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Abiotic stress upregulated TaZFP34 represses the expression of type-B response regulator and *SHY2* genes and enhances root to shoot ratio in wheat

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Abstract

Q-type C_2H_2 zinc finger proteins (ZFPs) are plant-specific DNA-binding proteins containing a conserved QALGGH motif. This study investigated the function of abiotic stress-inducible and predominantly root-expressed *Triticum aestivum* ZFPs (TaZFP22, TaZFP34 and TaZFP46) with a focus on TaZFP34. Expression of *TaZFP34* in roots was upregulated by high salinity, dehydration, oxidative and cold stresses. Overexpression of *TaZFP34* in wheat roots resulted in an increased root-to-shoot ratio, a phenomenon observed during plant adaptation to drying soil. Expression of a number of genes which are potentially involved in modulating root growth was significantly altered in the roots of *TaZFP34* overexpressing lines. In particular, the transcript levels of *TaRR12B*, *TaRR12D* and *TaSHY2* that are homologues of known negative regulators of root growth were significantly reduced. Expression of shoot growth-related genes, such as *GA3-ox* and *expansins,* was downregulated in the transgenic shoots. TaZFP34 bound to (C/G)AGT(G/A)-like elements in the promoters of TaZFP34 down-regulated *TaRR12D* and *TaSHY2* and transrepressed the reporter gene expression driven by *TaRR12D* and *TaSHY2* promoters. Expression of the above reporter genes was also repressed by TaZFP46 and TaZFP22. These data suggest that TaZFP34 is a transcriptional repressor and is involved in modulating the root-to-shoot ratio.

Key words: C_2H_2 zinc finger proteins, drought stress, root-specific expression, root and shoot growth, transcriptional repressor, wheat.

Abbreviations: ABA, abscisic acid; EAR, ERF-associated amphiphilic repression; GA, gibberellic acid; GFP, green fluorescence protein; PEG, polyethylene glycol; RFP, red fluorescence protein; RR, response regulator; ZFP, zinc finger protein.

1. Introduction

C_2H_2 zinc finger proteins (ZFPs) are characterised by the presence of two cysteines and two histidines in a zinc finger domain. Many ZFPs in plants contain a highly conserved QALGGH motif within the zinc finger domain [1-3], and these form a plant-specific Q-type C_2H_2 subfamily. A large number of Q-type TaZFP genes in wheat have been shown to be upregulated or downregulated in leaves and roots during drought stress and other abiotic stresses [3-4]. Similarly, many drought-responsive ZFP genes have been reported in other plant species, such as Arabidopsis, rice and petunia [1,5-9]. These findings indicate that ZFPs are involved in plant response to drought stress by regulating their target genes and potentially play an important role in plant drought adaptation. This idea has been pursued using a transgenic approach. For example, transgenic Arabidopsis plants overexpressing drought or dehydration inducible ZFP genes (e.g., Arabidopsis *AZF2*, *STZ*, *ZAT10* and *ZAT12*, and rice *ZFP36, ZFP179, ZFP182, ZFP245* and *ZFP252*) showed improvement in salt, dehydration or drought tolerance [2,5-12]. Improvement of abiotic stress tolerance by overexpression of a Q-type C_2H_2 gene has also been reported in other species such as petunia [1] and tomato [13,14].

Interestingly, the majority of drought upregulated ZFP genes in wheat are predominantly expressed in roots [3]. Root is an important organ for plant adaptation to drought stress. It has been shown in several plant species that root growth response to water deficit differs from shoot in that primary root elongation is essentially maintained in drying soil or under low water potential conditions (mild water deficit) when shoot elongation is markedly reduced [15-17]. Under severe dehydration stress when shoot growth is severely or completely inhibited, the primary roots are able to continue their elongation, though at a reduced rate [15]. This leads to an increased root-to-shoot ratio and helps to maintain plant water balance. An increase of over 50% in the ratio of root-to-shoot length under moderate and severe dehydration stress has also been documented in wheat [18]. However, molecular players modulating this differential response remain elusive. It is known that the elevation of abscisic acid (ABA) concentration at low water potentials is responsible for maintaining primary root elongation [15,17]. The exogenous application of ABA at a low concentration (0.1 μM) also promotes root elongation, although a high-level ABA application inhibits root elongation [19]. ABA is known to have a severe inhibitory effect on shoot growth [20] and therefore plays a central role in modifying plant architecture during drought stress by its differential regulation of root and shoot growth. The majority of drought upregulated genes

are known to be regulated by ABA. However, it is not clear at present how the ABA regulatory network interacts with root growth regulatory pathways.

ZFPs regulate gene expression through their sequence-specific binding to target genes. The DNA binding specificity of ZFPs is determined mainly by the amino acid sequence at the α-helix of the C_2H_2 domain. The QALGGH motif in the Q-type ZFPs is located in the N-terminus of the α-helix and is involved in determining the DNA-binding specificity of petunia ZPT2-2 [21]. Relatively little is known about the DNA-binding specificity of plant ZFPs, compared with mammalian ZFPs. The first reported DNA-binding sequence of plant ZFPs was the EP1 motif 5'-TTGACAGTGTCAC, present in the promoter of petunia *5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE*, recognised by a 2-fingered Q-type ZFP (EPF1/ZPT2-1) from petunia [22]. The AGT of the EP1 motif is the core sequence interacting with petunia EPF1/ZPT2-1 [23]. In most previous studies, the *in vitro* DNA-binding assay was performed using an artificial tetramer of EP1. A number of other 2-fingered Q-type ZFPs have been shown to be capable of binding to this artificial tetramer of EP1 *in vitro*, such as petunia EPF2-4 and EPF2-5 [23], petunia ZPT2-2 [21], Arabidopsis AZF and STZ [2]. These studies suggest that the DNA-binding specificity among Q-type ZFPs is likely to be similar due to the conservation of the QALGGH motif. The DNA-binding sequences of Q-type ZFPs from wheat are unknown to date, except for WZF1. Sakamoto et al. [24] showed that WZF1 was able to bind a CACTC (or GAGTG in reverse complementary) motif. However, the DNA-binding sequence specificity of WZF1 was not analysed in detail.

A number of abiotic stress upregulated ZFP proteins such as rice ZFP182, ZFP252, soybean ZF1, and *Glycine soja* ZFP1 have been shown to be positive regulators of stress defense genes [7,8,25,26]. Many abiotic stress-responsive ZFPs (e.g., Arabidopsis AZF1, AZF2, ZAT10, ZAT11 and ZAT12) contain an active ERF-associated amphiphilic repression (EAR) motif at the C-terminus with a core motif LxLxL or DNLxxP [27-28]. Arabidopsis AZF1 and AZF2 have been shown to possess repression activity [2] and are negative regulators of abiotic stress down-regulated genes [29]. However, the direct repression of target genes by these reported ZFPs has not been investigated. In addition, Arabidopsis EAR-containing ZAT10 and ZAT12 can act as a positive regulator of oxidative stress genes [10,11]. Overexpression of tomato EAR-containing SIZF2 leads to increased or reduced expression of a number of putative target genes in tomato [14], indicating that it acts as both a positive and a negative regulator. Interestingly, three abiotic stress-inducible C_2H_2 zinc finger

proteins from rice have been shown to repress the reporter gene expression driven by the *OsDREB1B* promoter in the protoplast system [30].

This study aims to investigate the role of drought-upregulated and root-predominantly expressed Q-type C_2H_2 transcription factors from *T. aestivum* (TaZFPs) in its adaptation to drought stress. Root systems play a vital role in nutrient and water acquisition. Under water limitation genotypes that prioritise assimilates from shoot growth to root growth for access to deep soil water can have an advantage in grain yield [31]. Identification of genes involved in modulation of this drought adaptive process would provide our understanding on the molecular basis of this trait. The expression of *TaZFP22* and *TaZFP34* in wheat roots was induced by various abiotic stresses. Both genes acted as transcriptional repressors, downregulating the expression of potential negative regulators of root growth. The overexpression of *TaZFP34* in wheat roots resulted in an increased ratio of root to shoot length by reducing shoot growth and maintaining root elongation, indicating that TaZFP34 is one of the molecular players in modulating the root-to-shoot ratio during drought stress.

2. Material and methods

2.1. Plant materials and general growth conditions

Bread wheat (*T. aestivum* L. cv. Fielder) control and transgenic plants were grown in a controlled-environment growth room in pots containing a 3:1:1 mix of sand:soil:peat under night/day conditions of 16-h light (500 µmol $m^{-2}s^{-1}$), 16/22°C and 80/60% relative humidity. For the observation of phenotypic changes of transgenic plants at the full developmental stage, control and transgenic plants were grown in 1.5-L size pots at the above conditions.

2.2. Measurement of root and shoot growth

For synchronising germination among transgenic lines and wild type control, seeds were imbibed on wet paper towel at 4°C for 5 days and were then placed at 12°C for 5 day for germination. Germinated seedlings were grown at 22°C for 3 days and then seeds were removed from the seedlings. The seedlings were subsequently grown hydroponically using Hoagland and Arnon nutrient solution No. 2 [32] under the above controlled-environment growth conditions. The root length was measured in the longest primary seminal root of each plant.

Observation of root growth in soil (3:1:1 mix of sand:soil:peat) was conducted using thin-soil-layer chambers (40 cm wide × 60 cm long × 1.2 cm thick). Thin-layer soil chambers were made from one Perspex sheet and another aluminium composite sheet which were held 1.2 cm apart. The bottom of the chambers was fitted with a fly screen mesh to hold soil in the chamber. The soil in the chambers was fully saturated with a 0.3g L^{-1} Aquasol fertiliser (Yates) solution before planting seedlings. Seeds were imbibed and germinated as described for hydroponic growth experiments. Germinated seedlings were grown at 22°C for 2 days. Each chamber was planted with one seedling with a shoot length of approximately 3 cm long. The chambers in a stack of 5 were placed over a tray at an angle of about 45° with the aluminium composite sheet side of each chamber in the upper position. Light entry to the Perspex sheet side of the chamber at the bottom of the 5 chamber stack was blocked by a black aluminium composite sheet. The top of each chamber stack was covered with a 3-cm thick white polyurethane foam sheet to prevent heat generated by direct exposure of the top aluminium composite sheet to light. Plants were grown in a controlled environmental growth room as described above. To replenish the water loss during growth, a solution of 0.15 g L^{-1} Aquasol fertiliser was added to the tray to a level of about 3 cm above the bottom of sheet chambers or from the top of the chamber every 4-7 days.

2.3. Abiotic stress treatments

For abiotic stress experiments used for expression analysis, 3-week-old wheat seedlings (cv. Babax) were treated with 200 mM NaCl, 15% (w/v) polyethylene glycol (PEG) or 1 mM H_2O_2 in Hoagland and Arnon nutrient solution No. 2. NaCl and PEG treatments were carried out in a tissue culture cabinet at 20°C with 16-h light (200 µmol $m^{-2}s^{-1}$) for 8 h and 48 h. Oxidative stress was performed by treatment of the seedlings with H_2O_2 for 1.5 h and 5 h in a controlled-environment growth room under light (500 µmol $m^{-2}s^{-1}$). Control seedlings for each abiotic stress were treated only with the Hoagland and Arnon nutrient solution in the same growth conditions. Cold treatment was performed by exposure of 3-week-old seedlings to 4°C for 4 h in a cold room with a light intensity of about 20 µmol $m^{-2}s^{-1}$ (use of the low light intensity here mainly due to the facility limitation) with the control plants under the same light intensity at 20°C. For a longer term of cold treatment in cold acclimation studies an appropriate light intensity for plant growth is required [33].

2.4. Expression analysis using quantitative RT-PCR

Frozen root samples were ground to a fine powder in liquid nitrogen. Total RNA was isolated from the samples using Plant RNA Reagent (Invitrogen, California, USA), according to the manufacturer's instruction. RNA was further purified through a Qiagen RNeasy column (Qiagen, Australia) after pre-treatment with RNase-free DNase I [34].

The mRNA levels of the genes of interest were quantified using real-time PCR and the gene-specific primers were designed as described previously [35]. The sequences of primer pairs used for real-time PCR are listed in Supplementary Table S1. Gene-specificity of the primers was confirmed for *TaZFP34* and the differentially expressed genes that are potentially involved in the modulation of root growth (*TaRR12B*, *TaRR12D*, *TaSHY2*, *TaIAA7*, *TaGID1*, *TaGA20-ox1* and *TaExpB18*) by sequencing of the cloned PCR products. A wheat house-keeping gene (*TaRP15*, RNA polymerase I, II and III, 15 kDa subunit) was selected as an internal reference gene for the calculation of relative transcript levels of the genes of interest [35]. Relative quantitation of mRNA levels was as described previously [35].

2.5. Plasmid construction

TaZFP-CELD fusion plasmids were constructed by cloning the coding region sequences of TaZFPs into a CELD fusion vector (pTacLCelD6xHis) [36]. Maize *Ubi1* promoter-driven TaZFP plasmids (Ubi1ZFP22, ZmUbi1:TaZFP22:OsrbcS3'; Ubi1ZFP34, ZmUbi1:TaZFP34:OsrbcS3'; and Ubi1ZFP46, ZmUbi1:TaZFP46:OsrbcS3') were constructed using pUbiSXYNA as a vector by replacing XynA cDNA with a TaZFP cDNA as described by Xue et al. [37]. The construction of an *OsRSP3*-promoter-driven *GREEN FLUORESCENCE PROTEIN* (*GFP*) (OsRSP3:GFP:OsrbcS3') expression cassette, OsRSP3GFP, was described previously [38]. An OsRSP3TaZFP34 construct (OsRSP3:TaZFP34:OsrbcS3') was made by inserting the *TaZFP34* coding sequence under the control of the rice *RSP3* (*OsRSP3*) promoter. The OsRSP3GFP and OsRSP3TaZFP34 expression cassettes were subsequently inserted into a VecBar2 binary vector according to Xue et al. [38]. *TaRR12D* and *TaSHY2* promoter-driven *GFP* constructs (TaRR12D:GFP:OsrbcS3' and TaSHY2:GFP:OsrbcS3') were made by inserting the *TaRR12D* (a 1668-bp fragment from -1670 to -3 bp upstream of its translation start) or *TaSHY2* (a 1273-bp fragment from -1292 to -19 bp upstream of its translation start) promoter upstream of the *GFP* coding region. TaZFP-GFP fusion protein constructs (Ubi1GFPZFP22, Ubi1GFPZFP34 and Ubi1GFPZFP46) were constructed by inserting the *GFP* coding sequence at the N-terminal region of TaZFP22, TaZFP34 and TaZFP46 in *ZmUbi1* promoter-

driven TaZFP plasmids. A rice *Act1* promoter-driven *RED FLUORESCENCE PROTEIN* (*RFP*) (OsAct1:TagRFP:OsrbcS3'), Act1RFP, was constructed using a *TagRFP* coding sequence from pSITEII-6C1 [39].

2.6. In vitro DNA-binding site selection and DNA-binding activity assays

Production of recombinant TaZFP-CelD proteins tagged with 6×His residues was essentially as described by Xue [40] with following modification: (1) 1 mM cysteine and 10 μM $ZnCl_2$ were added to the induction medium (LB containing 1 mM IPTG and 1 % glycerol) and the culture was grown at 29°C for 8 h after adding the induction medium; (2) crude TaZFP-CELD protein preparations contained 10 μM $ZnCl_2$. TaZFP-CelD fusion proteins were purified using Ni-NTA magnetic beads (Qiagen), according to the manufacturer's instruction.

TaZFP46 DNA-binding sites were determined initially using a CELD reporter-based *in vitro* DNA-binding site selection method [36] with the addition of 10 μM $ZnCl_2$ in binding/washing buffer as it is required for zinc finger proteins. The measurement of DNA-binding activity of TaZFP-CELD proteins was performed essentially as described by Xue [40] using StreptaWell High Bind and biotinylated probes. Binding/washing buffer used in DNA-binding assays contained 10 μM $ZnCl_2$. The cellulase activity of the TaZFP-CELD fusion proteins bound to immobilised biotinylated probes was measured by incubation in 100 μl of the CELD substrate solution (1 mM methylumbelliferyl β-D-cellobioside in 50 mM Na-citrate buffer, pH 6.0) at 40°C for 4-16 h. A biotin-labelled double-stranded oligonucleotide without a TaZFP-binding site was used as a control of background activity in DNA-binding assays. Biotinylated double-stranded oligonucleotide probes were synthesised as described previously [36].

2.7. Production of transgenic wheat overexpressing TaZFP34 and GFP in roots

The root promoter-driven *TaZFP34* (OsRSP3:TaZFP34:OsrbcS 3') and *GFP* (OsRSP3:GFP:OsrbcS 3') constructs in the binary vector were introduced into wheat (cv. Fielder) embryos using *Agrobacterium*-mediated transformation as described by Ishida et al. [41]. Transgenic plants were selected with the herbicide phosphinothricin and grown in a controlled environment growth room [42]. The transgenic plants expressing the *TaZFP34* transgene were verified by quantitative RT-PCR using the following primers: 5'-GGTGGCAACTAAGCCGTCATCG (forward) and 5'-

ACACACGAAACAAGGTGGGAGACA (reverse), targeting the rice *rbcS* 3' region sequence. The transgenic plants expressing the *GFP* transgene were verified by the presence of GFP fluorescence in roots. The homozygous state of transgenic lines was assessed by analysis of the Basta® herbicide resistance of 20 seedlings from each parental line using leaf painting with 0.2% (v/v) Basta® in a 0.1% (v/v) Tween-20 solution.

2.8. Subcellular localisation of TaZFP proteins

GFP-TaZFP fusion plasmids and pUbi1GFP control were bombarded into wheat leaves and roots (cv. Bobwhite) by particle bombardment [43]. The bombarded tissues were kept at room temperature (22°C) in dark for about 20 h. The Act1RFP construct was co-introduced with a GFP-TaZFP construct for illustration of cell shape, Bombarded tissues were examined in both RFP (excitation/emission: 545/620 nm) and GFP (480/510 nm) channels of a fluorescence microscope (Leica MZ16 FA) for subcellular localisations of RFP, GFP and GFP-TaZFP fusion proteins.

2.9. Transrepression or transactivation assays

Transrepression or transactivation of reporter genes by an effector construct was analysed as described for transactivation assays [43]. Constructs were introduced into the wheat leaves (cv. Bobwhite) by particle bombardment [43]. The effector gene was co-introduced with a *GFP* reporter gene driven by the promoter of interest to determine the transrepression or transactivation activity. The reporter genes without an effector construct were used as a control. For each bombardment 0.4 µg reporter construct and 0.2 µg effector construct or 0.2 µg herring sperm DNA for the reporter gene assay without an effector gene were used. Act1RFP (0.4 µg) was also included in each bombardment to verify the successful transformation of leaf cells. The bombarded tissues were kept at 22°C except where it is indicated and each leaf section was examined in both GFP and RFP channels for GFP and RFP expression about 20 h after bombardment.

3. Results

3.1. TaZFP22, TaZFP34 and TaZFP46 from T. aestivum are a group of abiotic stress-inducible C_2H_2 zinc finger proteins, localised in the nucleus

The full-length coding sequences of three C_2H_2 ZFP genes [TaZFP22 (EU408222), TaZFP34 (EU408224) and TaZFP46 (EU650398)] were isolated from *T. aestivum*.

TaZFP22, TaZFP34 and TaZFP46 were all 2-fingered proteins (Supplementary Fig. S1), but their sequences differed in the following aspects: overall sequence length, linker length between two C_2H_2 domains and EAR motif. All three ZFP proteins contained two Q-type (QALGGH motif) C_2H_2 domains (Supplementary Fig. S1). The C_2H_2 domains of TaZFP34 and TaZFP46 shared more sequence similarity with each other than that of TaZFP22.

It is generally considered that the transcriptional activity (activation or repression) of C_2H_2 zinc finger proteins is determined by the presence or absence of an active repression motif such as an EAR-motif [28]. TaZFP22 had an EAR-like motif (FDLNLPA) and TaZFP46 contained a typical EAR motif (LDLNHPP) (Supplementary Fig. S1). TaZFP34 did not possess an EAR-like motif or other known repression motifs: TLLLFR, R/KLFGV, LxLxPP and LVFY [28,44].

A BLAST search in the NBCI protein database was performed to examine potential orthologs of these TaZFP genes in model plant species (Arabidopsis and rice). TaZFP22 shares the highest homology with rice drought upregulated OsZFP252 (AY219847), with an amino acid sequence identity of 59.6% and similarity of 75.3%. The highest homologue of TaZFP22 in Arabidopsis is ZAT10 (40.9% amino acid identity and 64.3% similarity). The highest homologous genes of TaZFP34 in rice and *Arabidopsis thaliana* are an uncharacterised ZFP (Os03g0820300) and ZAT12 (AT5G59820), respectively. However, the amino acid sequence homology between TaZFP34 and AtZAT12 is low (28.5% identity and 49.7% similarity). TaZFP46 shows the closest sequence relationship with an uncharacterised ZFP in rice (Os05g0114400) and the highest sequence homologue in Arabidopsis is ZAT11 (AT2G37430, 34.5% amino acid identity and 52.7% similarity).

A previous study has shown that *TaZFP22*, *TaZFP34* and *TaZFP46* are all markedly upregulated in wheat leaves by drought stress and *TaZFP22* and *TaZFP34* are also drought-upregulated in the roots [3]. In this study we analysed expression responses of these three genes in the roots to various abiotic stresses: cold, salt, PEG-induced dehydration and oxidative stresses. Expression of *TaZFP34* in the roots was upregulated by cold, salt, dehydration and oxidative stress (Fig. 1A). Upregulation in the roots by cold, salt and oxidative stress was also seen in *TaZFP22*. No significant changes in *TaZFP46* expression in the roots were observed.

To determine the subcellular localisation of TaZFP22, TaZFP34 and TaZFP46, translational fusion constructs of these TaZFP proteins with GFP were made, and were subsequently introduced into wheat tissues using a transient expression system. As shown in Figure 1B, TaZFP34-GFP fusion protein was localised in the nucleus of wheat leaf and root

epidermal cells, which is consistent with their putative function as transcription factors. Similarly, TaZFP22- and TaZFP46-GFP fusion proteins were also localised in the nucleus (Supplementary Figure S2).

3.2. The DNA-binding specificity of TaZFP22, TaZFP34 and TaZFP46

An *in vitro* DNA-binding site selection method was used to get an idea of the target sequences of these TaZFP proteins using TaZFP46, which was expressed at a sufficient level in *E. coli* to perform this assay. Sequence analysis of twelve TaZFP46-selected oligonucleotides showed a consensus sequence of SAGTR (S = C or G, R = A or G) (Supplementary Fig. S3A), which is similar to the EP1 motif from the promoter of petunia *5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE* [22]. Each TaZFP46-selected oligonucleotide contained at least two SAGTR (or YACTS) motifs with a spacer of 3-9 nucleotides.

To define the TaZFP46 binding sequence, we performed mutagenesis analysis of the binding site using the EP1 motif (TTGA**CAGTG**TCAC) as a starting sequence, since a number of previous studies have shown that Q-type C_2H_2 proteins are capable of binding to the EP1 motif repeats. This analysis revealed that AGT was the core motif for TaZFP46, as base substitution at the AGT resulted in complete or almost complete abolition of binding activity (Supplementary Fig. S3B). However, some bases flanking the core sequence markedly influenced the binding activity.

To examine the optimal length of spacer between the two high affinity binding motifs (GGGAGTGA), base deletion or insertion between the two binding motifs in EP1m23 was performed. The binding activity was gradually reduced by progressive increase or decrease in the spacer length (Supplementary Fig. S3B). The spacer length of seven base pairs between two SAGTR motifs in the EP1m23 was preferred for TaZFP46 (Supplementary Fig. S3B). Results from *in vitro* binding site selection and mutagenesis analyses are summarised in Supplementary Table S2. It appeared that TaZFP46 preferentially bound to a G-rich motif (GGGAGTGA).

As TaZFP22, TaZFP34 and TaZFP46 are all Q-type 2-fingered C_2H_2 proteins, a number of representative oligonucleotide probes used for analysis of TaZFP46 DNA-binding activity were selected for comparative analysis of their DNA-binding specificity. As shown in Table 1, AGT was also the core binding motif for TaZFP22 and TaZFP34. TaZFP34 showed a similar pattern of DNA-binding specificity to TaZFP46. The DNA-binding specificity of TaZFP22 was less similar.

3.3. TaZFP34 functions as a negative regulator for shoot growth and enhances the ratio of root to shoot length

Among three drought-upregulated TaZFP genes, *TaZFP34* appears to be most interesting, as this gene was markedly induced by drought in both roots and leaves of wheat [3] and were upregulated by osmotic, salt, cold and oxidative stresses in roots. In particular, this gene was predominantly expressed in roots [3]. It was speculated that the function of this gene might be mainly in the roots. Therefore, the promoter of a putative root-specific protein from rice (*OsRSP3*) was used to drive *TaZFP34* expression in the roots. Based on Affymetrix expression profiling data, *OsRSP3* is exclusively expressed in the root of rice [38]. To confirm its root specificity, a *GFP* reporter construct driven by the *OsRSP3* promoter was introduced into wheat. Analysis of OsRSP3GFP expression in various organs of transgenic wheat plants showed that visible GFP green fluorescence was found only in wheat roots including a connection tissue between the shoot and roots of a seedling (Supplementary Fig. S4). Within the seminal roots, expression of OsRSP3GFP was found in the epidermal, cortical and endodermal cells.

Quantitative RT-PCR analysis of eight T_2 homozygous transgenic lines transformed with OsRSP3TaZFP34 identified 4 lines with *TaZFP34* expression in the roots at a level more than 5 times higher than that in wild-type roots (Fig. 2A). In particular *TaZFP34* mRNA levels in the roots of transgenic lines, ZFP34-2 and ZFP34-13, were increased by 40-fold or more, in comparison with the wild-type plants. *TaZFP34* mRNA levels in the shoots were also examined in two high expressor lines (ZFP34-2 and ZFP34-13). The *TaZFP34* expression level in the shoots of these high expressor lines was very low, compared to that in the roots (Supplementary Fig. S5A). The average level of *TaZFP34* transcripts in the shoots of these two high expressor lines was 4.8 times higher than that of the wild type plants. However, in the wild type plants the expression levels of *TaZFP34* in the shoots was about 4 times lower than that in the roots (Supplementary Fig. S5A). Analysis of dark-grown seedlings of OsRSP3GFP plants, which eliminates the interference of chlorophyll auto-fluorescence in the GFP channel, showed that weak GFP fluorescence was seen in the coleoptile and no GFP fluorescence was visible in the emerging leaves (Supplementary Fig. S5B). These analyses indicate that expression of the *OsRSP3* promoter-driven transgenes is essentially root-specific in transgenic wheat.

Transgenic wheat plants overexpressing *TaZFP34* showed slower shoot growth than wild-type plants in the early growth stage (Fig. 2D). Despite the reduced shoot growth, which

is expected to reduce photo-assimilates for supply to the roots, *TaZFP34* lines had similar or slightly faster growth of primary seminal roots under hydroponic growth conditions (Fig. 2C). As a result, the ratio of root-to-shoot length was significantly increased by up to 33% in the transgenic lines after 19-day growth (Fig. 2B). The reduced shoot growth and increased ratio of root-to-shoot length in the *TaZFP34* overexpressing lines were also observed in plants grown in soil-filled Perspex chambers (Fig. 2E and 2F). Analysis of root and shoot biomass of hydroponically grown seedlings also showed an increase in the ratio of root to shoot weight, compared to wild type plants (Fig. 2G-2I).

The *TaZFP34* overexpressing lines showed delayed flowering time by 2-3 days, had a shorter final plant height than the wild-type plants (Fig. 3A) and shorter spikes (Fig. 3B) with no significant differences in grain number per spike except the ZFP34-13 line (Fig. 3C). The straw dry weights at the maturity were significantly reduced in the transgenic lines except the ZFP34-14 line (Fig. 3D). The OsRSP3TaZFP34 transgenic lines showed a slight, but not statistically significant, increase in tiller number at the maturity (Fig. 3E). The grain yield per plant was significantly reduced in the high *TaZFP34*-expressing lines (ZFP34-2 and ZFP34-13), but not in the moderate-expressing lines (ZFP34-14 and ZFP34-25) (Fig. 3F). The grain size (hundred grain weight) was significantly decreased in all four OsRSP3TaZFP34 lines (Fig. 3G). These data show that *TaZFP34* overexpression has a negative impact on yield performance, mimicking the negative impact of drought stress on wheat productivity.

The maturity root biomass of two transgenic lines (ZFP34-13 and ZFP34-25) were also analysed in comparison with that of the wild type plants. The maturity root dry weight expressed as gram per plant (mean ± SD, n = 6) for the wild type, ZFP34-25 and ZFP34-13 were 2.49 ± 0.26, 2.93 ± 0.24 and 2.66 ± 0.27, respectively. The maturity root biomass was significantly increased in the moderately expressed OsRSP3TaZFP34 line (ZFP34-25, $P < 0.05$), but not in the highly expressed line (ZFP34-13).

3.4. Overexpression of TaZFP34 represses expression of transcriptional repressor IAA and type-B response regulator genes in roots and shoot growth-related genes in shoots

To understand whether TaZFP34 regulates the expression of root growth-related genes, we analysed the expression of genes that are homologous to known root growth-related genes from other species initially in two transgenic lines (ZFP34-2 and ZFP34-13) that expressed high-level *TaZFP34* in the roots. A number of transcriptional repressor IAA genes (*TaSHY2*, *TaIAA7* and *TaIAA27*) from the auxin/indole-3-acetic acid inducible gene family (Aux/IAA) were significantly down-regulated in the roots of both high *TaZFP34*-

overexpressing lines (Supplementary Table S3). SHORT HYPOCOTYL2 (SHY2) or IAA3 from Arabidopsis is a negative regulator of root meristem size and root growth [45]. Expression levels of three type-B two-component response regulators (MYB-related proteins: TaRR1L1, TaRR12B, TaRR12D) homologous to Arabidopsis response regulators (ARR1 and ARR12) were also significantly lower in the roots of at least in one of these two *TaZFP34* lines than the wild-type. ARR1 and ARR12 in Arabidopsis are known to be transcriptional activators of SHY2 [45-47]. The expression levels of *GA 20-oxidase* (*TaGA20-ox1*) that is involved in the gibberellic acid (GA) synthetic pathway and an *EXPANSIN B* gene (*TaExpB18*) were also significantly reduced at least in one of the two high-expressing lines. No significant expression changes were observed for cell cycle genes and auxin signalling pathway genes (Supplementary Table S3). The expression of *TaGID1* (a GA receptor) was upregulated in the *TaZFP34* lines. We also analysed the expression of some genes involved in ABA metabolism (*NCED3*, *NCED9* and *CYP707A1*) and ABA-upregulated genes (*ABI1*, *NAC69-1*, *WRAB1*, *WRAB18* and *LEA1*). No significant changes in the expression of these genes were observed in the *TaZFP34* overexpressing lines (Supplementary Table S3). Some antioxidant enzyme genes (*APX1*, *APX2* and *FSD2*) involved in the protection of oxidative stress were also analysed. Among these three genes the expression level of *TaAPX2* was significantly reduced in both transgenic lines (Supplementary Table S3).

Based on above expression data, we performed further expression analysis in four transgenic lines (two high expressors and two moderate expressors) on the following genes: *TaRR12B*, *TaRR12D*, *TaIAA7*, *TaSHY2*, *TaGA20-ox1*, *TaExpB18* and *TaGID1*, which are the homologues of Arabidopsis genes involved in modulating root growth. As shown in Figure 4A and 4B, the expression levels of *TaRR12B*, *TaRR12D*, *TaIAA7* and *TaSHY2* were all significantly reduced in the roots of both high (ZFP34-2 & 13) and moderate (ZFP34-14 & 25) *TaZFP34*-overexpressing lines. *TaGA20-ox1* expression level was also lower in these four lines. However, the expression level of *TaExpB18* was significantly reduced only in the ZFP34-2 line. *TaGID1* expression was significantly upregulated in lines ZFP34-2, ZFP34-13 and ZFP34-14.

In order to understand the molecular basis of the reduced shoot growth of *TaZFP34* overexpressing lines, the expression levels of shoot growth-related genes were determined in the shoots of two high-level *TaZFP34*-expressing lines (ZFP34-2 and ZFP34-13) in comparison with wild type plants. A literature survey indicates that reduced shoot growth involves expression changes in GA biosynthesis and signalling pathway genes [48,49].

Therefore, some GA pathway genes and expansin genes involved as positive regulators of shoot growth were chosen for analysis. The mRNA levels of *TaGA3-ox2*, involved in the final step of active GA synthesis, a *GA-INDUCED PROTEIN* (*TaGIP*) and two expansin genes (*TaExpA4* and *TaExpB1*) were significantly lower in the shoots of the transgenic lines than the wild type (Fig. 5). No statistical differences were observed in other GA pathway genes (*TaGA20-ox1*, *TaGID1*, *TaRht1* and *TaSLRL1*) in the shoots between the transgenic and wild type plants. *TaExpB18* was also analysed, but its mRNA level was too low in the shoots to be reliably determined.

3.5. TaZFP34 binds to SAGTR-like motifs in the promoters of its downregulated genes and represses the expression of reporter genes driven by its target promoters

Downregulation of a number of potential root growth repression genes in the transgenic lines with overexpression of *TaZFP34* in the roots indicates that TaZFP34 may act as a transcriptional repressor. To investigate whether TaZFP34 can serve as a direct repressor of the expression of these target genes, the promoter region sequences of two of the TaZFP34 down-regulated genes, *TaRR12D* and *TaSHY2*, were isolated. Sequence analysis showed that these isolated promoters contained the putative TaZFP34 binding motifs (Supplementary Fig. S6). DNA-binding analysis revealed that TaZFP34 was capable of binding to the SAGTR-like motifs in these promoters and the TaZFP34 binding affinity to one of the elements from the *TaRR12D* promoter (RR12DBS1) was higher than that of EP1 (Fig. 6A).

Next, *GFP* reporter genes were constructed under the control of *TaRR12D* and *TaSHY2* promoters and transrepression assays were performed by bombarding a *GFP* reporter gene with or without a *TaZFP34* effector construct driven by a constitutive maize *Ubi1* promoter (Fig. 6B). Wheat leaves were used for transient expression assays, although root tissue would be ideal for this analysis. This is because the transformation efficiency of wheat roots using particle bombardment is too low, due to their small surface area, to obtain a large number of transformed cells necessary for transrepression analysis. However, as the TaZFP34 repression activity involves its direct binding to the promoters of its target genes, therefore, it is unlikely to require the presence of a root-specific factor for its transrepression activity to the target genes. In addition, *TaZFP34* is also expressed in the leaves during drought stress [3]. A RFP construct was added in each transformation for verification of successful transformation. The co-transformation efficiency of *GFP* and *RFP* constructs in the transient expression assays was very high (Supplementary Fig. S7). GFP foci of the *GFP* reporter gene driven by the promoter of *TaRR12D* or *TaSHY2* were observed in wheat leaf

cells in the absence of the *TaZFP34* effector gene (Fig. 6C). Co-introduction of the *TaZFP34* effector gene with these reporters resulted in no GFP expression (Fig. 6C, Supplementary Fig. S7), whereas the expression of a co-transformed *RFP* construct was clearly visible in the RFP channel, indicating that these GFP negative leaf sections were transformed. These data demonstrate that TaZFP34 functions as a transcriptional repressor. To test whether TaZFP22 and TaZFP46 are transcriptional repressors, transrepression assays were also conducted for *ZmUbi1* promoter-driven *TaZFP22* and *TaZFP46* effector genes. Transrepression of *TaRR12D* and *TaSHY2* promoter-driven GFP reporter genes by TaZFP22 and TaZFP46 was also observed (Fig. 6C).

We also examined the expression of a *GFP* reporter gene driven by a SAGTR motif-containing promoter in wheat cells, using a ZFP46E-miniDhn6 promoter, which is a barley Dhn6 minimal promoter (MiniDhn6) with the addition of two TaZFP22, TaZFP34 and TaZFP46 preferred binding motifs (GGGAGTGAn5GGGAGTGA) in direct repeats (Fig. 7). No GFP foci were observed in wheat seedlings under control and PEG-induced dehydration conditions with introduction of the ZFP46E-miniDhn6GFP reporter construct. Co-introduction of a *TaZFP34* or *TaZFP46* effector construct also did not result in the expression of the ZFP46E-miniDhn6GFP reporter gene. For positive controls we tested two *GFP* reporter constructs using the MiniDhn6 promoter with the addition of known stress transcription factor binding motifs. GFP expression of the reporter constructs with the addition of barley dehydration-responsive factor 1 binding motifs (BDRF1-E [34]) or wheat heat shock elements from the *TaHSP90* promoter (HSE90 [35]) was observed under PEG-induced dehydration (BDRF1E-MiniDhn6GFP) and heat stress (HSE90-MiniDhn6GFP) conditions or co-introduction of a barley dehydration-responsive factor 1 (*HvDRF1*) and a wheat heat shock factor A6f (*TaHsfA6f*) effector gene (Fig. 7). These analyses suggest that these ZFP proteins do not possess transactivation activity and that the activity of these two SAGTR-binding ZFP transcription factors in wheat cells does not serve as transcriptional activators of SAGTR-containing genes under normal and dehydration stress conditions.

3.6. TaZFP34 targets of root growth repression genes are downregulated during salt and dehydration stresses

To investigate further the regulator and target relationship between TaZFP34 and its downregulated genes, we examined the expression patterns of *TaRR12B*, *TaRR12D*, *TaSHY2*, *TaIAA7*, *TaGA20-ox1* and *TaExpB18* in the roots during salt and PEG-induced dehydration stresses. As shown in Figure 4C, all these six genes were downregulated by salt or

dehydration stresses in the roots, although downregulation by 15% PEG was not statistically significant for some of these genes. In contrast, *TaZFP34* expression was upregulated during these abiotic stresses (Fig. 1).

4. Discussion

This study investigated the function of three root-predominantly expressed C_2H_2 zinc finger transcription factors (TaZFP22, TaZFP34 and TaZFP46) in wheat, which are upregulated in the leaves by drought stress and two of these (TaZFP22 and TaZFP34) are also upregulated by drought stress in the roots [3]. Functional analyses revealed that all three TaZFP proteins acted as transcriptional repressors. Overexpression of *TaZFP34* in wheat resulted in an enhanced ratio of root to shoot, an important phenotypic change for reducing water loss from shoot and maintenance of root growth for access to deep soil water, which is a common drought adaptation process in plants.

TaZFP22, TaZFP34 and TaZFP46 proteins contain two highly conserved QALGGH-type DNA binding domains [3]. The QALGGH sequence is known to act as base determinants in interaction with DNA [21,50]. Extensive DNA-binding specificity analysis revealed that all three TaZFPs bound to similar SAGTR motifs with a preferred binding sequence of GGGAGTGA. TaZFP22 and TaZFP46 contain an EAR-repression motif, while no known repression motifs were present in TaZFP34. Sequence homology analysis revealed that the highest homologues of TaZFP22, TaZFP34 and TaZFP46 with Arabidopsis ZFPs are AtZAT10, AtZAT12 and AtZAT11, respectively. These three Arabidopsis ZFPs are all known to be upregulated by abiotic stresses, such as drought, osmotic, cold, salt and oxidative stresses [10,11,51]. These Arabidopsis ZFPs contain an EAR repression motif at the C-terminus as TaZFP22 and TaZFP46 do [27]. AtZAT10 and AtZAT12 have been shown to be positive regulators for upregulating oxidative stress genes in Arabidopsis [10,11].

Cellular localisation analysis using the translational fusion of these TaZFPs with GFP showed that they were exclusively localised in the nucleus. Expression analysis revealed that *TaZFP34* was upregulated by dehydration, salt, cold and oxidative stresses in wheat roots and a similar expression response was observed for *TaZFP22*, indicating their potential role in modulating expression of genes in the nucleus involved in wheat responses to a diverse range

of abiotic stresses. These expression patterns of *TaZFP22* and *TaZFP34* in response to abiotic stresses are similar to those of their closest Arabidopsis homologues (*ZAT10* and *ZAT12*) [11].

The overexpression of *TaZFP34* in wheat roots resulted in a negative effect on shoot growth. The negative impact of overexpression of an abiotic stress-inducible Q-type ZFP gene on plant growth has also been observed in poplar plants overexpressing *PtaZFP2*, which is a homologue of Arabidopsis ZAT12 [52]. Interestingly, the primary seminal root elongation in the *TaZFP34* transgenic lines was maintained. As a result, the ratio of the root to shoot length was markedly increased in the transgenic plants with root overexpression of *TaZFP34*. Under given growth conditions a reduction in shoot biomass is likely to decrease the total plant capacity for photosynthesis, which would reduce the supply of photoassimilate for root growth and consequently may have a negative impact on root growth. However, it appears that the expression levels of potential root growth negative regulator genes (*TaSHY2*, *TaIAA7*, *TaIAA27*, *TaRR12B* and *TaRR12D*) were significantly reduced in the *TaZFP34* overexpressing lines. The expression level of *TaRR1L1* was also reduced in high *TaZFP34*-expressing plants, although a statistically significant reduction was observed only in one of the high-expressing lines. TaSHY2, TaIAA7 and TaIAA27 are members of the Aux/IAA family genes, which are generally known to be active repressors [53]. In Arabidopsis, SHY2 functions as a negative regulator of root meristem size and root growth [45,54]. SHY2 is regulated by Arabidopsis type-B RESPONSE REGULATORs 1 and 12 (ARR1 and ARR12) transcriptional activators through the cytokinin regulatory circuit [45-47]. TaRR1L1 and TaRR12s (TaRR12B and TaRR12D) are close homologues of Arabidopsis ARR1 and ARR12. In addition, the roots of *TaZFP34* lines had an increased expression level of the *TaGID1* gene, which is a positive regulator of root and shoot growth through its recruiting growth-repressing DELLA (Rht1 in wheat) protein to degradation [55]. Changes in the expression of these genes may explain the maintenance of root length in *TaZFP34* lines despite a significant reduction of shoot length.

However, the effects of *TaZFP34* overexpression on root growth-related genes were complex. One of the enzyme genes (*TaGA20-ox1*) that are involved in the GA synthetic pathway was downregulated in *TaZFP34* lines, although the expression of a key GA downstream gene (*TaGID1*) was increased. Therefore, a decrease in *TaGA20-ox1* expression in these *TaZFP34* lines may not have a significant impact on root growth through the GA signalling pathway. The expression of another gene (*TaExpB18*) that may have a positive impact on root growth was reduced in high *TaZFP34*-expressing lines, but not in moderately

expressing lines. *TaExpB18* is a root-predominantly expressed gene with a very low mRNA level in the shoots (data not shown). A reduction in *TaExpB18* expression in high *TaZFP34* expressing lines may compromise the effect of its repression of root growth negative regulator genes on root growth. This may explain why the root length of two moderately expressing lines was longer than two high expressing lines (Fig. 2C).

Analysis of the expression patterns of *TaRR12B* and *TaRR12D*, *TaSHY2*, *TaIAA7*, *TaExpB18* and *TaGA20-ox1* genes during salt and dehydration stresses showed that the expression levels of these TaZFP34 downregulated genes were generally reduced in wheat roots during these stresses, thus bearing an inverse relationship with the expression pattern of *TaZFP34*. TaZFP34 was able to bind to SAGTR-like motifs in the promoters of the *TaRR12D* and *TaSHY2*. In particular, the TaZFP34 binding affinity of the SAGTR-like element (RR12DBS1) in the *TaRR12D* promoter was relatively high, compared to those in the *TaSHY2* promoter. Transient expression analysis showed that TaZFP34 repressed the expression of reporter genes driven by the promoters of *TaRR12D* and *TaSHY2*. These analyses showed that TaZFP34 acted as a transcriptional repressor, although TaZFP34 did not contain any known repressor motifs. Repression of *TaRR12D* and *TaSHY2* promoter-driven reporter genes by EAR-repression motif-containing TaZFP22 and TaZFP46 was also observed. These data suggest that TaZFP34 and other stress-inducible ZFP repressors such as TaZFP22 may be involved in downregulation of *TaRR12* and *TaSHY2* genes in roots during salt and dehydration stresses.

A significant reduction in shoot growth as well as final plant height in the transgenic lines overexpressing *TaZFP34* in the roots indicates that the expression of shoot growth-related genes was affected in the shoots of the transgenic plants. Analysis of the GA signalling pathway and expansin genes showed that *TaGA3-ox2* that converts GA from inactive forms to active ones, two expansins (*TaExpA4* and *TaExpB1*) and a GA-induced protein (*TaGIP*) were downregulated in the high *TaZFP34*-expressing lines (ZFP34-2 and ZFP34-13). GA is known to play an important role in shoot growth and plant height [48]. Positive relationships between plant height and the expression levels of *TaGA3-ox2* and *TaGIP* have been shown in wheat [49]. As *TaZFP34* transgene expression was very low in the shoots, whether the impact of *TaZFP34* overexpression in OsRSP3TaZFP34 transgenic wheat on reduction of shoot growth and some shoot growth related gene expression is via root-to-shoot signalling awaits future study.

Cytokinin is known to have a profound effect on root growth [56]. Root-expressed cytokinin oxidase that reduces the root cytokinin level markedly enhances root growth in

both eudicot and monocot species [57,58]. The exogenous application of cytokinin reduces root growth [46]. Type-B response regulators such as ARR1 and ARR12 are transcription factors, mediating cytokinin signal transduction [59]. Arabidopsis *arr1*, *arr10* and *arr12* loss-of-function mutants show reduced sensitivity to cytokinin-mediated inhibition of root growth [56,60]. *TaZFP34* is known to be upregulated by drought stress via the ABA signalling pathway [3]. Downregulation of response regulator genes (*TaRR12B* and *TaRR12D*) in wheat roots by ABA-upregulated TaZFP34 may represent one point of cross-talk between ABA and cytokinin signalling pathways. It is expected that a number of ABA upregulated transcription factors are likely to have interactions with root growth regulatory pathways during drought stress. This is based on reports that root growth is positively affected by enhanced expression of some drought upregulated transcription factors [61,62]. Through this type of cross talk during drought stress, plants are able to maintain their root growth in drying soil, while shoot growth is reduced or completely inhibited. Although the overexpression of *TaZFP34* resulted in the downregulation of potentially negative regulators of root growth in wheat roots, it did not enhance the root elongation in high *TaZFP34*-expressing lines. This is probably because *TaZFP34* overexpression reduced shoot growth (hence reduced photo-assimilates available for root growth) and also repressed the expression of some root-growth genes such as *TaExpB18* in the roots. Overall results from this study implicate that TaZFP34 plays a role in enhancing the root to shoot ratio during drought stress by reducing shoot growth and maintaining root growth. These morphological changes in *TaZFP34*-overexpressing transgenic plants mimic the effect of drought stress on wheat plant growth, suggesting that the TaZFP34 transcriptional repressor participate in these morphological adaptations to drought stress. Further investigations into how drought upregulated transcription factors interact with known root and shoot growth regulatory pathways will elucidate the molecular basis of how roots maintain their growth and how the root to shoot ratio is modulated in drying soil.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Table 1. Comparison of the DNA-binding specificity of TaZFP46, TaZFP34 and TaZFP22 using synthetic oligonucleotides.

Oligo name	Oligo sequence	Relative binding activity		
		TaZFP46	TaZFP34	TaZFP22
Base substitution				
EP1	GGTTGA**CAGTG**TCACATGA**CAGTG**TCATT	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.12
EP1m1	GGTaGA**CAGTG**TCACAaGA**CAGTG**TCATT	0.97 ± 0.11	1.37 ± 0.13	0.84 ± 0.07
EP1m2	GGTTaA**CAGTG**TCACATaA**CAGTG**TCATT	0.46 ± 0.12	1.15 ± 0.03	0.67 ± 0.18
EP1m3	GGTTGc**CAGTG**TCACATCc**CAGTG**TCATT	0.52 ± 0.04	0.79 ± 0.14	0.56 ± 0.13
EP1m4	GGTTGAa**AGTG**TCACATGAa**AGTG**TCATT	0.17 ± 0.02	0.41 ± 0.19	0.29 ± 0.03
EP1m5	GGTTGA**C**c**GTG**TCACATGA**C**c**GTG**TCATT	0	0	0
EP1m6	GGTTGA**CA**a**TG**TCACATGA**CA**a**TG**TCATT	0	0	0
EP1m7	GGTTGA**CAG**a**G**TCACATGA**CAG**a**G**TCATT	0	0	0
EP1m9	GGTTGA**CAGTG**aCACATGA**CAGTG**aCATT	1.91 ± 0.17	1.59 ± 0.28	1.16 ± 0.31
EP1m11	GGTTGA**CAGTG**aaACATGA**CAGTG**aaATT	1.01 ± 0.09	0.48 ± 0.04	0.77 ± 0.08
EP1m12	GGTTGAt**AGTG**aCACATGAt**AGTG**aCATT	1.23 ± 0.16	0.88 ± 0.08	1.02 ± 0.08
EP1m13	GGTTGAg**AGTG**aCACATGAg**AGTG**aCATT	2.43 ± 0.23	1.98 ± 0.26	1.37 ± 0.13
EP1m16	GGTTGAg**AGT**aaCACATGAg**AGT**aaCATT	0.80 ± 0.17	0.15 ± 0.02	0
EP1m17	GGTTGAg**AGT**caCACATGAg**AGT**caCATT	0.05 ± 0.01	0	0
EP1m18	GGTTGgg**AGT**taCACATGgg**AGT**taCATT	0.22 ± 0.03	0.10 ± 0.02	0
EP1m23	GGTTGgg**AGTG**aCACATGgg**AGTG**aCATT	3.07 ± 0.28	3.16 ± 0.33	1.41 ± 0.04
EP1m24	GGTTGtg**AGTG**aCACATGtg**AGTG**aCATT	1.71 ± 0.16	1.80 ± 0.22	1.01 ± 0.14
Variation in spacer length between SAGTR motifs				
EP1m23	GGTTGgg**AGTG**aCACATGgg**AGTG**aCATT	3.11 ± 0.14	3.19 ± 0.17	1.38 ± 0.04
EP1m28	GGTTGgg**AGTG**aCACaATGgg**AGTG**aCATT	1.91 ± 0.02	2.15 ± 0.33	1.13 ± 0.09
EP1m29	GGTTGgg**AGTG**aCACaaATGgg**AGTG**aCATT	1.37 ± 0.07	1.75 ± 0.26	nd*
EP1m30	GGTTGgg**AGTG**aCACaaaATGgg**AGTG**aCATT	1.33 ± 0.09	1.61 ± 0.21	0.97 ± 0.21
EP1m31	GGTTGgg**AGTG**aCACaaaaATGgg**AGTG**aCATT	0.52 ± 0.03	nd	nd
EP1m32	GGTTGgg**AGTG**aCACaaaaaATGgg**AGTG**aCATT	0.49 ± 0.08	1.16 ± 0.23	0.93 ± 0.16
EP1m33	GGTTGgg**AGTG**aCAATGgg**AGTG**aCATT	1.53 ± 0.04	1.55 ± 0.19	1.08 ± 0.13
EP1m34	GGTTGgg**AGTG**aCATGgg**AGTG**aCATT	1.68 ± 0.09	1.99 ± 0.30	nd
EP1m35	GGTTGgg**AGTG**aCTGgg**AGTG**aCATT	1.92 ± 0.10	2.21 ± 0.15	1.02 ± 0.15
EP1m36	GGTTGgg**AGTG**aCGgg**AGTG**aCATT	0.49 ± 0.04	nd	nd
EP1m37	GGTTGgg**AGTG**aGgg**AGTG**aCATT	0.52 ± 0.10	nd	1.11 ± 0.09

Values are means ± standard deviation (SD) of 2-3 assays. These values are expressed as relative to EP1. SAGTR in EP1 is typed in bold letters and substituted or inserted bases to EP1 in lower-case letters.

* nd, not determined.

Figure legends

Figure 1. Expression response of *TaZFP22*, *TaZFP34* and *TaZFP46* to abiotic stress in the roots of 3-week-old wheat seedlings and the nuclear localisation of TaZFP34-GFP fusion protein in wheat leaves and roots.
(A) Expression of *TaZFP22*, *TaZFP34* and *TaZFP46* in wheat roots in response to cold (4^oC), H_2O_2, salt and PEG-mediated dehydration stresses. Values are means + SD of 3-4 biological replicates. Statistical significance of differences between control and treated groups is indicated by an asterisk (* $P < 0.05$ using Student's *t*-test).
(B) Subcellular localisation of TaZFP34-GFP fusion protein. The TaZFP34-GFP construct (Ubi1GFPZFP34) was co-bombarded with an Act1RFP construct. The RFP red fluorescence illustrates the shape of transformed leaf and root epidermal cells (shown at the right) in the RFP channel of a fluorescence microscope. Ubi1GFP was used as a control, which showed distribution of GFP green fluorescence in the whole leaf and root epidermal cells, whereas N-terminal GFP-fused TaZFP34 protein was localised in the nucleus.

Figure 2. *TaZFP34* expression levels in the roots of transgenic wheat lines (ZFP34-2, 13, 14 and 25) with root overexpression of *TaZFP34* and enhancement of root-to-shoot ratios.
(A) Relative *TaZFP34* expression levels in wheat roots. T_2 homozygous transgenic lines were used for analysis. Values are means + SD of 3-4 biological replicates.
(B-D) Root and shoot lengths measured at 15 and 19 days after seedlings were grown hydroponically. Values are means + standard errors of the means (SEM) of 15-20 seedlings.
(E& F) Root and shoot lengths measured at 14 days after planting in thin-soil-layer chambers. Values are means + SEM of 5 seedlings.
(G-I) Dry weights of roots and shoot measured at 20 days after germination. Seedlings were grown under hydroponic conditions. Values are mean + SD of 4-5 biological replicates and each replicate contained 4 seedlings.
Statistical significance of differences between control and transgenic lines is indicated by an asterisk (* $P < 0.05$).

Figure 3. The above-ground phenotypic changes of transgenic lines (ZFP34-2, 13, 14 and 25) with root overexpression of *TaZFP34*. T_2 homozygous transgenic lines were used for analysis. Transgenic lines showed short final plant height (**A**), short spikes (**B**), essentially no differences in grain number per spike (**C**), reduced straw weight at the maturity (**D**), no significant changes in tiller number at the maturity (**E**), reduced grain yield in highly expressing lines (**F**) and reduced hundred grain weight (**G**). Values are means + SD of 6-8 plants. Statistical significance of differences between control and transgenic lines is indicated by an asterisk (* $P < 0.05$).

Figure 4. Expression of TaZFP34 target genes in the roots of three-week-old *TaZFP34* transgenic lines (ZFP34-2, 13, 14 and 25 at the T_2 stage) (**A** & **B**) and expression of TaZFP34 downregulated genes during salt (0.2 M NaCl) and dehydration (15% PEG) stresses in wheat roots (**C**). Values are means + SD of 3-4 biological replicates. Statistical significance of differences between control and transgenic lines is indicated by an asterisk (* $P < 0.05$).

Figure 5. Expression levels of shoot growth-related genes in the shoots of two high *TaZFP34*-expressing T_3 transgenic lines and wild type plants. Shoots of three-week-old transgenic (ZFP34-2 and ZFP34-13) and wild type plants were used for expression analysis. Values are means + SD of four biological replicates and expression levels are expressed as

relative to those of the wild type plants. Statistical significance of differences between control and transgenic lines is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$).
TaGA3-ox2, GA 3-oxidase 2 (catalysing the conversion of inactive GA to active forms); TaGIP, GA-induced protein; TaSLRL1, slender 1-like (a repressor of the GA signalling, which negatively regulates plant height in rice); TaExpA4, expansin A4 (expansins are involved in plant growth); TaExpB1, expansin B.

Figure 6. Binding of TaZFP34 to *TaRR12D* and *TaSHY2* promoter elements and repression of *TaRR12D* and *TaSHY2* promoter-driven *GFP* reporters by TaZFP34, TaZFP22 and TaZFP46.
(A) Binding of TaZFP34 to the SAGTR-like elements in the promoters of *TaRR12D* and *TaSHY2* in comparison with the EP1 element. RBA, relative binding activity.
(B) Illustration of reporter and effector constructs.
(C) Transrepression of *TaRR12D* and *TaSHY2* promoter-driven *GFP* reporters by TaZFP34, TaZFP22 and TaZFP46 in a transient expression system. The GFP foci in the leaf sections of wheat plants indicate the expression of reporter genes. Act1RFP was included in each transient expression assay to demonstrate that leaf cells were transformed. Each leaf section was examined in both GFP and RFP channels. The red background in the GFP channel is chlorophyll auto-fluorescence.

Figure 7. Transient expression analysis of *GFP* reporter genes driven by the minimal *HvDhn6* promoter (MiniDhn6) with addition of TaZFP46-binding motifs or other *cis*-acting elements in wheat leaves. Constructs with the addition of two TaZFP46-preferred binding motifs (TaZFP46E: GGGAGTGAn5GGGAGTGA) in direct repeats, three barley DRF1 binding motifs (BDRF1E) or three heat shock elements from the promoter of wheat HSP90 (HSE90) to the minimal *HvDhn6* promoter are shown at the top. The GFP foci in wheat leaf sections indicate the expression of reporter genes. Act1RFP was included in each transient expression assay to demonstrate that leaf cells were transformed. Each leaf section was examined in both GFP and RFP channels.