Characterization and expression quantitative trait loci analysis of TaABI4, a pre-harvest sprouting related gene in wheat

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Abstract

Pre-harvest sprouting (PHS) induced by the absence of seed dormancy causes a severe reduction in crop yield and flour quality. In this study, we isolated and characterized TaABI4, an ABA-responsive transcription factor that participates in regulating seed germination in wheat. Sequence analysis revealed that TaABI4 has three homologues, located on chromosomes 1A/1B/1D. TaABI4 contains a conserved AP2 domain, and AP2-associated, LRP and potential PEST motifs. Putative cis-acting regulatory elements (CE1-like box, W-box, ABRE elements and RV elements) were identified in the TaABI4 promoter region that showed high conservation in 17 wheat cultivars and wheat-related species. Expression profiling of TaABI4 indicated that it is a seed-specific gene accumulating during the middle stages of seed development. Transcript accumulation of TaABI4 in wheat cultivar Chuanmai 32 (CM32, PHS susceptible) was 5.07-fold and 1.39-fold higher than that in synthetic hexaploidy wheat SHW-L1 (PHS resistant) at 15 and 20 DPA, respectively. Six expression quantitative trait loci (eQTL) of TaABI4 on chromosomes 2A, 2D, 3B and 4A were characterized based on the accumulated transcripts of TaABI4 in SHW-L1 and CM32-derived recombinant inbred lines. These QTLs explained 10.7 to 46.1% of the trait variation with 4.53–10.59 of LOD scores, which contain genes that may affect the expression of TaABI4.

Introduction

Pre-harvest sprouting (PHS) is the germination of grain prior to ripening in the spike when there is excessive moisture before harvest. PHS has become a recurring worldwide problem since it causes a severe reduction in crop yield and flour quality due to starch and protein degradation (Olaerts et al., 2016). Seed dormancy accounts for up to 60% of the variation in PHS tolerance, and PHS in wheat is mainly caused by the lack of adequate seed dormancy (DePauw and McCaig, 1991; Li et al., 2004). The level of wheat grain dormancy partly depends on abscisic acid (ABA) sensitivity before and after the grain reaches physiological maturity (Gubler et al., 2005; Sun et al., 2005; Shu et al., 2016). One well-characterized positive regulator of ABA signalling, ABSCISIC ACID-INSENSITIVE 4 (ABI4), was initially identified in screens for mutants exhibiting ABA-resistant germination (Finkelstein, 1994). ABI4 is a member of the APETALA 2 (AP2/ERF) transcription factor family and can activate or repress gene expression by binding to specific cis-elements in gene promoters via its AP2 DNA-binding domain (Wind et al., 2013). It has been documented that ABI4 interacts with target genes to regulate seed dormancy and germination. For example, ABI4-dependent temporal regulation of PTR2 expression influences water status during seed germination, promoting the germination of imbibed grain (Choi et al., 2020). ABI4 is indispensable for inhibiting the expression of three family members of Arabidopsis response regulators (ARRs), namely, ARR6/7/15, which are involved in seed dormancy (Huang et al., 2017). Moreover, ABI4 is a primary positive regulator of ABI4, ABI5 and starch branching enzyme 2.2 (SBE2.2), activating transcription by binding the CACCG-box (CE1-like) in the promoter regions during seed development (Bossi et al., 2009). Apart from ABI4 itself, various transcription factors may regulate ABI4 transcription, including several WRKY transcription factors that can bind to the W-box sequence in the ABI4 promoter region (Shang et al., 2010; Antoni et al., 2011; Liu et al., 2012). MYELOBLASTOSIS 96 (MYB96) induces ABI4 expression by binding to its promoter during seed germination and seedling development (Lee and Seo, 2015).
In addition to ABI4, two other transcription factors (ABI3/VPI1 and ABI5) have been characterized that regulate ABA response during seed development (Finkelstein, 1994; Finkelstein and Lynch, 2000; Osa et al., 2003). It has been reported that some cross-regulation of expression existed among ABI3, ABI4 and ABI5, which function in a combinatorial network, rather than a regulatory hierarchy, controlling seed development and ABA response (Soderman et al., 2000). Moreover, ABI3, ABI4 and ABI5 have similar effects on seed dormancy and the expression of maturation-specific seed proteins (Finkelstein, 1994). However, ABI4 is a focal point in the signal transduction pathways of ABA (Niu et al., 2002). Orthologues of ABI4 have been reported in many other plant species, including maize, rice and lotus (Niu et al., 2002; Ming et al., 2013; Wang et al., 2015). In maize, ZmABI4 is seed specific, reaching maximum expression at 20 days post-anthesis (DPA) (Niu et al., 2002). In the rice database, a single sequence shares significant homology with the AtABI4 AP2 domain, indicating that a single ABI4 homologue exists in rice (Yu et al., 2002). However, there is limited information available for ABI4 orthologues in wheat.

Synthetic hexaploid wheat SHW-L1 obtained from the hybridization of Triticum turgidum and Aegilops tauschii is a useful genetic resource and shows significant tolerance to PHS (Yang et al., 2014). To investigate the regulatory factors that interact with TaABI4 and the role of TaABI4 in the ABA-induced seed dormancy pathway, we performed a conservation analysis on ABI4 in wheat ancestral species and modern cultivars and subsequently cloned this gene. We analysed the expression pattern of TaABI4 at different grain developmental stages. Furthermore, we carried out expression QTL analysis (eQTL) to detect regions regulating the expression of TaABI4 in recombinant inbred lines (RILs). Finally, candidate genes were also predicted and evaluated in the eQTL interval, providing further insight into the role of TaABI4 in ABA signal transduction pathways and into the regulatory framework that controls seed germination in wheat.

Materials and methods

Plant material

Chuanmai32 (CM32, PHS susceptible), synthetic hexaploid wheat (SHW-L1, PHS-resistant) and their derived RILs (138 lines) were grown under glasshouse conditions (16 house light at 22°C, 8 h dark at 12°C, 70% relative humidity). Days to flowering was measured for each spikelet based on the anther extrusion at 50% of the spike. Developing grains from 5 to 30 DPA were collected at 5-d intervals from the center florets for subsequent gene expression profiling. Young leaves of SHW-L1 and CM32 were used for DNA extraction. Each sample had biological replicates and was immediately frozen into liquid nitrogen and stored at −80°C for RNA extraction.

Sequence characterization and in silico promoter analysis

Based on the results of BLASTP searches, we obtained coding sequences of TaABI4 in Chinese Spring using EnsemblPlants (http://plants.ensembl.org/index.html). Protein domains of genes were predicted using the SMART tool (http://smart.embl-heidelberg.de/). The coding sequences of TaABI4 were used to query the target database (VirgoBLAST, http://202.194.139.32/blast/viroblast.php, The Wheat ‘Pan Genome’, http://www.10wheatgenomes.com/data-repository/, and The Aegeilops tauschii genome, http://aegilops.wheat.ucdavis.edu/ATGSP/data.php) to download homologous genes and 2 kb upstream sequences from translational initiation codon in 17 wheat cultivars and 3 wheat ancestors (Altschul et al., 1997; Luo et al., 2017; Ling et al., 2018; Zhu et al., 2019; supplementary Table S1).

Amino-acid sequences were aligned using DNAMAN (Version 5.2.10, Lynnon Biosoft, Quebec, Canada). Putative cis-acting regulatory elements located in the promoter regions were predicted using PLANTCARE (Lescot et al., 2002; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (Higo et al., 1999; http://www.dna.affrc.go.jp/PLACE/). An analysis of conserved motifs in 17 wheat cultivars was obtained using the Meme suite (Bailey et al., 2009; http://meme-suite.org/tools/meme). This program was used to search for the top five cis-motifs with consensus patterns of 6–50 base width and E-value < 0.01, on the forward strand of the input sequences only.

Prediction of proteins and PEST motifs

Generated coding sequences were translated to predicted proteins using DNAMAN with default parameters. Searches for potential PEST sequences were performed using the ePESTFind (http://www.bioinformatics.nl/cgi-bin/emboss/epestfind). We used the input parameters in all cases and defined that a score above zero denoted a possible PEST sequence (Gregorio et al., 2014).

PCR amplification

According to the TaABI4 nucleotide sequences of Chinese Spring, specific primers for the gene were designed online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and shown in supplementary Table S2. Genomic DNA was isolated from SHW-L1/CM32 young leaves using the CTAB method (Zhang et al., 2013) and was used as templates to amplify the DNA sequences of TaABI4. PCR was performed using high-fidelity Prime STAR Polymerase (TaKaRa, Dalian, China) under the following conditions: 98°C for 3 min, 35 cycles of 98°C for 50 s, 50 s and 72°C for 90 s, followed by a final extension step of 72°C for 10 min. The PCR amplification products were ligated into the pEASY-blunt Cloning Vector (TransGen, Beijing, China), and the resulting ligation mixtures were transformed into E. coli Trans1-T1 chemically competent cells (TransGen, Beijing, China) to obtain positive clones for sequencing.

RNA extraction and expression analysis

Primer pairs in the relevant conserved exon regions of TaABI4 among A, B and D genomes in SHW-L1 and CM32 were used to amplify 151 bp amplicons (supplementary Table S2). The expression level of TaABI4 was measured in the parents at six seed development stages (5, 10, 15, 20, 25 and 30 DPA). RNA was extracted from each sample using the total RNA extraction kit (Biofit, China), and genomic DNA was removed with DNaseI. Three seed-developing stages (10, 20 and 30 DAP) of SHW-L1/CM32 were selected to carry out RNA sequencing (RNAseq). RNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Fremont CA, USA) and checked for integrity on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto CA, USA) by denaturing agarose gel electrophoresis with ethidium bromide staining. Equimolar amounts of the libraries were constructed and sequenced by BerryGenomics (Beijing, China) using the Illumina HiSeq-2000 and HiSeq X Ten platform.
Genes transcript levels were estimated using transcripts per million (TPM; Zhao et al., 2020). First-strand cDNA was synthesized using a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). cDNA sampling was performed in duplicate and SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules CA, USA) was used for real-time quantitative PCR (RT-qPCR) (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad, USA). Each reaction contained approximately 50 ng first-strand cDNA, 0.5 μl, 10 μmol l⁻¹ gene-specific primers and 10 μl real-time PCR SYBR Green (TIANGEN, Beijing, China). Amplification conditions were 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, 40 s at 72°C and a final extension of 10 min at 72°C. Seven 1/10 dilutions of the recombinant plasmid cDNA template were used to make a standard curve for amplification efficiency (E) calculation. Three housekeeping genes, TaActin, Ta.14126.1 and Ta.7894.3.a1_at, were used as internal controls (Long et al., 2010). Gene expression data were analysed using the Bio-Rad CFX Manager (Bio-Rad, Hercules CA, USA) software. The expression profile of the target gene was normalized to that of the internal control genes, and the geometric mean was calculated. The relative gene expression quantity of each sample was calculated using the E−ΔΔCt method (Pfaffl, 2001).

Expression QTL (eQTL) analysis

In order to characterize regions that regulate TaABI4 expression levels, we conducted eQTL mapping analysis within the RIL population using the previously constructed high-density genetic map (Yang et al., 2019). eQTL analysis was achieved using the WinQTLcart2.5 software (North Carolina State University, Raleigh, NC, USA) with the composite interval mapping (CIM) method (Wang and Basten, 2007). The analysis was done by setting the control parameters to model 6 (standard model), forward regression, 10-cm windows and five makers as the control. The threshold was set at 4.0 to detect eQTLs. The wheat reference genome ‘Chinese Spring’, IWGSC RefSeq v1.0 (International Wheat Genome Sequencing Consortium, 2018), was used to query marker positions using the blastn2.2.26+ package (Camacho et al., 2009). On the basis of eQTL intervals, the gene annotation was conducted using Wheat Gmap (http://www.wheatgmap.org.cn/tools/gene/information/). Subsequent candidate genes were validated by querying all of the predictions against the Nr-NCBI (http://www.ncbi.nlm.nih.gov/) and EnsemblPlants (http://plants.ensembl.org). Genes relating to the ABA signalling pathway were compared and mapped to the genome reference sequence of Chinese Spring v1.0 to identify candidate genes that may underlie eQTL. The CORREL function in Excel was used to calculate the correlation coefficient between the expression pattern of TaABI4 and RNAseq data of candidate genes in SHW-L1 and CM32. The correlation coefficient was used to measure the strength of the relationship between two variables.

Results

Sequence characterization of TaABI4

The DNA sequence of ABI4 (AT2G40220) from Arabidopsis was used as a query sequence to carry out BLAST searches in EnsemblPlants. Three homologues of TaABI4 were identified on the A, B and D sub-genomes of 18 wheat cultivars (TaABI4-1A, TaABI4-1B and TaABI4-1D). All the 50 TaABI4 sequences were found to be represented by a single exon. The coding sequences (CDS) of three homologues of Chinese Spring were conserved with 97.53% nucleotide identity. Compared with TaABI4-1A, TaABI4-1B and TaABI4-1D had two 3–6 bp deletions as well as 28 single-nucleotide polymorphisms (SNPs), 13 SNPs of which caused non-synonymous mutations (supplementary Fig. S1). The three homologue-encoded proteins with 260, 256 and 257 amino-acid residues, respectively. The proteins have highly conserved AP2 domains that were also found in previously annotated AtABI4 in Arabidopsis and ZmABI4 in maize (Zea mays) (Fig. 1).
In addition, ten amino acids (KGGPENAKFR) were contiguous to the AP2 domain (designated as the AP2-associated motif). Additionally, a stretch of eight amino acids (LRPLLPRP) identified as the LRP motif was located nearby (Fig. 1). TaABI4 proteins revealed 100% identity in these common regions, while TaABI4-1A contained three additional amino acids, His171, Leu196 and Ala197 (Fig. 1). Putative proteins were predicted from Ae. tauschii, T. dicoccoides cv. Zavitan and T. urartu. The protein sequence of AetABI4 obtained from Ae. tauschii showed 100% identity with the TaABI4-1D sequence. TuABI4-1A obtained from T. urartu showed 99.23% amino acid identity with TaABI4-1A. TdABI4-1B of T. dicoccoides cv. Zavitan shared 98.08% identity with TaABI4-1B (Fig. 1).

### Table 1. Conservation of putative PEST sequences in ABI4 proteins from wheat cultivars

| Name    | Length | Score value | Position (N/C-termini) | Identity (%) | Motif logo |
|---------|--------|-------------|------------------------|--------------|------------|
| ABI4-1A | 21AA   | 0.44        | 219–260(C)             | 99.8         | ![Logo](https://doi.org/10.1017/S0960258521000015) |
| ABI4-1B | 20AA   | 2.08        | 196–256(C)             | 100          | ![Logo](https://doi.org/10.1017/S0960258521000015) |
|         |        | 3.52        | 4–32(N)                | 100          | ![Logo](https://doi.org/10.1017/S0960258521000015) |
| ABI4-1D | 19AA   | 3.13        | 197–257(C)             | 100          | ![Logo](https://doi.org/10.1017/S0960258521000015) |

### ABI4 proteins and putative motif analysis in wheat cultivars

The AP2 domains, the AP2-associated motifs and the LRP motifs were conserved in 50 putative ABI4 homologous proteins in terms of their position and sequence identity (Fig. 2). Putative PEST degradation signals at the terminus of wheat ABI4 proteins with a positive probability value (>0) were detected using the PEST-find program (Rice et al., 2000), which were in agreement with a previous report (Gregorio et al., 2014). This demonstrated that potential PEST sequences were detected in all of these proteins, with probability scores ranging from +0.44 (ABI4-1A) to +3.52 (ABI4-1B) (Table 1). For ABI4-1B and ABI4-1D proteins, one PEST sequence was detected at the C-terminal with a length of 60AA. For ABI4-1A proteins, a shorter PEST motif of 41 amino acids was detected, sharing 99.8% identity among the 17 cultivars in addition to another PEST sequence predicted at the N-terminus that was also identified in TaABI4-1B. Although some variant amino acids were detected in the proteins of each genome, as shown in grey boxes in Fig. 2, they did not locate in the region of crucial motifs. This demonstrates that the ABI4 proteins are conserved in their protein architecture, coinciding with their central role in wheat hormone signalling.

### Potential cis-acting regulatory elements of ABI4 promoters in wheat ancestors

The presence of potential cis-regulatory elements in the upstream (≥2000 bp) region of TaABI4 homologues from wheat cultivar Chinese Spring was analysed. Eleven types of potential cis-acting regulatory elements were identified in the upstream region (Fig. 3). This region was also isolated from T. dicoccoides cv. Zavitan, T. urartu and Ae. tauschii. A putative TATA-box was detected 190 bp upstream of the start codon. A binding site (CE1-like motif, CACCGCCCC) was present immediately downstream from a putative W-box (TTGACY). In addition, MY elements with CATGCATG involved in seed-specific regulation were predicted. ABRE elements known to be involved in ABA response, with CACGTG core motif, were recognized nearby the 5′-termini. ARE elements with an AAACCA core motif that are essential for the anaerobic induction also existed in all ABI4 proteins. Additionally, conserved motifs such as CAAT-box, CAT-box and A-box were detected. One Myb and one Myc

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**Fig. 2.** Protein structure schematic diagram of ABI4 in wheat cultivars. The grey boxes indicate the polymorphism of amino-acid sequences and the black boxes are highly conserved amino-acid sequences. The boxes filled with twill are conserved domains and motifs, and the boxes filled with dots are potential PEST motifs.
element, known to be involved in ABA signalling (Lin, 2009), were predicted in the TaABI4-1D and AetABI4 promoter regions. The detected cis-acting regulatory elements were conserved among the wheat and its ancestral species.

**The putative motif analysis of ABI4 genes in wheat cultivars**

The top five motifs identified by this analysis were found in almost all of the ABI4 genes in wheat cultivars and were highly conserved in terms of number and position (Fig. 4). Although motif 2 did not exist in the A sub-genome of Kronos, it shared 99.4% identity among 50 upstream regions and was regarded as a novel cis-motif with no current description in the PLACE database (Table 2). As shown in Table 2, motif 1 with a W-box as its core element was also conserved in all sequences with 100% identity. Although there were some variable SNPs in motif 3, motif 4 and motif 5, they did not exist in the core region of each motif. Overall, putative motifs within the upstream of ABI4 genes were almost completely conserved in wheat cultivars.

**Cloning and qRT-PCR analysis of TaABI4 in SHW-L/CM32 developing seeds**

The TaABI4 sequences were cloned from SHW-L and CM32, which were highly conserved in these two cultivars (supplementary Fig. S2). According to RNAseq analysis, the expression level of TaABI4 in CM32 was higher than that in SHW-L at each detected stage (Fig. 5A). Then, RT-qPCR assays were performed using cDNA from five time points (5, 15, 20, 25 and 30 DPA) to detect the expression level variation of TaABI4 between SHW-L1 and CM32. During seed development, TaABI4 expression began as early as 10 DPA, increasing between 10 and 15 DPA as the transition from growth to storage phase of grain development (starting after 12 DPA) took place, and peaked at 20 DPA, with a decline in expression until 30 DPA. The expression of TaABI4 in CM32 was higher than that in SHW-L1 in most of the measured stages. Two significant differences in relative expression were detected at 15 DPA (5.07-fold) and 20 DPA (1.39-fold) (Fig. 5B).

**eQTL mapping**

The significant difference between CM32 and SHW-L1 in the expression levels of TaABI4 at 15 and 20 DPA enabled the detection of eQTLs. Based on the consensus genetic map and corresponding SNP marker positions, six significant eQTLs (P < 0.05, LOD > 4) were identified (Table 3 and Fig. 6). One eQTL detected on chromosome 2A at 15 DAP was designated as eQABI4.15DPA.2A.1, with LOD scores at 4.53. Two eQTL regions located on chromosome 2D designated as eQABI4.20DPA.2D.1 and eQABI4.20DPA.2D.2 were detected at 20 DAP, showing 9.63 and 6.38 LOD scores, respectively. eQABI4.20DPA.4A.1 and eQABI4.20DPA.4A.2 were located on chromosome 4D with negative alleles from SHW-L1. They explained 38.2 and 46.1% of the phenotypic variation, respectively. The physical mapping of 3B eQTL was designated as eQABI4.20DPA.3B.1, showing that the corresponding interval location was Chr.3B: 667902308-669428443. All identified eQTLs had negative additive effects, indicating that eQTLs that could reduce the expression of TaABI4 were derived from synthetic wheat SHW-L1. Genes involved in regulatory processes, including hormone response and biosynthesis, signal transduction, protein phosphorylation, starch and sucrose metabolism, were selected in those eQTL regions. Subsequently, genes expressed in seeds and ABA related were highlighted, resulting in five candidate genes being identified (Table 4). Further correlation coefficient analysis of gene expression was carried out (Table 4). Correlation coefficients between the expression of TaABI4 and candidate genes ranged from 0.61 to 0.9. TaABI4 expression positively correlated with the expression of TraesCS2A02G089400, TraesCS2A02G093600, TraesCS4A02G094300 and TraesCS4A02G114400, while it negatively correlated with the expression of TraesCS4A02G093600.

**Discussion**

In this study, we presented the characterization of the wheat ABI4, a gene involved in ABA responsiveness during seed development and germination. TaABI4 proteins from three wheat sub-genomes
were conserved with an AP2 domain required for nuclear localization (AP2-associated motif), as well as regions for transcriptional activation (LRP motif). The conserved domains are used as hallmarks to identify ABI4 orthologues in different species (Gregorio et al., 2014). Although the protein sequences for the three homologues had slight polymorphisms, the overall identity was high (96.9%). Our results suggested that the AP2 proteins presented in wheat are the orthologues of the *Arabidopsis* ABI4.
and should be considered as TaABI4-1A, TaABI4-1B and TaABI4-1D.

Compared with the ABI4 from *T. urartu*, *Ae. tauschii* and *T. dicoccoides*, the amino-acid variation existed only in TaABI4-1A (Thr15/Leu15, Gly218/Arg218) and TaABI4-1B (Ser108/Pro108) and was not located in core regulatory regions (Fig. 1). This indicated that TaABI4 was highly conserved during the polyploidization and domestication processes of wheat. In *Arabidopsis*, the low accumulation of ABI4 resulted from both post-transcriptional and post-translational regulation (Finkelstein et al., 2011). PEST sequences are degradation motifs that can affect protein stability (Gregorio et al., 2014) and are characterized by regions enriched in the amino-acid proline, glutamic acid, serine and threonine (Rogers et al., 1986). Based on the available pan-genome data, we analysed 50 putative ABI4 proteins from 18 wheat cultivars to predict potential PEST motifs. Most of the possible PEST sequences were located in the N-terminal region of the protein and were longer than AtABI4 (Table 1). These differences may cause divergence in post-translational mechanisms compared with *Arabidopsis*. In fact, ZmABI4 also has two PEST motifs located in the N-terminus and C-terminus, showing score values of +3.04 and +0.68, respectively (Gregorio et al., 2014).

The discovery of *cis*-acting regulatory elements in the promoter regions is essential to understanding the spatial and temporal expression patterns of ABI4 genes. The six *cis*-acting regulatory elements were conserved in terms of position and sequence identity (Fig. 3). TATA-box is regarded as the core promoter element, and transcription factors bind to TATA-proximal regions (W-box, CE-1 like) having been shown to regulate downstream gene transcription (Heins et al., 1992; Busk et al., 1997; Phukan et al., 2016). Additionally, A-box and RY element are *cis*-acting regulatory elements, and CAT-box is related to meristem expression in *Arabidopsis* (Sakata et al., 2010). ABRE (ABA-responsive elements) motifs are known to participate in response to ABA (Sarkar and Lahiri, 2013). TaABI4-1D contained

![Fig. 5. The expression pattern of TaABI4 in SHW-L1 and CM32. (A) The expression assays using RNAseq. The y axis denotes TPM (transcripts per kilobase million). (B) The expression assays using qRT-PCR.](https://doi.org/10.1017/S0960258521000015)
two classical ABRE elements that are necessary to constitute an active ABA-responsive complex because a single ABRE is not sufficient to confer ABA responsiveness (Hobo et al., 1999; Zhang et al., 2005; Ganguly et al., 2011). The identification of conserved cis-acting regulatory elements in ABI4 promoters of wheat revealed that other transcription factors might regulate those homologues.

The expression pattern of TaABI4 was variable between modern wheat cultivar CM32 and synthetic wheat SHW-L1. It is noteworthy that TaABI4 showed a higher transcript accumulation in weakly dormant material (CM32) than in dormant material (SHW-L1) during most periods of seed development (Fig. 5). By contrast, seeds of the Arabidopsis abi4 mutant germinated significantly more quickly than wild type (Shu et al., 2013), indicating that the presence of functional ABI4 is important for resistance to PHS. The expressions of both TaABI3 and TaABI5 in SHW-L1 were significantly higher than those in CM32 (Zhou et al., 2016). These results are consistent with the corresponding research results in Arabidopsis and maize, finding that ABI3 and ABI5 are positive regulators of seed dormancy (McCarty et al., 1991; Hoecker et al., 1995; Finkelstein and Lynch, 2000). The gene expression patterns of TaABI3 and TaABI5 were similar to that of TaABI4 in the early and middle stages of seed development (5–15 DPA), signifying that TaABI4 associated the ABA biosynthetic pathway with TaABI3 and TaABI5 as found in Arabidopsis (Lopez-Molina et al., 2002). From these results, other regulatory factors interacting with TaABI4 are required to complete our understanding of the gene networks involving seed germination.

### Table 3. eQTL mapping results of TaABI4 in SHW-L1 and CM32

| eQTLs | eQTL IDs | Chromosome | Position (cM) | LOD | R² | Phenoype | Physical location (bp) |
|-------|----------|-------------|---------------|-----|-----|----------|------------------------|
| eQABI4.15DPA.2A.1 | AX-110535525 | 2A | 241.49 | 4.53 | 0.223 | 15 DAP | 46861724–48957480 |
| eQABI4.20DPA.2D.1 | AX-110535525 | 2D | 179.81 | 9.63 | −9.36 | 20 DAP | 613440336–616018344 |
| eQABI4.20DPA.2D.2 | AX-110535525 | 2D | 181.66 | 6.38 | 0.384 | 20 DAP | 616018344–618242947 |
| eQABI4.20DPA.3B.1 | AX-110535525 | 3B | 243.92 | 4.34 | −4.91 | 20 DAP | 667902308–669428443 |
| eQABI4.20DPA.4A.1 | AX-110535525 | 4A | 20.97 | 7.16 | 0.382 | 20 DAP | 104670825–107567689 |
| eQABI4.20DPA.4A.2 | AX-110535525 | 4A | 31.15 | 10.59 | 0.382 | 20 DAP | 107567689–110374428 |

Fig. 6. eQTL genetic locations in the genetic map. The size of the circles means LOD values. The x axis denotes different chromosomes. The yellow/blue circles indicate eQTLs for TaABI4 at 15 DPA/20 DPA.
eQTL mapping is an efficient approach to identify genetic loci controlling complex crop traits (Chen et al., 2010; Motomura et al., 2013). In this study, we chose 15 and 20 DPA, which are the middle periods of seed development, to identify six significant eQTLs associated with TaABI4 expression variation on chromosomes 2A, 2D, 3B and 4A (Table 3), suggesting that the observed differences in TaABI4 expression in the RIL population were regulated in part by trans-acting factors (Doss et al., 2005). Several previous studies mapped the major QTLs for seed dormancy and PHS tolerance to chromosomes 4A (Mares et al., 2005; Torada et al., 2005; Chen et al., 2008). In this study, two major eQTLs located on chromosome 4A accounted for 38.2 and 46.1% of the phenotypic variance. This result further confirmed that the chromosome 4A harbours QTL and eQTL associated with the PHS resistance is important for wheat. The eQTL regions detected in this study may provide candidate genes that play potential roles in regulating PHS through effects on TaABI4 expression. Thus, eQTLs detected in this study suggested that unidentified genes or indirect regulation genes would affect TaABI4, which causes the different expression patterns of TaABI4 compared with Arabidopsis.

Five putative candidate genes were detected in eQTL internals, and the correlation between the expression of each candidate gene and the expression of TaABI4 was analysed according to the available RNAseq database of SHW-L1 and CM32. A correlation of |−1.0| shows a perfect negative correlation, while a correlation of 1.0 shows a perfect positive correlation.

Table 4. Candidate genes expressed in seeds and ABA-related genes in eQTL interval

| Gene ID       | Chromosome location     | Orthologous genes            | Description in Wheat Gmap | \( r \) |
|---------------|-------------------------|------------------------------|---------------------------|-------|
| TraesCS2A02G089400 | 2A:42494117–42495524   | Ply4 (AT2G38310)             | Abscisic acid receptor    | 0.61  |
| TraesCS2A02G091900 | 2A:52391814–5239012   | OsbZIP62 (Os07g0686100)      | bZIP transcription factor | 0.90  |
| TraesCS4A02G093600 | 4A:101402669–101405632 | OsPP1 (Os03g0268000)         | Serine/threonine-protein phosphatase | −0.74 |
| TraesCS4A02G094300 | 4A:10244587–102447774 | OsPP2C30 (Os03g0268000)      | Protein phosphatase 2C     | 0.83  |
| TraesCS4A02G114400 | 4A:13883230–138833256 | OsPyl (Os03g0297600)         | Abscisic acid receptor     | 0.71  |

*Correlation coefficient \( (r) \) was calculated between the expression pattern of TaABI4 and RNAseq data of candidate genes in SHW-L1 and CM32. A correlation of |−1.0| shows a perfect negative correlation, while a correlation of 1.0 shows a perfect positive correlation.

Fig. 7. The locations of QTL associated with the pre-harvest sprouting and seed dormancy and eQTL of TaABI4 were mapped on a physical map of Chinese spring. The location numbers and the corresponding locations can be found in Tables 3 and 4.
in ABA signalling pathway during seed germination (Kim et al., 2012). TraesCS4A02G114400 located in the internals of eQABI4.20DPA.4A.2 was the orthologue of OsPYL, which positively regulated the ABA response during the seed germination (Tian et al., 2015). Together, these results suggested that five candidate genes may have a regulatory relationship with TaABI4.

In this study, the characterization of TaABI4, including its conserved protein domains and cis-acting regulatory elements analysis, provides information on the critical nucleotide and amino-acid residues of this gene. Meanwhile, high conservation was found in the amino-acid sequences and promoter regions, but the different expression level of TaABI4 in two wheat cultivars drove us to identify regions linked to candidate genes that function upstream of TaABI4 transcripts. Six potential eQTL regions that may regulate the expression of TaABI4 were detected. Five potential upstream candidate genes that may influence the expression of TaABI4 were also detected. These results can be utilized for future TaABI4 studies on interactions with other transcription factors in response to ABA and the establishment of the co-expressed networks relating to seed germination, which will successfully boost the efficiency of wheat breeding with sufficient seed dormancy to prevent PHS.

Supplementary material. To view supplementary material for this article, please visit: https://doi.org/10.1017/S0960258521000015.

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Conflict of interest. The authors declare no conflicts of interest.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Antoni R, Rodriguez I, Gonzalez-Guzman M, Pizzio GA and Rodriguez PL (2011) News on ABA transport, protein degradation, and ABEs/WRKYs in ABA signaling. Current Opinion in Plant Biology 14, 547–553.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW and Noble WS (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Research 37, W202–W208.

Bassi F, Cordoba E, Dupré P, Mendoza MS, Román CS and León P (2009) The Arabidopsis ABA-INSENSITIVE (ABI) 4 factor acts as a central transcription activator of the expression of its own gene, and for the induction of ABI5 and SBE2.2 genes during sugar signaling. Plant Journal 59, 359–374.

Busk PK, Jensen AB and Pagès M (1997) Regulatory elements in vivo in the promoter of the abscisic acid responsive gene rab17 from maize. Plant Journal 11, 1285–1295.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K and Madden TL (2009) BLAST+: architecture and applications. BMC Bioinformatics 10, 421.

Chen C, Cai S and Bai G (2008) A major QTL controlling seed dormancy and pre-harvest sprouting resistance on chromosome 4A in a Chinese wheat landrace. Molecular Breeding 21, 351–358.

Chen X, Hackett CA, Nikes RE, Hedley PE, Booth C, Druka A, Marcel TC, Vels A, Bayer M, Milne I, Morris J, Ramsay L, Marshall D, Cardle L and Waugh R (2010) An eQTL analysis of partial resistance to Puccinia hordei in barley. PLoS ONE 5, e8598.

Choi M-G, Kim EJ, Song J-Y, Choi S-B, Cho S-W, Park CS, Kang C-S and Park Y-I (2020) Peptide transporter2 (PTR2) enhances water uptake during early seed germination in Arabidopsis thaliana. Plant Molecular Biology 102, 615–624.

DePauw RM and McCaig TN (1991) Components of variation, heritabilities and correlations for indices of sprouting tolerance and seed dormancy in Triticum spp. Euphytica 52, 221–229.

Doss S, Schadt EE, Drake TA and Lusis AJ (2005) cis-acting expression quantitative trait loci in mice. Genome Research 15, 681–691.

Finkelestein RR (1994) Mutations at two new Arabidopsis ABA response loci are similar to the abci2 mutations. Plant Journal 5, 765–771.

Finkelestein RR and Lynch TJ (2000) The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell 12, 599–609.

Finkelestein R, Lynch T, Reeves W, Petitfils M and Mostachetti M (2011) Accumulation of the transcription factor ABA-insensitive (ABI4) is tightly regulated post-transcriptionally. Journal of Experimental Botany 62, 3971–3979.

Ganguly M, Roychoudhury A, Sarkar SN, Sengupta DN, Datta SK and Datta K (2011) Inducibility of three salinity/abscisic acid-regulated promoters in transgenic rice with gusA reporter gene. Plant Cell Reports 30, 1617–1625.

Gregorio J, Hernandez-Bernal AF, Cordoba E and Leon P (2014) Characterization of evolutionarily conserved motifs involved in activity and regulation of the ABA-INSENSITIVE (ABI) 4 transcription factor. MolecularPlant 7, 422–436.

Gubler F, Millar AA and Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. Current Opinion in Plant Biology 8, 183–187.

Heins I, Frohberg C and Gatz C (1992) The Tn10-encoded Tet repressor blocks early but not late steps of assembly of the RNA polymerase II initiation complex in vivo. Molecular and General Genetics 232, 328–331.

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

Hobo T, Asada M, Koyama Y and Hattori T (1999) AGGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant Journal 19, 679–689.

Hoecker U, Vasil IK and McCarty DR (1995) Integrated control of seed maturation and germination programs by activator and repressor functions of viviparous-1 of maize. Genes and Development 9, 2459–2469.

Huang X, Xiaoayon Z, Zhihong G, Shuuhu Y and Yiting S (2017) ABI4 represses the expression of type-A ARRs to inhibit seed germination in Arabidopsis. Plant Journal 89, 354–365.

International Wheat Genome Sequencing Consortium (2018) Shifting the limits in wheat research and breeding through a fully annotated and anchored reference genome sequence. Science 361, eaar7791.

Kim H, Hwang H, Hong JW, Lee YN, Ahn IP, Yoon IS, Yoo SD, Lee S, Lee SC and Kim BG (2012) A rice orthologue of the ABA receptor, OsPYL/RACARS, is a positive regulator of the ABA signal transduction pathway in seed germination and early seedling growth. Journal of Experimental Botany 63, 1013–1024.

Lee HG and Seo PJ (2015) The MYB96–HHP module integrates cold and abscisic acid signaling to activate the CBF–COR pathway in Arabidopsis. Plant Journal 82, 962–977.

Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P and Robauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Research 30, 325–327.

Li C, Ni P, Franci M, Hunter A, Zhang Y, Schibeci D, Li H, Tarr A, Wang J, Cakir M, Yu J, Bellgard M, Lance R and Appels R (2004) Genes controlling seed dormancy and pre-harvest sprouting in a rice-wheat-barley comparison. Functional and Integrative Genomics 4, 84–93.

Lin Y (2009) Isolation and analysis of glucose-6-phosphate dehydrogenase (G6PDH) promoter from poplar. Genomics and Applied Biology 28, 445–449.

Ling HQ, Ma B, Shi X, Liu H, Dong L, Sun H, Cao Y, Gao Q, Zheng S, Li Y, Yu Y, Du H, Qi M, Li Y, Lu H, Yu H, Cui Y, Wang N, Chen C, Wu H, Zhao Y, Zhang J, Li Y, Zhou W, Zhang B, Hu W, van Eijk M, Tang J,
Witsenboer HMA, Zhao S, Li Z, Zhang A, Wang D and Liang C (2018) Genome sequence of the progenitor of wheat A subgenome *Triticum urartu*. Nature 557, 424–428.

Liu Y, Yan L, Wu Z, Mei C, Lu K, Yu Y, Liang S, Zhang X, Wang X and Zhang D (2012) Cooperation of three WRKY-domain transcription factors WRKY18, WRKY40, and WRKY60 in repressing two ABA-responsive genes ABI4 and ABI5 in Arabidopsis. Journal of Experimental Botany 63, 6571–6592.

Long XY, Wang JR, Ouette T, Rocheleau H, Wei YM, Pu ZE, Jiang QT, Lan XJ and Zheng YLJPMB (2010) Genome-wide identification and evaluation of novel internal control genes for Q-PCR based transcript normalization in wheat. Plant Molecular Biology 74, 307–311.

Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT and Chua NH (2010) The viviparous-1 developmental gene of maize encodes a novel transcriptional activator. *Cell* 166, 895–906.

Mares DJ, Mrva K, Cheong J, Williams K, Watson B, Storlie E, Sutherland M and Zou Y (2002) A QTL located on chromosome 4A associated with dormancy in white- and red-grained wheats of diverse origin. *Theoretical and Applied Genetics* 111, 1357–1364.

McCarty DR, Hattori T, Carson CB, Vasili V, Lazar M and Vasil IK (1991) The viviparous-1 developmental gene of maize encodes a novel transcriptional activator. *Cell* 66, 895–905.

Ming R, Vanburen R, Liu Y, Mei Y and Shen-Miller JGGB (2013) Genome of the long-living sacred lotus (*Nelumbo nucifera Gaertn.*). *Genome Biology* 14, R41.

Motomura Y, Kobayashi F, Ichha JCM and Takumi S (2013) A major quantitative trait locus for cold-responsive gene expression is linked to frost-resistance gene Fr-A2 in common wheat. *Breed ing Science* 63, 58–67.

Niu X, Helentjaris T and Bate NJ (2002) Maize ABI4 binds coupling element1 to abscisic acid and sugar response genes. *Plant Cell* 14, 2565–2575.

Olaerts H, Roye C, Derde LJ, Sinnaeve G, Meza WR, Bodson B and Courtin T (2016) Impact of preharvest sprouting of wheat (*Triticum aestivum*) in the field on starch, protein, and arabinoxylan properties. *Journal of Agricultural and Food Chemistry* 64, 8324–8332.

Osa M, Kato K, Mori M, Shindo C, Torada A and Miura H (2003) Mapping QTLs for seed dormancy and the Vp1 homologue on chromosome 3A in wheat. *Theoretical and Applied Genetics* 106, 1491–1496.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29, e45–e45.

Phukan UJ, Jeena GS and Shukla RK (2016) WRKY transcription factors: molecular regulation and stress responses in plants. *Frontiers in Plant Science* 7, 760.

Rice P, Longden I and Bleasby A (2000) EMBOSS, the European molecular biology open software suite. Trends in Genetics 16, 276–277.

Rogers S, Wells R and Rechsteiner M (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234, 364–368.

Sakata Y, Nakamura I, Taji T, Tanaka S and Quatrano RS (2010) Regulation of the ABA-responsive Em promoter by ABI3 in the moss *Physcomitrella patens*: role of the ABA response element and the RY element. *Plant Signalling Behaviour* 5, 1061–1066.

Sarkar AK and Lahiri A (2013) Specificity determinants for the abscisic acid response element. *F.E.B.S Open Bio* 3, 101–105.

Shang Y, Yan L, Liu Z, Cao Z, Mei C, Xin Q, Wu F, Wang X, Du S, Jiang T, Zhang X, Zhao R, Sun H, Liu R, Yu Y and Zhang D (2010) The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* 22, 1909–1935.

Shu K, Zhang H, Wang S, Chen M, Wu Y, Tang S, Liu C, Feng Y, Cao X and Xie Q (2013) ABI4 regulates primary seed dormancy by regulating the biogenesis of abscisic acid and gibberellins in Arabidopsis. *PLoS Genetics* 9, e1003577.

Shu K, Liu XD, Xie Q and He ZH (2016) Two faces of one seed: hormonal regulation of dormancy and germination. *Molecular Plant* 9, 34–45.

Soderman EM, Brocard IM, Lynch TJ and Finkelstein RR (2000) Regulation and function of the Arabidopsis ABA-insensitive4 gene in seed and abscisic acid response signaling networks. *Plant Physiology* 124, 1752–1765.

Sun G, Zhang X and Xiao S (2005) Maternal effect on sensitivity of embryo to ABA and grain dormancy in wheat. *Journal of Triticeae Crops* 25, 37–41.

Tian X, Wang Z, Li X, Lv T, Liu H, Wang L, Niu H and Bu Q (2015) Characterization and functional analysis of pyrabactin-resistance-like abscisic acid receptor family in rice. *Rice* 8, 28.

Tora A, Ikeuchi S and Koike M (2005) Mapping and validation of PCR-based markers associated with a major QTL for seed dormancy in wheat. *Euphytica* 143, 251–255.

Wang S and Basten CJ (2007) Windows QTL cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC.

Wang L, Liu K, Mao S, Li Z, Yu W, Wang J, Liu Y, Wei Y and Zheng Y (2015) Large-scale screening for *Aegilops tauschii* tolerant genotypes to phosphorus deficiency at seedling stage. *Euphytica* 204, 571–586.

Wind JJ, Peviani A, Snel B, Hanson J and Smeekens SCJ (2013) ABI4: versatile activator and repressor. *Trends in Plant Science* 18, 125–132.

Yang J, Liu Y, Pu Z, Zhang L, Yuan Z and Chen G (2014) Molecular characterization of high pl α-amylase and its expression QTL analysis in synthetic wheat RILs. *Molecular Breeding* 34, 1075–1085.

Yang S, Xu K, Chen S, Li T, Xia H, Chen L, Liu H and Luo L (2019) A stress-responsive bZIP transcription factor OsZIP62 improves drought and oxidative tolerance in rice. *BMC Plant Biology* 19, 260.

Yang J, Tan C, Lang J, Tang H, Hao M, Tan Z, Yu H, Zhou Y, Liu Z, Li M, Zhou Y, Cheng M, Zhang L, Liu D and Wang J (2019) Identification of qPH5.sicau-IB and qPH5.sicau-3D from synthetic wheat for pre-harvest sprouting wheat improvement. *Molecular Breeding* 39, 132–143.

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, Cao M, Liu J, Sun J, Tang J, Chen Y, Huang X, Liu W, Ye C, Tong W, Cong L, Geng J, Han Y, Li L, Li W, Hu G, Huang X, Li W, Li J, Liu Z, Liu L, Ji Q, Liu J, Li L, Li T, Wang X, Lu H, Wu T, Zhu W, Mi P, Han H, Dong W, Ren X, Feng C, Cui P, Li X, Wang H, Xu X, Zhai W, Xu Z, Zhang J, He S, Zhang J, Xu J, Zhang K, Zheng X, Dong J, Zeng W, Tao L, Ye J, Tan J, Ren X, Chen X, He J, Liu D, Tian W, Tian C, Xia H, Bao Q, Li G, Gao H, Cao T, Wang J, Zhao W, Li P, Chen W, Wang X, Zhang Y, Hu J, Wang J, Liu S, Yang J, Zhang G, Xiong Y, Li Z, Mao L, Zhou C, Zhu Z, Chen R, Hao B, Zheng W, Chen S, Guo W, Li G, Liu S, Tao M, Wang J, Zhu L, Yuan L and Yang H (2002) A draft sequence of the rice genome (*Oryza sativa L.* spsp. indica). *Science* 296, 79–92.

Zhang Y, Zhao F and Zhao J (2005) Effects of exogenous ABA on the seed germination of rice (*Oryza sativa L.*) and the expression of relative genes. *Journal of Wuhan Botanical Research/Plant Science Journal* 23, 203–210.

Zhang L, Wang B, Pan L and Peng J (2013) Recycling isolation of plant DNA, a novel method. *Journal of Genetic Genomics* 40, 45–54.

Zhang S, Ye Z and Stanton R (2020) Misuse of RPKM or TPM normalization when comparing across samples and sequencing protocols. *RNA* 26, 903–909.

Zhou K, Yang J, Wang Z and Wang J (2016) Sequence analysis and expression profiles of TaABI5, a pre-harvest sprouting resistance gene in wheat. *Genes & Genomics* 39, 161–171.

Zhu Z, Chen R, Hao B, Zheng W, Chen S, Guo W, Li G, Liu S, Tao M, Wang J, Zhu L, Yuan L and Yang H (2002) A draft sequence of the rice genome (*Oryza sativa L.* spsp. indica). *Science* 296, 79–92.