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

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A thesis submitted to The University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

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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

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Date: 12/02/2018
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### Abbreviation

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


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, Morrán 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:

Statement of authorship

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>Transcriptional Network Involved in Drought Response and Adaptation in Cereals</th>
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| Publication Status | ![Published](^


**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.

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

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

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**Kovalchuk N**
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*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.*

Signed... ...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

http://dx.doi.org/10.5772/62336

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/CFB) [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 Ca²⁺ 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 heterodimerization 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 subunit of Mg-chelatase (CHLH/ABAR), which regulates the lipid metabolism linked to drought tolerance in plants [30].

![Figure 1](image-url)  
**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$_2$O$_2$), 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$_2$O$_2$ 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 AREF/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/CFB, 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.
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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/EREBP 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 barley 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-helices [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-I [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 Ile, 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 barley, 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 TAGCGT (A-box), GACGTC (C-box), CACGTT (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 HvzZIP 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 START-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, Oshox8, Oshox12-14, Oshox16, and Oshox20-25 [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 Osbox6, −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, TaHDZipl-1 and TaHDZipl-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.](image)

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/TTAACCC) 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 OsTIP 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 (CAGC) 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 ABI2 [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 ANAC019 [113, 114] were shown to form homodimers. 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 heterodimers [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, AtWRKY18, 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 adapt to abiotic stresses.

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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>
<thead>
<tr>
<th>Construct</th>
<th>ID of transgenic wheat</th>
<th>Generations</th>
<th>Number of independent homozygous lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUbi-TaHDZipI-5</td>
<td>GL167</td>
<td>T2/T3/T4</td>
<td>3</td>
</tr>
<tr>
<td>pWRKY71-TaHDZipI-5</td>
<td>GL245</td>
<td>T3/T4</td>
<td>3</td>
</tr>
<tr>
<td>pCor39-TaHDZipI-5</td>
<td>GL246</td>
<td>T3/T4</td>
<td>3</td>
</tr>
<tr>
<td>pHDZI-3-TaCBF5L</td>
<td>GL180</td>
<td>T2/T3/T4</td>
<td>3</td>
</tr>
<tr>
<td>pHDZI-4-TaCBF5L</td>
<td>GL181</td>
<td>T2/T3/T4</td>
<td>4</td>
</tr>
</tbody>
</table>

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 °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 H2O, total volume 1 L) was added into each tube, incubated at 65 °C for half an hour, and then kept in a 4 °C refrigerator for 15 minutes. When the tubes were cooled down to the room temperature, 300 μl of 6 M ammonium acetate (4 °C) was added into each tube, mixed vigorously with a vortex, and then kept at 4 °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>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Length of time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Denaturation</td>
<td>95</td>
<td>4 minutes</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95</td>
<td>30 seconds</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>55</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72</td>
<td>48 seconds</td>
</tr>
<tr>
<td>Cycling 39 times from step 2 to 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Final extension</td>
<td>72</td>
<td>1 minute</td>
</tr>
<tr>
<td>6. Cooling</td>
<td>15</td>
<td>Unlimited time</td>
</tr>
</tbody>
</table>

3.5 Homozygous sublines selection in transgenic wheat

3.5.1 T₀ generation transgenic wheat growth, sampling and harvest

Leaves of T₀ 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 T₁ progeny seeds were harvested and dried at 37 °C for one week, and then stored at 4 °C in darkness.

3.5.2 T₁ generation transformed sublines selection, growth, sampling and harvest

T₁ progeny seeds from T₀ 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 T2 progeny seeds were harvested and dried at 37 °C for one week, and then stored at 4 °C in darkness.

3.5.3 T2/T3 homozygous sublines selection

T2/T3 progeny seeds from three or more T1/T2 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 °C 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 °C.

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·3H₂O / 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 added 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 °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 °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\textsuperscript{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 °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 °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 °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 °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 °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, and 3.6 µl RNase free water. The mixture was kept at 65 °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™ III RT (200 U/µl) and 1.15 µl RNase free water were added into each tube. The mixture was at 50 °C for 1 hour for cDNA synthesis reaction, and the incubation was terminated at 70 °C for 15 minutes. Each cDNA sample was diluted for 20 times and stored at -20 °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>
<thead>
<tr>
<th>Housekeeping gene name</th>
<th>Primer type</th>
<th>Primer forward (5'-3')</th>
<th>PCR product length without introns (bp)</th>
<th>PCR product length with introns (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaCRT</td>
<td>Primer forward</td>
<td>GGCGGAAGGATCAGGTGCATGCAG</td>
<td>184</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>Primer reverse</td>
<td>GTTGTGCTACCGGAAGCGAAAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Name of Q-PCR component</th>
<th>volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer forward (5 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Primer reverse (5 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Kapa Sybr Fast Universal 2X qPCR Master Mix</td>
<td>5</td>
</tr>
<tr>
<td>(Geneworks)</td>
<td></td>
</tr>
<tr>
<td>50X ROX Low (Geneworks)</td>
<td>0.2</td>
</tr>
<tr>
<td>H2O</td>
<td>2</td>
</tr>
<tr>
<td>cDNA sample</td>
<td>2</td>
</tr>
<tr>
<td>Total volume</td>
<td>10</td>
</tr>
</tbody>
</table>

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™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 °C for 30 seconds and then added with 250 µl SOC media (0.5 % yeast extract, 2 % tryptone, 10 mM MgCl₂, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄ and 20 mM glucose). The tubes were incubated in Orbital mixer incubator (Ratek, Australia) in 37 °C 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 °C overnight (12–16 hours) and then stored at 4 °C 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 °C 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 °C. 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 °C for 1 hour and the enzyme was deactivated by 1 µl protein K solution at 37 °C 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 °C for 30 seconds and then added with 250 µl SOC media. The tubes were incubated in Orbital mixer incubator (Ratek, Australia) in 37 °C 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 °C overnight (12–16 hours) and then stored at 4 °C 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 °C 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 °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 °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 OSM media (½-strength Murashige-Skoog (MS) medium with 40 µL GuSO₄, 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°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:

Statement of Authorship

| Title of Paper | Overexpression of the class I homeodomain transcription factor TaHDZIP1-5 increases drought and frost tolerance in transgenic wheat |
| Publication Status | Published | Accepted for Publication |

**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 to 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 TaHDZIP1-5 dimerisations with the HDZIP cis-element and defined the foundation of the domain. |

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

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Summary
Characterization of the function of stress-related genes helps to understand the mechanism of plant responses to environmental conditions. The findings of this work defined the role of the wheat TaHDZipl-5 gene, encoding a stress-responsive homeodomain–leucine zipper class I (HDZIpl-5) transcription factor, during the development of plant tolerance to frost and drought. Strong induction of TaHDZipl-5 expression by low temperatures, and the elevated TaHDZipl-5 levels of expression in flowers and early-developing grains in the absence of stress, suggests that TaHDZipl-5 is involved in the regulation of frost tolerance at flowering. The TaHDZipl-5 protein behaved as an activator in a yeast transactivation assay, and the TaHDZipl-5 activation domain was localized to its C-terminus. The TaHDZipl-5 protein homo- and hetero-dimerizes with related TaHDZipl-3, and differences between DNA interactions in both dimers were specified at 3D-molecular levels. The constitutive overexpression of TaHDZipl-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 TaHDZipl-5 overexpression.

Keywords: 3D protein modelling, abiotic stress, activation domain, phenotypic features, protein homo- and hetero-dimerization, stress-inducible promoters.

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 AU$360 million in direct and indirect losses annually (GRDC National Frost Initiative, gco.gov.au/KW34). Thus, identification, characterization and application of candidate genes for the molecular breeding of crop acclimation to both frost and drought are of 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; Hvang 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 physiologic processes under stress, including cuticular wax biosynthesis (Alharimi et al., 2004; Bi et al., 2016, 2017; Borisjuk et al., 2014; Seo et al., 2011), stomatal closure (Ben 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.
involved in the same physiological processes, and therefore, this intervention draws the attention of plant biotechnologists (Agerwal et al., 2017; Gahlaut et al., 2016; Hrnova and Lopato, 2016).

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; Szlák 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; Patena and González, 1999; Szlák 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; Chen et al., 1998; Mukherjee and Bürgin, 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 CAATNATGG (Weijer 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; Hrnova and Lopato, 2016).

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 post-transcriptional levels (Bhattacherjee 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 AtHd1 and AtHd12 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 Hdh1 from sunflower was responsive to low temperatures (Cabello et al., 2012), while Hdh4 was activated by desiccation (Dezar et al., 2005a,b). Transcription of wheat TaHd2pi-2 was not influenced by ABA and was partially suppressed by low temperatures; however, the transcription activity of the TaHd2pi-2 protein was strongly increased by the addition of exogenous ABA (Kovalchuk et al., 2016). In contrast, TaHd2pi-4 and TaHd2pi-5 were activated by ABA on both transcriptional and post-transcriptional 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 (Bhattacherjee 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 (Bhattacherjee et al., 2016, 2017, Zhang et al., 2012). In contrast, overexpression of the similar ZmHd24 gene (HD-Zip I γ-clade) from maize in transgenic rice enhanced plant tolerance to drought, despite an increased sensitivity to ABA. ZmHd24-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 γ-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 TaHd2pi-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 TaHd2pi-1 to TaHd2pi-5, were isolated from wheat and partially characterized (Harris et al., 2016; Kovalchuk et al., 2016; Lopato et al., 2006). TaHd2pi-1 (γ-clade) expression was detected in seedlings and mature vegetative tissues, while TaHd2pi-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). TaHd2pi-3 was demonstrated to function as a regulator of plant growth, flowering time and frost tolerance (Kovalchuk et al., 2016). Overexpression of TaHd2pi-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 TaHd2pi-3 gene (γ-clade) was initially identified as a close homologue of AtHb7 and AOhb12 from Arabidopsis, and its induction of transcription by drought was demonstrated by Harris et al. (2016). In contrast to the homologous genes from Arabidopsis, TaHd2pi-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 TaHd2pi-4 and TaHd2pi-5. In contrast to TaHd2pi-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. TaHd2pi-5 was selected for further characterization because it was more strongly induced by cold and drought than the two...
other genes from the wheat HD-Zip I γ-clade (Harris et al., 2016). In this work, we studied the expression levels of TaHDZiP1-5 in a variety of wheat tissues, analysed the TaHDZiP1-5 transactivation properties and revealed the determinants of homo- and heterodimerized TaHDZiP1-5 and TaHDZiP3-3 in complex with a defined cis-element at the 3D molecular level. TaHDZiP1-5 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 TaHDZiP1-5 significantly improved plant tolerance to both stress; however, it negatively influenced plant growth and grain yields. Stress-inducible expression of TaHDZiP1-5 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 TaHDZiP1-5 (Table S2). The phylogenetic tree (Figure 1) shows that TaHDZiP1-5 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 TaHDZiP1-5 expression in a variety of unستressed bread wheat tissues

Expression of the endogenous TaHDZiP1-5 gene was analysed in a variety of tissues of unстressed wheat plants. TaHDZiP1-5 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 TaHDZiP1-5 were detected in coleoptiles; these were about 30-fold lower than those in the endosperm.

Functional cis-elements responsible for ABA-dependent TaHDZiP1-5 promoter activation

Promoter sequences of two homeologous genes, TdrHDZiP1-SA and TdrHDZiP1-SB, were isolated from a durum wheat BAC library (Cenci et al., 2003), because the respective broad wheat sequences were not yet available at the time when this work commenced. The comparison of durum wheat promoter sequences (TdrHDZiP1-SA and TdrHDZiP1-SB) with corresponding sequences from broad 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 TdrHDZiP1-5 promoter regions (PLACE software; Higo et al., 1999). TdrHDZiP1-SA 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 mx 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 TdrHDZiP1-SA 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 TaHDZiP1-5 activation domain using an in-yeast activation assay

Results of the in-yeast activation assay showed that the yeast strains carrying pG3K7+TaHDZiP1-5 grew well on SDX-Trp
Figure 2 Characterization of the TaHD2zip-5 gene. (a) Transcript numbers of the TaHD2zip-5 gene in wheat tissues were estimated by qRT-PCR. (b) Mapping of cis-elements responsible for the abscisic acid (ABA)-dependent activation of the TaHD2zip-5A promoter, using a transient expression assay in wheat cell culture. Depicted is a schematic representation of ABA-responsive element (ABRE) and MYB-responsive (MYBRI) cis-elements in four promoter deletions (D1-D4) of the TaHD2zip-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.

Figure 3 Identification of the TaHD2zip-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 positive homeodomain, leucine zipper and AD, respectively. (b) Conserved sequences of the identified AD in TaHD2zip-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.

Table 3 The position of the TaHD2zip-5 AD, different truncated variants of the TaHD2zip-5 protein were tested with the in-yeast activation assay. The empty pGBK7 vector was transformed into medium (confirming transformation) and the SD7-Trp-His medium containing 5 mM 3-AT (confirming the yeast HIS3 reporter gene activation by a plant activation domain-AD) (Figure 3a). To

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yeast and used as a negative control. After 4 days of cultivation, the yeast carrying the 26–255 residue fragment grew well on SDT-Tip-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 TaHDZip1-5 homologues from other monocot plants (Figure 3b).

Based on molecular model predictions of TaHDZip1-5, homo-dimerization and hetero-dimerization influence the DNA binding specificity.

Harris et al. (2016) have recently shown that the expression levels of TaHDZip1-4 and TaHDZip1-5 increased under cyclic drought conditions, while those of TaHDZip1-3 remained low. These authors proposed a model explaining how TaHDZip1-3 binds to DNA cis-elements in a homo-dimeric form and that a hetero-

![Figure 4](image_url)

**Figure 4** Molecular features of homeodomains (HDs) of TaHDZip1-3 and TaHDZip1-5 in homo- and hetero-dimeric forms in complex with the HDZ1 cis-element. (a) A sequence alignment of TaHDZip1-3 and TaHDZip1-5 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 TaHDZip1-3 and TaHDZip1-5, and hetero-dimeric TaHDZip1-3/TaHDZip1-5 models in complex with the HDZ1 cis-element; blue descriptions and atomic colour representations are used for HDZ1. The ribbons of TaHDZip1-3 and TaHDZip1-5 are coloured in blue and yellow, respectively. DNA-interacting residues are shown in sticks, and DNA sugar-phosphate backbones are coloured in pink-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 TaHDZip1-3 and TaHDZip1-5.

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dimeric form of TaHDZipl-4 and TaHDZipl-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 TaHDZipl-3 and TaHDZipl-5, and of hetero-dimeric TaHDZipl-3/TaHDZipl-5, in complex with a HD21 cis-element to seek whether DNA interactions differed between homo- and hetero-dimeric structural models.

Structural bioinformatic comparisons of wheat TaHDZipl-3 and TaHDZipl-5 proteins showed the presence of HD domains with well-defined boundaries. An alignment of the template (PDB: 1KGG) used for structural modelling and TaHDZipl-3 and TaHDZipl-5 indicated that there was not a high level of sequence identity between the investigated proteins (Figure 4, top panel). HDs of TaHDZipl-3 shared respective 26% identity and 53% similarity to the template (PDB: 1KGG), while TaHDZipl-3 and TaHDZipl-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 TaHDZipl-3 and TaHDZipl-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 (TaHDZipl-3/TaHDZipl-3 or TaHDZipl-5/TaHDZipl-5) and hetero-dimeric (TaHDZipl-3/TaHDZipl-5) HD models in complex with HD21 (5′-CAATCATTCG-3′/ 5′-CAATCATTCG-3′), interacting nucleobases are underlined (Soll and Blandalt, 1993) were constructed to understand the differences in binding of DNA. The structural models of HDs of TaHDZipl-5 showed the presence of three α-helices, interconnected with loops similarly to TaHDZipl-3 (Figure 4, bottom panel); however, minor structural differences were observed between TaHDZipl-3 and TaHDZipl-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 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 TaHDZipl-5 at the N-terminus of the first HD monomer bound directly to nucleobases T7 and G16, while Gin105 and Asn106 contacted the T8 nucleobase indirectly through a water molecule (Figure 4, bottom panel). In addition, Asn106 in TaHDZipl-5, which corresponds to Asn73 in TaHDZipl-3, bound to the nucleobase A3. Further, the analyses of HD21-binding modes in TaHDZipl-3/TaHDZipl-5 showed that two positively charged residues of the TaHDZipl-3 monomer at the N-terminus bound to the nucleobases T7 and T15 via Arg26 and to the nucleobase G16 through Lys27. Additionally, Gin73 at the major groove α-helix bound to the nucleobase T8, while Gin72 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 TaHDZipl-5 HD monomer with HD21 were similar to those of the second HD monomer in the homo-dimeric form.

A detailed analysis of hydrogen bond patterns in homo-dimeric (TaHDZipl-3/TaHDZipl-3) and hetero-dimeric (TaHDZipl-3/TaHDZipl-5) DNA complexes (Table S3) showed that three nucleobases (A2, T4 and T8) were bound to Asn73 (participating through both monomers) and Gin72 (Hams et al., 2016). Besides, four nucleobases (A3, T7, T8 and G16) were bound to Arg58, Gin105 and Asn106 (participating through both monomers) in homo-dimeric TaHDZipl-5. The analysis of interactions in hetero-dimeric TaHDZipl-3/TaHDZipl-5 showed the participation of seven nucleobases (A2, A3, T7, T8, G11, T15 and G16) that were contacted through Arg26, Lys27, Gin72, Asn73 of TaHDZipl-3 and through Asn106 of TaHDZipl-5; Arg26 and Gin72 residues formed bidentate interactions with DNA. Free energies of homo-dimeric TaHDZipl-5 (290 kcal/mol) and hetero-dimeric TaHDZipl-3/TaHDZipl-5 (244 kcal/mol), calculated through FoldX (Schymkowitz et al., 2005), indicated that hetero-dimeric DNA interactions in TaHDZipl-5 was more stable than its TaHDZipl-5 homo-dimeric form.

Evaluation of transgenic weat plants constitutively expressing TaHDZipl-5

Initially, transgenic wheat plants (cv. Gladius) were generated using a construct where expression of the transgene TaHDZipl-5 was driven by a constitutive maize polyubiquitin promoter. Three independent transgenic T1, 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, T1 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 T2 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 T2 lines, L1-3-9, L2-7-9 and L4-8-9 (pUb1-TaHDZipl-5), were identified to be homozygous for the transgene. The phenotypic data of transgenic wheat lines were compared to those of WT plants (Figure S5). The data obtained from the well-watered bin correlated well with those obtained for T1 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 S5). 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 S5).

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

Three-week-old transgenic wheat seedlings (T2, progenies of sublines L2-7 and L4-8 transformed with pUB1-TaHDZipl-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 the 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).
Transgenic wheat seedlings tolerate frost better than WT plants

Three-week-old T1 (Figure 6b) and T2 (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 5d) based on the earlier established protocol for barley (Kovačchuk et al., 2013). This included a 6.5-h exposure to −7 °C for the T1 generation and the same exposure time to −8 °C for T2 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).

Evolution of transgenic wheat plants with stress-inducible expression of TaHDZipl-5

With the aim to avoid or minimize the influence of TaHDZipl-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 TaCor39 genes. These stress-inducible promoters were previously employed to avoid the negative influences of TaDREB3 gene expression on phenotype and yield in transgenic barley (Kovačchuk 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 pUb1-TaHDZipl-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 TaHDZipl-5 cDNA was isolated in a Y2H screen using TaHDZipl-5 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). TaHDZipl-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 superfamily and conserved intron-exon structure, the TaHDZipl-5 gene was identified as a monocot-specific member of the γ-clade (Harris et al., 2016). The closest homologues of TaHDZipl-5 from rice and maize, OsXox22 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 Osxox22 and another monocot-specific γ-clade member from rice, Osxox24, leads to
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, ZmHdZ1, positively regulated plant responses to stress in transgenic Arabidopsis and conferred tolerance to drought in transgenic rice (Wu et al., 2016).

Regulation of Hd-1 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 a clade HD-Zip class I TEs conferred cold/frost tolerance to transgenic Arabidopsis and barley (Cabello et al., 2012; Kovachchuk et al., 2016), however, the influence on the overexpression of monoclonal-specific γ-class members on cold or frost tolerance of transgenic plants remains to be determined.

The molecular characterization of the TaHdZip-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 TaHdZip-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/spo/climate/averages/frost/index.jsp).

The expression of TaHdZip-5 in wheat 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 TaHdZip-5 transcripts in vegetative tissues was low (Figure 3a). Expression of TaHdZip-5 in flowers shortly before fertilization, and during the early stages of grain development, and the strong induction of TaHdZip-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 TaHdZip-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 TaHdZip-5 were not available in databases, we analysed the TaHdZip-5A and TaHdZip-5B...
promoters of homeologous genes from durum wheat. Firstly, several concentrations of ABA were tested to select the minimal endogenous concentration (0.5 μM) leading to a strong promoter activation (data not shown). Secondly, we analysed the 1055-bp sequence of the TaHDZipl-5A promoter (including 5'UTR), which could activate ABA-dependent TFs. Mapping revealed that the promoter was activated through the proximal MYB element GGATA (−310 bp from the translation start), which was identified by Baranovskiy et al. (1994) as the binding site for MYBS1, a MYB-like protein with an endosperm-related function (Mercy et al., 2003). Additionally, the activity of the TaHDZipl-5A promoter was enhanced by two ABREs and/or one MYB element, situated upstream of the proximal MYB element (Figures 2b and S3). All mapped cis-elements were found in the promoters of both homeologous durum wheat 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 TaHDZipl-5 acted as a transcriptional activator. In this work, the transactivation TaHDZipl-5 domain was defined in an in-yeast activation assay. A series of CDS deletions encoding truncated variants of TaHDZipl-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), TaHDZipl-5 was found to contain an AD at the C-terminal region of the protein (Figure 3a), which is a highly
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- and hetero-dimerize and also interact with other members through hetero-dimerization. We revealed that TaHDZipl-5 displayed an equal propensity to form homo-dimeric TaHDZipl-5 and hetero-dimeric TaHDZipl-3-TaHDZipl-5 complexes. However, the DNA interaction differences between TaHDZipl-5 homo- and hetero-dimers remained unclear. Hence, we constructed the 3D models of homo-dimers of TaHDZipl-5 or TaHDZipl-3 and hetero-dimeric TaHDZipl-3-TaHDZipl-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 TaHDZipl-3-TaHDZipl-5 hetero-dimer were more stable than those in the TaHDZipl-5 homo-dimer. These models suggested that the TaHDZipl-3/TaHDZipl-5 hetero-dimer is more efficient in binding DNA. This may indicate that the TaHDZipl-3/TaHDZipl-5 hetero-dimer could be more efficient also in vivo, during the activation of target promoters, than the TaHDZipl-5 homo-dimer.

Initially, we overexpressed the TaHDZipl-5 gene in transgenic wheat plants using a constitutive polyubiquitin promoter from maize. Homozygous T1 or T2 sublines were selected in a pilot experiment using T2 and T3 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 TaHDZipl-5 transgene negatively influenced plant phenotypes by

Figure 8 Characteristics of transgenic wheat transformed with pCor39-TaHDZipl-5. (a) Comparison of plant growth and yield characteristics of wild-type (WT) and transgenic T2 plants, under well-watered conditions (black boxes) and mild drought (grey boxes). (b) Transgene expression levels in WT and transgenic T2 plants at 24 °C (control) and 4 °C (cold). (c) Frost tolerance of WT and transgenic wheat transformed with pCor39-TaHDZipl-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).
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 S5). Differences in flowering time resulted in 1- to 3-day delays in T$_2$ sublines compared to the average flowering times of control plants. The differences in flowering times of selected T$_2$ plants amounted up to 2 weeks (Figure S6), but these differences decreased in two subsequent generations of transgenic lines. In contrast, the growth and yield characteristics in the T$_3$ 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$_2$ 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 S5a). 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 Zmhd24, overexpressed in transgenic Arabidopsis and rice (Wu et al., 2016). For these discrepancies, we offer the following explanation. Both Zmhd24 and TaHDZip1-5 originate from plants which have different physiological responses to drought compared to rice. Therefore, Zmhd24 and TaHDZip1-5 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.

Abiotic stress 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$_2$ 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 TaHDZip1-5 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 TaHDZip1-5 transgene revealed the up-regulation of TaCOR148 (GenBank: AF207546) and TaABF15 (GenBank: X591133) transcripts in several transgenic lines (Figure S7). However, this up-regulation did not correlate with the levels of TaHDZip1-5 transgene transcripts (Figure 6d).

Constitutive overexpression of TaHDZip1-5 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 phenopd
Constructs were transformed in the Australian elite bread wheat cv. Glatius 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 Shvyrkov et al. (2010). Transgene integration was confirmed by PCR using a forward primer from the 3′ end of the TaH502glp-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 T0 and/or T1 progenies of selected transgenic lines by quantitative real-time PCR (Q-PCR), based on the 2^−ΔΔCT method (Kovalchuk et al., 2013; Li et al., 2004). Alox terminator primers and a specific DNA probe (Yadav et al., 2015) were used for transgene amplification, and endogenous Purinol-doline-b (Pm-b) gene primers and probe (Li et al., 2004; Yadav et al., 2015) were used for template loading normalization (Table S1). T0 lines with a single copy number were selected for further analysis. The selection of homozygous T1 lines were conducted in pilot experiments using the T2 progeny of transgen T1 lines with two copies of transgene or T2 progeny of T2 lines. In these experiments, transgene integration was assessed by PCR in twelve seedlings of each line. The line was considered homogenous if the expected PCR product was observed for all twelve plants. Seeds of homozygous T1 (or T2) 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 ABAP-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.

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Enhancement of wheat frost tolerance using the HD-Zip II factor


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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 TaHDZip-5A and TaHDZip-5B promoters 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 TaHDZip-5A (5A) and TaHDZip-5B (5B) promoters. LAUGN (Huang and Millor, 1991) was used to find the best local alignments.

Figure S5 (a) Transgenic copy numbers in T1 transgenic plants estimated by Q-PCR. Plants seeds used in analyses are indicated by arrows. (b) Examples of selection of homoygous lines by PCR using transgene-specific primers.

Figure S6 Growth characteristics and yield components of control wild-type (WT) and transgenic T1 wheat (Triticum aestivum cv. Claudio) plants transformed with pBi31-TaHDZip-5.

Figure S7 Expression levels of three stress-inducible LEA (Late Embryogenesis Abundant)/COR (cold-responsive)/DRE (dehydrin) genes (TaWzy2, GenBank: EU395844; TaCOR148, GenBank: AF207546; TaRab15, GenBank: X591333) and the TaDREB3 (GenBank: DQ353835) regulatory gene, in leaves of unstressed control WT plants and T3 sublines of tree independent transgenic lines.

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 y-clade TIs from Arabidopsis and selected monocots, shown in Figure 1.

Table S3 Hydrogen bonds of homo-dimeric TaHDZip-3 and TaHDZip-5, and hetero-dimeric TaHDZip-3/TaHDZip-5 with HDZ1 (5’-CCAATCATGTC-3’/5’-GCAATGATG-3’).

Table S4 Characteristics of the T1/T2 progenies of TaHDZip-5 transgenic lines analysed in large containers under well-watered or mild drought condition.

Appendix S1 Materials and methods.

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

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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 determine the transgene expression level, Q-PCR was performed with primers of the target gene *TaHDZipl-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 *TdhHDZipl-5A* and *TdhHDZipl-5B* promoters and the identification of abscisic acid (ABA)-responsive cis-elements**

A fragment of the coding region of *TaHDZipl-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 *TaHDZipl-5* gene were identified by PCR using DNA of selected BAC clones as templates, and primers were derived from the coding region of *TaHDZipl-5* cDNA. Genes of the *T. turgidum* ssp *durum*, orthologues of *TaHDZipl-5*, are designated as *TdhHDZipl-5A* and *TdhHDZipl-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 *TdhHDZipl-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 pGBKKT7 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 heterodimeric 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'-CAATCATGTC-3'/5'-GCAAATGATTG-3') was constructed from the AT-rich cis-element (5'-TAATTGAGATT-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 T₁ 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 T₁ 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 T₃ 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 T₃ 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 T₃ 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, Tuttingen, 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 TaHDZipl-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 T₃ sublines of each of three independent transgenic lines with the constitutive overexpression of TaHDZipl-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 TdHDZipl-5A and TdHDZipl-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 TdHDZipl-5B (5B) and TdHDZipl-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 T1 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 T1 sublines of two independent single-transformation-event lines were selected using the analysis of the transgene (TahHDZipl-5) presence in the T2 progeny. H2O - 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<sub>1</sub> 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<sub>3</sub> 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 TaHDZipl-3 and TaHDZipl-5, and hetero-dimeric TaHDZipl-3/TaHDZipl-5 with HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3').

Table S4. Characteristics of the T2/T3 progenies of TaHDZipl-5 transgenic lines analysed in large containers under well-watered or mild drought condition.

Supplementary references


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Fig. S5. (a) Transgene copy numbers in T1 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 T1 sublines of two independent single-transformation-event lines were selected using the analysis of the transgene (TalHIDZipL-5) presence in the T2 progeny. H2O - 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.
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Table S1. List of PCR primers and DNA probes used in this study.

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<th>Short name</th>
<th>Purpose</th>
<th>Designation</th>
<th>Nucleotide sequence (5’–3’)</th>
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Table S3. Hydrogen bonds in homo-dimeric TaHDZIpl-3 and TaHDZIpl-5, and hetero-dimeric TaHDZIpl-3/TaHDZIpl-5 with HDZ1 (5'-CAATCATTGC-3'/5'-GCAATGATTG-3').

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Table S4. Characteristics of the T\textsubscript{2}/T\textsubscript{3} progenies of *TaHDZipI-5* transgenic lines analysed in large containers under well-watered or mild drought condition.

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

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<tr>
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<td>Overall percentage (%)</td>
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<td>Certification:</td>
<td>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.</td>
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## 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.

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

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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 messengers 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 HindIII–KpnII 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 T₀ and T₁ 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−ΔΔCt 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 transcribed 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 T₂ 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 \( \alpha^{32}\text{P} \) dCTP was labelled using a random primer labelling method (Cho et al., 2003). Purified RNA was denatured and separated on an agarose gel with 6% (v/v) formaldehyde, transferred into a nylon membrane 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 °C for 16 hours and a night temperature of 19 °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 °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 T₃ transgenic sublines and WT plants grown under severe drought conditions
Experiments were conducted in a growth chamber with 25/19 °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 T4 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 °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 °C) to -8 °C, and remained at -8 °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 °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/RPAGRxKFxEKHP) 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 T2 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 T4 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 those controlled by the HDZI-3 promoter in most transgenic plants (Fig. 3A and B).

**Phenotypic evaluation of T₃ transgenic wheat with pHDZI-3 or pHDZI-4 promoters under moderate drought and well-watered treatments during the flowering stage**

Four sublines of T₃ 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 T₄ 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 shown). 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 T4 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 T4 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 °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 conclusion, 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 PKKPAAGR 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--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


**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 monococcum*; 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 “♦”.

**Fig. 2** Wheat *HDZI*-3 (A) and *HDZI*-4 (B) promoters induced in three-week-old control and TaCBF5L transgenic T₂ wheat seedling leaves before and after six hours of dehydration. N: WT plants with endogenous TaCBF5L gene only show weak band under well-watered and dehydration conditions, and were used as negative control; P: Transgenic wheat plants with TaCBF5L transgene showing strong band under dehydration were used as positive control; W: Well-watered; D: Drought.

**Fig. 3** Transgene TaCBF5L expression controlled by the promoter *HDZI*-3 (A) and the promoter *HDZI*-4 (B) under different drought stages: well-watered condition (leaf water potential 12–15 bar), leave wilting point (leaf water potential 15–20 bar), moderate drought (leaf water potential 20–30 bar) and drought condition (leaf water potential >40 bar). The error bars represent ±SD of three technical replicates.

**Fig. 4** Growth characteristics and yield components of control wild-type (WT) and transgenic wheat (*Triticum aestivum* cv. Gladius) transformed with pHDZI-3-TaCBF5L (A), and pHDZI-4-TaCBF5L (B) under well-watered (black boxes) and moderate drought (grey boxes) conditions. Flowering time of transgenic plants was compared with the average flowering time of 16 control WT plants, which is represented as day 0. Values represent means ±SE (n varies for each column and is shown in each case directly on the graphs) at ‘∗’ P < 0.05, ‘∗∗’ for P < 0.01 and ‘∗∗∗’ for P < 0.001, which were calculated by the Student’s t-test (unpaired, two-tailed).

**Fig. 5** Growth characteristics and yield components of control wild-type (WT) and transgenic wheat (*Triticum aestivum* cv. Gladius) transformed with pHDZI-3-TaCBF5L (A), and pHDZI-4-TaCBF5L (B) under severe drought. Flowering time of transgenic plants was compared with the average flowering time of 12 control WT plants, which is represented as day 0. Values represent means ±SE (n varies for each column and is shown in each case directly on the graphs) at ‘∗’ P < 0.05, ‘∗∗’ for P < 0.01 and ‘∗∗∗’ for P < 0.001, which were calculated by the Student’s t-test (unpaired, two-tailed).
**Fig. 6** Survival rate of transgenic plants with pH Dzi-3-TaCBF5L (A) and pH Dzi-4-TaCBF5L (B) subjected to frost and compared with WT plants. Error bars represent ±SD for three technical replicates. Differences between transgenic sublines and WT plants were tested in the unpaired Student’s t-test (‘*’ P < 0.05).

**Fig. 7** Transgene TaCBF5L expression controlled by the HDZI-3 (A) and HDZI-4 (B) promoters under well-watered condition (control) and cold treatment of 4 °C. The error bars represent ±SD of three technical replicates.

**Fig. 8** Expression of the TaCBF5L transgene and stress-inducible LEA/CORD/HN genes in transgenic wheat plants with inducible over-expression of TaCBF5L controlled by HDZI-3 or HDZI-4 promoters. (A) Expression of the transgene TaCBF5L under well-watered condition with leaf water potential 12–15 bar and severe drought with leaf water potential over 40 bar. (B) Up- or down-regulation of stress responsive genes in transgenic plants expressed as fold up- or down-regulation by severe drought relative to well-watered and normalised WT plants.

**Fig. 9** Expression of the TaCBF5L transgene and stress-inducible LEA/CORD/HN genes in transgenic wheat plants with over-expression of TaCBF5L controlled by HDZI-3 or HDZI-4 promoters (A). Expression of the transgene TaCBF5L under room temperature (control) and cold treatment at 4 °C; (B) Down-regulation of stress responsive genes in transgenic plants expressed as a fold down-regulation by cold, relative to room temperature conditions and normalised against WT plants.

**Legends to Supplementary Figures**

**Fig. S1** A copy number of the TaCBF5L transgene using Q-PCR in T1 transgenic wheat. Arrows indicate selected sublines.

**Fig. S2** 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. The arrow (marked as stop watering) indicates the point at which watering was withdrawn. Flowering period is marked by two vertical dashed lines.

**Fig S3** Details of drought tolerance experiments. (A) Position of transgenic plants with the HDZI-3 promoter and WT plants in a pot. (B) Position of transgenic plants with the HDZI-4 promoter and WT plants in a pot. (C) Soil moisture content at a field capacity (%) in a pot is shown under different days of drought. Leaf samples with stress-inducible promoters were
collected under four different stages, and the sampling time points are marked with arrows. C1: well-watered (12–15 bar); C2: leaf wilting point (15–20 bar); C3: moderate drought (20–30 bar); C4: severe drought (> 40 bar).

**Fig. S4** Pot soil water content for plant flowering under severe drought. (A) Position of transgenic plants with the *HDZI*-3 promoter and WT plants in a pot. (B) Soil moisture content at a field capacity (%) in a pot was shown under different days of drought. The arrow (marked no watering) shows the point at which watering was withdrawn. The two vertical dashed lines indicate the period of flowering date.

**Fig. S5** Pot soil water content for plant flowering under severe drought. (A) Position of transgenic plants with the *HDZI*-4 promoter and WT plants in a pot. (B) Soil moisture content at a field capacity (%) in a pot is shown under different days of drought. The arrow (marked no watering) shows the point at which watering was withdrawn. The two vertical dashed lines indicate the period of flowering date.

**Fig. S6** 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.

**Fig. S7** Multiple sequence alignment of TaCBF5L and six homologous proteins with a close evolutionary relationship to TaCBF5, than other homologues from wheat, maize, rice, barley and *Arabidopsis*. AP2 DNA-binding domains, PKKR/PAGR and LWSY motifs are marked with rectangles.

**Legends to Supplementary Tables**

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

**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.
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Fig. 2 Wheat *HDZI-3* (A) and *HDZI-4* (B) promoters induced in three-week-old control and *TaCBF5L* transgenic T2 wheat seedling leaves before and after six hours of dehydration. N: WT plants with endogenous *TaCBF5L* gene only show weak band under well-watered and dehydration conditions, and were used as negative control; P: Transgenic wheat plants with *TaCBF5L* transgene showing strong band under dehydration were used as positive control; W: Well-watered; D: Drought.
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Fig. 5 Growth characteristics and yield components of control wild-type (WT) and transgenic wheat (*Triticum aestivum* cv. Gladius) transformed with pHDZI-3-TaCBF5L (A), and pHDZI-4-TaCBF5L (B) under severe drought. Flowering time of transgenic plants was compared with the average flowering time of 12 control WT plants, which is represented as day 0. Values represent means ±SE (*n* varies for each column and is shown in each case directly on the graphs) at ‘*’ *P* < 0.05, ‘**’ for *P* < 0.01 and ‘***’ for *P* < 0.001, which were calculated by the Student’s *t*-test (unpaired, two-tailed).
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Supplementary figures and tables

**Fig. S1** Copy number of the *TaCBF5L* transgene using Q-PCR in T<sub>1</sub> transgenic wheat. Arrows indicate selected sublines.
**Fig. S2** 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. The arrow (marked as stop watering) indicates the point at which watering was withdrawn. Flowering period is marked by two vertical dashed lines.
**Fig. S3** Details of drought tolerance experiments. (A) Position of transgenic plants with the $\textit{HDZI}$-3 promoter and WT plants in a pot. (B) Position of transgenic plants with the $\textit{HDZI}$-4 promoter and WT plants in a pot. (C) Soil moisture content at a field capacity (%) in a pot is shown under different days of drought. Leaf samples with stress-inducible promoters were collected under four different stages, and the sampling time points are marked with arrows. C1: well-watered (12–15 bar); C2: leaf wilting point (15–20 bar); C3: moderate drought (20–30 bar); C4: severe drought (> 40 bar).
Fig. S4 Pot soil water content for plant flowering under severe drought. (A) Position of transgenic plants with the *HDZI*-3 promoter and WT plants in a pot. (B) Soil moisture content at a field capacity (%) in a pot was shown under different days of drought. The arrow (marked no watering) shows the point at which watering was withdrawn. The two vertical dashed lines indicate the period of flowering date.
**Fig. S5** Pot soil water content for plant flowering under severe drought. (A) Position of transgenic plants with the *HDZI-4* promoter and WT plants in a pot. (B) Soil moisture content at a field capacity (%) in a pot is shown under different days of drought. The arrow (marked no watering) shows the point at which watering was withdrawn. The two vertical dashed lines indicate the period of flowering date.
**Fig. S6** 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.
Fig. S7 Multiple sequence alignment of TaCBF5L and six homologous proteins with a close evolutionary relationship to TaCBF5, than other homologues from wheat, maize, rice, barley and Arabidopsis. AP2 DNA-binding domains, PKKR/PAGR and LWSY motifs are marked with rectangles.
Table S1. The list of PCR primers, Q-PCR primers and Northern hybridisation primers and probes used in this study.

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<th>Purpose</th>
<th>Designation</th>
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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.

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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 TaHDZip1-5 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 TaHDZip1-5 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 TaHDZip1-5 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 TdHDZip1-5A, as a homolog of TaHDZip1-5, using transient expression assay, helped to indicate what potential TF might participate in upregulation of the gene TdHDZip1-5.

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
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$_2$O—MiliQ water was used as negative control as well.
Fig. A2 Agarose gel (1 %) electrophoresis of PCR product of the transgene TaHDZI-5 in T₃ 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; H₂O—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$_2$O—MiliQ water was used as negative control as well.
References (excluding Chapters 2, 4 and 5)


