INTRODUCTION

Auxin, a plant hormone, plays important roles in plant growth and development (Guilfoyle, 2007; Guan et al., 2017; Luo et al., 2018). According to a model of the auxin-mediated signalling pathway in arabidopsis (Dharmasiri et al., 2005; Chapman and Estelle, 2009) at low auxin level, auxin response factor (ARF) binds to an auxin response element (AuxRE) together with Aux/IAA. Aux/IAA represses ARF activity and auxin-responsive gene expression. With high auxin concentrations, auxin receptor E3 ubiquitin ligases TIR1/AFB and Aux/IAA form a complex with auxin (Gray et al., 2001; Tan et al., 2007; Calderón Villalobos et al., 2012). The complex initiates degradation of the Aux/IAA proteins by the 26S proteasome, then de-represses ARF activity and enables auxin-responsive gene activation (Zenser et al., 2001; Kepinski and Leyser, 2005).

Previous studies demonstrated that ARFs as transcription factors could bind to TGTCCTC-containing AuxREs in the promoters of auxin-responsive genes to activate or repress their expression (Ulmasov et al., 1997, 1999a; M. Zhang et al., 2017). ARF proteins are conserved, and most of them consist of three domains, i.e. an N-terminal DNA-binding domain (DBD), variable middle regions as activation (AD) or repression (RD) domains, and a C-terminal domain (CTD) (Tiwari et al., 2003; Korasick et al., 2014). The ARF DBD is a plant-specific B3 type that binds to AuxREs. Its nuclear magnetic resonance (NMR) solution structure has also been determined (Yamasaki et al., 2004). The variable middle regions determine whether it is a transcription activator or a repressor. The AD is enriched in glutamine (Q), serine (S) and leucine (L), whereas the RD is enriched in serine (S), proline (P), leucine (L) and/or glycine (G) (Chandler, 2016; Nanao et al., 2014). The CTD is a dimerization domain for combining with ARFs or Aux/IAA proteins (Kim et al., 1997; Ulmasov et al., 1999b).

The ARF family in arabidopsis contains 22 members and one pseudogene (ARF23) (Guilfoyle and Hagen, 2007; Remington et al., 2004), and rice contains 25 ARF members (Wang et al., 2007; Zhang et al., 2018). The functions of ARFs in arabidopsis were revealed mainly by study of arf mutants. AtARF2 has...
roles in seed size, fertility, senescence and hormone cross-talk (Okushima et al., 2005; Promchuea et al., 2017). AtARF3 functions in pattern development of floral meristems and reproductive organs (Sessions et al., 1997), while AtARF5 works in embryo axis formation and vascular tissue development (Hardtke and Berleth, 1998). AtARF7 is involved in aerial tissue growth (Harper et al., 2000). AtARF8 functions in fruit initiation (Goetz et al., 2006) and AtARF19 is active in hormone cross-talk (Li et al., 2006). Some AtARFs have redundant roles in plant development (Hardtke et al., 2004). However, research into ARF family members in wheat is lacking.

Wheat is a staple food crop worldwide, and its root and plant architectures strongly affect grain yield (Li et al., 2016). As a hexaploid species, wheat has a large and complex genome (AABBDD) that poses a challenge for gene discovery and determining gene function (International Wheat Genome Sequencing Consortium, 2014; Luo et al., 2017; Zhao et al., 2017; Appels et al., 2018; Ling et al., 2018). Association analysis is an efficient approach to reveal relationships between genes and traits (Bradbury et al., 2007; Wang et al., 2016). Pyramiding elite alleles through marker-assisted selection (MAS) greatly enhances the efficiency of wheat breeding (Collard and Mackill, 2008). Thus, finding elite alleles and developing functional markers are fundamental to genetic improvement of the crop.

In this study, three copies of TaARF4 were isolated from wheat and characterized. Ectopic expression of TaARF4 in arabidopsis resulted in reduced H833 expression and response to abscisic acid (ABA). TaARF4-overexpressing plants also had shorter primary roots and reduced plant height; it is proposed that this is due to repressing Gricthen Hagen 3 (GH3) gene expression that mediates indole-3-acetic acid (IAA) homeostasis. Association analysis showed that TaARF4-B was strongly associated with plant height (PH) and root depth of wheat. The geographic distribution and allelic frequencies demonstrated that TaARF4-B haplotypes were selected in the history of Chinese wheat breeding.

MATERIALS AND METHODS

Identification of TaARF4 and construction of a phylogenetic tree

Using the URG1 website tBLASTn program and arabidopsis ARF2 N-terminal DBD as a query sequence, we isolated an ARF member (named TaARF4) with cDNA (AK335756.1) and protein (CDM85878.1) sequence accession numbers in GenBank; nothing was known about its function. The full-length cDNAs of the homoeologous genes TaARF4-A, TaARF4-B and TaARF4-D were cloned by reverse transcription–PCR (RT–PCR) from wheat cultivar Hanxuan 10. ARF family members were identified from different plant species by a protein BLAST search in the NCBI database. The full-length sequence was used to construct a phylogenetic tree by the maximum likelihood method through the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.2.

Expression pattern analysis of TaARF4 in wheat

Hanxuan 10, a drought- and heat-tolerant common wheat cultivar, was used for genomic sequence isolation of TaARF4 and gene expression pattern analysis. Root and leaf tissues from 2-week-old seedlings and different tissues at the flowering stage (stem, leaf and spike, and root tissues from different depths) were sampled for spatio-temporal expression pattern analysis. Two-week-old wheat seedlings were sprayed with 50 μM ABA solution, then whole plants were sampled for ABA-induced expression analysis at 0, 1, 3, 6, 12, 24 and 48 h after treatment. Total RNAs were extracted using Trizol reagent (Invitrogen, 15596-018). cDNA was synthesized with a reverse transcription kit (TiangEN, KR104). SYBR Premix Ex Taq (TaKaRa, DRR820A) was used for real-time PCR on an ABI QuantStudio® 7 Flex analyser according to the manufacturer’s instructions. Because the nucleotide sequences of TaARF4-A, TaARF4-B and TaARF4-D were highly conserved, it was difficult to distinguish them; therefore, a common primer pair for all three copies was designed by Primer Premier 5 software (Supplementary Data Table S1). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal control. Three biological replicates and three technical replicates were assayed for each sample.

Sub-cellular localization of TaARF4 protein and production of TaARF4–GFP transgenic lines

The full-length cDNA of TaARF4-A was cloned into the modified vector pCAMBIA1300 at the XbaI and SpeI sites under control of the 35S promoter and with a green fluorescent protein (GFP) tag. Primers are listed in Supplementary Data Table S2. The constructs and empty vectors were separately transfected into Agrobacterium tumefaciens strain GV3101 by electroporation. For transient expression, Agrobacterium was cultured in 5 mL of YEB medium supplemented with 100 mg mL−1 rifampicin, 50 mg mL−1 kanamycin then infiltrated into the abaxial side of 5-week-old Nicotiana benthamiana leaves. The Agrobacterium-infected tobacco plants were grown in a greenhouse for 3 d before GFP fluorescence in leaves was observed with a confocal laser scanning microscope (Zeiss LSM700). At the same time, stable transgenic arabidopsis lines were produced by infection with Agrobacterium by the floral dip method (Clough and Bent, 1998). Transformants were selected on agar plates containing 50 μg mL−1 hygromycin B. GFP fluorescence in homoyzgyous T1 generation plants was detected in 1-week-old transgenic plant roots by a Zeiss LSM700 microscope, and DAPI (4’,6-diamidino-2-phenylindole) was used to stain nuclei.

Purification of TaARF4-Δ protein and electrophoretic mobility shift assays (EMSAs)

The TaARF4-Δ N-terminus containing the DBD (TaARF4-Δ, amino acids 1–350) was fused with a glutathione S-transferase (GST) tag and expressed in Escherichia coli BL21 cells. The recombinant protein was induced by 0.2 mM isopropyl-β-D-thiogalactoside (IPTG), and the E. coli were incubated at 18 °C for 12 h. TaARF4-Δ protein was purified by glutathione–Sepharose 4B (GE Healthcare, 52-2303-00). P3 containing an inverted repeat of AuxRE was used as a
probe. Cold (unlabelled) P3, cold mutated P3, biotin-labelled P3, biotin-labelled mutated P3 and their reverse complementary sequences were synthesized and annealed. EMSA was performed using a LightShift® Chemiluminescent EMSA Kit (Thermo Scientific, 20148) according to the manufacturer’s instructions. Briefly, probes and protein in binding buffer were incubated at room temperature for 20 min. The binding reaction mixes were separated in 5% native polyacrylamide gels. DNA fragments in the gels were transferred to nitrocellulose membranes. After cross-linking, the membranes were incubated in blocking buffer, and then transferred to conjugate/blocking buffer. After washing, membranes were incubated in Substrate Equilibration Buffer, followed by Substrate Working blocking buffer. After incubation for 2 d at 4 °C, the plates were transferred to a growth chamber with a 23 h light (21 °C)/1 h darkness (19 °C) cycle. Numbers of seedlings with cotyledon greening were scored after cultivation for 1 week.

Root length and plant height. Seeds for root growth assays were sown on MS plates and grown in a chamber with 23 h light (21 °C)/1 h darkness (19 °C) for 5 d. Seedlings were then transferred to MS medium vertical culture with root tips placed at the same level. Photographs were taken after 7 d, and new growth parts of the primary roots were measured and analysed by Image J software. The growth rate for each genotype was calculated. At least 15 plants from three Petri dishes were measured for each experiment, and three independent biological experiments were performed. Two-month-old seedlings growing in a forest soil:vermiculite (1:1) mixture with 16 h light (21 °C)/8 h darkness (19 °C) in a greenhouse were used to assess plant height. At least 15 seedlings were measured from three pots for each experiment, and three independent biological experiments were performed.

Detection of gene expression

Total RNAs were extracted from 2-week-old Columbia and transgenic arabidopsis; cDNA synthesis and real-time PCR were performed as mentioned above. \( HB33, GH3.2 \) and \( GH3.5 \) gene expression patterns were determined using \( Tubulin \) as the control. Primers are listed in Supplementary Data Table S1.

Chromatin immunoprecipitation (ChIP) analyses

Chromatin immunoprecipitation was carried out on 2 g of 2-week-old transgenic arabidopsis plants (GFP and TaARF4-GFP) according to the method of Saleh et al. (2008). Briefly, after DNA and protein were cross-linked with 1% formaldehyde, chromatin was isolated, and DNA was sheared on ice by sonicating five times for 15 s at 1 min intervals using an LTRASONIC PROCESSOR-500. Salmon sperm DNA/protein A agarose (Millipore, 16-157) and anti-GFP antibody (Abcam, AB290) were used to precipitate the DNA and protein complex. Then DNA and protein were reverse cross-linked, and DNA was precipitated. The precipitates were separately dissolved in 500 μL of TE to carry out real-time PCR using 5 μL of ChIP product for each one. Three independent biological experiments, each with three technical replicates, were performed. Primers are shown in Supplementary Data Table S3.

Free IAA extraction and analysis

Three independent replicates were performed. For each replicate, about 200 mg of 2-week-old fresh seedling tissue were ground in liquid nitrogen, weighed and IAA was extracted at −20 °C for 24 h with 2 mL of cold methanol. Antioxidant and internal standard (2 H 2 -IAA, CDN Isotopes) were added and samples were purified using an Oasis MAX solid-phase extract cartridge (150 mg 6 mL−1; Waters), and analysed by a UPLC-MS/MS system (ACQUITY UPLC; Waters and Quattro Premier XE; Waters) as previously described (Wang et al., 2015). This part of the work was conducted on a plant hormone platform at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

Gene sequence polymorphism and functional marker development

Thirty-two wheat accessions with wide variation identified by simple sequence repeat (SSR) markers were chosen to sequence TaARF4 fragments for analysis of polymorphisms. Genome-specific primers were designed for amplification of TaARF4-A, TaARF4-B and TaARF4-D fragments from the A, B and D genomes (Supplementary Data Table S4). Purified PCR products were ligated into pEASY-Blunt vectors and transformed into E. coli top 10 competent cells. Positive clones were selected and sequenced with an ABI 3730 DNA Analyzer. Sequence polymorphism was analysed by SeqMan software. Derived cleaved amplified polymorphic sequence (dCAPS) primers for target genes were developed based on dCAPS Finder 2.0 software (http://helix.wustl.edu/dcaps/dcaps.html). The genotypes of the wheat accessions were identified as follows: first, TaARF4 genes in A, B and D genomes were amplified using genome-specific primers; second, 1 μL of PCR product as template was subjected to a second round of PCR; third, the second-round PCR products were digested by restriction endonucleases and electrophoresed in 4% agarose gels.

Wheat populations used in the study

Five wheat germplasm populations were used for different research purposes. Accession names in the populations are listed in Supplementary Data Table S5.
Population 1 consisted of 150 doubled haploid (DH) lines derived from the cross Hanxuan 10 × Lumai 14. This population was employed for gene mapping and analysis of the effects of different haplotypes. Population 2 consisted of 262 accessions mainly from the Northern Winter Wheat Zone and Yellow and Huai River Valleys Facultative Wheat Zone, and was used for association analysis between agronomic traits and genotypes. Population 3 consisted of 323 accessions from the same region and was used for association analysis between root traits and haplotypes. Population 4 consisting of 157 landraces and Population 5 consisting of 348 modern cultivars from all of ten major wheat zones of China were used to determine the temporal and spatial distributions of haplotypes.

Agronomic and root traits

Population 1 and Population 2 were planted at Shunyi (40°23′N, 116°56′E) and Changping (40°13′N, 116°13′E), Beijing, over 3 years (2010–2012) for measurement of plant height under two water regimes, drought-stressed (DS) and well-watered (WW). The amounts of rainfall during the three growing seasons were 131, 180 and 158 mm, respectively. DS plots were rain-fed, while WW plots were irrigated with 750 m³ ha⁻¹ (75 mm) at the pre-overwintering, booting, flowering and grain fill stages when the amounts of rainfall were insufficient during each corresponding period. A greenhouse covered with polythene at the flowering stage to increase temperature and simulate heat stress (HS) was used at Shunyi. Seven agronomic traits, i.e. PH, length of penultimate internode (LPI), spike length (SL), 1000 grain weight (TGW), number of spikes per plant (NSP), number of spikelets per spike (NSS) and numbers of grain per spike (NGS), were measured under both water regimes, with and without heat stress. Population 3 was planted in PVC tubes, and root phenotypes were recorded at the seedling, tillering, jointing and grain fill stages. Population 5 for investigation of agronomic traits was planted at Luoyang (34°61′N, 112°45′E) in Henan province in 2002 and 2005, and at Shunyi, Beijing in 2010.

Association analysis

Population structure was examined by software Structure v2.3.2 (B. Zhang et al., 2017). Association analysis was performed by the mixed linear model (MLM) in TASSEL 5, in which population structure parameter Q was used. Associations at P < 0.05 were considered significant. Statistical analyses were conducted using SPSS 16.0 software.

Phosphorylated site prediction

TaARF4 protein sequences were submitted to the NetPhos 3.1 Server website (http://www.cbs.dtu.dk/services/NetPhos/). Serine, threonine or tyrosine phosphorylation sites were predicted, and both generic and kinase-specific predictions were performed.

RESULTS

Cloning and structural analysis of TaARF4 genes

Three full-length cDNA sequences of TaARF4 were cloned from wheat cultivar Hanxuan 10, and named TaARF4-A, TaARF4-B and TaARF4-D according to their genomic origins. The three TaARF4 genes were highly similar in sequence, encoding 797, 796 and 796 amino acids, respectively, and very similar in protein structure identity at 99.08 %. Like other ARF family members, TaARF4s consisted of a DBD at the N-terminus, an RD that is usually enriched in serine (S), proline (P), leucine (L) and/or glycine (G) (SPL-RD) in the middle region, and a CTD (Supplementary Data Fig. S1A). The phylogenetic tree (Supplementary Data Fig. S1B) showed that the TaARF4s were more similar to ARF-4s in monocotyledons than those in arabidopsis.

Expression pattern of TaARF4 in wheat tissues

Real-time PCR was performed to identify expression patterns of the TaARF4 genes. TaARF4 was constitutively expressed in wheat tissues (roots and leaves of seedlings; roots, stems, leaves and spikes at flowering). The highest expression level was detected in the spikes (Fig. 1A), and in roots expression levels were lower at greater depths. Constitutive expression of TaARF4 genes suggested that they might have roles in growth of all tissues.

Subcellular localization of TaARF4 protein

Fluorescence was detected in all cell parts of tobacco epidermal leaf cells and root cells of T. aestivum arabiadopsis lines with the empty vector carrying the GFP gene, whereas TaARF4-GFP was specifically distributed in the nucleus (Fig. 1B, C).

TaARF4 protein binds to the AuxRE cis-element in vitro

The promoters of auxin response genes share a consensus sequence (TGTCTC) known as the AuxRE. EMSAs were performed to test whether TaARF4 binds to AuxRE cis-acting elements in vitro. Since we could not purify the entire TaARF4 protein, the TaARF4 N-terminus with the DBD (TaARF4-D, amino acids 1–350) of the corresponding cDNA was cloned into a pGEX-4T1 vector (GST tag). Expression and purification of protein were carried out using E. coli BL21 cultures (Fig. 2A). A DNA-binding band detected with addition of TaARF4-Δ and biotin-labelled P3 probes had slower migration compared with the free probe (Fig. 2B, lane 3), whereas TaARF4-Δ could not bind to biotin-labelled mutated P3 probes (Fig. 2B, lane 7), and no target band was visible in the GST control (Fig. 2B, lane 2). Along with the gradual increase in cold P3 probe concentrations, the biotin-labelled DNA-binding band was diminished (Fig. 2B, lanes 4–6). The cold mutated P3 (mp3) probe did not compete with labelled P3 probes (Fig. 2B, lane 8). These results showed that the TaARF4 N-terminal DBD binds to AuxRE in vitro.
TaARF4 regulates plant growth

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A

![Graph showing relative expression of genes](image)

B

![Fluorescence images of TaARF4 and GFP](image)

C

![DNA abundance relative to input](image)

Fig. 1. Expression patterns of TaARF4 and sub-cellular localization of TaARF4 protein. (A) Tissue expression patterns of TaARF4 were detected by real-time PCR. GAPDH was used as the control. Three independent biological experiments, each with three technical replicates, were carried out. Error bars are 2 x s.e. ‘0–30’ indicates the root section from the ground to 30 cm depth at flowering stage; ‘30–50’ indicates the root section from 30 to 50 cm; ‘50–70’ indicates the root section from 50 to 70 cm; ‘70–90’ indicates the root section from 70 to 90 cm; and ‘90–100’ indicates the root section from 90 to 100 cm. Significant differences were calculated based on ANOVA. (B) GFP and TaARF4–GFP were transiently expressed in transformed tobacco leaf cells. Green fluorescence images are on the left, bright field images are in the middle and merged images are on the right. Scale bars = 100 μm.

Overexpression of TaARF4 enhances ABA resistance in arabidopsis

TaARF4-A, TaARF4-B and TaARF4-D have highly similar sequences, therefore TaARF4-A was chosen to represent all three TaARF4 genes for characterization of functions. TaARF4-A in a vector in which its expression was driven by the 35S promoter was transformed into arabidopsis. The Col and empty vector line (GFP) were used as controls. All six transgenic lines showed higher TaARF4 expression levels than the controls (Supplementary Data Fig. S2). We selected transgenic lines OE3 and OE4 to investigate stay green in cotyledons in response to ABA. Under normal conditions (MS medium), there was no significant difference between the controls and TaARF4 overexpression lines. Following culture on MS medium with 0.5 μM ABA for 7 d, the cotyledon greening ratio was about 67% for the controls, compared with about 97% for the two overexpression lines (Fig. 3A, B). We also detected the expression level of TaARF4 under ABA treatment. As shown in Fig. 3C, the transcription level of TaARF4 was upregulated by ABA and reached its highest level on exposure to ABA for 12 h, a level which was about 5-fold that of the non-treated control. At 48 h, the expression level declined to a similar level to that of the control. These results indicated that overexpression of TaARF4 led to resistance to ABA, and that TaARF4 negatively regulates ABA response in arabidopsis.

TaARF4 takes part in ABA response by binding to the HB33 promoter and regulating its expression

It was reported that lots of homeobox gene family members took part in abiotic stress response and one member HB33 had an important role in ABA response (Söderman et al., 1996; Tan and Irish, 2006; Wang et al., 2011; Bhattacharjee et al., 2016). Therefore, we analysed HB33 expression in controls and TaARF4-OE transgenic plants. Expression was lower in TaARF4-OE plants than in the controls (Fig. 4A). There are two AuxREs in the HB33 promoter region, one at −441 bp in the forward direction and the other in the reverse direction at −396 bp (Fig. 4B). ChiP assays were carried out to determine whether TaARF4 could directly bind to the HB33 promoter regions in vivo. GFP antibody was used for precipitations of protein and DNA. DNA abundance relative to the input was detected by real-time PCR. As shown in Fig. 4C, TaARF4–GFP bound to the HB33 promoter region, where there are two AuxRE cis-elements (HB33-F1R1), but could not bind to the Tubulin gene promoter region and HB33 coding region (HB33-F2R2) that lack AuxRE. These results indicated that TaARF4 can take part in the ABA pathway by binding to the HB33 promoter region to regulate HB33 expression.

Overexpression of TaARF4 in arabidopsis led to shorter primary roots and plant height

Since many arf mutants were reported to affect plant development, we investigated the morphological characteristics of TaARF4-overexpressing arabidopsis plants. When 5-day-old seedlings overexpressing TaARF4 were transferred to MS medium in vertical orientation for 7 d, they developed shorter primary roots and longer lateral roots than Col and transgenic GFP controls (Fig. 5A). Primary root growth of the transgenic lines was reduced to about 82% of that of the controls (Fig. 5C). The PHs of 2-month-old transgenic lines planted in soil were about 85% of that of the controls (Fig. 5B, D). Collectively, these data indicate that TaARF4 has roles in root growth and PH.
TaARF4 binds to the GH3 promoters, and regulates GH3 gene expression and IAA homeostasis in arabidopsis

Three kinds of genes respond to auxin, namely Aux/IAA, SAUR and GH3 (Nemhauser et al., 2006). The GH3 gene was first discovered as an auxin response gene in Glycine max (Hagen et al., 1984). It was shown to conjugate various amino acids to jasmonic acid (JA) and auxin, facilitating hormone activation, storage or transport, and helping to maintain hormone homeostasis (Staswick et al., 2005). However, the relationship of TaARF4 and GH3 is not clear. We therefore checked GH3 gene expression and IAA homeostasis of TaARF4-overexpressing arabidopsis plants. Expression levels of GH3.2 and GH3.5 were lower in TaARF4 plants compared with the controls (Fig. 6A). In the GH3.2 promoter region there is one AuxRE at −248 bp; and in the GH3.5 promoter region, there are two AuxREs at −172 bp and −671 bp (Fig. 6B). ChIP assays using GFP antibody were carried out to determine whether TaARF4 could directly bind to the GH3 promoter regions in vivo. Real-time PCR results indicated that TaARF4-GFP bound to the AuxRE regions, not the coding regions. In the absence of the GFP antibody, we could not detect TaARF4-GFP bound to DNA fragments (Fig. 6C). Free IAA contents of OE3 and OE4 were 12.1 and 14.9 % higher than in the Col wild type (Fig. 6D). Thus, it is proposed that TaARF4 can bind to the promoter region of GH3 genes to repress GH3 gene expression, and further repress free IAA changing into other forms, leading to higher free IAA content in OE plants. This suggests that the shorter primary root length and reduced height of TaARF4-overexpressing arabidopsis plants might be due to higher free IAA content, which inhibits apical dominance.

Sequence polymorphism and genetic mapping

Genome-specific primers were designed based on the polymorphism detected in the flanking regions of TaARF4 genes in three genomes. Using these primers, TaARF4-A, TaARF4-B and TaARF4-D genomic fragments were separately cloned from A, B and D genomes; their lengths were 4584, 4375 and 4123 bp, respectively. We sequenced genomic fragments from 32 diverse accessions, and no nucleotide polymorphism was detected in the flanking regions of TaARF4 genes.

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Fig. 3. Overexpression of TaARF4 enhances arabisopsis resistance to ABA. (A) Cotyledon greening of Columbia (Col), GFP and two TaARF4-OE lines on MS or MS supplemented with 0.5 μM ABA. After sterilization, seeds were imbibed on MS medium plates with or without ABA and cultured in a growth chamber for 7 d. Scale bar = 2 cm. (B) Statistical analyses of cotyledon greening rates. There were three independent experiments, each with three replicates (in different plates), and in each replicate at least 25 seeds were counted. Error bars are 2× s.e. *P < 0.05, **P < 0.01 (Student’s t-test). (C) Expression patterns of TaARF4 following ABA treatment were detected by real-time PCR. Two-week-old wheat seedlings were sprayed with 50 μM ABA solution, and whole plants were harvested at 0, 1, 3, 6, 12, 24 and 48 h after treatment. GAPDH was used as the control. Three independent biological experiments, each with three technical replicates, were carried out. Error bars are 2 × s.e. Significant differences were calculated based on ANOVA.

Fig. 4. HB33 expression in TaARF4-OE plants and ChIP assay on the promoter of HB33. (A) Expression of HB33 was negatively regulated by TaARF4. Expression of HB33 was detected in 2-week-old seedlings of Col, GFP and two TaARF4-OE plants. Three biologically independent experiments, each with three technical replicates, were performed. Error bars are 2 × s.e. *P < 0.05, **P < 0.01 (Student’s t-test). (B) Bars indicate putative TaARF4-binding sites of HB33. The ATG translation start site is indicated at position +1. Primers used for ChIP PCR are listed in Supplementary Data Table 3. (C) ChIP assay of TaARF4 binding to the promoter of HB33. Col and empty GFP seedlings were used as negative controls. Two transgenic lines (TaARF4-GFP-OE3 and TaARF4-GFP-OE4) and GFP antibody were used for the ChIP assay. Real-time PCR was carried out to show DNA abundance relative to input. Three biological replicates were performed, and each biological replicate had three technical replicates. Error bars are 2 × s.e. Significant differences were calculated based on ANOVA.
Association analysis of TaARF4-B haplotypes and agronomic traits

To investigate the phenotypic effects of the two TaARF4-B haplotypes, Population 2 consisting of 262 accessions was used for association analysis of a range of agronomic traits. Among 16 environments (years × sites × water regimes × heat treatment), significant associations between TaARF4-B and LPI were identified in 14 environments (except 2010CPDS and 2012CPWW). TaARF4-B was strongly associated with PH in 14 environments (except 2011SYWW and 2012SYWW) (Table 1). The phenotypic variation in LPI explained by TaARF4-B ranged from 2.44 to 10.54 %, and the phenotypic variation in PH explained by TaARF4-B ranged from 4.26 to 8.82 %. Accessions with HapI had significantly shorter LPI than those with HapII in all the environments except 2010CPDS. HapI reduced LPI by 1.47–2.75 cm compared with HapII (Fig. 8A). Accessions possessing HapI had significantly shorter PH than those with HapII in all 16 environments. HapI reduced PH by 5.97–10.35 cm compared with HapII (Fig. 8B).

The effects of TaARF4-B haplotypes were confirmed in two other populations. Accessions possessing TaARF4-B HapI in Population 1 also had significantly shorter PH than those with HapII in 13 of the 16 environments. HapI reduced PH by 3.67–9.10 cm compared with HapII (Fig. 8C). Similar results were obtained for Population 5, where HapI reduced PH from 5.23 to 6.67 cm relative to HapII (Fig. 8D). The results suggested that the TaARF4 haplotype affected plant growth, and TaARF4 HapI was considered a superior allele for reducing PH.

Association analysis between TaARF4-B haplotypes and root phenotype

We also performed an association analysis between TaARF4-B haplotypes and root depth phenotype at the seedling, tillering, jointing and grain fill stages. Significant associations were identified between haplotypes and root depth at tillering \( (P = 0.035, PVE = 4.50 \%) \), jointing \( (P = 0.020, PVE = 5.48 \%) \) and grain fill \( (P = 0.015, PVE = 5.94 \%) \) stages. HapI accessions had shallow roots, which were 2.46 cm shorter compared with those of HapII at tillering, 4.71 cm at jointing and 7.00 cm at grain fill stages (Fig. 8E).

Geographic distribution of TaARF4-B haplotypes in ten Chinese wheat zones

Chinese wheat production regions are divided into ten ecological zones based on growing season and ecological conditions. Landraces (Population 4) and modern cultivars (Population 5) were used to investigate the distribution of TaARF4-B haplotypes (Fig. 9A, B). For the landraces, HapII was the dominant haplotype in seven zones (except Zone II...
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The average frequency of Hap I was 56%. For modern cultivars, Hap I was the dominant haplotype in almost all zones, with the only exception being Zone IV (45%). The average frequency of Hap I was 67%. From Chinese landraces to modern cultivars, the frequencies of Hap I increased across eight zones [with the exception of Zone V (from 60 to 50 %) and Zone X (from 44 to 40 %)]. Comparisons of landraces and modern cultivars indicated that TaARF4-B Hap I was positively selected in wheat breeding.

**TaARF4-B haplotypes were selected during wheat breeding**

Wheat breeding is a process of accumulating favourable haplotypes, and leaves footprints in genomes. In order to verify further that selection had occurred for a particular haplotype, we determined haplotype frequency changes according to cultivar release dates. PH continually declined from pre-1960s to post-1990s, whereas the frequency of Hap I increased from 57.1 % pre-1960s to 74.1 % in the 1960s (Fig. 9C). After the 1960s, the frequency of Hap I remained stable while PH continued to decline, implying selection of reduced height based on other genes.
Table 1. TaARF4-B haplotypes associated with agronomic traits in 16 environments

| Year | Site | Environment | LPI  | PH  |
|------|------|-------------|------|-----|
|      |      |             | P-value | PVE (%) | P-value | PVE (%) |
| 2010 | CP   | DS          | n.s.  | 2.44 | 0.0247* | 7.16 |
|      | CP   | WW          | 0.0022*** | 6.00 | 0.0374* | 5.26 |
|      | SY   | DS          | 0.0185* | 7.42 | 0.0472* | 6.27 |
|      | SY   | WW          | 0.0105* | 5.42 | 0.0288* | 4.64 |
|      | SY   | DS–HS       | 0.0184* | 7.55 | 0.0401* | 5.53 |
|      | SY   | WW–HS       | 0.0115* | 6.76 | 0.0361* | 6.52 |
|      | SY   | DS          | 0.0327* | 8.32 | 0.0498* | 6.21 |
|      | SY   | WW          | 0.0161* | 7.28 | n.s.    | 5.34 |
|      | SY   | DS–HS       | 0.0148* | 8.31 | 0.0305* | 5.89 |
|      | SY   | WW–HS       | 5.79E-04*** | 7.75 | 0.0130* | 4.88 |
| 2011 | CP   | DS          | 0.0129* | 6.21 | 0.0141* | 7.20 |
|      | CP   | WW          | n.s.   | 3.85 | 0.0406* | 4.88 |
|      | SY   | DS          | 0.0204* | 3.59 | 0.0422* | 4.26 |
|      | SY   | WW          | 0.0126* | 5.82 | n.s.    | 4.75 |
|      | SY   | DS–HS       | 0.0075** | 10.19 | 0.0107* | 8.12 |
|      | SY   | WW–HS       | 0.0046*** | 10.54 | 0.0143* | 8.82 |

LPI, length of penultimate internode; PH, plant height; n.s., not significant; *P < 0.05, **P < 0.01 and ***P < 0.001; PVE, phenotypic variation explained.

The environments were at Changping (CP) and Shunyi (SY) under well-watered (WW), drought-stressed (DS) and/or heat-stress (HS) conditions in 2010–2012.

**DISCUSSION**

Optimizing root and plant architecture is regarded as an important objective for wheat breeding. Therefore, it is essential to understand the molecular mechanisms of root growth and plant development. Here, we identified a novel ARF gene family member, TaARF4, and demonstrated that TaARF4 overexpression in arabidopsis caused shorter primary root length and PH. We propose that this effect occurs by repressing GH3 gene expression to mediate IAA homeostasis. Meanwhile, association analysis results showed that variation in TaARF4-B was significantly associated with root depth and PH. Therefore, TaARF4 may be an important gene resource for regulating wheat growth, and dCAPS markers of TaARF4 might be useful for the selection of wheat genotypes with optimal plant architecture.

**ARFs and their target genes**

ARFs as transcription activators or repressors participate in the auxin pathway, and affect auxin-responsive gene expression (Hagen and Guilfoyle, 2002; Weijers et al., 2018). Identification of the target genes for ARFs is a major step in studying their function. Previous research by ChIP, EMSA and gene expression analyses proved that Aux/IAA genes are direct targets of ARF5/MP transcriptional regulation (Krogan et al., 2014). ATHB8 (Arabidopsis thaliana Homeobox Gene 8) is a target of ARF5/MP which regulates pre-procambial cell state acquisition by auxin signalling in arabidopsis leaves (Donner et al., 2009). DRN (DORNROSCHEN) is another target of ARF5/MP in the arabidopsis embryo (Cole et al., 2009). PLT (PLETHORA) transcription requires ARF5/MP and NPH4/ARF7 to mediate embryonic root patterning (Aida et al., 2004). In rice, CRL1 (crown rootless 1) is a target of ARF in crown root formation (Inukai et al., 2005). Thus, ARFs target different genes and have different functions. Our research indicates that overexpression of TaARF4 leads to ABA insensitivity by targeting HB33, and reduces root length and PH by targeting GH3 genes.

**TaARF4, GH3s and IAA homeostasis**

Indole-3-acetic acid, as the major auxin form in plants, plays an important role in plant growth (Benková et al., 2003; Mockaitis and Estelle, 2008). Biosynthesis, catabolism and conjugation of IAA significantly affect plant development (Ljun et al., 2002; González-Lamothe et al., 2012). IAA has many conjugate forms, such as ester conjugates with sugars, and amide conjugates with amino acids (Penick et al., 2009). IAA amide forms IAA-Ala, IAA-Leu and IAA-Phe are forms that store IAA, whereas IAA-Asp and IAA-Glu are degradation precursors (Ludwig-Müller, 2011). These forms maintain IAA homeostasis. GH3s have roles in multiple processes (Ludwig-Müller et al., 2009). There are 19 GH3 family members in arabidopsis, and at least seven members (GH3.2–GH3.6, GH3.9 and GH3.17) are encoded by group II genes and can catalyse IAA amide synthesis (Staswick et al., 2005). In this study, we showed that TaARF4 overexpression in arabidopsis led to low GH3 gene expression and higher free IAA content. The high free IAA that inhibits apical dominance also reduced primary root length and PH.

**TaARF4-B haplotypes and phosphorylation status**

Thirteen variants were identified among two haplotypes of TaARF4-B, but only one amino acid change was discovered, threonine to alanine at amino acid position 158. This amino acid is located in a conserved DBD. Threonine can be phosphorylated whereas alanine is dephosphorylated site. Using the NetPhos 3.1 Server, we predicted phosphorylated sites in TaARF4 and found that this site can be phosphorylated by...
DNA-dependent protein kinase (Supplementary Data Fig. S4). This predicts that TaARF4-B HapI might be phosphorylated at amino acid position 158, whereas HapII cannot be phosphorylated at this site. In plants, phosphorylation of transcription factors is an important regulatory mechanism, especially in hormone signalling pathways (Hill, 2015). For example in arabidopsis, the phosphorylation status of BZR1/2 affects target gene expression in the brassinosteroid (BR) signalling pathway (Wang et al., 2012). In the ABA signalling pathway, the phosphorylation status of RAV1 affects expression of ABI2, ABI4 and ABI5 (Feng et al., 2014). Similarly, the phosphorylation status of ARF2 is important to ARF2 function. In the BR signalling pathway, ARF2 can be phosphorylated by BIN2, resulting in loss of DNA binding and repression activities (Vert et al., 2008). Low potassium triggers a Ser689 phosphorylation in ARF2 and relieves its repression on HAK5 (Zhao et al., 2016). So different TaARF4-B haplotypes in wheat accessions may lead to a difference in TaARF4-B phosphorylation status, which in turn may lead to different root depth and PH.
SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aoab and consist of the following. Figure S1: structural analysis and phylogenetic tree of TaARF4 protein and homologous ARF proteins. Figure S2: semi-quantitative PCR detecting TaARF4-A expression levels in arabidopsis. Figure S3: mapping of TaARF4-B on wheat chromosomes. Figure S4: potential phosphorylation sites in TaARF4-B predicted by the NetPhos 3.1 Server. Table S1: primers used for real-time PCR. Table S2: primers used for vector construction. Table S3: primers used for ChIP assays. Table S4: primers used for genomic fragment isolation, sequencing and marker development. Table S5: accession names in Populations 2, 3, 4 and marker development.

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