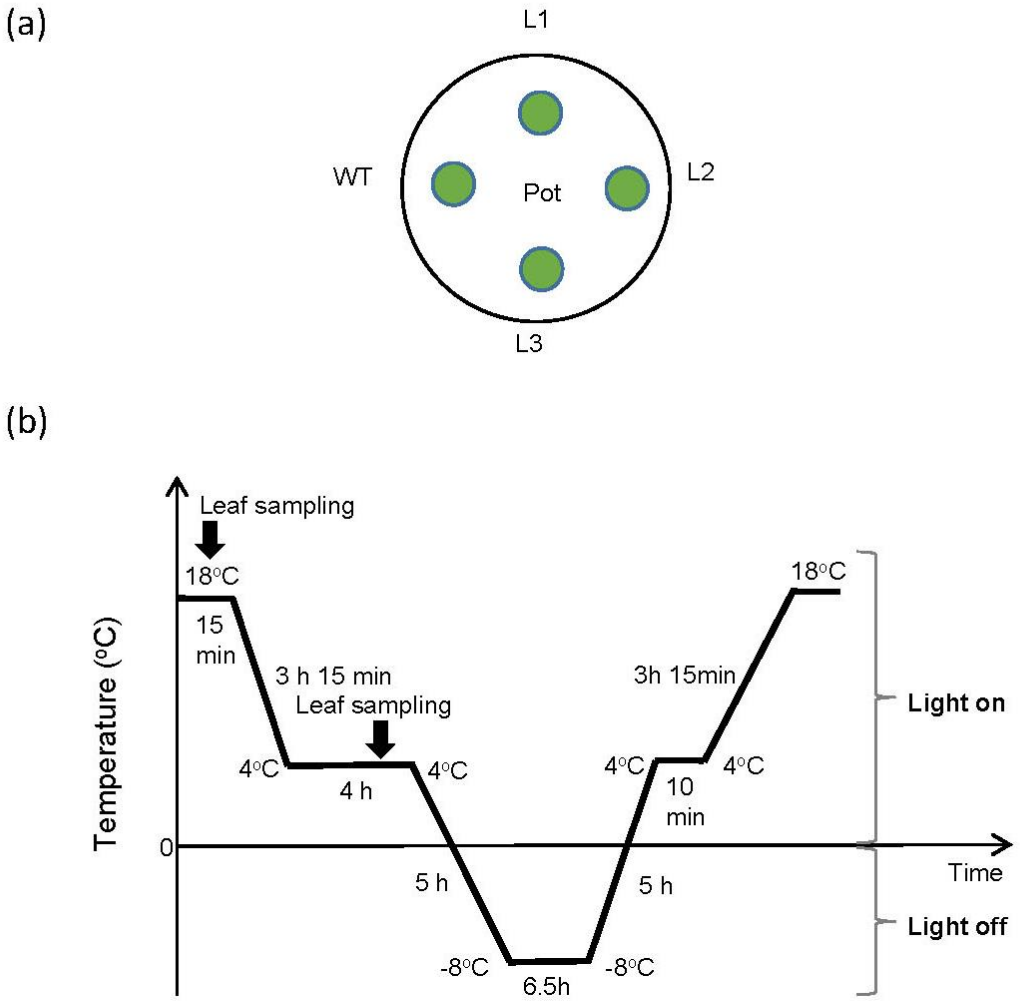

Fig. S2. Details of frost tolerance experiments. (a) Position of seedlings in pots during frost tolerance tests. (b) Temperature and light conditions during frost tolerance experiments in a semi-automated cold cabinet.

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Strand = Plus / Plus

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Fig. S3. Alignments of *TdHDZipI-5A* and *TdHDZipI-5B* promoter sequences and sequences of corresponding genes of *Triticum aestivum* cv. Chinese Spring, identified in the Whole Genome Reference Assembly Pseudomolecules v1.0 databases of the International Wheat Genome Sequencing Consortium, using the BLAST software (Altschul *et al.*, 1997).

Fig. S4. Alignment of *TdHDZipI-5B* (5B) and *TdHDZipI-5A* (5A) promoters. LALIGN (Huang and Miller, 1991) was used to find the best local alignments. Primers used for generation of promoter deletions are underlined. Characteristic elements present in sequences of both promoters are indicated with boxes of different colours. MYBR - MYB recognition element; ABRE - abscisic acid responsive element; ATG - translational start.

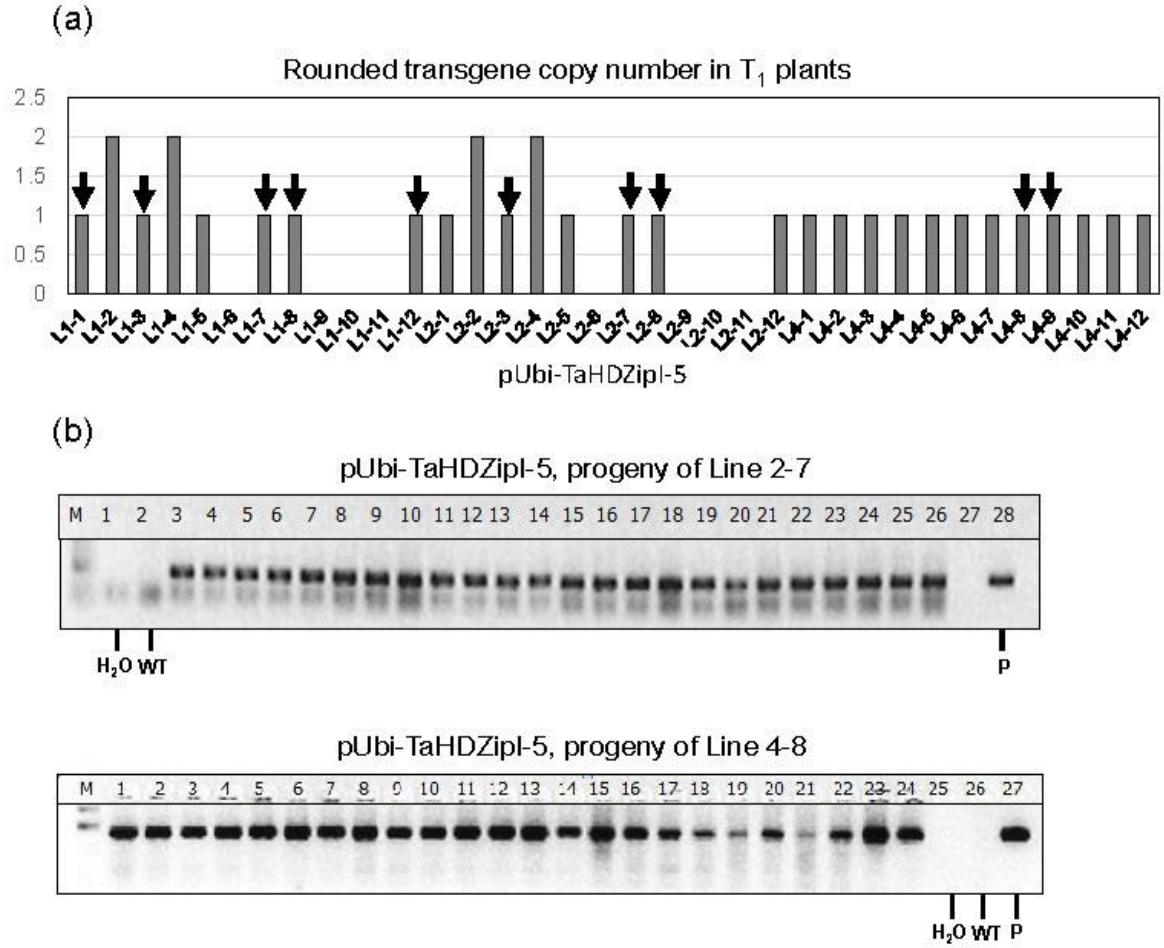

Fig. S5. (a) Transgene copy numbers in T_1 transgenic plants estimated by Q-PCR. Plants seeds used in analyses are indicated by arrows. (b) Examples of selection of homozygous lines by PCR using transgene-specific primers. Homozygous T_1 sublines of two independent single-transformation-event lines were selected using the analysis of the transgene (*TaHDZipI-5*) presence in the T_2 progeny. H_2O - sample containing no DNA, WT - sample containing DNA isolated from WT untransformed plant, P - positive control, where 1000-fold diluted plasmid DNA was used as a template.

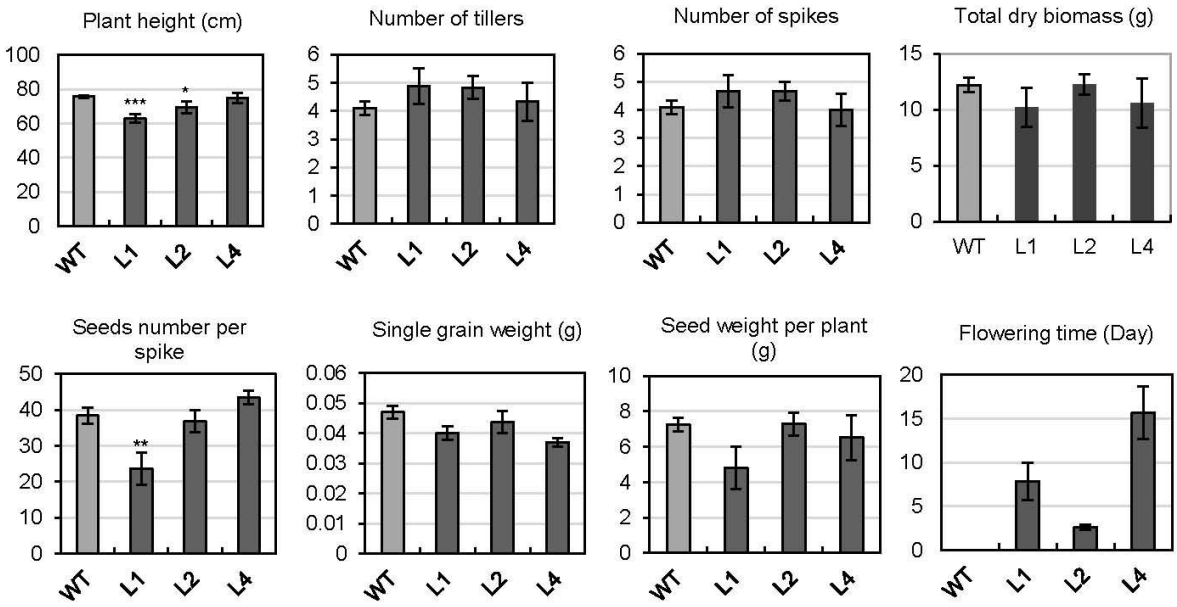

Fig. S6. Growth characteristics and yield components of control wild-type (WT) and transgenic T_1 wheat (*Triticum aestivum* cv. Gladius) plants transformed with pUbi-TaHDZipI-5. Plants were grown under well-watered conditions. Flowering time of transgenic plants was compared with the average flowering time of 12 control WT plants, which is represented as day 0. Differences between transgenic lines and WT plants were tested in the unpaired Student's t-test (* $P<0.05$, ** $P<0.01$, *** for $P < 0.001$).

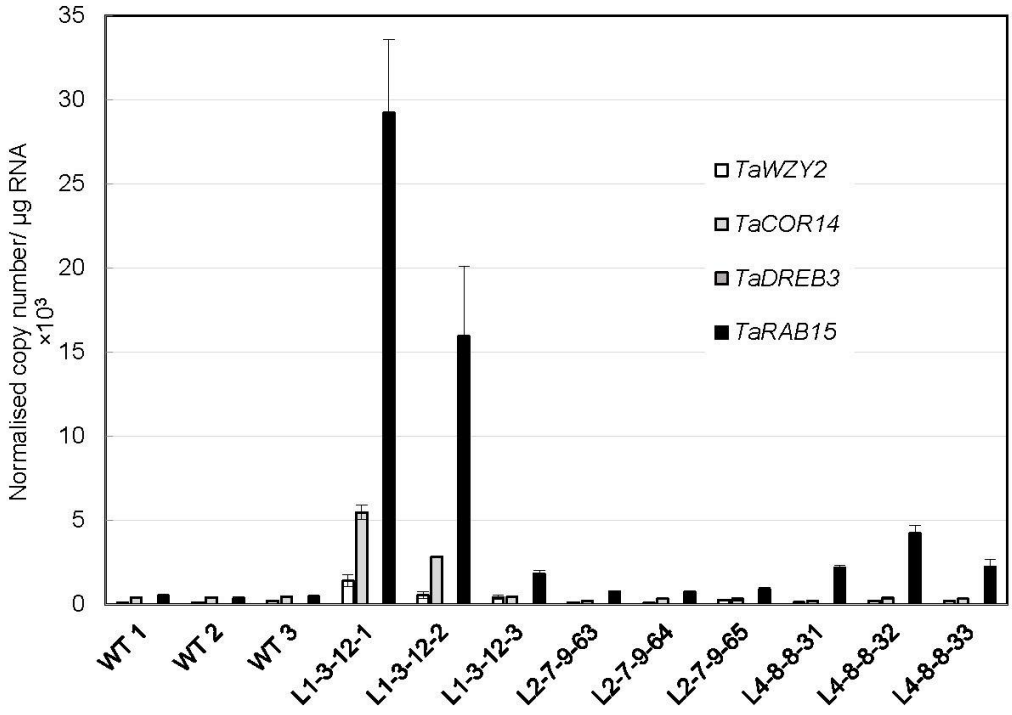

Fig. S7. Expression levels of three stress-inducible LEA (Late Embryogenesis Abundant)/COR (Cold-Responsive)/DHN (Dehydrin) genes (*TaWZY2*, GenBank: EU395844; *TaCOR14B*, GenBank: AF207546; *TaRab15*, GenBank: X59133) and the TaDREB3 (GenBank: DQ353853) regulatory gene, in leaves of unstressed control WT plants and T_3 sublines of tree independent transgenic lines. Expression levels of the *TaHDZipI-5* transgene in the same lines are shown in Fig. 6D. No correlation was found between the expression levels of the *TaHDZipI-5* transgene and downstream genes. Error bars represent ± SD of three technical replicates.

Table S1. List of PCR primers and DNA probes used in this study.

Short name	Purpose	Designation	Nucleotide sequence (5′–3′)
TaHDZipI-5, transgene	PCR, transgene specific primers	Forward primer	GGTCTTCCCGGAGTCCTTCTGC
		Reverse primer	CATCGCAAGACCGGCAACAGG
Nos terminator	Q-PCR, copy number	Forward primer	CTTAAGATTGAATCCT
		Reverse primer	CGAATTCAGTAACATAGA
		TaqMan probe	AGCGCGCAAACTAGGAT
Pin-b	Q-PCR, copy number	Forward primer	ATTTTCCAGTCACCTGGCCC
		Reverse primer	TGCTATCTGGCTCAGCTGC
		TaqMan probe	ATGGTGGAAGGGCGGCTGTGA
TaCRT	RT-qPCR	Forward primer	CGATGATGATGATGGCATATGG
		Reverse primer	TACCTGCCAAACTTCAATTCC
TaHDZipI-5, endogene	Q-PCR, gene expression	Forward primer	GAGAGCTGCGTGCTC
		Reverse primer	CGAACCACTTTGTACAAG
TaActin	Q-PCR, gene expression	Forward primer	GACAATGGAACCGGAATGGTC
		Reverse primer	GTGTGATGCCAGATTTTCTCCAT
TaCyclophilin	Q-PCR, gene expression	Forward primer	CAAGCCGCTGCACTACAAGG
		Reverse primer	AGGGGACGGTGCAGATGAA
TaGAPdH	Q-PCR, gene expression	Forward primer	TTCAACATCATTCCAAGCAGCA
		Reverse primer	CGTAACCCAAAATGCCCTTG
TaEFA	Q-PCR, gene expression	Forward primer	CAGATTGGCAACGGCTACG
		Reverse primer	CGGACAGCAAAACGACCAAG
TaWZY2	Q-PCR, gene expression	Forward primer	TACGGACAGCAAGGTCATACG
		Reverse primer	TTCTGCAGAGGTGTCCCAGAC
TaCOR14	Q-PCR, gene expression	Forward primer	TTCTGCAGAGGTGTCCCAGAC
		Reverse primer	ACCAACGTTGTAGCTCTTATAC
TaDREB3	Q-PCR, gene expression	Forward primer	CTCGATTCGCTTGCTCCTCAG
		Reverse primer	TCCTGATGACAAGCTGTAGTGTGC
TaRab15	Q-PCR, gene expression	Forward primer	ACAGGGACTGGAGGAGCCTAC
		Reverse primer	TGGAACTGGAAGGCTTTGACC
TdHDZipI-5 D1	PCR, promoter deletion fragment amplification	Forward primer	CACCCTTGCCTGATCTGCTC
		Reverse primer	GCCACTGCTCTGCTCCGAC
TdHDZipI-5 D2	PCR, promoter deletion fragment amplification	Forward primer	CACCCAAGATCTTTGCACCCGTG
		Reverse primer	GCCACTGCTCTGCTCCGAC
TdHDZipI-5 D3	PCR, promoter deletion fragment amplification	Forward primer	CACCGGTGCCGATCTCGAGG
		Reverse primer	GCCACTGCTCTGCTCCGAC
TdHDZipI-5 D4	PCR, promoter deletion fragment amplification	Forward primer	CACCGAAGACCACATGTTCG
		Reverse primer	GCCACTGCTCTGCTCCGAC

Table S2. A sequence alignment of 14 entries (with GenBank accessions) used to generate a phylogenetic tree displaying the evolutionary relationships of HD-Zip I γ-clade TFs from *Arabidopsis* and selected monocots, shown in Fig. 1. An asterisk (*) indicates positions with a single conserved residue; a colon (:) indicates conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix); a full stop (.) indicates conservation between groups of weakly similar properties (scoring ≤ 0.5 in the Gonnet PAM 250 matrix).

```
AtHB7_AY091364            ----------------MTEGGEY----------------------------SPAMMSAE
AtHB12_AY087187           ----------------MEEGDFF----------------------------NCCFSEIS
Zmhdz4_KJ728250           ----------------MDRPDH----QQQQFFMPTTVQV-----PQPQQQQ-QQLCVPM-
Oshox22_AY224440          HASDGRTQLASWARIAMDRGDHHHLQQQHQFLMPPPAPV----VP-------PQLCMPAM
TaHDZipI-5_KT224376       ----------------MDY------HQQQQFLMPPPASL-----P----AQ-QQLCAPMM
Zmhdz6_EU972354           ----------------MERG-------DCQFTVVPPRQY----------DE-AQFMHQLM
Sb04g033380_XP_002452838  ----------------MERGD------DCQFMVV--HQY----------DEAAQLMHQLM
Oshox24_AK063685          ----------------MES--------DCQFLVAPPQPHMYYDTAAAAVDE-AQFLRQMV
TaHDZipI-4_KT224375       ----------------MES--------DRQFLLAPPPPM-----HAAPGDD-GQFLQQQQ
Zmhdz9_BT087258           ----------------MEG-D----------------------------DDGPEWMMEV-
Sb02g030660_XP_002462710  ----------------MDG-E----------------------------DDVPEWMMEV-
Zmhdz12_NM_001156038      ----------------MDGAE----------------------------DDGTEWMMH--
Oshox6_AK103160           ----------------MDG-E----------------------------ED-SEWMMMDV
TaHDZipI-3_KT224374       ----------------MEQGE----------------------------ED-GDWMMEP-
                                          *

AtHB7_AY091364            PFLTM-------------------KK--MKKSNHN--KNNQRRFSDEQIKSLEMMF-ESE
AtHB12_AY087187           SGMTMN-----------------KKK--MKKS------NNQKRFSEEQIKSLELIF-ESE
Zmhdz4_KJ728250           ----------LDEPPSSF-----LAG--RGGGGASGRGERKRRFTEEQIRSLESMFHAHH
Oshox22_AY224440          --MADEQYMDL--------GGGGAAA--APGRGGA--GERKRRFTEEQIRSLESMFHAHH
TaHDZipI-5_KT224376       -GMGMEMGMEMEMEEQLC-----FVG--RGGGGRG--AERKRRFTEEQTRSLESMFHAHH
Zmhdz6_EU972354           --AAGDQ-----QDP--A-----GAG--RGAAGGG--GERKRRFTEEQVRSLETTFHARR
Sb04g033380_XP_002452838  VAAAGDQ-----QDPNAG-----AAG--RGAGGGG--GERKRRFTEEQVRSLETTFHARR
Oshox24_AK063685          --AAADH-----HAAAAGRGGGDGDG--GGGGGGG--GERKRRFTEEQVRSLETTFHARR
TaHDZipI-4_KT224375       --LS--------------------------GGGA--GERKRRFTEEQVRSLESTFHTRR
Zmhdz9_BT087258           -------------------GGAGATG--KGKGGAL--DKNKKRFSEEQIKSLESMF-ATQ
Sb02g030660_XP_002462710  -------------------GGAGGKGGKGGGGGAL--DKNKKRFSEEQIKSLESMF-ATQ
Zmhdz12_NM_001156038      --------------------GAGGKG---KGGGAL--DKNKKRFSDEQTKSLESMF-ATQ
Oshox6_AK103160           -------------------GGKGGKG---GGGGGA--ADRKKRFSEEQIKSLESMF-ATQ
TaHDZipI-3_KT224374       ----------------------AAG---KKGGAM--IDRKKRFSEEQIKSLESMF-ATQ
                                                             ..:::**::** :***  *   .

AtHB7_AY091364            TRLEPRKKVQLARELGLQPRQVAIWFQNKRARWKSKQLETEYNILRQNYDNLASQFESLK
AtHB12_AY087187           TRLEPRKKVQVARELGLQPRQVAIWFQNKRARWKTKQLEKEYNTLRANYNNLASQFEIMK
Zmhdz4_KJ728250           AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQLEHDYALLRAKFDDLHAHVESLK
Oshox22_AY224440          AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQLEHDYAALRSKYDALHSRVESLK
TaHDZipI-5_KT224376       AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQLEHDFTALRADYDALHSRVESLK
Zmhdz6_EU972354           AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQLEHDYAALRAQFDAMHARVESLR
Sb04g033380_XP_002452838  AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQLEHDYAALRARYDALHARVDSLR
Oshox24_AK063685          AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQIEHDYAALRAQYDALHARVESLR
TaHDZipI-4_KT224375       AKLEPREKAELARELGLQPRQVAIWFQNKRARWRSKQLEQDFAELRGHYDALRARVESLK
Zmhdz9_BT087258           TKLEPRQKLQLARELGLQPRQVAIWFQNKRARWKSKQLERDYSALRDDYDALLCSYESLK
Sb02g030660_XP_002462710  TKLEPRQKLQLARELGLQPRQVAIWFQNKRARWKSKQLEREYSALRDDYDALLCSYESLK
Zmhdz12_NM_001156038      AKLEPRQKLQLARELGLQPRQVAIWFQNKRARWKSKQLEREYSALRDDYHALLCSYESLK
Oshox6_AK103160           TKLEPRQKLQLARELGLQPRQVAIWFQNKRARWKSKQLEREYSALRDDYDALLCSYESLK
TaHDZipI-3_KT224374       TKLEPRQKLQLARELGLQPRQVAIWFQNKRARWKSKQLERQYAALRDDYDALLSSYDQLK
                          ::****:* ::**********************::**:* ::  **  :. : .  : ::

AtHB7_AY091364            KEKQALVSELQRLKEAT---QKKTQEEER-----QCSGD--------------------Q
AtHB12_AY087187           KEKQSLVSELQRLNEEM---QRPKEEKHH-----ECCGD--------------------Q
Zmhdz4_KJ728250           QDKLALTTQLSELSERL---RE-RDDRA--------------AAAGGGGR---------E
Oshox22_AY224440          QEKLALTVQLHELRERL---RE-REER----------------SGNGGAA---------T
TaHDZipI-5_KT224376       HEKLALAAQLQELSERL---RE-RDG-----------------GGGGAA---------T
Zmhdz6_EU972354           QEKIALAAQVDELRGRL---NE-RQDQ-----------------------------------
Sb04g033380_XP_002452838  EEKLALAKQVDELRGRLQSVSE-RQDQ-----------------------------------
Oshox24_AK063685          QEKLALADQVDELRGKL---NE-RQDQ-----------------------------------
TaHDZipI-4_KT224375       QEKLTLAAQLEELKKKL---NE-RQDQ-----------------------------------
Zmhdz9_BT087258           KEKHTLLKQLEKLAEML---HE-PRGKYSGNADAAGAGDDVRS-GVGGMK--DEFAD--A
```

```
Sb02g030660_XP_002462710 KEKHALLKQLEKLAEML---HE-PRGKYGGNAD-AGAGDDVRS-GVGGMK--EEFTDA-A
Zmhdz12_NM_001156038     DEKRALLKQLEKLAEML---HEPPQGKYYGG-----NADDVRSGGVGGTKEEEESTDACA
Oshox6_AK103160          KEKLALIKQLEKLAEML---QE-PRGKYGDN-----AGDDARSGGVAGMK-KEEFVGAGG
TaHDZipI-3_KT224374      KDKQALLNQLEKLAEML---RE-PGGAKCGDNAGAAARDDVRL-AVAGMSMKDEFVDAGG
                         .:* :*  :: .*         .

AtHB7_AY091364           AVVALSSTHHESENEENRRRKPEEVRPEMEMKDDKGH-HGVMCDHHDYE------DDDNG
AtHB12_AY087187          GLALSSST--ESHNG----------KSEPEGRLDQG---SVLC------------NDGD
Zmhdz4_KJ728250          TMASSSSC--IGGGGEEEA-----------EDDKRN--VLLFGCVDMEPPAESCVL-VG
Oshox22_AY224440         TAASSSSC--NGSGSEEVDD----------DDDKRN---AAAGCLDLEPP-ESCVL-GG
TaHDZipI-5_KT224376      ATASSSSC--NGGGREL-------------DDDKRN-------VVDVEPP-ESCVL-GG
Zmhdz6_EU972354          ----SGSC--EVNDAEAA------------ADDKRN-NSTSS----------LVQ-DD
Sb04g033380_XP_002452838 ----SGSC--EVNDAA--------------DDGKRNLNSTTTTCLV-------LVQ-ED
Oshox24_AK063685         ----SGSC--DGGGAEGD------------DDDKRN--SVMNASSS------GLVE-ED
TaHDZipI-4_KT224375      ----SASC--D---AAAE------------VDDKSN-NVSSC----------IVA-KD
Zmhdz9_BT087258          GAAPYSSE--GGGGG--------KF-AHF-TDDDV---GALFRPSS-----PQPSA--AG
Sb02g030660_XP_002462710 GAALYSSE--GGGGG--------KF-AHF-TDDDV---GALFRPSA------QPTA--AG
Zmhdz12_NM_001156038     GAALYSSE--CAGGG--------RFIAHFLADDDVGA-ALFRPPSS-----PQPTA--GL
Oshox6_AK103160          AATLYSSA--EGGGTTTTTTA--KLMPHF-GSDDVDA-GLFLRPSSQHHPPPPHAG--AG
TaHDZipI-3_KT224374      ASKLYSAS--EGCGGSG------KL-SLF-GEDDDDA-GLFLRPSL-----QLPTAHDGG
                              .:                          .

AtHB7_AY091364           YS--------NNIKREYFG-----GFEEEPD--H-LMNIVEPA-DSCLTSSDDWRGFKSD
AtHB12_AY087187          YN--------NNIKTEYF------GFEEETD--HELMNIVEKADDSCLTSSENWGGFNSD
Zmhdz4_KJ728250          ST-CAALA---DVSV-----------ESECDDQH-LH-------------------YDDE
Oshox22_AY224440         AT-CATPA---DVSVE----------SDQCDD-Q-LD-------------------YDEG
TaHDZipI-5_KT224376      TA-CGTPA---DVSAS---------VESECDD-H-LH-------------------YDGA
Zmhdz6_EU972354          GA-TPPPA-AVDA--SEDSAAT----GEYYD--H-VA-------------------YEYD
Sb04g033380_XP_002452838 DG-ATPPA-AVDASGSEDSAATEYGYGYDYD--HVVA-------------------YGEG
Oshox24_AK063685         YV-SCLAVPVVDV--SEDGSAACGGSSYEYDH-H-LD-------------------YLGG
TaHDZipI-4_KT224375      ES-AAPAA---DV---SDGSTP---GWYEYDN-H-LA-------------------YGVD
Zmhdz9_BT087258          FT-SSGPP---EHQ--------------PFQF-H-SG---------C---------WPSS
Sb02g030660_XP_002462710 FTSSSGPP---EHQ--------------PFQF-H-SS--------SC---------WPSS
Zmhdz12_NM_001156038     LT-SSGPP---EHQ--------------PFQF-H-SG--------CC---------WPSS
Oshox6_AK103160          FT-SSEPA--ADHQ--------------SFNF-H-SS-------------------WPSS
TaHDZipI-3_KT224374      FT-ASGPA---EYQQQ---------SPSSFPF-H-SN-------------------WPSS
                                    :                    :                        :

AtHB7_AY091364           TTTLLDQSSNNYP-WRDF-WS----------
AtHB12_AY087187          --SLLDQSSSNYPNWWEF-WS----------
Zmhdz4_KJ728250          ---FPES-YCAMPELWEP-WPL--VEWNAVA
Oshox22_AY224440         L--FPES-FCATPELWEP-WPL--VEWNAVA
TaHDZipI-5_KT224376      V--FPES-FCATPELWEP-WPWPPVEWNAVA
Zmhdz6_EU972354          G--LHDPFVCATPDLWDT-WPL--LEWNAVA
Sb04g033380_XP_002452838 ---LHDP-LCATPDLWDT-WPL--LEWNAVA
Oshox24_AK063685         G-QLPDP-FCGMPDLWEI-WPM--VEWNAVA
TaHDZipI-4_KT224375      ---LQEP-FCATPELWETSWPL--VEWNAVA
Zmhdz9_BT087258          ----TEQ-TCSSSQWWEF-ESL--SE-----
Sb02g030660_XP_002462710 ---TTEQ-TCSSSQWWEF-ESL--SE-----
Zmhdz12_NM_001156038     ---SAEQ-TCSGSQWWEL-ESL--SE-----
Oshox6_AK103160          ----TEQ-TCSSTPWWEF-ESE---------
TaHDZipI-3_KT224374      ---AAEQ-TCSSSQWWEF-ESP--SE-----
                              :      .   :   .
```

Table S3. Hydrogen bonds in homo-dimeric TaHDZipI-3 and TaHDZipI-5, and hetero-dimeric TaHDZipI-3/TaHDZipI-5 with HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3').

Residues	Number of hydrogen bonds and distances in Å[1]								Water	DNA phospho-diester backbone	Total number
	HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3')										
	A2	A3	T4	T7	T8	G11	T15	G16			
TaHDZipI-3											
Gln66	-	-	-	-	-	-	-	-	1 (3.0)	-	1
Asn73	-	-	-	-	2 (3.0, 3.4)	-	-	-	1 (2.8)	-	3
Lys79	-	-	-	-	-	-	-	-	-	1 (3.4)	1
TaHDZipI-3											
Gln66	-	-	-	-	-	-	-	-	2 (3.0, 2.8)	2 (3.4, 3.4)	4
Gln72	-	-	1 (3.2)	-	-	-	-	-	-	-	1
Asn73	1 (3.5)	-	-	-	-	-	-	-	-	-	1
Total	1	0	1	0	2	0	0	0	4	3	11

Residues	Number of hydrogen bonds and distances in Å[1]								Water	DNA phospho-diester backbone	Total number
	HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3')										
	A2	A3	T4	T7	T8	G11	T15	G16			
TaHDZipI-5											
Arg58	-	-	-	1 (2.9)	-	-	-	1 (3.5)	-	-	2
Gln99	-	-	-	-	-	-	-	-	1 (3.0)	1 (2.6)	2
Gln105	-	-	-	-	1 (3.4)	-	-	-	1 (3.1)	-	2
Asn106	-	-	-	-	1 (3.4)	-	-	-	1 (3.2)	-	2
TaHDZipI-5											
Arg60	-	-	-	-	-	-	-	-	-	1 (2.3)	1
Gln99	-	-	-	-	-	-	-	-	1 (3.0)	1 (3.3)	2
Asn106	-	2 (3.0, 3.1)	-	-	-	-	-	-	1 (2.8)	-	3
Total	0	2	0	1	2	0	0	1	5	3	14

Residues	Number of hydrogen bonds and distances in Å[1]								Water	DNA phospho-diester backbone	Total number
	HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3')										
	A2	A3	T4	T7	T8	G11	T15	G16			
TaHDZipI-3											
Arg26	-	-	-	1 (3.1)	-	-	1 (3.5)	-	-	-	2
Lys27	-	-	-	-	-	-	-	1 (3.0)	-	-	1
Arg65	-	-	-	-	-	-	-	-	-	1 (3.2)	1
Gln66	-	-	-	-	-	-	-	-	1 (3.0)	2 (2.6, 3.2)	3
Gln72	-	-	-	-	1 (3.4)	1 (2.9)	-	-	1 (2.8)	-	3
Asn73	-	-	-	-	1 (2.3)	-	-	-	1 (3.2)	-	2
Arg77	-	-	-	-	-	-	-	-	-	1 (3.2)	1
TaHDZipI-5											
Arg60	-	-	-	-	-	-	-	-	-	1 (2.8)	1
Gln99	-	-	-	-	-	-	-	-	2 (3.0, 3.2)	1 (3.4, 3.4)	3
Asn106	1 (2.9)	2 (3.2, 3.3)	-	-	-	-	-	-	1 (2.8)	-	4
Arg110	-	-	-	-	-	-	-	-	-	1 (3.1)	1
Total	1	2	0	1	2	1	1	1	6	7	22

Table S4. Characteristics of the T_2/T_3 progenies of *TaHDZipI-5* transgenic lines analysed in large containers under well-watered or mild drought condition.

Construct	Container No.	Condition	Lines	Total number of plants	Number of dead plants	Number of nulls	Number of plants with expressed transgene
pUbi-TaHDZipI-5	17	Drought	WT	16	0	-	-
			L1-3-9	16	3	0	13
			L2-7-9	16	0	0	16
			L4-8-9	16	0	0	16
	18	Well-watered	WT	16	0	-	-
			L1-3-9	16	3	0	13
			L2-7-9	16	0	0	16
			L4-8-9	16	0	0	16
pWRKY71-TaHDZipI-5	21	Drought	WT	16	0	-	-
			L14-7	16	0	4	12
			L46-3	16	0	3	13
			L48-7	16	0	5	11
	22	Well-watered	WT	16	0	-	-
			L14-7	16	0	2	14
			L46-3	16	2	1	13
			L48-7	16	0	8	8
pCor39-TaHDZipI-5	23	Drought	WT	16	0	-	-
			L23-4	16	1	4	11
			L39-7	16	0	0	16
			L55-4	16	0	6	10
	24	Well-watered	WT	16	0	-	-
			L23-4	16	4	0	12
			L39-7	16	0	0	16
			L55-4	16	0	2	14

Chapter 5 Characterisation of drought-responsive *HD-Zip I* promoters in transgenic wheat plants

Statement of authorship

Title of Paper	Characterization of drought-responsive HD-Zip I promoters in transgenic wheat plants
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Yang Y, Riboni M, Li Y, Ismagul A, Bazanova N, Chirkova L, Hussain S, Hrmova M, Haefele S, Lopato S, Kovalchuk N, Characterization of drought-responsive HD-Zip I promoters in transgenic wheat plants.

Principal Author

Name of Principal Author (Candidate)	Yunfei Yang		
Contribution to the Paper	Designed the experiment with supervisors, performed experiments, analysed data, wrote and edited the manuscript.		
Overall percentage (%)	70 %		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/07/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Matteo Riboni		
Contribution to the Paper	Performed Q-PCR to determine the transgene *TaCBF5L* expression under different abiotic stresses.		
Signature		Date	19/07/2017
Name of Co-Author	Yuan Li		
Contribution to the Paper	Performed Q-PCR to determine the copy number of the transgene *TaCBF5L* in T_0/T_1 transgenic plants.		
Signature		Date	19/07/2017

Name of Co-Author	Ainur Ismagul		
Contribution to the Paper	Performed plant transformation experiments. On behalf of Ainur Ismagul		
Signature		Date	19/07/2017
Name of Co-Author	Natalia Bazanova		
Contribution to the Paper	Performed a Y1H assay for isolation of TaCBF5L from wheat.		
Signature		Date	19/07/2017
Name of Co-Author	Larissa Chirkova		
Contribution to the Paper	Performed northern blot hybridization experiments.		
Signature		Date	19/07/2017
Name of Co-Author	Syed Sarfraz Hussain		
Contribution to the Paper	Performed homozygous lines selection of TaCBF5L transgenic wheat.		
Signature		Date	19/07/2017
Name of Co-Author	Maria Hrmova		
Contribution to the Paper	Provided bioinformatics guidance and performed substantial editing of the manuscript.		
Signature		Signature	19/07/2017
Name of Co-Author	Stephan Haefele		
Contribution to the Paper	Supervised experiments and performed substantial editing of the manuscript		
Signature		Signature	19/07/2017

Name of Co-Author	Sergiy Lopato		
Contribution to the Paper	Conceived, designed and supervised experiments, analysed the data and edited the manuscript.		
Signature		Signature	19/07/2017
Name of Co-Author	Nataliya Kovalchuk		
Contribution to the Paper	Supervised and performed guidance on experiments, edited the manuscript and acted as the corresponding author.		
Signature		Signature	19/07/2017

Characterisation of drought-responsive HD-Zip I promoters in transgenic wheat plants

*Yunfei Yang, Matteo Riboni, Yuan Li, Ainur Ismagul, Natalia Bazanova[†], Larissa Chirkova, Syed Sarfraz Hussain, Maria Hrmova, Stephan Haefele[††], Sergiy Lopato, Nataliya Kovalchuk**

University of Adelaide, School of Agriculture, Food and Wine, Glen Osmond, SA 5064, Australia

*Correspondence: nataliya.kovalchuk@adelaide.edu.au; Tel: +61883037180;

E-mails:

yunfei.yang@adelaide.edu.au

matteo.riboni@csiro.au

yuan.li@adelaide.edu.au

ainur.ismagul@adelaide.edu.au

natasha.bazanova@csiro.com

larissa.chirkova@adelaide.edu.au

maria.hrmova@adelaide.edu.au

stephan.haefele@rothamsted.ac.uk

nataliya.kovalchuk@adelaide.edu.au

Current addresses:

[†]Commonwealth Scientific and Industrial Research Organisation, Glen Osmond, SA 5064, Australia

[††]Rothamsted Research, West Common Harpenden, Hertfordshire, Al5 2JQ, UK

GenBank accession numbers of the gene *TaCBF5L* are MF406152.

Summary

Drought and frost are major environmental stresses that negatively affect crop production globally. Regulatory networks of transcription factors (TFs) are involved in diverse physiological processes in plants to ensure that plants respond rapidly and effectively to abiotic stresses. In this study, we identified a novel CBF/DREB TFs protein, TaCBF5L, isolated from roots of stress-stressed wheat. We defined the role of TaCBF5L in abiotic stress responses using transgenic wheat. We also characterised the activities of *HDZI-3* and *HDZI-4*, which are the promoters of genes encoding the γ-clade TFs of the HD-Zip class I subfamily. We found that these promoters were stress-inducible under drought and frost, using transgenic wheat transformed with *TaCBF5L*. Both promoters, particularly *HDZI-3,* improved the frost survival rate of transgenic seedlings, while *HDZI-4* significantly increased the grain yield of plants under severe drought; although no improvement of plant yield phenotype was seen under well-watered conditions or moderate drought. The mechanism of the *TaCBF5L* transgene, driven by the *HDZI-3* and *HDZI-4* promoters, was further explored by finding six downstream genes of *TaCBF5L* under severe drought, and four downstream genes of *TaCBF5L* under frost.

Key words: Abiotic stress tolerance, yield, phenotype, *HDZI-4* and *HDZI-3* promoters, wheat C-repeat binding factor 5 like protein (TaCBF5L).

Abbreviations: ABA — abscisic acid; HD-Zip I — homeodomain-leucine zipper class I; AP2 — APETALA2; CBF — C-repeat-binding factor; CRT — C-repeat; DREB — dehydration-responsive binding protein; DRE — dehydration-responsive element; ERF — ethylene-responsive element-binding; LEA — late embryogenesis abundant; Q-PCR — quantitative real-time PCR; RT-PCR — reverse transcription PCR; Ta — *Triticum aestivum*; TF(s) — transcription factor(s); TaCBF5L — wheat C-repeat binding factor 5 like protein; WT — Wildtype; Y1H — yeast-1-hybrid.

Introduction

Drought and low temperature are two significant abiotic stresses limiting the yields of staple crops globally. To survive harsh environments, plants need to provide rapid responses to stress factors. The environmental stimuli are perceived by receptors and sensors such as cytoskeleton and hydroxyproline-rich and arabinogalactan glycoproteins (Humphrey *et al.*, 2007; Luan, 2002; Śniegowska-Świerk *et al.*, 2015; Thion *et al.*, 1996). These stimuli are converted into

intracellular signals by second massengers such as Ca^{2+} (Cao *et al.*, 2017; Cheong *et al.*, 2003; Klimecka and Muszynska, 2007; Knight *et al.*, 1997; Sanders *et al.*, 2002; Urao *et al.*, 1994), that trigger regulatory networks through ABA-dependent and ABA-independent pathways, which guide diverse physiological changes in metabolism to provide resistance for plants (Heidarvand and Amiri, 2010; Kidokoro *et al.*, 2017; Shinozaki *et al.*, 2003; Todaka *et al.*, 2017; Yang *et al.*, 2011).

There are two groups of genes involved in abiotic stresses regulatory networks (Gong *et al.*, 2015; Hu *et al.*, 2007; Sazegari *et al.*, 2015; Yang *et al.*, 2016). One group comprises regulatory genes encoding TFs that function in up- or down-regulation of downstream gene expressions (Harris *et al.*, 2011; Pujol and Galaud, 2013; Raza *et al.*, 2016; Smith, 2000). The other group is represented by functional genes, whose expression can be activated or repressed by corresponding TFs, and the products of these genes are directly involved in biochemical and physiological changes for stress acclimations (Nakashima *et al.*, 2014; Novillo *et al.*, 2011; Shinozaki *et al.*, 2003). Environmentally regulated gene transcription in response to environmental stresses is an effective strategy adopted by plants to deal with unfavorable growth conditions.

The APETALA2 (AP2)/ethylene-responsive element-binding (ERF) group is a superfamily of TFs involved in abiotic stresses. The members of AP2/ERF contain AP2 or ERF binding domains, which recognise six nucleotides A/GCCGAC of the dehydration-responsive element/C-repeat (DRE/CRT) motif, located on promoter regions of the corresponding downstream genes (Hrmova and Lopato, 2014; Sakuma *et al.*, 2002). AP2/ERF TFs regulate the expression levels of these genes by interacting with their DRE/CRT *cis*-elements (Bouaziz *et al.*, 2015). The superfamily of AP2/ERF is classified into five groups, which are represented by the following subfamilies: AP2, ERF, RAV, CBF/DREB, and the subfamily of other TFs (Sakuma *et al.*, 2002).

Numerous TFs belonging to the CBF/DREB subfamily have been reported to enhance the stress endurance of transgenic plants by regulating the related downstream genes under the control of strong constitutive promoters (Ban *et al.*, 2011; Chen *et al.*, 2007; Sarkar *et al.*, 2014; Xianjun *et al.*, 2011). However, such constitutive overexpression of these TFs often leads to severe growth retardation or a low grain yield under normal growth conditions (Kasuga *et al.*, 1999; Lopato and Langridge, 2011; Morran *et al.*, 2011). Several stress-inducible promoters of such genes such as *rd29A* (Kasuga *et al.*, 2004; Mallikarjuna *et al.*, 2011), *ZmRab17* (Morran *et al.*, 2011), *Oshox24* (Nakashima *et al.*, 2013) and *OsWRKY71* (Kovalchuk *et al.*, 2013) were proved

to minimise the negative effects of overexpression of TFs on plant growth or a grain yield. Hence, finding novel stress-inducible promoters is one of the critical methodologies to improve plant development and growth, and even grain yields with the optimised expression levels of transgenes (Hrmova and Lopato, 2014).

HDZI-3 and *HDZI-4* promoters belong to the γ-clade of genes and encode HD-Zip I proteins from wheat, TdHDZipI-3 and TdHDZipI-4, respectively (Harris, 2014; Harris et al., 2016). *HDZI-3* was identified to be ABA-independent, while *HDZI-4* is ABA-dependent (Harris et al., 2014). According to the previous study, the homologues genes *TaHDZipI-3* and *TaHDZipI-4* are inducible by drought and cold (Harris, 2014; Harris *et al.*, 2016) and hence, *HDZI-3* and *HDZI-4* were stress-inducible promoter candidates. In our study, the *HDZI-3* and *HDZI-4* promoters were used to optimise *TaCBF5L* transgene expression in transgenic wheat under abiotic stresses. We also isolated a novel CBF/DREB TF protein TaCBF5L from wheat, and characterised its role in abiotic stress responses using transgenic wheat under drought and cold stresses, driven by either *HDZI-4* or *HDZI-3* promoter.

Experimental procedures

Isolation and identification of the TaCBF5L gene

A full-length cDNA of *TaCBF5L* was isolated from the roots of drought-stressed *Triticum aestivum* L. cv. Chinese Spring, using a modified yeast-one hybrid approach (Lopato *et al.*, 2006) with DRE *cis*-element TACCGAC as a bait. The homologous proteins to TaCBF5L were searched from a variety of species such as *Arabidopsis*, wheat, rice, maize and barley, using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from a non-redundant protein sequences database. Protein encoding nucleotide sequences were confirmed via EST database of the National Center for Biotechnology Information (NCBI). A multiple sequence alignment of the homologous proteins and TaCBF5L was conducted using MAFFT version 7 (Katoh and Standley, 2013). A phylogenetic tree was reconstructed based on the alignment results using Molecular Evolutionary Genetics Analysis (MEGA 6.06) (Tamura *et al.*, 2013) with Neighbour Joining and p-distance specifications and 1000 bootstrap replications.

Plasmids construction and transformation

A 1915-bp-long fragment of the promoter sequence upstream of the translation start codon of *TdHDZipI-3*, and a 2142-bp-long fragment of the promoter sequence upstream of the translation

start codon of *TdHDZipI-4*, were isolated by PCR using genomic DNA of *Triticum turgidum* ssp. *durum* as a template (Harris *et al.*, 2016). Each fragment of promoters was inserted into the pMDC32 vector (Curtis and Grossniklaus 2003) with a *2x35S* promoter, which was excised using *Hin*dIII–*Kpn*II restriction sites (Busk *et al.*, 1997); these DNA constructs were designated as pTdHDZI-3 and pTdHDZI-4, respectively. The full-length of the coding region of *TaCBF5L* was cloned into the pENTR-D-TOPO vector (Invitrogen, Melbourne, Victoria, Australia), and confirmed by sequencing. *TaCBF5L* was subcloned into pTdHDZI-3 and pTdHDZI-4 vectors, and designated as pTdHDZI-3-TaCBF5L and pTdHDZI-4-TaCBF5L, respectively. These constructs were transformed into the Australian elite wheat (*Triticum aestivum* L. cv. Gladius) using a biolistic bombardment method (Ismagul *et al.*, 2014). PCR was used for demonstrating of transgene integration with a forward primer on the 3' end of the transgene *TaCBF5L* coding region and a reverse primer on the 5' end of the *nopaline synthase* gene (*NOS) terminator* (Table S1).

Determination of copy number and gene expression by quantitative real-time PCR (Q-PCR)

Q-PCR was used for estimating the genomic copy number of the transgene *TaCBF5L* in T_0 and T_1 progenies of transgenic wheat. Genomic DNA was extracted using a freeze-dry method (Shavrukov *et al.*, 2010). The Q-PCR was performed with primers and probes of a termination sequence of the *NOS terminator* and a reference gene *Puroindoline-b* (*Pin-b*) following the $2^{-\Delta\Delta Ct}$ method (Kovalchuk *et al.*, 2013; Yuan *et al.*, 2008). Total RNA from leaf tissues was isolated using TRIzol® kit (Life Technologies, Grand Island, NY, USA) and reverse transcripted into cDNA. An intron-spanning primer of a wheat house-keeping gene *calreticulin (TaCRT)* (Table S1) was used for an assessment of non-genomic DNA contamination in cDNA samples by performing reverse transcription PCR (RT-PCR). To determine the expression level of transgene *TaCBF5L* and stress-related genes under abiotic stresses, Q-PCR was performed (Ferdous *et al.*, 2015), using specific primers of target genes (Table S1). Relative quantities of a transgene was normalised using three out of four reference genes *Taactin*, *Tacyclophilin*, *TaGAPdH* and *TaEFA*. All experiments were conducted in technical triplicates.

Northern blot hybridization for detecting transgene expression

A Northern blot hybridisation method was used for showing the transgene *TaCBF5L* expression in T_2 sublines of transgenic wheat with each of the *HDZI-3* and *HDZI-4* promoters under well-watered and dehydration conditions. RNA was extracted from wheat leaves using Direct-zol™ RNA MiniPrep (Zymo Research, USA), following the manufacturer's introduction. The probe

was synthesised with a denatured full-length coding sequence (CDS) of the *TaCBF5L* transgene as a template and [α–^{32}P] dCTP was labelled using a random primer labelling method (Cho *et al.*, 2003). Purified RNA was denatured and seperated on an agarose gel with 6 % (v/v) formaldehyde, transferred into a nylon memberane and hybridised with a radioactive labeled probe (Cho *et al*., 2003)

T_1 transgenic wheat sublines grown under well-watered conditions for seeds multiplication

Eight T_1 seeds from each T_0 transgenic line were sown in 12-cm square pots filled with cocopeat. Wildtype (WT) plants (control plants) were grown under the same conditions. One plant was grown per pot. Plants in pots were well-watered in a greenhouse with a day temperature of 23 ^{o}C for 16 hours and a night temperature of 19 ^{o}C for 8 hours. Flag leaves of three-week-old plants were collected for genomic DNA isolation. The isolated DNA was further used for copy number and transgene integration detection. T_1 independent sublines with low copy number (Fig. S1) driven by *HDZI-4* or *HDZI-3* promoters were selected for seed multiplication and a primary phenotypic characterisation (Yadav *et al.*, 2015).

Comparison of growth and yield components of T_3 transgenic sublines and WT plants grown under well-watered and moderate drought conditions

Four sublines of transgenic plants with pHDZI-4-TaCBF5L or pHDZI-3-TaCBF5L were planted into two deep containers (190 × 68 × 60 cm) filled with soil mixtures simulating field conditions (Yadav *et al.*, 2015). WT plants were grown under the same conditions. Different conditions were established in the two containers: one was well-watered all the time and the other one was well-watered until an early-tillering stage, and the water was withheld. Soil water potential was monitored with the Magpie-3 (Measuring Engineering Australia) continuously (Fig. S2), using sensors in two depths (10 and 30 cm). Plants in the second container suffered moderate drought at the start of the flowering stage. In each container, 16 control plants and 16 transgenic plants from each subline were grown randomly in rows, with 8 plants in a row. Flowering time started for the moderate drought treatment when the water potential curve raised to the middle of the peak for the sensor at the 30 cm depth (Fig. S2). Phenotypic data of all plants from each container were recorded at the end of reproductive stages. The data of each measured parameter for each subline were statistically analysed using the Student t-test (unpaired, two-tails).

Sustainable drought tolerance test or estimations of survival rates of seedlings under terminal drought

Three transgenic sublines were used in a drought survival test, with WT plants as control plants. Experiments were conducted in a growth chamber (25/19 ^{o}C temperature of day/night; 16/8 hours of light/dark length). Weights of seven empty pots (6-inch in a diameter) were measured before tests started. Pots were filled with a soil mixture of field clay and cocopeat (2:1 ratio). Each pot was fully watered and immersed in a plastic tray covered with water overnight. The pots were then moved out of the tray and saturated weights of each pot were measured after a 24-hour-free drainage. A small amount of saturated soil from pots was removed to ensure the same weight of each pot. All empty pots had a similar weight, so minor differences (0.1 %–0.01 %) of weight between pots were neglected. One pot was randomly chosen from seven pots and marked as pot A. The soil in pot A was used for field capacity measurements, following the method described by Samarah (2005). Two plants from each subline and two WT plants were grown in each of remaining six pots (Fig. S3A and B), fully filled with saturated soil. The weight of each pot was measured every two or three days to establish the curve of soil water moisture as a percentage of a field capacity (Fig. S3C). One pot was randomly chosen from remaining six pots and marked as pot B. Plants in pot B were used for the leaf water potential test through Decagon WP4C Water Potential Meter (Decagon Devices, Inc, Pullman, WA) by following the manufacturer's protocol. After six-day growth, the leaf water potential test was performed every two or three days until a recovery stage. Both the soil water moisture curve and the leaf water potential data were used to indicate the drought degree, which the plants suffered from. Leaves from plants in the other five pots were sampled during well-watered conditions (>70 % field capacity), leaf wilting point (50–60 % field capacity), moderate drought (40–50 % field capacity) and severe drought (< 40 % field capacity) with a leaf water potential at 12–15 bars, 15–20 bars, 20–30 bars and over 40 bars, respectively. Gene expression levels from leaves in four different drought stages were determined by Q-PCR. Plants were determined to be recovered, when the pot weight had no further reduction for 2–3 days, and when most plants suffered obvious withering and their whole leaves turned yellow. Survival rates were estimated after 2–3 weeks of recovery. All experiments were repeated three times. Survival rates of each subline and WT plants were statistically analysed using the Student t-test (unpaired, two-tails).

Comparison of growth and yield components of T_3 transgenic sublines and WT plants grown under severe drought conditions

Experiments were conducted in a growth chamber with 25/19 ^{o}C day/night temperature and a 16 hours day length. Six empty pots (8-inch in a diameter) were used. Each pot was filled with a saturated soil mixture made from field clay and cocopeat (2:1 ratio). The method for making water-saturated soil was the same as described above. Extra soil was removed from each pot to ensure that all pots had the same weight. One pot was randomly chosen from six pots and used for a field capacity calculation. Two transgenic seeds from each subline and two WT seeds were sown into each of remaining five pots (Fig. S4A and S5A). All five pots were well-watered until plants were grown for 3–4 weeks. Water was then withheld and the weight of each pot was measured every two or three days to establish a soil moisture curve. Nearly all plants (99 %) survived this initial stress and entered into a reproductive stage whereby they were exposed to severe drought (< 30 % field capacity) (Fig. S4B and S5B); at this stage of the experiment, the flowering time was noted. Phenotypic analyses were performed on a plant height, tiller and spike numbers, a total dry biomass, a number of seeds, a seed weight per plant and a single grain weight when plants were at a full ripening stage. Data of each measured parameter for each subline were statistically analysed using the Student t-test (unpaired, two-tails).

Frost tolerance test or determination of survival rates of seedlings under terminal frost

Frost tolerance of three T_4 sublines of transgenic wheat and WT plants, as control plants was tested. Seeds were sown and germinated in twelve 6-inch pots filled with cocopeat, placed in a growth chamber (24/16 ^{o}C of day/night temperature; 16 hour day length). One plant from each subline and one WT plant were grown together in each pot and well-watered for three weeks (Fig. S6A). Pots with 3-week-old seedlings were placed into a cold cabinet (BINDER, Tuttlingen, Germany). The temperature in the cabinet had a slow reduction from a room temperature (18 ^{o}C) to -8 ^{o}C, and remained at -8 ^{o}C for 6.5 hours, and raised back slowly to a room temperature (Fig. S6B). Thereafter, plants in pots were placed back to a growth chamber for recovery. Leaf tissues were sampled from each plant before a cold treatment and at 4 ^{o}C (Fig. S6B). These leaf samples were used for transgene expression determination by Q-PCR. An ice nucleating solution, SNOMAX® (Sno-Quip Pty Ltd, Mittagong, NSW, Australia) (2 g/L), was used to spray plants below zero to induce extra-cellular ice formation in plants (Guenther et al., 2006). Survival rates were calculated 2~3 weeks after recovery. Experiments were repeated three times. Survival rates of each subline and those of WT plants were statistically analysed using the Student t-test (unpaired, two-tails).

Results

Isolation and the structure of the TaCBF5L gene

A 687-bp full encoding sequence of *TaCBF5L* was isolated from drought-stressed roots of bread wheat (*Triticum aestivum* L. cv. Chinese Spring) using yeast-one-hybrid (Y1H) with the drought-responsive element (DRE) sequence as a bait. The phylogenetic reconstruction of DREB TFs was performed with the Neighbour Joining Algorithm in MEGA 6.06 (Tamura *et al.*, 2013). This analysis showed clear sub-divisions among different subgroups of DREB TF from wheat, maize, rice, barley and *Arabidopsis* (Fig. 1), whereby the TaCBF5L TF protein belonged to subgroup C of CBF/DREB proteins.

Based on the reconstruction of phylogeny (Fig. 1), TaCBF5L shows a closer evolutionary relationship with TaCBF5 (0.84 % sequence identity), TdDREB3 (0.77 % sequence identity), HvCBF5 (0.76 % sequence identity), TaDREB3 (0.74 % sequence identity), TmCBF5 (0.74 % sequence identity) and ZmDBP4 (0.64 % sequence identity) compared to the other entries in the tree (Fig.1, Table S2). The analysis of the multiple sequence alignment of six close homologous proteins with TaCBF5L revealed that the TaCBF5L protein contained an AP2 DNA-binding domain of 35 amino acid residues and the well-conserved PKKPAGR motif (PKK/RPAGRxKFxETRHP) positioned at the N-terminus. In addition, the LWSY motif was another conserved domain that was found at the C-terminus (Fig. S7).

Inducible expression of TaCBF5L under dehydration and different drought conditions in transgenic sublines driven by HDZI-3 or HDZI-4 promoters

Expression levels of *TaCBF5L* in T_2 sublines of transgenic wheat during well-watered and 6-hour dehydration conditions were detected, and compared with those of WT plants, using a Northern blot hybridisation method (Fig. 2). The results of this comparison showed that the *TaCBF5L* transgene expression, controlled by either of two promoters *HDZI-3* or *HDZI-4*, was much stronger under dehydration than under well-watered conditions (Fig. 2). The *TaCBF5L* endogenous gene of WT plants showed no or weak expression before and after the dehydration treatment.

The expression levels of *TaCBF5L* in T_4 transgenic sublines under four different drought stages were determined using the Q-PCR method. Evaluation of the data showed that the expression levels of *TaCBF5L*, controlled by the promoter *HDZI-3* or *HDZI-4*, showed no or a little increase during the leaf wilting point (15–20 bar) and moderate drought (20–30 bar), compared

to those with the basal level of expression under well-watered (12–15 bar) conditions (Fig. 3A and B). However, the *TaCBF5L* expression was obviously up-regulated during the severe drought stress stage (>40 bar). In addition, the expression levels of *TaCBF5L* controlled by the *HDZI-4* promoter were higher than that those controlled by the *HDZI-3* promoter in most transgenic plants (Fig. 3A and B).

Phenotypic evaluation of T_3 transgenic wheat with pHDZI-3 or pHDZI-4 promoters under moderate drought and well-watered treatments during the flowering stage

Four sublines of T_3 transgenic and WT wheat plants were planted in two deep containers and subjected to moderate drought and well-watered conditions during the flowering stage. Grain yields and yield components of these plants were evaluated at the end of their reproduction stages.

Transgenic sublines L13-7-8 and L14-5-3 with pHDZI-3-TaCBF5L showed similar phenotypic features such as tiller, spike, seed number, single grain weight, plant height and total dry biomass, compared to those of WT plants (Fig. 4A). However, two other transgenic sublines L3-7-3 and L3-8-8, derived from the same line L3, showed significantly smaller sizes of plants, fewer seeds, less biomass and grain yield than those of WT under moderate drought (Fig. 4A). In addition, all sublines subjected to well-watered conditions and the subline L14-5-3 exposed to moderate drought conditions flowered 2–3 days earlier than WT plants.

For the transgenic sublines with pHDZI-4-TaCBF5L, two sublines grown under well-watered conditions and one subline exposed to mild drought showed lower spike numbers and grain yields. Three sublines of transgenic plants with pHDZI-4-TaCBF5L flowered 2–3 days earlier than WT plants, although one subline L20-3-2 was significantly delayed in growth and flowered five days later than WT plants (Fig. 4B). The same subline L20-3-2 showed lower tiller and seed numbers, less biomass and a lower plant height in comparison to WT plants. However, most of sublines showed similar tiller numbers as those that were characteristic for WT plants (Fig. 4B).

Phenotypic evaluation of T_4 transgenic wheat with HDZI-4 or HDZI-3 promoters under severe drought during the flowering stage

Two independent lines of transgenic wheat with pHDZI-4-TaCBF5L or pHDZI-3-TaCBF5L were grown along WT plants in pots with water-saturated soil for 3–4 weeks. After this time interval, water was withheld and the phenotypic evaluation was performed at the end of the

reproductive stage. Over 95 % of the transgenic and WT wheat plants survived the seedling stage and proceeded to the reproductive stage (data not show). The soil water moisture curve indicated that plants were exposed to severe drought (with 25–35 % of field capacity) during flowering time (Figs. S4 and S5).

Transgenic sublines L13-7-8-11 and L14-5-3-3 containing the *HDZI-3* promoter had similar numbers of spikes and tillers compared to those of WT plants (Fig. 5A). However, both sublines showed delayed flowering and the subline L13-7-8-11 showed smaller plant size, fewer seeds, less biomass and lower grain yields than the control WT plants (Fig. 5A). Transgenic L14-5-3-1 and L24-5-2-1 sublines with the *HDZI-4* promoter also showed a similar spike number and tiller numbers than WT plants (Fig. 5B). However, both transgenic and WT plants showed significantly larger plant sizes and produced more biomass and grain yields than WT plants (Fig. 5B). Both transgenic L14-5-3-1 and L24-5-2-1 sublines flowered 3–4 days earlier than WT plants (Fig. 5B).

Stress-inducible expression of TaCBF5L driven by HDZI-3 or HDZI-4 promoters improves frost tolerance of transgenic wheat seedlings

Frost tolerance of three T_4 sublines with pHDZI-3-TaCBF5L, was compared to that of WT plants. Based on the evaluation of survival rates (Fig. 6A), WT plants did not grow well and no more than 5 % of WT plants survived the harsh conditions of frost. However, all transgenic sublines showed strong resistance to frost, with a survival rate that was 3–4 times higher than that of WT plants (Fig. 6A). Importantly, the survival rates of two transgenic sublines L3-8-8-11 and L14-5-3-3 were significantly higher than those of WT plants (Fig. 6A).

We have also compared frost tolerance of three T_4 sublines with pHDZI-4-TaCBF5L with those of WT plants. After frost treatment, all transgenic sublines showed a stronger recovery than those of WT plants. Around 14–23 % of transgenic sublines survived, with a survival rate of 1.2–2 times higher than that of WT plants (Fig. 6B). In addition, the survival rates of L24-5-2-9 showed a significant difference compared to that of WT plants (Fig. 6B).

In most plants of each subline with the *HDZI-3* promoter, the expression levels of the *TaCBF5L* transgene increased after frost was applied*;* these levels increased between 2–4 times compared to basal levels (Fig. 7A). In contrast, the activity of the *HDZI-4* promoter was not stable under a low temperature since not all transgenic sublines showed an increase of transgene over-expression driven by *HDZI-4* under frost. The *TaCBF5L* transgene driven by the *HDZI-4* promoter was up-regulated in L17-7-1-2 and L24-5-2-9 transgenic sublines, but down-regulated

in the L14-5-3-8 subline by frost (Fig. 7B). Further, the *HDZI-3* promoter led to higher cold-inducible over-expression levels and lower basal levels of *TaCBF5L* compared to the *HDZI-4* promoter (Fig. 7A, B). Hence, the *HDZI-3* promoter showed a more stable and better performance to improve the cold tolerance of transgenic wheat than the *HDZI-4* promoter.

Activation of stress-inducible genes by over-expression of TaCBF5L TF under severe drought or a low temperature

The expression of the *TaCBF5L* transgene, in all tested transgenic sublines with *HDZI-3* and *HDZI-4* promoters, was only up-regulated by severe drought (> 40 bar) and showed no or very low expression levels at the leaf wilting point (15–20 bar) or moderate drought (20–30 bar), compared to well-watered conditions (12–15 bar) (Fig. 3A, B). Hence, cDNA samples of transgenic and WT wheat plants exposed to severe drought or well-watered conditions were used to search for potential downstream genes (Fig. 8A). Expression of six *LEA/COR/DHN* genes, *Wcor410*, *TaRab17*, *Wlt10*, *TaRab15*, *Wcor18* and *Wcs19*, as the cold/drought inducible genes, was examined in the transgenic sublines and WT plants. The ratios of expression levels of six *LEA/COR/DHN* genes under severe drought relative to well-watered condition were calculated, and used to measure changes in induction of the drought-inducible over-expression of *TaCBF5L* in transgenic plants relative to those of WT plants with an endogenous *TaCBF5L* gene. The data indicated that an additional induction on these genes occurred in response to severe drought, and potentially excluded the effect of the endogenous *TaCBF5L* gene. *TaCor18, TaRab15, TaRab17* and *TaWlt10* genes were up- or down-regulated by *TaCBF5L* in all transgenic sublines (Fig. 8B). *TaCor18, TaRab17* and *TaWlt10* were up-regulated by drought and the induction of each target gene in transgenic sublines with the *HDZI-4* promoter was higher than that in transgenic sublines with the *HDZI-3* promoter (Fig. 8B). Noteworthy, expression of *TaRab15* led to a 0.03-fold repression of *TaCBF5L* in all the transgenic sublines (Fig. 8B; lower left-hand side panel).

Expression of six *LEA/COR/DHN* stress-related genes was also detected in transgenic wheat under 4 ^{o}C compared to that at room temperature conditions. A relative increase/decrease in induction of the expression of *LEA/COR/DHN* stress-related genes in transgenic plants before and after cold treatment were calculated and compared to those of WT plants. All six stress-related genes were found to be down-regulated in all tested transgenic sublines (Fig. 9B). Repression of *LEA/COR/DHN* genes appeared to be different in transgenic sublines with *HDZI-3* or *HDZI-4* promoters. In transgenic sublines with the *HDZI-3* promoter, *TaCor410* expression was more strongly repressed than that of the other five genes, by the 0.0625-fold factor (Fig.

9B). However, in transgenic sublines with the *HDZI-4* promoter, the *TaWlt10* gene was repressed stronger than the other five genes, with a 0.0313-fold repression (Fig. 9B). Notably, expressions of six *LEA/COR/DHN* genes showed a strong correlation with transgene expression levels under the *HDZI-4* promoter, compared to that of the *HDZI-3* promoter (Fig. 9A, B).

Discussion

Identification of the wheat TaCBF5L gene

A novel wheat gene *TaCBF5L* was isolated from the cDNA library of stress-stressed wheat roots by using a Y1H method with the DRE *cis*-element as a bait. A phylogenetic tree was derived from an alignment of CBF/DREB proteins, particularly using TaCBF5L homologues, from *Arabidopsis* and a variety of monocot species. Based on the BLAST search, most homologous proteins to TaCBF5L were from *Triticum monococcum* and *Hordeum vulgare*, and a few homologues were from other monocot plants, such as maize and rice, and a dicot *Arabidopsis*. The phylogenetic analysis of the investigated CBF/DREB proteins revealed three major subgroups (Fig. 1). Based on the CBF/DREBs classification method suggested by Miller *et al.* (2006), the three subgroups were designated as subgroups A, B and C, which also corresponded to similar subgroups identified in barley (Skinner *et al.*, 2005). In cocnlcusion, the TaCBF5L protein was classified to subfamily C, similarly to TmCBF5 and HvCBF5.

In subfamily C, TaCBF5L had a closer relationship to six other TFs TaCBF5, TdDREB3, HvCBF5, TaDREB3, TmCBF5 and ZmDBP4 (Fig. 1). The roles of three of the TaCBF5L homologues, TaCBF5, TdDREB3, HvCBF5 were not yet specified in stress responses. However, the roles of other three homologues of TaCBF5L on the adaptive response to abiotic stresses were identified. Both TaDREB3 and ZmDBP4 were demonstrated to confer both drought and frost tolerance in transgenic plants (Kovalchuk *et al.*, 2013; Morran *et al.*, 2011; Wang *et al.*, 2011). Further, TmCBF5 showed a high expression level that was induced by cold acclimation (Sutton *et al.*, 2009). The multiple sequence alignment TaCBF5L with six homologous proteins (Fig. S7) showed that TaCBF5L contained conserved AP2 DNA binding domains, and PKKPAGR and LWSY motifs, which shared the common features with CBF/DREBs proteins (Navarrete-Campos *et al.*, 2017; Peng *et al.*, 2015; Wisniewski *et al.*, 2014).

The HDZI-4 promoter improves the grain yield of transgenic wheat under severe drought during the flowering stage

Drought may impair plant growth and development at any time point of a plant life cycle. However, the sensitivity to drought is especially acute during reproductive stages because of the plant-water status changes, leading to a high transpiration rate and a declining soil moisture (Saini and Westgate, 1999). *HDZI-4,* as an ABA-dependent promoter was found to confer the tolerance of transgenic wheat to severe drought during the flowering stage. Transgenic sublines driven by the *HDZI-4* promoter produced more seeds, a higher biomass and grain yield, and showed early flowering, compared to WT plants. However, the *HDZI-3* promoter, as an ABA-independent promoter, did not show any improvement in drought tolerance in transgenic wheat. According to the expression data evaluation of the *TaCBF5L* transgene driven by *HDZI-4* and *HDZI-3* under different drought stages, the *TaCBF5L* transgene showed relatively high expression levels under severe drought (> 40 bar) compared to those of basal levels under well-watered conditions (12–15 bar). Further, *TaCBF5L* expression driven by *HDZI-4* was induced strongly than that driven by *HDZI-3* under severe drought, which means that *HDZI-4* performed better under drought in transgenic wheat than *HDZI-3.*

However, both *HDZI-3* and *HDZI-4* promoters did not improve the survival rates of transgenic seedlings compared to those of WT plants under severe drought conditions (data not show). Based on the transgene expression data evaluation, we found that the expression of *TaCBF5L* driven by each of the two promoters was not induced under the conditions of a leaf wilting point stage (15–20 bar) or moderate drought (20–30 bar) compared with basal levels. It is likely that the two *HDZI-3* and *HDZI-4* promoters are not activated during mild or moderate drought stages. Transgenic and WT plants may suffer detrimental damage, when exposed to mild or moderate drought before transgressing from a vegetative to a reproductive phase, and exert low recovery rates, even when the promoters are strongly activated under severe drought.

Four wheat *LEA*/*DHN*/*COR* genes *TaRAB15*, *TaRab17*, *TaCor18* and *TaWLT10* were found to be up- or down-regulated by *TaCBF5L* under severe drought. *TaRAB15* was down-regulated and the other three genes were up-regulated by *TaCBF5L* driven by each of the two *HDZI-3* and *HDZI-4* promoters under severe drought. These four potential downstream genes were reported to be respondent to drought and/or cold stresses. *TaRAB15* was firstly isolated from a water-stressed wheat root cDNA library (King *et al.*, 1992). Two domains were identified on the protein sequence of TaRAB15, and these were found to be homologous regions which exist in other RAB, DHN and LEA proteins from resurrection plant, tomato, cotton, rice, barley and maize (King *et al.*, 1992). As one of the ABA responsive (RAB) genes, TaRAB15 was hypothesised to play the role in response to desiccation in plants (King *et al.*, 1992). *TaRab17*

was identified to be ABA-dependent and inducible by drought and a low temperature (Egawa *et al.*, 2006; Kobayashi *et al.*, 2006; Kobayashi *et al.*, 2004; Talanova *et al.*, 2011). *TaCor18* is assumed to be induced by cold acclimation (Rinalducci *et al.*, 2011) and *TaWLT10* was found to improve plant tolerance to frost (Ohno and Takumi, 2015). However, except of *TaRab17*, the roles of *TaRab15, TaCor18* and *TaWlt10* in drought tolerance of plants have not yet been clarified. Additional work is required to identify unknown genes functioning in drought and to explain how *TaCBF5L* confers drought tolerance to plants.

The pHDZI-3 promoter has a stable activity to improve frost tolerance of TaCBF5L transgenic wheat under the seedling stage

Overexpression of *TaCBF5L,* under each of the *HDZI-3* and *HDZI-4* promoters, conferred cold tolerance to transgenic wheat by improving their survival rates compared to those of WT plants. Notably, the *HDZI-3* promoter exhibited a more stable activity in enhancing the frost tolerance of transgenic plants compared to the *HDZI-4* promoter.

Six *COR*/*DHN*/*LEA* genes, *TaCor410* (GenBank accession: L29152), *TaWcs19* (L13437), *TaWLT10, TaRab15, TaRab17,* and *TaCor18* were found to be down-regulated by *TaCBF5L* driven by each of the *HDZI-3* and *HDZI-4* promoters. *TaCor410* was strongly up-regulated upon the exposure to frost, as it is assumed that it participates in plasma membrane protection and stabilisation under dehydration and frost according to its abundance, localisation and biochemical properties (Danyluk *et al.*, 1994; Hrmova and Lopato, 2014). *TaWcs19* (L13437), as an ABA-independent gene, was not found to be responsive to drought, heat, salt, wounding, or anaerobic stresses except of cold, which suggested that the *TaWcs19* gene was specifically induced by cold (Chauvin *et al.*, 1993; Sarhan *et al.*, 1998). *TaWLT10,* as a cold-responsive gene, encodes a cereal-specific low temperature protein. It is assumed that its N-terminal motif functions in an extracellular trafficking (Kobayashi *et al.*, 2004; Ohno and Takumi, 2015). The authors suggested that the movement of the TaWLT10 protein, through endoplasmic reticulum (ER) or a Golgi pathway into an extracellular space, is presumed to protect plants from tissues disrupting under freezing conditions (Ohno and Takumi, 2015). In addition *TaRab17* was demonstrated to be cold-inducible while *TaCor18* was assumed to be inducible by freeze acclimation (Kobayashi *et al.*, 2004; Rinalducci *et al.*, 2011).

It is notable that all potential cold-related downstream genes showed decreased expression levels in transgenic plants compared to WT plants at a low temperature. However, the potential downstream genes regulated by *TaCBF5L* showed different expression under cold and severe

drought conditions. The explanation lies in different roles of these genes in regulatory networks under a variety of abiotic stresses. It is likely that *TaCBF5L* co-co-operates with other TFs under drought and frost conditions, which could result in divergent expression of downstream genes. Future work is required to explore these possibilities and to dissect the precise details of regulatory systems controlling frost and drought abiotic stresses.

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References

Alexandrov, N.N., Brover, V.V., Freidin, S., Troukhan, M.E., Tatarinova, T.V., Zhang, H., Swaller, T.J., *et al*. (2009) Insights into corn genes derived from large-scale cDNA sequencing. *Plant Mol. Biol*. **69**, 179–194.

Ban, Q., Liu, G. and Wang, Y. (2011) A DREB gene from *Limonium bicolor* mediates molecular and physiological responses to copper stress in transgenic tobacco. *J. Plant Physiol.* **168**, 449–458.

Bouaziz, D., Charfeddine, M., Jbir, R., Saidi, M.N., Pirrello, J., Charfeddine, S., Bouzayen, M., *et al*. (2015) Identification and functional characterization of ten AP2/ERF genes in potato. *Plant Cell Tissue Organ Cult.***123**, 155–172.

Cao, X. Q., Jiang, Z. H., Yi, Y. Y., Yang, Y., Ke, L. P., Pei, Z. M. and Zhu, S. (2017) Biotic and abiotic stresses activate different Ca2+ permeable channels in *Arabidopsis*. *Front. Plant Sci.* **8**, 1–12.

Chauvin, L. P., Houde, M. and Sarhan, F. (1993) A leaf-specific gene stimulated by light during wheat acclimation to low temperature. *Plant Mol. Biol.* **23**, 255–265.

Chen, M., Wang, Q.Y., Cheng, X.G., Xu, Z.S., Li, L.C., Ye, X.G., Xia, L.Q., *et al*. (2007) GmDREB2, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. *Biochem. Biophys. Res. Commun*. **353**, 299–305.

Cheong, Y.H., Kim, K. N., Pandey, G.K., Gupta, R., Grant, J.J. and Luan, S. (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *Plant Cell*, **15**, 1833–1845.

Cho, M.H., Lee, E.K., Kang, B.G. and Cseke, L.J. (2003) Northern Blot Hybridization. In: *Handbook of Molecular and Cellular Methods in Biology and Medicine*. (Cseke L.J., et al. eds), pp: 105–111, Florida: CRC Press.

Choi, D. W., Rodriguez, E.M. and Close, T.J. (2002) Barley Cbf3 gene identification, expression pattern, and map location. *Plant Physiol.* **129**, 1781–1787.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant physiology **133**, 462–469.

Danyluk, J., Houde, M., Rassart, É. and Sarhan, F. (1994) Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant gramineae species. *FEBS Lett.* **344**, 20–24.

Egawa, C., Kobayashi, F., Ishibashi, M., Nakamura, T., Nakamura, C. and Takumi, S. (2006) Transcript accumulation and alternative splicing of a DREB2 homolog are differentially regulated by cold and drought stresses in common wheat. *Genes Genet. Syst*. **81**, 77–91.

Ferdous, J., Li, Y., Reid, N., Langridge, P., Shi, B. J. and Tricker, P.J. (2015) Identification of reference genes for quantitative expression analysis of MicroRNAs and mRNAs in barley under various stress conditions. *PloS One*. **10**, 1–20.

Gong, L., Zhang, H., Gan, X., Zhang, L., Chen, Y., Nie, F., Shi, L., *et al*. (2015) Transcriptome profiling of the potato (Solanum tuberosum L.) plant under drought stress and water-stimulus conditions. *PloS One*. **10**, 1–20.

Guenther, J. F., Seki, S., Kleinhans, F., Edashige, K., Roberts, D. M. & Mazur, P. (2006). Extra- and intra-cellular ice formation in Stage I and II Xenopus laevis oocytes. *Cryobiology*, **52**, 401-416.

Harris, J.C. (2014) *The role of homeodomain-leucine zipper class I transcription factors in the drought response of wheat*. (Doctoral dissertation).

Harris, J.C., Hrmova, M., Lopato, S. and Langridge, P. (2011) Modulation of plant growth by HD-Zip class I and II transcription factors in response to environmental stimuli. *New Phytol.* **190**, 823–837.

Harris, J.C., Sornaraj, P., Taylor, M., Bazanova, N., Baumann, U., Lovell, B., Langridge, P., *et al*. (2016) Molecular interactions of the γ-clade homeodomain-leucine zipper class I transcription factors during the wheat response to water deficit. *Plant Mol. Biol.* **90**, 435–452.

Heidarvand, L. and Amiri, R.M. (2010) What happens in plant molecular responses to cold stress? *Acta Physiol. Plant.* **32**, 419–431.

Hrmova, M. and Lopato, S. (2014) Enhancing abiotic stress tolerance in plants by modulating properties of stress responsive transcription factors. In: *Genomics of Plant Genetic Resources* pp. 291-316. Springer.

Hu, Z., Killion, P.J. and Iyer, V.R. (2007) Genetic reconstruction of a functional transcriptional regulatory network. *Nature Genet*. **39**, 683–687.

Humphrey, T.V., Bonetta, D.T. and Goring, D.R. (2007) Sentinels at the wall: cell wall receptors and sensors. *New Phytol.* **176**, 7–21.

Ismagul, A., Iskakova, G., Harris, J.C. and Eliby, S. (2014) Biolistic transformation of wheat with Centrophenoxine as a synthetic auxin. In: *Crop Breeding: Methods and Protocols* (Fleury, D. and Whitford, R. eds), pp. 191–202. New York, NY: Springer New York.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol*. **17**, 287–291.

Kasuga, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2004) A combination of the *Arabidopsis* DREB1A gene and stress-inducible rd29A promoter improved drought-and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* **45**, 346–350.

Katoh, K. and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780.

Kidokoro, S., Yoneda, K., Takasaki, H., Takahashi, F., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2017) Different cold-signaling pathways function in the responses to rapid and gradual decreases in temperature. *Plant Cell*, **29**, 760–774.

King, S.W., Joshi, C. and Nguyen, H.T. (1992) DNA sequence of an ABA-responsive gene (rab 15) from water-stressed wheat roots. *Plant Mol. Biol.* **18**, 119–121.

Klimecka, M. and Muszynska, G. (2007) Structure and functions of plant calcium-dependent protein kinases. *Acta Biochim. Pol.* **54**, 219–233.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.* **12**, 1067–1078.

Knox, A.K., Dhillon, T., Cheng, H., Tondelli, A., Pecchioni, N. and Stockinger, E.J. (2010) CBF gene copy number variation at Frost Resistance-2 is associated with levels of freezing tolerance in temperate-climate cereals. *Theor. Appl. Genet.* **121**, 21–35.

Knox, A.K., Li, C., Vágújfalvi, A., Galiba, G., Stockinger, E.J. and Dubcovsky, J. (2008) Identification of candidate *CBF* genes for the frost tolerance locus Fr-A^{m}_{2} in *Triticum monococcum*. *Plant Mol. Biol.* **67**, 257–270.

Kobayashi, F., Takumi, S., Egawa, C., Ishibashi, M. and Nakamura, C. (2006) Expression patterns of low temperature responsive genes in a dominant ABA-less-sensitive mutant line of common wheat. *Physiol. Plant.* **127**, 612–623.

Kobayashi, F., Takumi, S., Nakata, M., Ohno, R., Nakamura, T. and Nakamura, C. (2004) Comparative study of the expression profiles of the *Cor*/*Lea* gene family in two wheat cultivars with contrasting levels of freezing tolerance. *Physiol. Plant.* **120**, 585–594.

Kovalchuk, N., Jia, W., Eini, O., Morran, S., Pyvovarenko, T., Fletcher, S., Bazanova, N., *et al.* (2013) Optimization of TaDREB3 gene expression in transgenic barley using cold-inducible promoters. *Plant Biotechnol. J.* **11**, 659–670.

Lin, Y.H., Hwang, S.Y., Hsu, P.Y., Chiang, Y.C., Huang, C.L., Wang, C.N. and Lin, T.P. (2008) Molecular population genetics and gene expression analysis of duplicated CBF genes of Arabidopsis thaliana. *BMC Plant Biol*. **8**, 1–16.

Liu, J., Wang, F., Yu, G., Zhang, X., Jia, C., Qin, J. and Pan, H. (2015) Functional analysis of the maize C-Repeat/DRE Motif-binding transcription factor CBF3 promoter in response to abiotic stress. *Int. J. Mol. Sci.* **16**, 12131–12146.

Lopato, S., Bazanova, N., Morran, S., Milligan, A.S., Shirley, N. and Langridge, P. (2006) Isolation of plant transcription factors using a modified yeast one-hybrid system. *Plant Methods*. **2**, 1–15.

Lopato, S. and Langridge, P. (2011) Engineering stress tolerance in cereals using *DREB*/*CBF* genes: outcomes, problems and perspectives. *ISB News Report*.

Luan, S. (2002) Signalling drought in guard cells. *Plant Cell Environ.* **25**, 229–237.

Mallikarjuna, G., Mallikarjuna, K., Reddy, M. and Kaul, T. (2011) Expression of OsDREB2A transcription factor confers enhanced dehydration and salt stress tolerance in rice (Oryza sativa L.). *Biotechnol. Lett.* **33**, 1689–1697.

Matsumoto, T., Wu, J., Kanamori, H., Katayose, Y., Fujisawa, M., Namiki, N., Mizuno, H., *et al*. (2005) The map-based sequence of the rice genome. *Nature*. **436**, 793–800.

Mayer, K., Schüller, C., Wambutt, R., Murphy, G., Volckaert, G., Pohl, T., Düsterhöft, A., *et al*. (1999) Sequence and analysis of chromosome 4 of the plant *Arabidopsis thaliana*. *Nature*, **402**, 761–769.

Miller, A.K., Galiba, G. and Dubcovsky, J. (2006) A cluster of 11 CBF transcription factors is located at the frost tolerance locus Fr-A^{m}_{2} in Triticum monococcum. *Mol. Genet. Genomics*, **275**, 193–203.

Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S., Shirley, N., Langridge, P. and Lopato, S. (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant Biotechnol. J.* **9**, 230–249.

Morrell, P.L. and Clegg, M.T. (2007) Genetic evidence for a second domestication of barley (*Hordeum vulgare*) east of the Fertile Crescent. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3289–3294.

Nakashima, K., Jan, A., Todaka, D., Maruyama, K., Goto, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2013) Comparative functional analysis of six drought-responsive promoters in transgenic rice. *Planta*, **239**, 1–14.

Nakashima, K., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2014) The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front. Plant Sci.* **5**, 1–7.

Navarrete-Campos, D., Le Feuvre, R., Balocchi, C. and Valenzuela, S. (2017) Overexpression of three novel CBF transcription factors from *Eucalyptus globulus* improves cold tolerance on transgenic *Arabidopsis thaliana*. *Trees*, **31**, 1041–1055.

Novillo, F., Medina, J., Rodríguez-Franco, M., Neuhaus, G. and Salinas, J. (2011) Genetic analysis reveals a complex regulatory network modulating *CBF* gene expression and *Arabidopsis* response to abiotic stress. *J. Exp. Bot.* **63**, 293–304.

Ohno, R. and Takumi, S. (2015) Extracellular trafficking of a wheat cold-responsive protein, WLT10. *J. Plant Physiol.* **174**, 71–74.

Pasquariello, M., Barabaschi, D., Himmelbach, A., Steuernagel, B., Ariyadasa, R., Stein, N., Gandolfi, F., *et al*. (2014) The barley Frost resistance-H2 locus. *Funct. Integr. Genomics*, **14**, 85–100.

Peng, Y. L., Wang, Y. S., Cheng, H. and Wang, L. Y. (2015) Characterization and expression analysis of a gene encoding CBF/DREB1 transcription factor from mangrove Aegiceras corniculatum. *Ecotoxicology*, **24**, 1733–1743.

Pujol, B. and Galaud, J. P. (2013) A practical guide to quantifying the effect of genes underlying adaptation in a mixed genomics and evolutionary ecology approach. *Acta Bot. Gallica*, **160**, 197–204.

Raza, G., Ahmad, N., Hussain, M., Zafar, Y. and Rahman, M. (2016) Role of Genetics and Genomics in Mitigating Abiotic Stresses in Soybeans. In: *Environmental Stresses in Soybean Production: Soybean Production*. **2**, 205.

Rinalducci, S., Egidi, M.G., Karimzadeh, G., Jazii, F.R. and Zolla, L. (2011) Proteomic analysis of a spring wheat cultivar in response to prolonged cold stress. *Electrophoresis*, **32**, 1807–1818.

Saini, H.S. and Westgate, M.E. (1999) Reproductive development in grain crops during drought. *Adv. Agron.* **68**, 59–96.

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration-and cold-inducible gene expression. *Biochem. Biophys. Res. Commun.* **290**, 998–1009.

Samarah, N.H. (2005) Effects of drought stress on growth and yield of barley. *Agron. Sustain. Dev.* **25**, 145–149.

Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, **14**, S401–S417.

Sarhan, F., Houde, M. and Laliberte, J. F. (1998) Freezing tolerance proteins Wcs19 and Wcor410 from gramineae. *U.S. Patent No. 5,731,419*. Washington, DC: U.S. Patent and Trademark Office.

Sarkar, T., Thankappan, R., Kumar, A., Mishra, G.P. and Dobaria, J.R. (2014) Heterologous expression of the AtDREB1A gene in transgenic peanut-conferred tolerance to drought and salinity stresses. *PloS One*. **9**, 1–25.

Sazegari, S., Niazi, A. and Ahmadi, F.S. (2015) A study on the regulatory network with promoter analysis for Arabidopsis DREB genes. *Bioinformation*. **11**, 101–106.

Schnable, P.S., Ware, D., Fulton, R.S., Stein, J.C., Wei, F., Pasternak, S., Liang, C., Zhang, J., Fulton, L. and Graves, T.A. (2009) The B73 maize genome: complexity, diversity, and dynamics. *Science*, **326**, 1112–1115.

Shavrukov, Y., Gupta, N.K., Miyazaki, J., Baho, M.N., Chalmers, K.J., Tester, M., Langridge, P., *et al*. (2010) HvNax3—a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Funct. Integr. Genomic.* **10**, 277–291.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol.* **6**, 410–417.

Skinner, J.S., Szűcs, P., von Zitzewitz, J., Marquez-Cedillo, L., Filichkin, T., Stockinger, E.J., Thomashow, M.F., *et al*. (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in *Arabidopsis*. *Theor. Appl. Genet.* **112**, 832–842.

Skinner, J.S., Zitzewitz, J., Szűcs, P., Marquez-Cedillo, L., Filichkin, T., Amundsen, K., Stockinger, E.J., *et al*. (2005) Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. *Plant Mol. Biol.* **59**, 533–551.

Smith, H. (2000) Phytochromes and light signal perception by plants--an emerging synthesis. *Nature*, **407**, 585-591.

Śniegowska-Świerk, K., Dubas, E. and Rapacz, M. (2015) Drought-induced changes in the actin cytoskeleton of barley (*Hordeum vulgare* L.) leaves. *Acta Physiol. Plant.* **37**, 73.

Sutton, F., Chen, D. G., Ge, X. and Kenefick, D. (2009) Cbf genes of the Fr-A2 allele are differentially regulated between long-term cold acclimated crown tissue of freeze-resistant and–susceptible, winter wheat mutant lines. *BMC Plant Biol.* **9**, 1-9.

Talanova, V., Titov, A., Topchieva, L. and Repkina, N. (2011) Expression patterns of ABA-dependent and ABA-independent genes during wheat cold adaptation. *Russ. J. Plant Physiol.* **58**, 1005–1010.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729.

Thion, L., Mazars, C., Thuleau, P., Graziana, A., Rossignol, M., Moreau, M. and Ranjeva, R. (1996) Activation of plasma membrane voltage-dependent calcium-permeable channels by disruption of microtubules in carrot cells. *FEBS Lett.* **393**, 13–18.

Todaka, D., Zhao, Y., Yoshida, T., Kudo, M., Kidokoro, S., Mizoi, J., Kodaira, K.S., *et al*. (2017) Temporal and spatial changes in gene expression, metabolite accumulation and phytohormone content in rice seedlings grown under drought stress conditions. *Plant J.* **90**, 61–78.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. (1994) Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis* thaliana. *Mol. Gen. Genet.* **244**, 331–340.

Wang, C. T. and Dong, Y. M. (2009) Overexpression of maize ZmDBP3 enhances tolerance to drought and cold stress in transgenic *Arabidopsis* plants. *Biologia*, **64**, 1108–1114.

Wang, C. T., Yang, Q. and Yang, Y. M. (2011) Characterization of the *ZmDBP4* gene encoding a CRT/DRE-binding protein responsive to drought and cold stress in maize. *Acta Physiol. Plant.* **33**, 575–583.

Wisniewski, M., Nassuth, A., Teulières, C., Marque, C., Rowland, J., Cao, P.B. and Brown, A. (2014) Genomics of cold hardiness in woody plants. *Crit. Rev. Plant Sci.* **33**, 92–124.

Xianjun, P., Xingyong, M., Weihong, F., Man, S., Liqin, C., Alam, I., Lee, B.H., *et al*. (2011) Improved drought and salt tolerance of *Arabidopsis thaliana* by transgenic expression of a novel *DREB* gene from Leymuschinensis. *Plant Cell Rep.* **30**, 1493–1502.

Yadav, D., Shavrukov, Y., Bazanova, N., Chirkova, L., Borisjuk, N., Kovalchuk, N., Ismagul, A., *et al*. (2015) Constitutive overexpression of the *TaNF-YB4* gene in transgenic wheat significantly improves grain yield. *J. Exp. Bot.* **66**, 6635–6650.

Yang, W., Liu, X. D., Chi, X. J., Wu, C. A., Li, Y. Z., Song, L. L., Liu, X.M., *et al*. (2011) Dwarf apple MbDREB1 enhances plant tolerance to low temperature, drought, and salt stress via both ABA-dependent and ABA-independent pathways. *Planta*, **233**, 219–229.

Yang, Y., Sornaraj, P., Borisjuk, N., Kovalchuk, N. and Haefele, S.M. (2016) Transcriptional network involved in drought response and adaptation in cereals. In: *Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives* (Shanker, A.K. and Shanker, C. eds), pp 3–29. Rijeka: InTech.

Yuan, J.S., Wang, D. and Stewart, C.N. (2008) Statistical methods for efficiency adjusted real-time PCR quantification. *Biotechnol. J.* **3**, 112–123.

Figure legends

Fig. 1 The phylogenetic tree of DREB TFs from a representative dicot *Arabidopsis* and monocots wheat and barley. The tree was constructed using the MAFFT alignment of protein sequences. Ta —*Triticum aestivum*; Tm — *Triticum monucoccum*; Td — *Triticum durum*; Hv — *Hordeum vulgare*; Os — *Oryza sativa*; Zm — *Zea mays*; At — *Arabidopsis thaliana*. TFs are classified into group A, B and C, and labelled on the root. TaCBF5L is pointed at by "♦".

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Legends to Supplementary Figures

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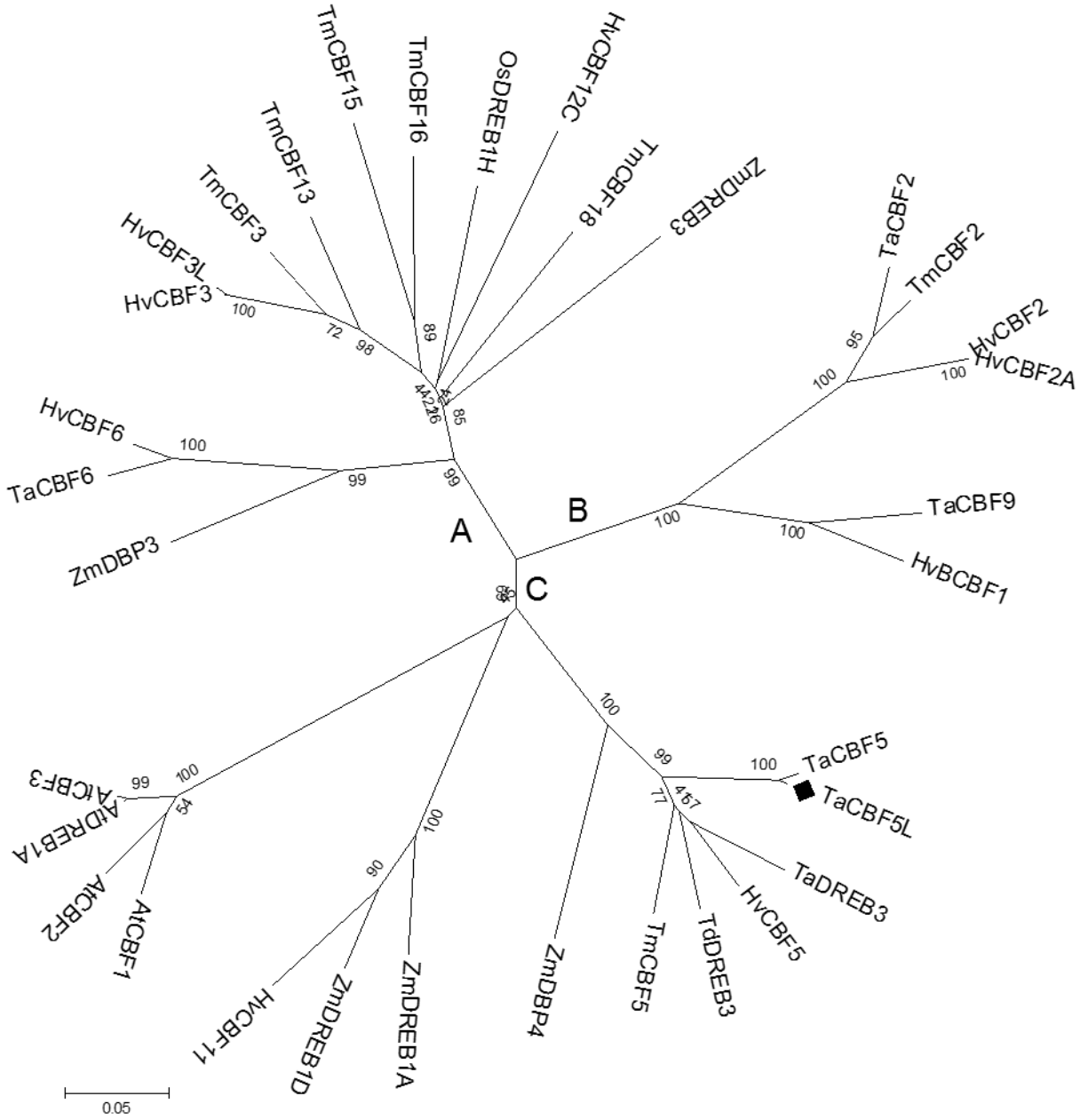
Figures



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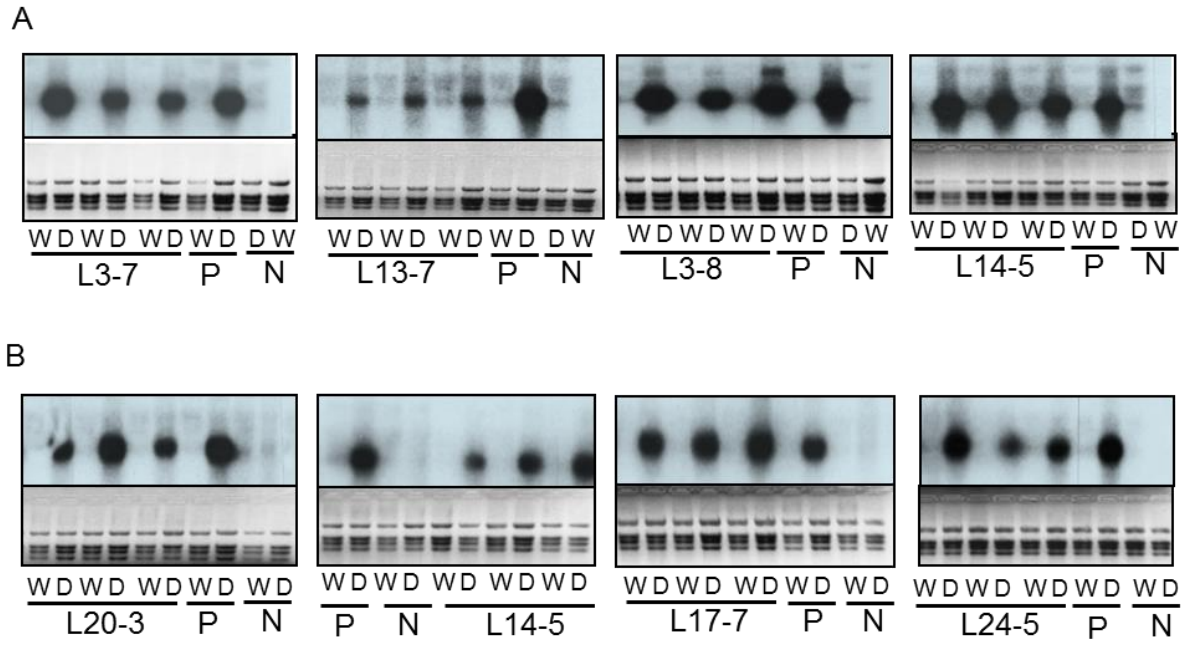

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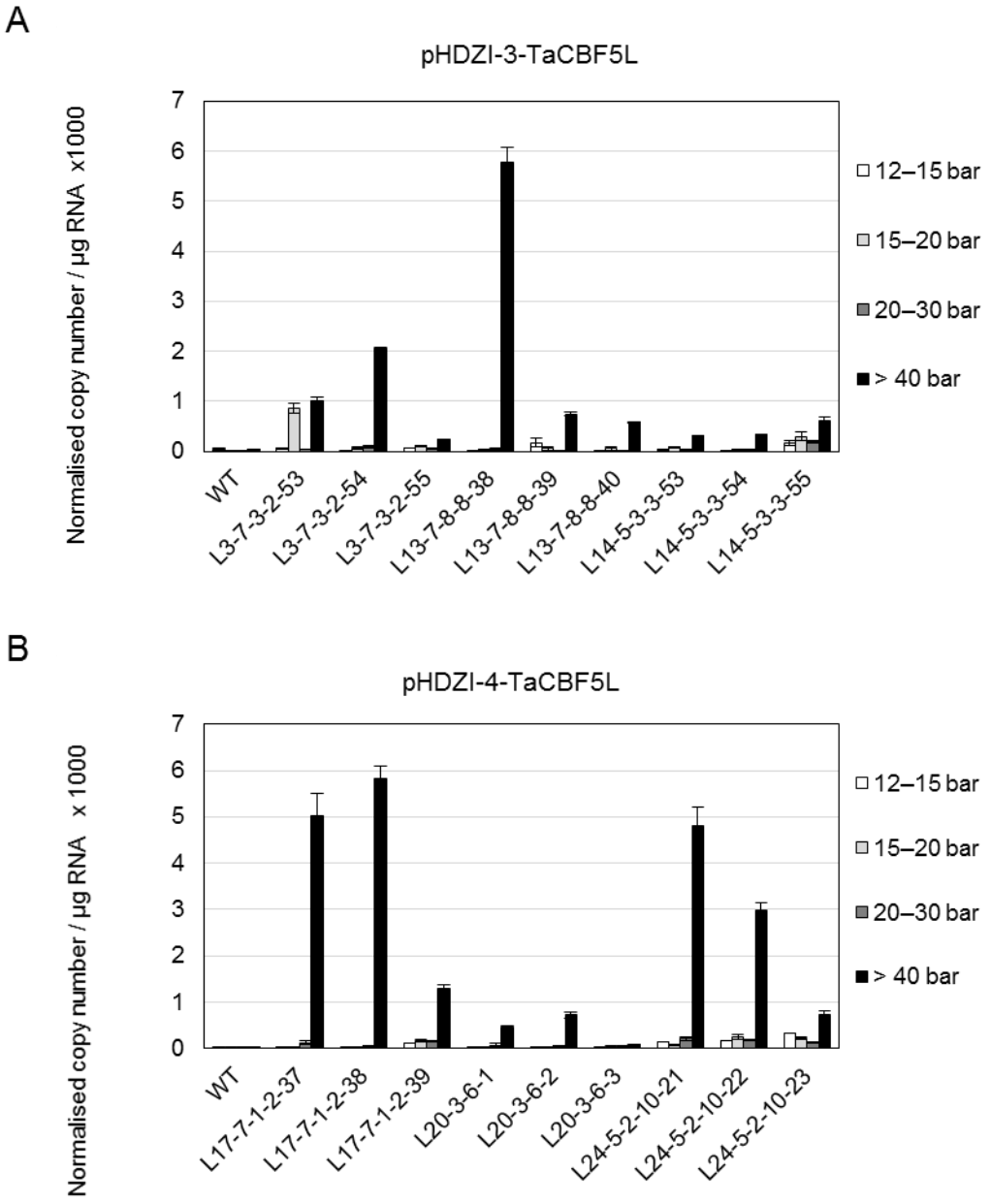

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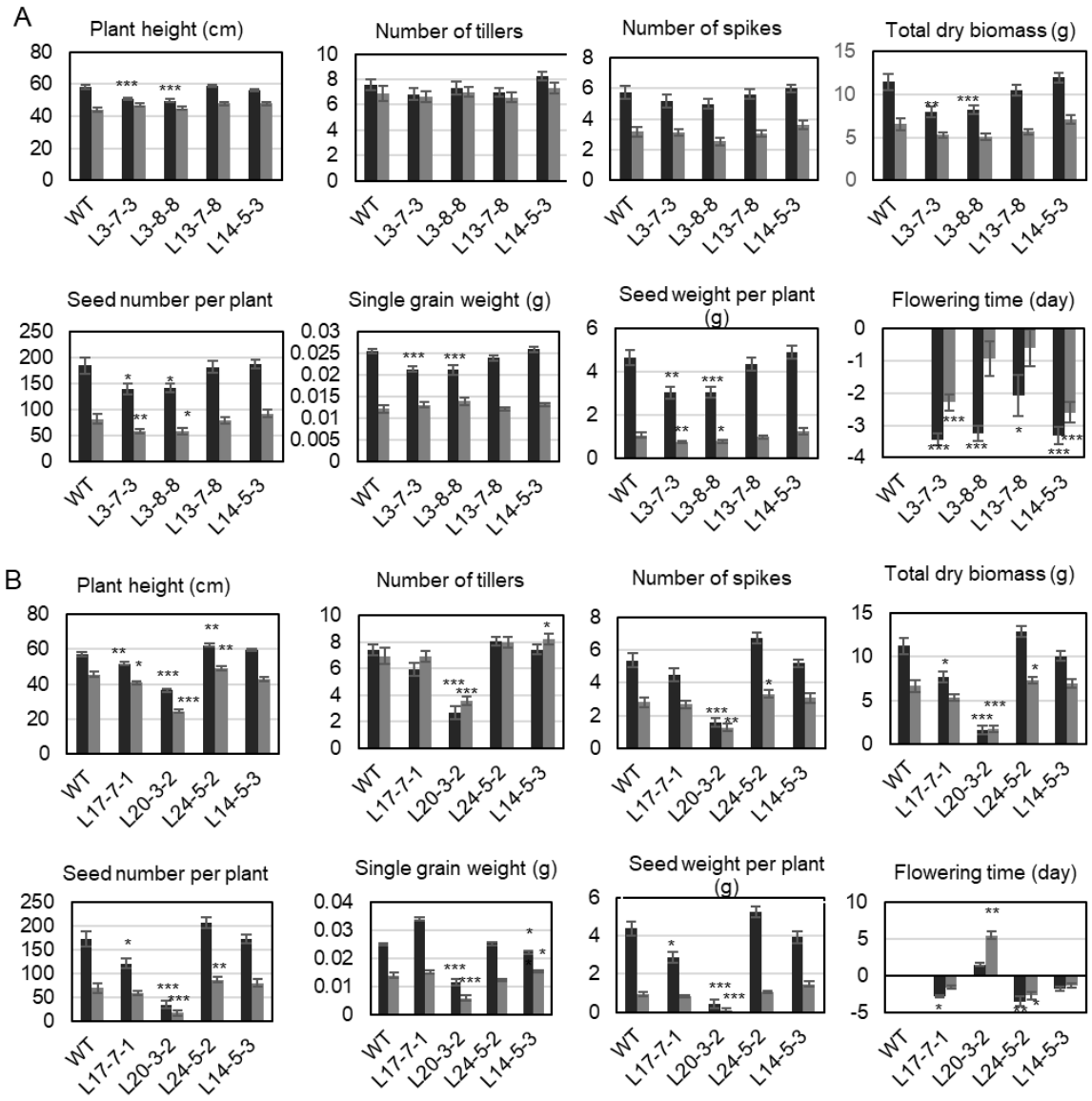

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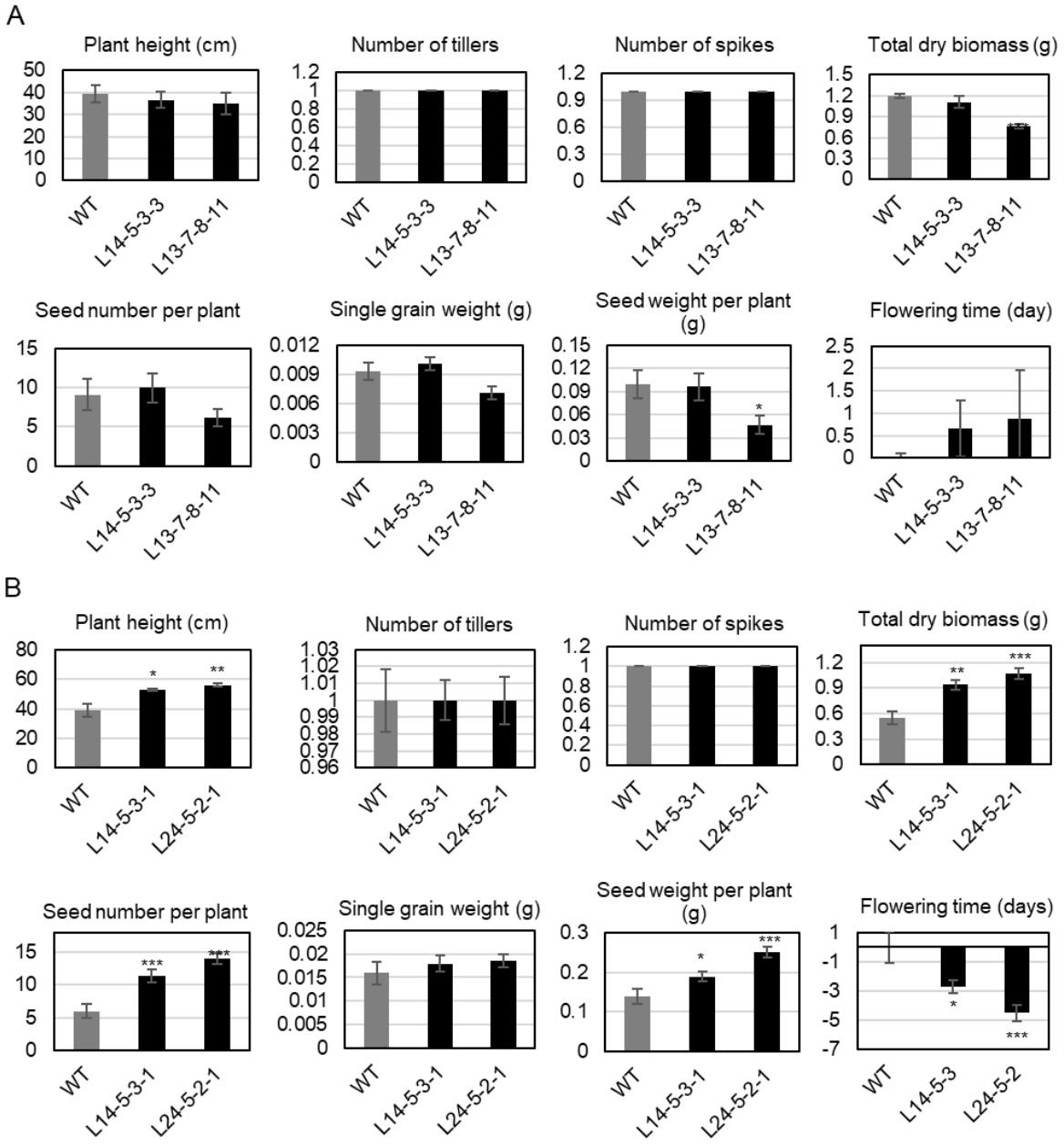

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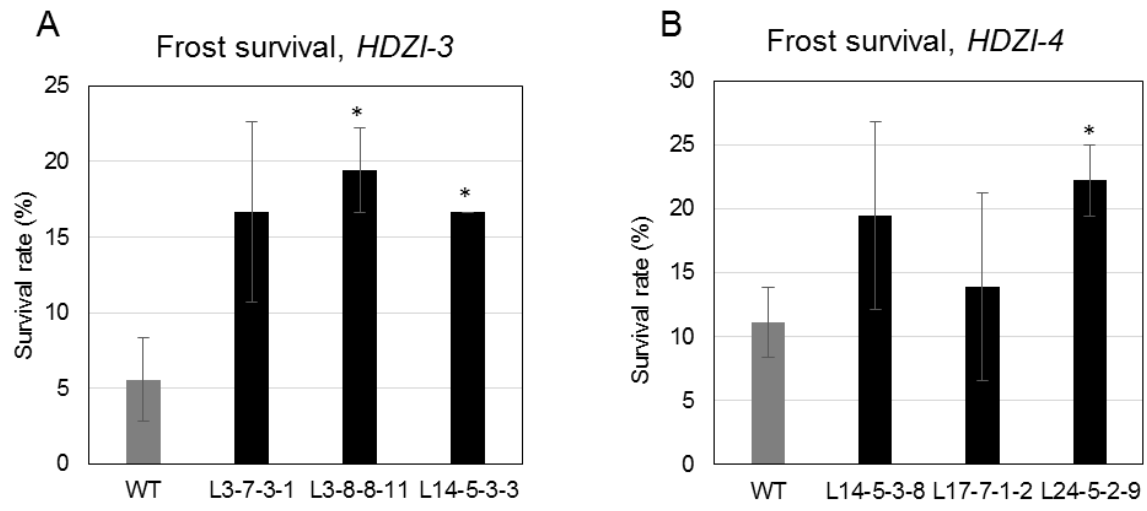

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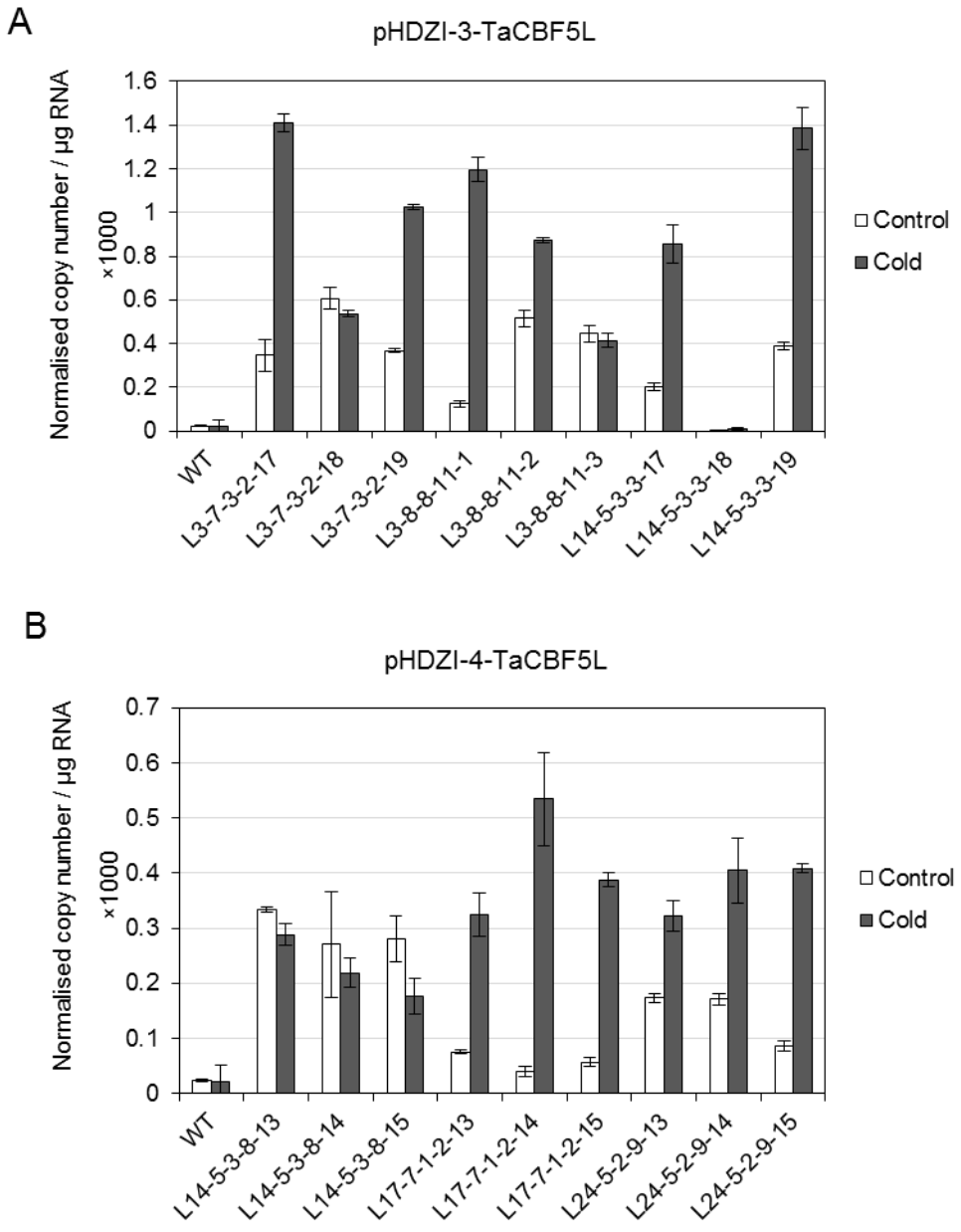

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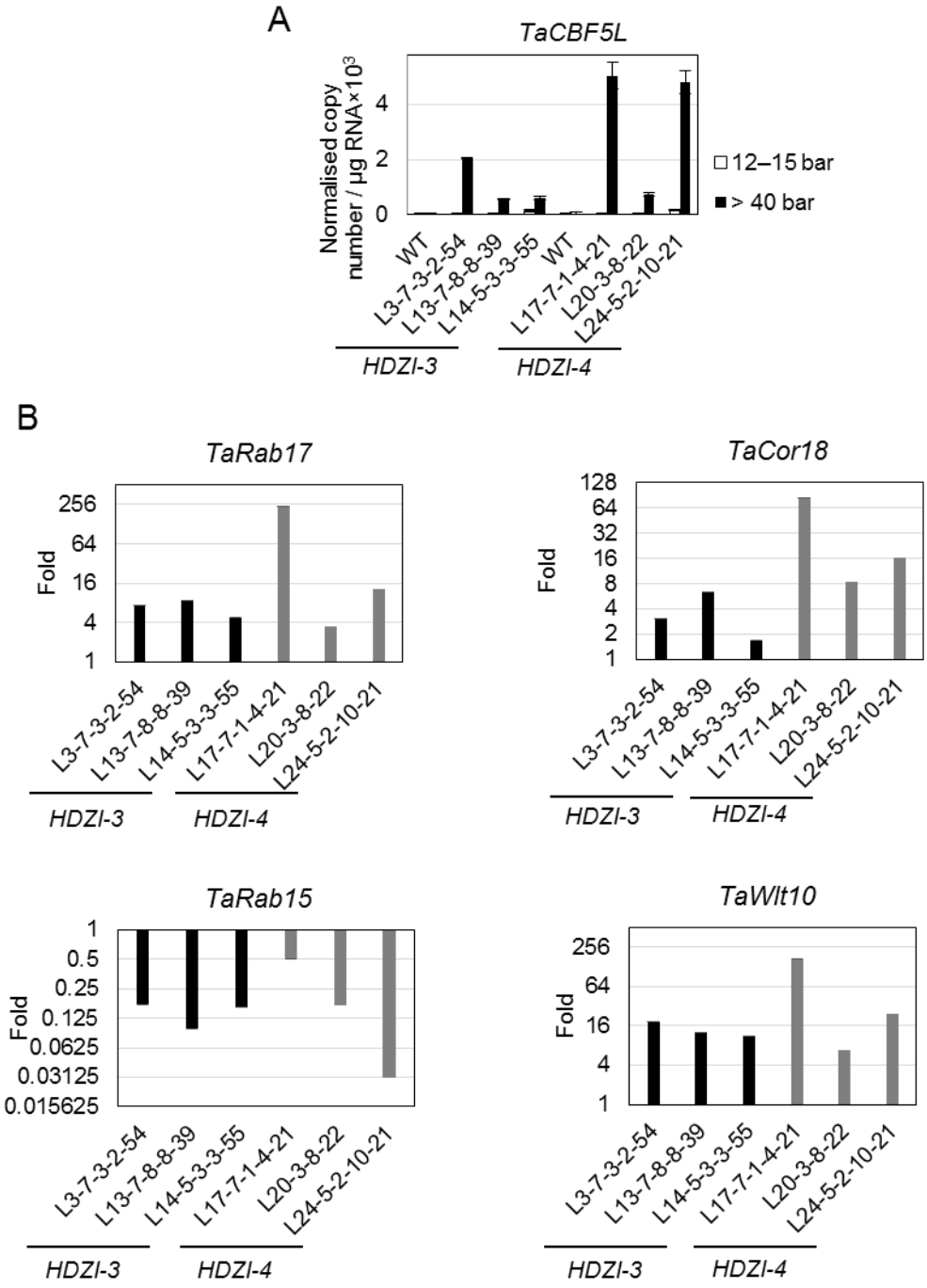

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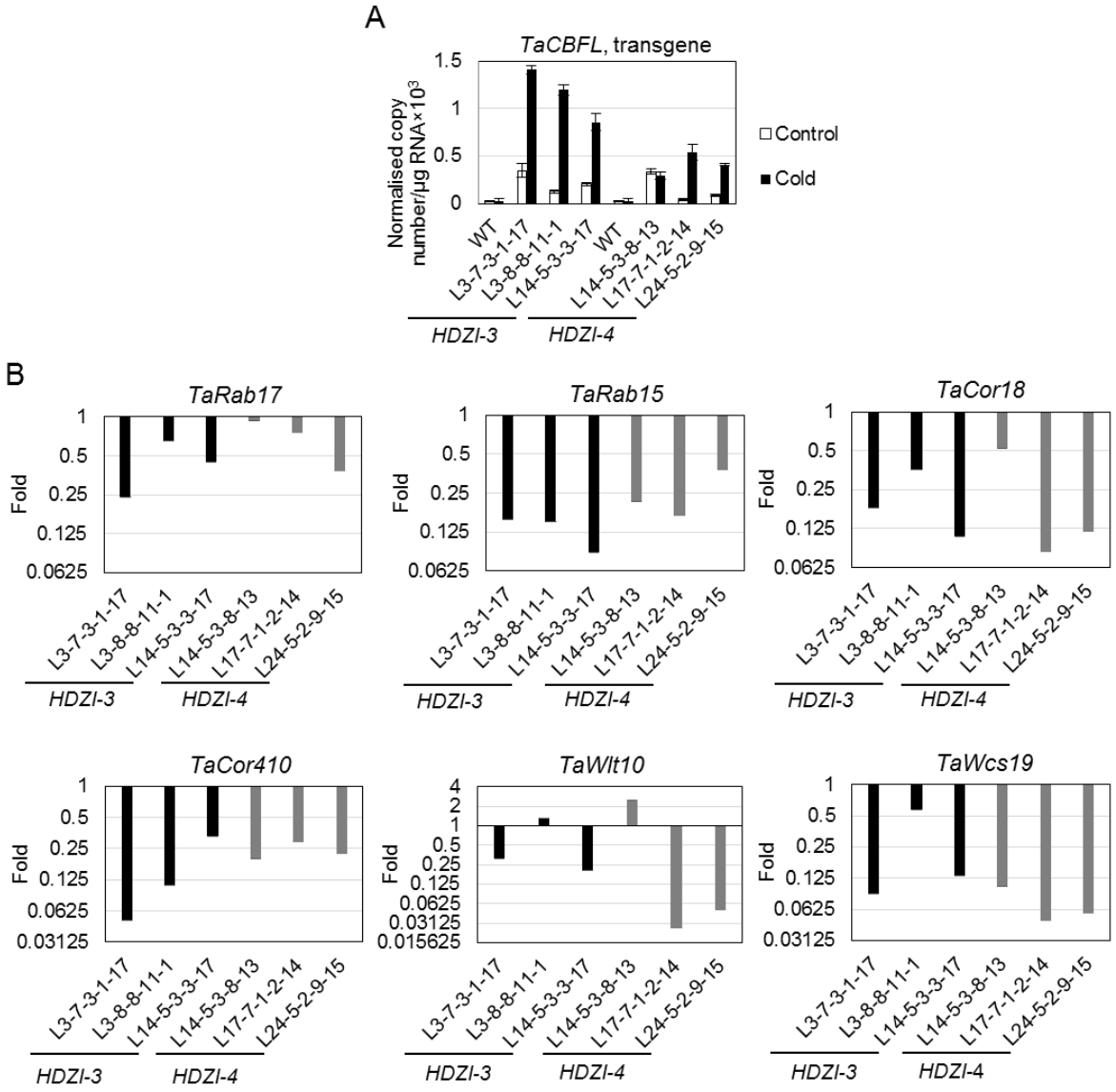

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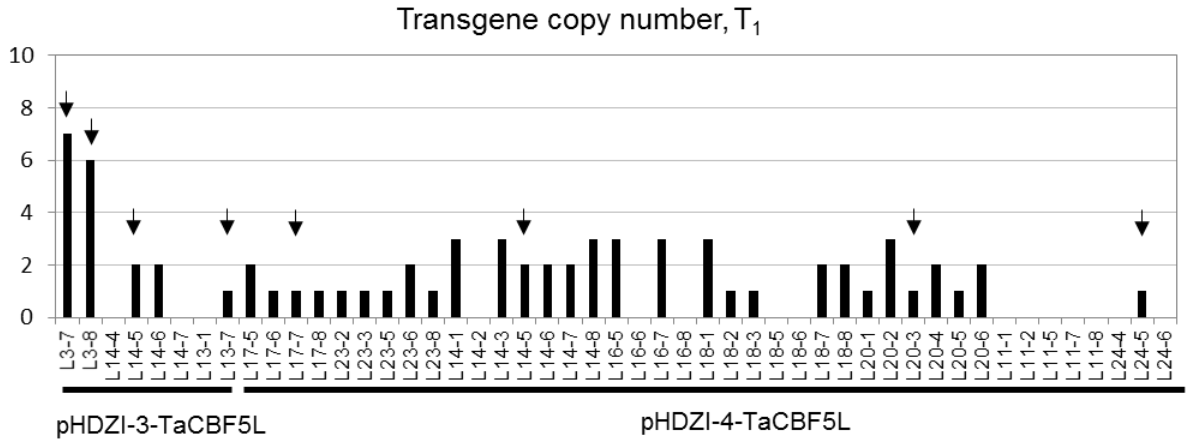
Supplementary figures and tables



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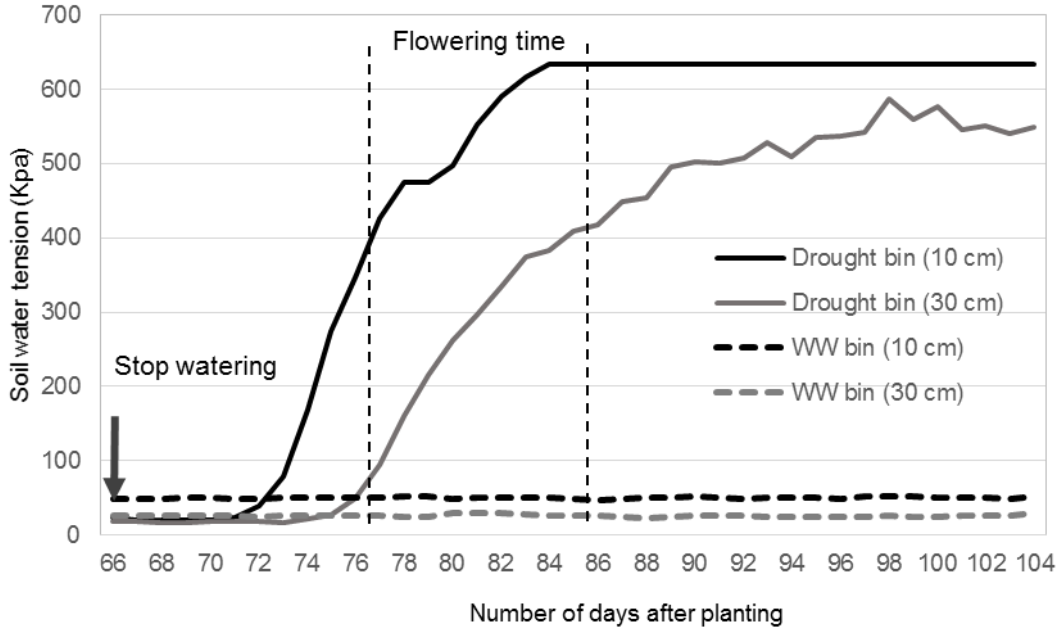

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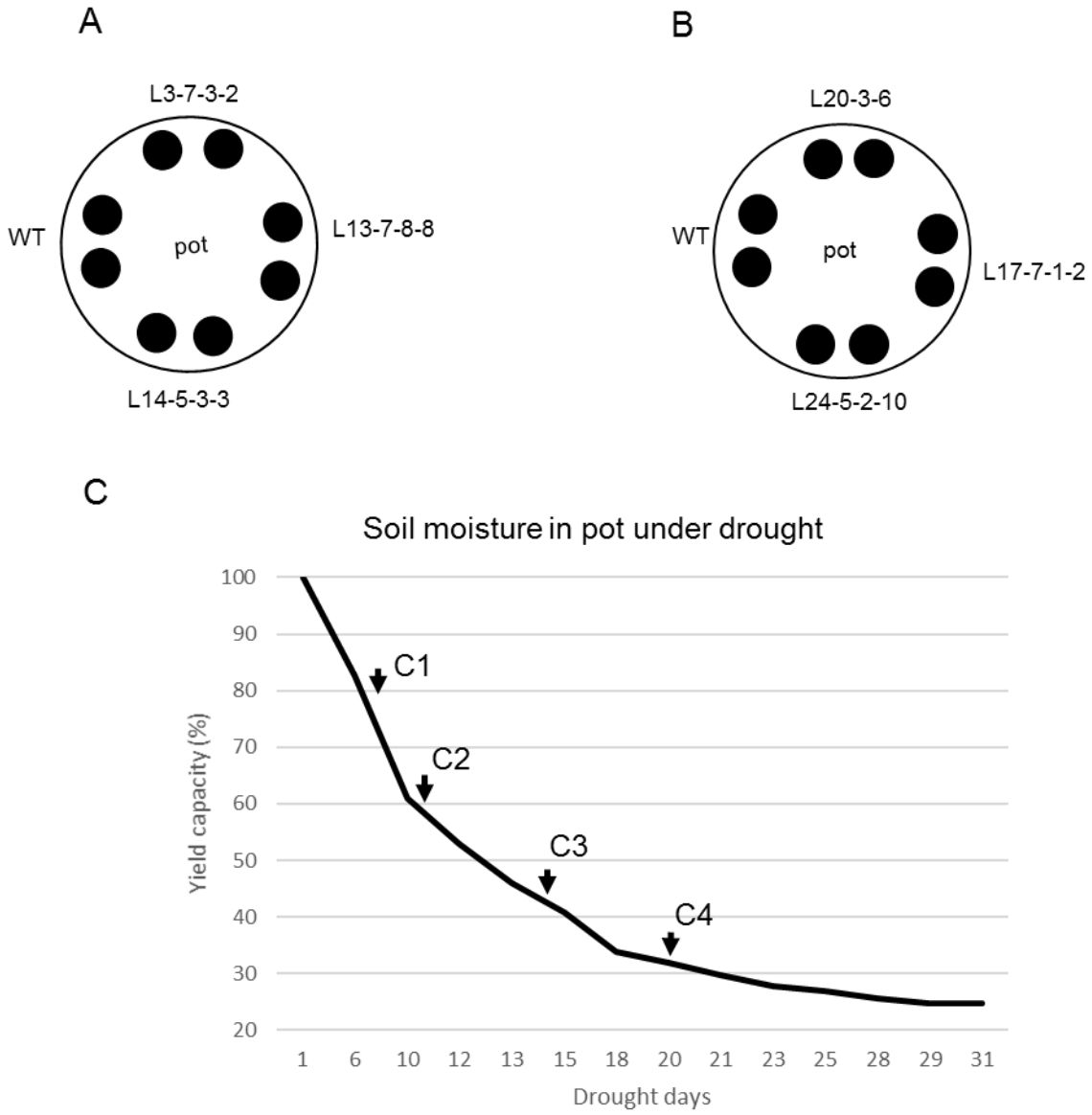

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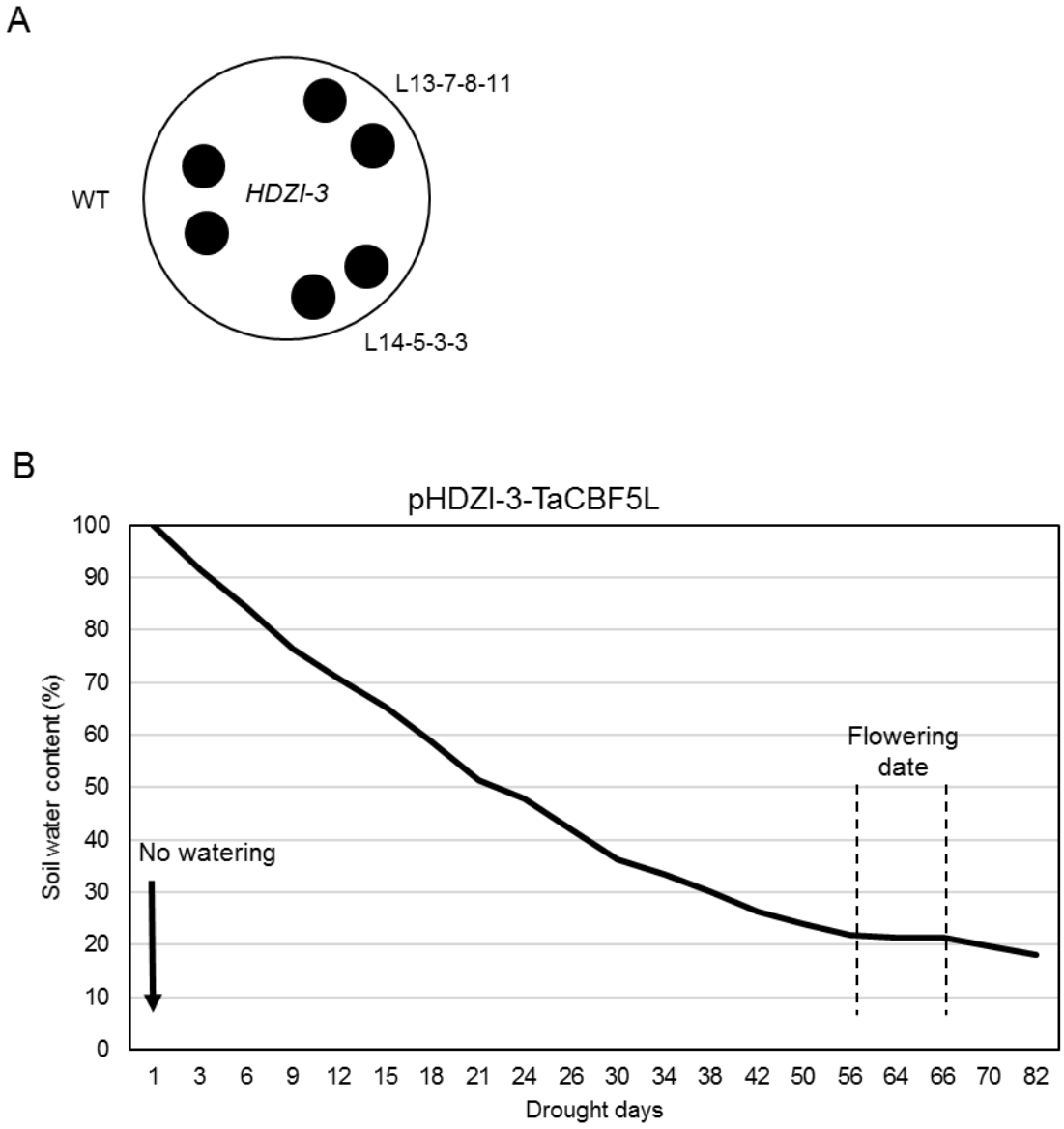

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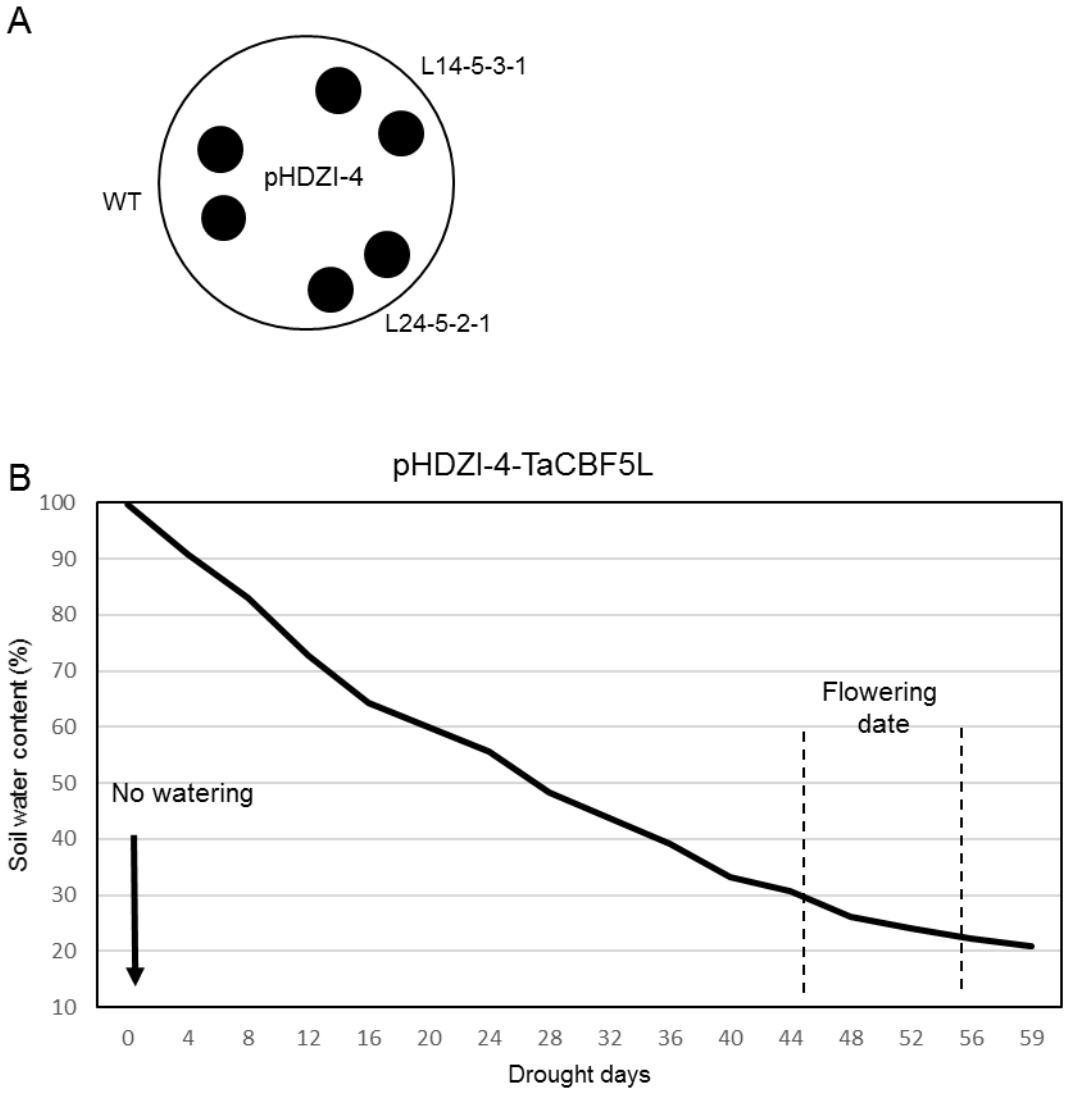

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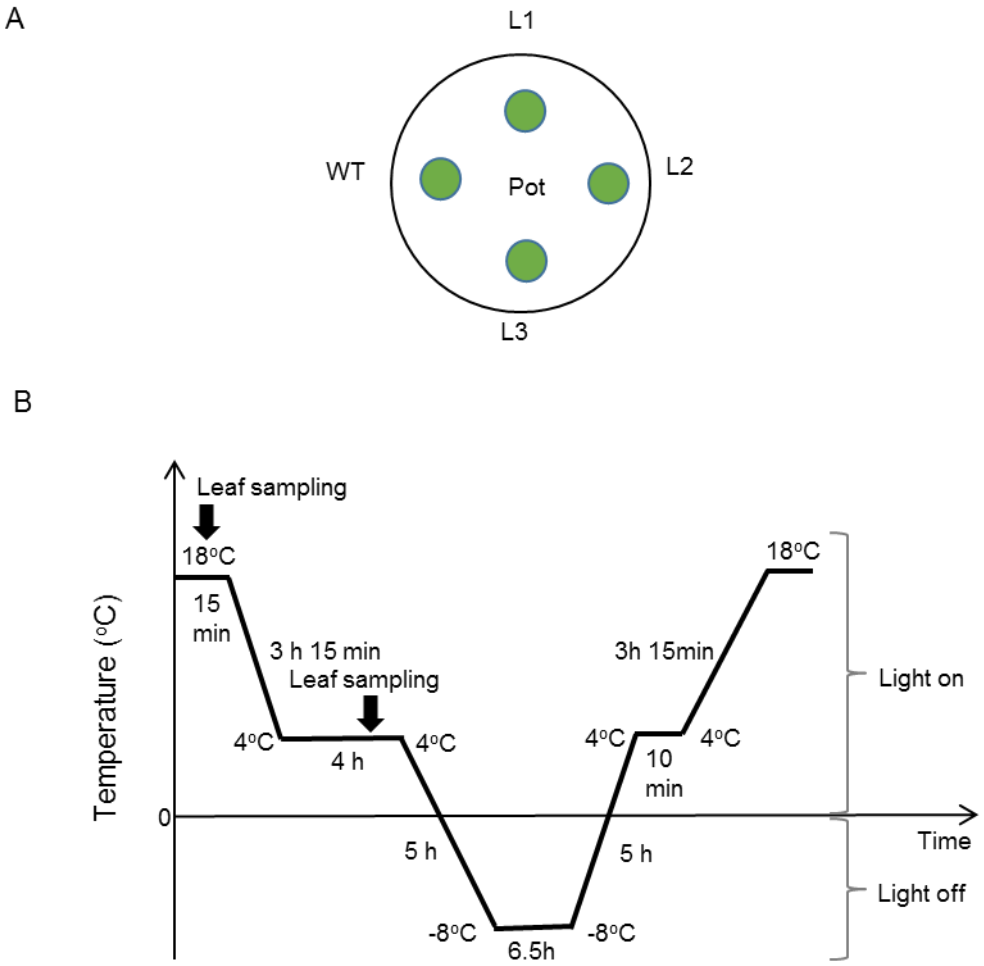

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Table S1. The list of PCR primers, Q-PCR primers and Northern hybridisation primers and probes used in this study.

Short name	Purpose	Designation	Nucleotide sequence (5′–3′)
TaCBF5L	PCR, transgene specific primers	Forward primer	TGATGCAGTTTGACGACTACGC
		Reverse primer	CATCGCAAGACCGGCAACAGG
TaCBF5L	Q-PCR, gene expression	Forward primer	GTCGCGCTCTGGAGCTACTGA
		Reverse primer	CGGCAACAGGATTCAATC
NOS terminator	Q-PCR, copy number	Forward primer	CTTAAGATTGAATCCT
		Reverse primer	CGAATTCAGTAACATAGA
		TaqMan Probe	AGCGCGCAAACTAGGAT
Pin-b	Q-PCR, copy number	Forward primer	ATTTTCCAGTCACCTGGCCC
		Reverse primer	TGCTATCTGGCTCAGCTGC
		TaqMan Probe	ATGGTGGAAGGGCGGCTGTGA
TaCRT	RT-qPCR	Forward primer	CGATGATGATGATGGCATATGG
		Reverse primer	TACCTGCCAAACTTCAATTCC
Taactin	Q-PCR, gene expression	Forward primer	GACAATGGAACCGGAATGGTC
		Reverse primer	GTGTGATGCCAGATTTTCTCCAT
Tacyclophilin	Q-PCR, gene expression	Forward primer	CAAGCCGCTGCACTACAAGG
		Reverse primer	AGGGGACGGTGCAGATGAA
TaGAPdH	Q-PCR, gene expression	Forward primer	TTCAACATCATTCCAAGCAGCA
		Reverse primer	CGTAACCCAAAATGCCCTTG
TaEFA	Q-PCR, gene expression	Forward primer	CAGATTGGCAACGGCTACG
		Reverse primer	CGGACAGCAAAACGACCAAG
TaRAB15	Q-PCR, gene expression	Forward primer	ACAGGGACTGGAGGAGCCTAC
		Reverse primer	TGGAACTGGAAGGCTTTGACC
Tacor410	Q-PCR, gene expression	Forward primer	TGCCTGGTTACCACAAGACAG
		Reverse primer	GACCTTCAAAGACGATCAACC
TaRab17	Q-PCR, gene expression	Forward primer	ACGCTGTCATGAACACGCTCG
		Reverse primer	AGTTACACCAAGGAGCATGTG
TaWcs19	Q-PCR, gene expression	Forward primer	GGAAGCTAGCTAAGCTAACAC
		Reverse primer	CAGCTATCTTATCTCTTGTAC
TaWlt10	Q-PCR, gene expression	Forward primer	AGCCATTACCGGTACCAGTAC
		Reverse primer	ACAGAGTTACAGACGCTCATC
Tacor18	Q-PCR, gene expression	Forward primer	GTGTTCTAAGGAGTTCTTGTG
		Reverse primer	AATTACAAAGGCTGCTCGTGG
TaCBF5L	Northern hybridisation, probe preparation	Forward primer	ATGGACCAGTACAACTACG
		Reverse primer	TCAGTAGCTCCAGAGCGCGAC

Table S2. TaCBF5L homologous proteins from wheat, maize, rice, barley and *Arabidopsis* to TaCBF5L were searched using the BLAST tool. The accession/locus numbers, query cover and sequence identity percentage to TaCBF5L and related references are listed below.

Name of TFs	Accession/locus number	Species	Query cover (%)	Identity (%)	Reference
TaCBF2	AY785900	*Triticum aestivum*	57	57	(Skinner et al., 2005)
TaDREB3	DQ353853	*Triticum aestivum*	92	74	(Lopato et al., 2006)
TaCBF5	AY785902	*Triticum aestivum*	87	84	(Skinner et al., 2005)
TaCBF6	AY785903	*Triticum aestivum*	61	55	(Skinner et al., 2005)
TaCBF9	AY785905	*Triticum aestivum*	39	76	(Skinner et al., 2005)
TmCBF2	AY951949	*Triticum monococcum*	69	49	(Miller et al., 2006)
TmCBF3	AY951949	*Triticum monococcum*	87	47	(Miller et al., 2006)
TmCBF5	AY951947	*Triticum monococcum*	89	74	(Miller et al., 2006)
TmCBF13	AY951951	*Triticum monococcum*	87	46	(Miller et al., 2006)
TmCBF15	AY951944	*Triticum monococcum*	52	60	(Miller et al., 2006)
TmCBF16	EU076384	*Triticum monococcum*	37	76	(Knox et al., 2008)
TmCBF18	AY951946	*Triticum monococcum*	84	48	(Miller et al., 2006)
TdDREB3	GU785009	*Triticum durum*	88	77	(Morran et al., 2011)
ZmDBP4	NM_001177010	*Zea mays*	87	64	(Schnable et al., 2009)
ZmDREB3	NM_001112181	*Zea mays*	45	69	(Liu et al., 2015)
ZmDREB1A	NM_001157386	*Zea mays*	62	62	(Alexandrov et al., 2009)
ZmDREB1D	NM_001154158	*Zea mays*	58	64	(Schnable et al., 2009)
ZmDBP3	FJ805751	*Zea mays*	48	66	(Wang and Dong, 2009)
OsDREB1H	LOC_Os09g35020	*Oryza sativa*	55	60	(Matsumoto et al., 2005)
HvBCBF1	AF298230	*Hordeum vulgare*	38	78	(Choi et al., 2002)
HvCBF2A	AY785840	*Hordeum vulgare*	69	50	(Skinner et al., 2006)
HvCBF2	DQ445249	*Hordeum vulgare*	69	50	(Knox et al., 2010)
HvCBF3	DQ445250	*Hordeum vulgare*	87	45	(Knox et al., 2010)
HvCBF3L	EF409434	*Hordeum vulgare*	87	45	(Morrell and Clegg, 2007)
HvCBF5	AY785855	*Hordeum vulgare*	89	76	(Skinner et al., 2005)
HvCBF6	AY785859	*Hordeum vulgare*	56	55	(Skinner et al., 2005)
HvCBF11	AY785889	*Hordeum vulgare*	60	62	(Skinner et al., 2006)
HvCBF12C	KF686739	*Hordeum vulgare*	86	46	(Pasquariello et al., 2014)
AtCBF1	EF522963	*Hordeum vulgare*	43	69	(Lin et al., 2008)
AtDREB1A	NM_118680	*Arabidopsis thaliana*	40	74	(Mayer et al., 1999)
AtCBF2	EF523057	*Arabidopsis thaliana*	40	70	(Lin et al., 2008)
AtCBF3	EF523110	*Arabidopsis thaliana*	86	44	(Lin et al., 2008)

Chapter 6 Discussion

6.1 General discussion

Drought and frost are two major environmental stresses which result in crop production losses all over the world. Studies in improvement of wheat stress tolerance and performance under frost and drought have been focussed on identification and analysis of drought-related genes encoding HD-Zip I γ-clade proteins participating in abiotic stress response and the optimisation of transgene expression with different stress-inducible promoters. However, limited studies were performed on elucidation of the role of the wheat HD-Zip I γ-clade proteins in resistance to drought and cold stress.

This thesis was designed to: 1) explore the function and activity of a γ-clade gene, which encodes the wheat HD-Zip I family, involved in drought and cold acclimation of transgenic plants; 2) optimise the transgene *TaHDZipI-5* expression by using different stress-inducible promoters and a constitutive promoter under different stresses; 3) characterise the promoters of genes encoding γ-clade of wheat HD-Zip I proteins in transgenic wheat under drought and cold conditions and explain the mechanisms of stress tolerance by searching for potential downstream genes. In this chapter, the significance of the work and potential directions for future research are discussed.

6.2 Significance of the work

Drought and frost significantly decrease yields of crops around the world. Characterisation of the function of stress-related genes helps to understand the mechanisms of plant responses to severe environmental conditions. The findings of this work confirmed the role of the wheat *TaHDZipI-5* gene, encoding a stress-responsive homeodomain-leucine zipper class I (HD-Zip I) TF, during the development of plant tolerance to frost and drought. The constitutive overexpression of *TaHDZipI-5* in bread wheat significantly enhanced frost and drought tolerance of transgenic wheat sublines during the seedling stage, though undesired phenotypic features occurred during the reproductive stage.

The analysis of *cis*-element promoter of *TdHDZipI-5A*, as a homolog of *TaHDZipI-5*, using transient expression assay, helped to indicate what potential TF might participate in upregulation of the gene *TdHDZipI-5*.

A novel TF, TaCBF5L, was isolated using a Y1H assay and was confirmed as one of CBF/DREB TFs by alignment with its homolog proteins and reconstruction of the phylogenetic

tree. The stress-inducible overexpression of the gene *TaCBF5L* was proven to confer the drought and cold tolerance to transgenic wheat under each of two promoters of wheat HD-Zip.

One of the two promoters, *HDZI-4*, improved the grain yield of transgenic sublines under severe drought. Besides, both promoters *HDZI-3* and *HDZI-4*, but especially *HDZI-3*, successfully enhanced the cold tolerance of transgenic wheat in the seedling stage.

In addition, six *Cor* genes expressions were determined in transgenic wheat. Four of them were up- or down-regulated by the drought-inducible overexpression of *TaCBF5L* and all the six tested genes were down-regulated by the cold-inducible overexpression of *TaCBF5L* under each of the two promoters, *HDZI-3* and *HDZI-4*. The finding of potential downstream genes may help to explain the mechanism of the overexpression of *TaCBF5L* under the wheat HD-Zip I promoters, improving the tolerance of transgenic wheat under different abiotic stresses.

6.3 Possible research directions

The constitutive expression of *TaHDZipI-5* contributed to the improvement of drought and cold tolerance of transgenic wheat, according to the results of survival in drought/cold experiments of transgenic plants in the vegetative stage. Both of two tested transgenic sublines demonstrated significantly higher survival rates under drought and one out of three tested transgenic sublines showed obviously higher survival rate under low temperatures compared with WT plants. However, the mechanism of plant drought and frost tolerance improvement by TaHDZipI-5 remains unclear at this stage. The exploration of more downstream genes up-regulated by TaHDZipI-5 might be helpful to explain the mechanism. According to the research of Bhattacharjee *et al.* (2016), over 809 scanned rice genes possessing AH1/AH2 motifs in their promoters were revealed to be the potential downstream gene of HD-Zip TFs. These genes were demonstrated to participate in several functional processes, including anti-oxidation, small molecule metabolism, lipid metabolism, cellular stimulus response, reproductive and anatomical structure development and hormone mediated signalling pathways (Bhattacharjee *et al.*, 2016). Therefore, TaHDZipI-5 might regulate the stress-related genes through the above physiological processes but more evidence needs to be collected.

In this project, the characterisation of HD-Zip I promoters in transgenic wheat suggested that the ABA-independent promoter *HDZI-3* and ABA-dependent promoter *HDZI-4* functioned differently in conferring drought and frost tolerance to transgenic wheat, and the downstream genes regulated by TaCBF5L were different under drought and frost. This suggested different TFs co-operation of downstream gene networks under different abiotic stresses. However, more

efforts are still required to explicate the complicated networks by finding more possible downstream genes and TFs.

Appendix

The homozygous sublines of transgenic wheat with pUbi-TaHDZipI-5/ pWRKY71-TaHDZipI-5/ pCor39-TaHDZipI-5 were confirmed using PCR method. The PCR products of the *TaHDZipI-5* transgene in each homozygous subline indicated to have positive bands through agarose gel electrophoresis and the results were shown in Fig. A1, A2 and A3 respectively.

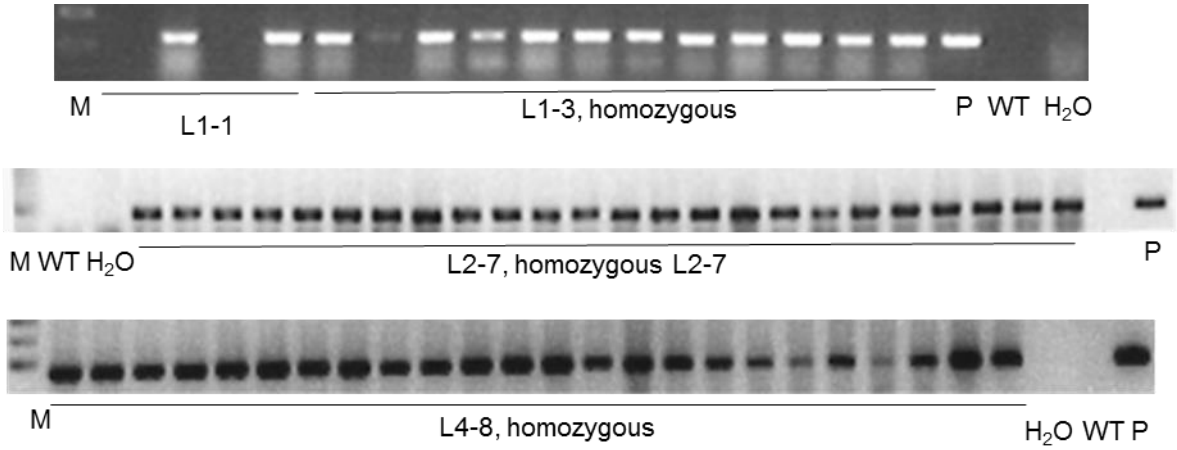

Fig. A1 Agarose gel (1.5 %) electrophoresis of PCR product of the transgene *TaHDZI-5* in T_2 homozygous sublines of transgenic wheat with promoter *ZmUbi*. M—100 bp DNA ladder*;* P—the plasmid with transgene band was used as positive control; WT—wildtype plants in which the transgene band was absent as negative control; H_2O— MiliQ water was used as negative control as well.

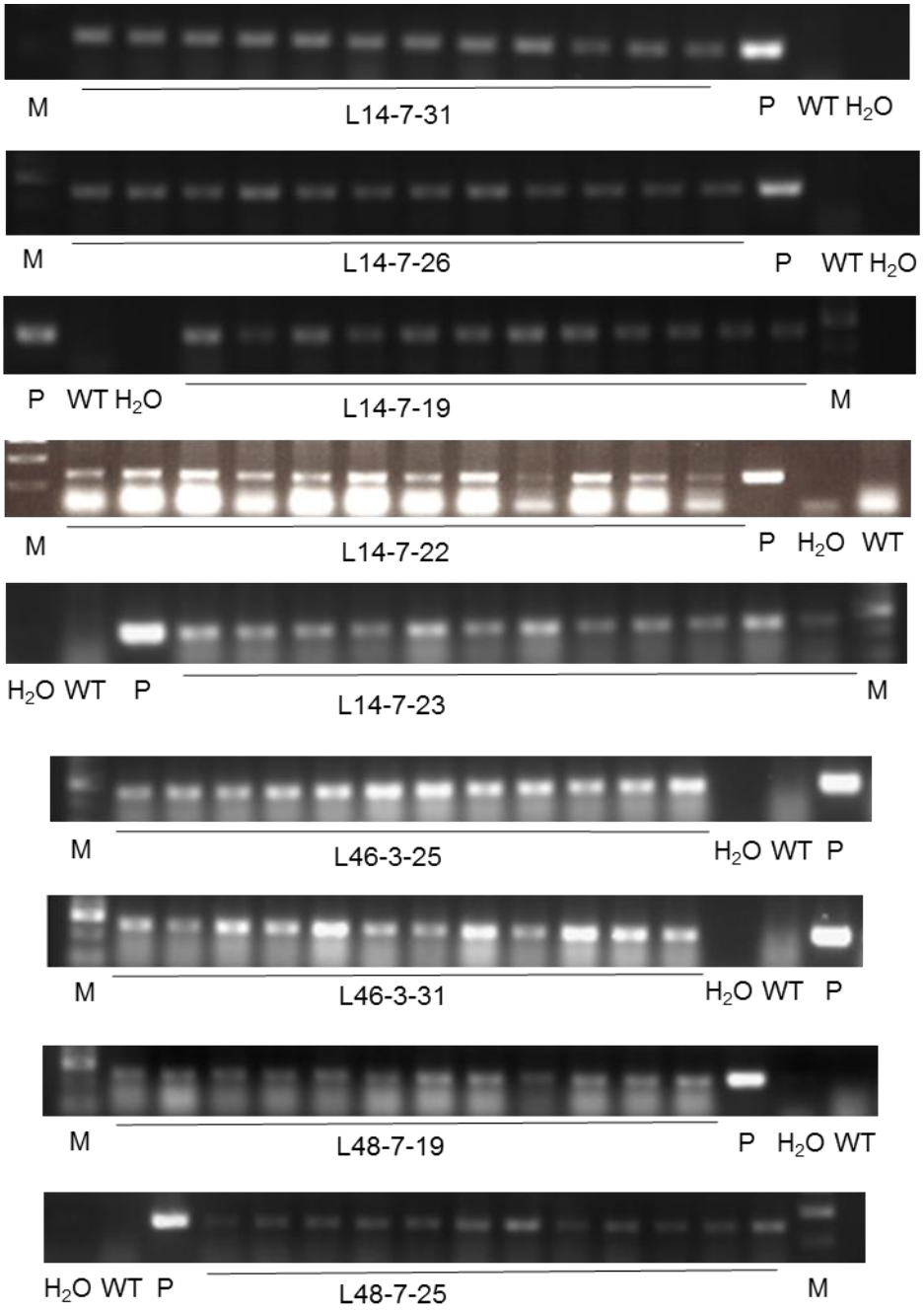

Fig. A2 Agarose gel (1 %) electrophoresis of PCR product of the transgene *TaHDZI-5* in T_3 homozygous sublines of transgenic wheat with promoter *OsWRKY71*. M—1kb DNA ladder*;* P—the plasmid with transgene band was used as positive control; WT—wildtype plants in which the transgene band was absent as negative control; H_2O— MiliQ water was used as negative control as well.

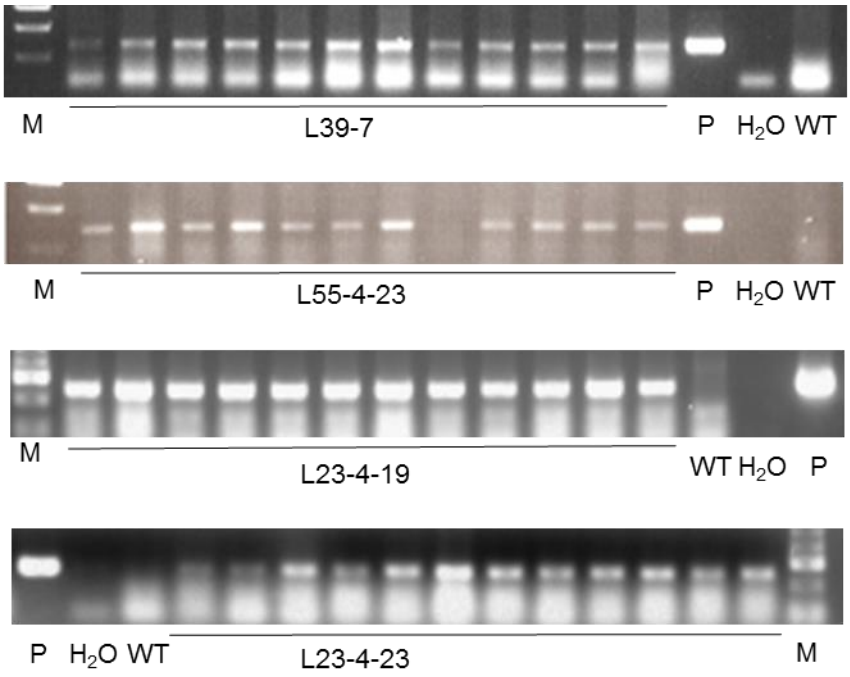

Fig. A3 Agarose gel (1 %) electrophoresis of PCR product of the transgene *TaHDZI-5* in T_2/T_3 homozygous sublines of transgenic wheat with promoter *TdCor39*. M—1kb DNA ladder*;* P—the plasmid with transgene band was used as positive control; WT—wildtype plants in which the transgene band was absent as negative control; H_2O— MiliQ water was used as negative control as well.

References (excluding Chapters 2, 4 and 5)

AGALOU, A., PURWANTOMO, S., ÖVERNÄS, E., JOHANNESSON, H., ZHU, X., ESTIATI, A., DE KAM, R. J., ENGSTRÖM, P., SLAMET-LOEDIN, I. H. & ZHU, Z. 2008. A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. *Plant molecular biology,* 66**,** 87-103.

AMAR, S. B., SAFI, H., AYADI, M., AZAZA, J., KHOUDI, H., MASMOUDI, K. & BRINI, F. 2013. Analysis of the promoter activity of a wheat dehydrin gene (DHN-5) under various stress conditions. *Australian Journal of Crop Science,* 7**,** 1875.

ARIEL, F. D., MANAVELLA, P. A., DEZAR, C. A. & CHAN, R. L. 2007. The true story of the HD-Zip family. *Trends in plant science,* 12**,** 419-426.

BARTON, K. A. & CHILTON, M. D. 1983. Agrobacterium Ti plasmids as vectors for plant genetic engineering. *Methods in Enzymology*, 101, 527-539.

BHATTACHARJEE, A., KHURANA, J. P. & JAIN, M. 2016. Characterization of Rice Homeobox Genes, OsHOX22 and OsHOX24, and Over-expression of OsHOX24 in Transgenic *Arabidopsis* Suggest Their Role in Abiotic Stress Response. *Frontiers in Plant Science*, 7.

BRINI, F., HANIN, M., LUMBRERAS, V., IRAR, S., PAGES, M. & MASMOUDI, K. 2007. Functional characterization of DHN-5, a dehydrin showing a differential phosphorylation pattern in two Tunisian durum wheat (*Triticum durum* Desf.) varieties with marked differences in salt and drought tolerance. *Plant Science,* 172**,** 20-28.

BRUCE, W. B., CHRISTENSEN, A. H., KLEIN, T., FROMM, M. & QUAIL, P. H. 1989. Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. *Proceedings of the National Academy of Sciences,* 86**,** 9692-9696.

CABELLO, J. V., ARCE, A. L. & CHAN, R. L. 2012. The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins. The Plant Journal, 69, 141-153.

CHANDRA BABU, R., ZHANG, J., BLUM, A., DAVID HO, T.-H., WU, R. & NGUYEN, H. 2004. HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. *Plant Science,* 166, 855-862.

CHINNUSAMY, V., OHTA, M., ANRAR, S., LEE, B.-H., HONG, X., AGARWAL, M. & ZHU, J.-K. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes & development,* 17, 1043-1054.

CHRISTENSEN, A. H. & QUAIL, P. H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research,* 5**,**213-218.

CHRISTENSEN, A. H., SHARROCK, R. A. & QUAIL, P. H. 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology,* 18**,** 675-689.

CORNEJO, M.J., LUTH, D., BLANKENSHIP, K. M., ANDERSON, O. D. & BLECHL, A. E. 1993. Activity of a maize ubiquitin promoter in transgenic rice. *Plant Molecular Biology,* 23**,** 567-581.

DEZAR, C. A., FEDRIGO, G. V. & CHAN, R. L. 2005. The promoter of the sunflower HD-Zip protein gene Hahb4 directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA. *Plant Science,* 169**,** 447-456.

DEZAR, C. A., GAGO, G. M., GONZÁLEZ, D. H. & CHAN, R. L. 2005. Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis* thaliana plants. *Transgenic research*, 14, 429-440.

EGAWA, C., KOBAYASHI, F., ISHIBASHI, M., NAKAMURA, T., NAKAMURA, C. & TAKUMI, S. 2006. Differential regulation of transcript accumulation and alternative splicing of a DREB2 homolog under abiotic stress conditions in common wheat. *Genes & Genetic Systems,* 81**,** 77-91.

FROMM, M., TAYLOR, L. P. & WALBOT, V. 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proceedings of the National Academy of Sciences*, 82, 5824-5828.

GUO, W., WARD, R. W. & THOMASHOW, M. F. 1992. Characterization of a cold-regulated wheat gene related to *Arabidopsis* cor47. *Plant Physiology,* 100**,** 915-922.

HARRIS, J. C., HRMOVA, M., LOPATO, S. & LANGRIDGE, P. 2011. Modulation of plant growth by HD-Zip class I and II transcription factors in response to environmental stimuli. *New Phytologist,* 190**,** 823-837.

HARRIS, J. C. 2014. The role of homeodomain-leucine zipper class I transcription factors in the drought response of wheat. University of Adelaide. Adelaide, SA.

HENRIKSSON, E., OLSSON, A. S., JOHANNESSON, H., JOHANSSON, H., HANSON, J., ENGSTRÖM, P. & SÖDERMAN, E. 2005. Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships. *Plant Physiology,* 139**,** 509-518.

JANSSEN, B.-J. & GARDNER, R. C. 1990. Localized transient expression of GUS in leaf discs following cocultivation with Agrobacterium. *Plant molecular biology,* 14, 61-72.

KASUGA, M., MIURA, S., SHINOZAKI, K. & YAMAGUCHI-SHINOZAKI, K. 2004. A combination of the *Arabidopsis* DREB1A gene and stress-inducible rd29A promoter improved drought-and low-temperature stress tolerance in tobacco by gene transfer. *Plant and Cell Physiology,* 45**,** 346-350.

KOBAYASHI, F., MAETA, E., TERASHIMA, A., KAWAURA, K., OGIHARA, Y. & TAKUMI, S. 2008. Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat. *Journal of Experimental Botany,* 59**,** 891-905.

KOVALCHUK, N., JIA, W., EINI, O., MORRAN, S., PYVOVARENKO, T., FLETCHER, S., BAZANOVA, N., HARRIS, J., BECK-OLDACH, K. & SHAVRUKOV, Y. 2013. Optimization of *TaDREB3* gene expression in transgenic barley using cold-inducible promoters. *Plant biotechnology journal*.

LEE, Y.H. & CHUN, J.Y. 1998. A new homeodomain-leucine zipper gene from *Arabidopsis* thaliana induced by water stress and abscisic acid treatment. *Plant molecular biology,* 37**,** 377-384.

LOPATO, S., BAZANOVA, N., MORRAN, S., MILLIGAN, A. S., SHIRLEY, N. & LANGRIDGE, P. 2006. Isolation of plant transcription factors using a modified yeast one-hybrid system. *Plant Methods,* 2**,** 3.

LOPATO, S. & LANGRIDGE, P. 2011. Engineering stress tolerance in cereals using DREB/CBF genes: outcomes, problems and perspectives. *ISB News Report*.

MARE, C., MAZZUCOTELLI, E., CROSATTI, C., FRANCIA, E. & CATTIVELLI, L. 2004. Hv-WRKY38: a new transcription factor involved in cold-and drought-response in barley. *Plant molecular biology,* 55**,** 399-416.

MCELROY, D. & BRETTELL, R. I. 1994. Foreign gene expression in transgenic cereals. *Trends in Biotechnology,* 12**,** 62-68.

MORRAN, S., EINI, O., PYVOVARENKO, T., PARENT, B., SINGH, R., ISMAGUL, A., ELIBY, S., SHIRLEY, N., LANGRIDGE, P. & LOPATO, S. 2011. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant biotechnology journal,* 9**,** 230-249.

MORIKAWA, H., IIDA, A. & YAMADA, Y. 1989. Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle-gun). *Applied microbiology and biotechnology,* 31, 320-322.

NAKAMURA, M., KATSUMATA, H., ABE, M., YABE, N., KOMEDA, Y., YAMAMOTO, K. T. & TAKAHASHI, T. 2006. Characterization of the class IV homeodomain-Leucine zipper gene family in *Arabidopsis*. *Plant Physiology,* 141**,** 1363-1375.

OHNO, R., TAKUMI, S. & NAKAMURA, C. 2001. Expression of a cold-responsive *Lt-Cor* gene and development of freezing tolerance during cold acclimation in wheat (*Triticum aestivum* L.). *Journal of experimental botany,* 52**,** 2367-2374.

RUAN, L., CHEN, L., CHEN, Y., HE, J., ZHANG, W., GAO, Z. & ZHANG, Y. 2012. Expression of *Arabidopsis* HOMEODOMAIN GLABROUS 11 Enhances Tolerance to Drought Stress in Transgenic Sweet Potato Plants. *Journal of Plant Biology,* 55**,** 151-158.

SAINT PIERRE, C., CROSSA, J. L., BONNETT, D., YAMAGUCHI-SHINOZAKI, K. & REYNOLDS, M. P. 2012. Phenotyping transgenic wheat for drought resistance. *Journal of Experimental Botany,* 63**,** 1799-1808.

SKRIVER, K. & MUNDY, J. 1990. Gene expression in response to abscisic acid and osmotic stress. *The Plant Cell,* 2**,** 503.

SÖDERMAN, E., MATTSSON, J. & ENGSTRÖM, P. 1996. The *Arabidopsis* homeobox gene ATHB-7 is induced by water deficit and by abscisic acid. The Plant Journal, 10, 375-381.

XIAO, B., HUANG, Y., TANG, N. & XIONG, L. 2007. Over-expression of a LEA gene in rice improves drought resistance under the field conditions. *Theoretical and Applied Genetics,* 115**,** 35-46.

YAMAGUCHI, S. K. & SHINOZAKI, K. 2001. Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Rice biotechnology: Improving yield, stress tolerance and grain quality,* 236**,** 176-189.

YAMAGUCHI, S. K. & SHINOZAKI, K. 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.,* 57**,** 781-803.

ZHANG, S., LI, N., GAO, F., YANG, A. & ZHANG, J. 2010. Over-expression of TsCBF1 gene confers improved drought tolerance in transgenic maize. *Molecular Breeding,* 26**,** 455-465.