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Wheat Dof transcription factor WPBF interacts with *TaQM* and activates transcription of an alpha-gliadin gene during wheat seed development

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Abstract Wheat prolamin-box binding factor (WPBF), a DOF transcription factor previously was isolated from wheat endosperm and suggested to function as an activator of prolamin gene expression during seed development. In this study, we showed that *WPBF* is expressed in all wheat tissues analyzed, and a protein, *TaQM*, was identified from a wheat root cDNA library, to interact with the Dof domain of WPBF. The specific interaction between WPBF and *TaQM* was confirmed by pull-down assay and bimolecular fluorescence complementation (BiFC) experiment. The expression patterns of *TaQM* gene are similar with that of *WPBF*. The GST-WPBF expressed in bacteria binds the Prolamin box (PB) 5′-TGTAAAG-3′, derived from the promoter region of a native *alpha-gliadin* gene encoding a storage protein. Transient expression experiments in co-transfected BY-2 protoplast cells demonstrated that WPBF *trans*-activated transcription from native *alpha-gliadin* promoter through binding to the intact PB. When WPBF and *TaQM* are co-transfected together the transcription activity of *alpha-gliadin* gene was six-fold higher than when WPBF was transfected alone. Furthermore, the promoter activities of *WPBF* gene were observed in the seeds and the vascular system of transgenic *Arabidopsis*, which was identical to the expression profiles of WPBF in wheat. Hence, we proposed that WPBF functions not only during wheat seed development but also during other growth and development processes.

Keywords *WBPF* · Dof · Transcription factor · *TaQM* · Protein interaction · Wheat · Expression pattern

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Introduction

Many factors are necessary in the regulation of spatial and temporal gene expression, including interactions between sequence-specific DNA-binding proteins and the corresponding *cis*-elements (Lemon and Tjian 2000; Levine and Tjian 2003), and between proteins and other proteins. A large body of information has been produced to further understand seed development in cereal grains. However, detailed knowledge of the regulation of gene expression in endosperm is lacking.

The expression patterns of seed-specific genes are accurately regulated. The regulation involves *cis*-elements in promoters and *trans*-acting factors (Diaz et al. 2002). Previous studies identified three conserved *cis*-motifs in the endosperm-specific promoters from barley, wheat and rice, namely the GLM motif, Prolamin box (PB) and the 5′-AACA/TA-3′ motif, respectively. The GCN4-like motif (GLM: 5′-ATGAG/CTCAG-3′) is bound by bZIP proteins that belong to the Opaque2 subfamily (Albani et al. 1997; Onate et al. 1999; Vicente-Carbajosa et al. 1998; Wu et al. 1998). The PB (5′-TGTAAAG-3′) is recognized by DOF (DNA

binding with one finger) transcription factors (Vicente-Carbajosa et al. 1998; Mena et al. 1998; Isabel-LaMoneda et al. 2003; Yanagisawa, 2004;). The 5′-AACA/TA-3′ motif is the binding site of GAMYB and OsMYB5 in barley and rice (Diaz et al. 2002; Suzuki et al. 1998). Deletion and point mutation experiments revealed that PB is important for the regulation of expression of the endosperm-specific genes (Mena et al. 1998; Diaz et al. 2005).

Several *trans*-acting factors, including bZIP and DOF family members, jointly function in regulating expression of storage proteins during seed development (Isabel-LaMoneda et al. 2003; Mena, et al. 2002; Mena et al. 1998; Vicente-Carbajosa et al. 1997). The interaction between maize PBF and O2 (opaque 2) was involved in the zein protein expression during the seed development (Vicente-Carbajosa et al. 1997), and it is conserved in many promoters of cereal storage-protein genes (Yanagisawa, 2002). Recent study shows that BPBF (Barley PBF) interacts with HvGAMYB protein by its C-terminal to activate the endosperm-specific genes during seed development (Diaz et al. 2002). The Dof protein from barley, SAD, also interacts with HvGAMYB in plant nuclei which was confirmed by a BiFC (bimolecular fluorescent complex) approach (Diaz et al. 2005). Taken together, these studies demonstrated that the Dof proteins not only bind the *cis*-element but also mediate protein-protein interaction through its Dof domain or C-terminal.

Wheat PBF(WPBF), first isolated from wheat endosperm as a homologue of BPBF, was proposed to function by binding to the PB-box of *Hor2* (prolamin gene in barley) promoter (Mena et al. 1998). The gliadin is one of the major seed storage proteins in wheat endosperm, which are extractable in aqueous alcohol, accumulate specifically during seed development, and serve as nitrogen sources during seed germination and seedling growth (Shewry et al. 1995). A PB-box motif was found in promoter region of wheat *alpha-gliadin* gene by sequence analysis (Vicente-Carbajosa et al. 1997). However, binding of WPBF to the native promoter of *alpha-gliadin* gene has not been confirmed by direct experimental evidencein wheat.

In our previous study, a *WPBF* gene was found to be up-regulated in the root of a wheat hybrid compared to its parental inbred lines (Wang et al. 2006). In the present study, we showed that *WPBF* gene is constitutively expressed in various wheat tissues, including roots, leaves and seeds. By yeast two-hybrid screening, we obtained a protein, *TaQM*, from the wheat root cDNA library. The in vitro and in vivo interaction between WPBF and *TaQM* was confirmed by pull-down assay and BiFC approach, respectively. The transient expression assays in cotransfected BY-2 protoplast cells showed that WPBF *trans*-activates native *alpha-gliadin* (*Gli*) promoter through interaction with the 5′-TGTAAAG-3′ motif. When WPBF and *TaQM* are cotransfected together the transcription activity of *Gli* gene showed a six-fold increase than the WPBF alone. Moreover, the activities of *WPBF* promoter were observed in the seeds and the vascular system of transgenic *Arabidopsis*, suggesting that WPBF function not only in seed development, but also during other growth and development stages.

Materials and methods

Plant materials

The wheat line 3338 was used in this study. Wheat was grown as described (Yao et al. 2005). For yeast-two hybrid library construction, the roots from vigorous tillering stage seedlings were harvested. The tissues, including seeds during germination, roots and leaves of 2–4 days after germination, leaves of jointing stage and developing seeds, were collected for RT-PCR analysis.

Isolation of the full-length cDNA of WPBF and its upstream region

The 3′ and 5′ end region of the *WPBF* cDNA was isolated by 3′ and 5′ rapid amplification of cDNA ends (RACE) using a *WPBF*-specific primer.

WPBF 3′A, 5′-TGGCAGCCTCAGTCAGAACAATG-3′;
WPBF 3′B, 5′-GGCTACTACTATGGTGGGCC-3′;
WPBF 5′A, 5′-GGACGTTCGCAAAGTTCATCCC-3′;
WPBF 5′B, 5′-ACAGTCGTGGGTTCCGGTGAGG-3′.

The 3′ end region of *WPBF* cDNA was cloned by one-step reverse transcribed-PCR on total RNA from the roots of vigorous tillering stage with WPBF 3′A and an oligo dT-based anchored primer (5′-CTGATCTAGAGGTACCGGATCC$(T)_{18}$–3′). The 5′-RACE PCR was carried out using a SMART™ cDNA amplification kit (Clontech).

The promoter region of *WPBF* was isolated by inverse PCR (Triglia et al. 1988). The PCR product was cloned and sequenced. Basic local alignment search tool (BLAST) of this fragment identified a known sequence of AF385139.

Expression profile

Total RNA was extracted using TRIzol reagent (Tianwei, China) and treated with DNase I

(Invitrogen) to remove DNA contamination. Two microgram total RNA of each sample was used to synthesize first-strand cDNA by using random hexamers primer for developing and germination seeds, or using oligo(dT)$_{18}$ primer for other tissues with M-MLV reverse transcriptase (Promega, USA) according to the manufacture's instruction. One μL of the first-strand cDNA was used as template in a 20 µl RT-PCR reaction subjected to 25–30 cycles of amplification. Gene-specific primers were designed according to the cDNA sequences. A wheat 18S ribosomal RNA fragment or a 350 bp *tubulin* gene fragment was amplified as an internal control. The RT-PCR products were sequenced to verify the specificity of PCR amplification.

WPBFs,5′-GCCAGCGGTGTCATTTG-3′, WPBFas, 5'-CGCCGCTGTCGTTATTGT-3'; TaQMs, 5′-TCC AGACTGGTATGAGGGGTG-3′, TaQMas, 5′-TCC GAGCAGCTTAGCGTTG-3′; Glis, 5′-AGCCGCA ACTACCATATTCA-3′, Glias, 5′-CTGCGACTGC TCAGGGAT-3′; 18Ss, 5′-GTGACGGGTGACGGA-GAATT-3′, 18Sas, 5′-GACACTAATGCGCCCGG-TAT-3′; Tatubs, 5′-AGAACACTGTTGTAAGGC TCAAC -3′, Tatubas, 5′-GAGCTTTACTGCCTCGA ACATGG -3′.

Localization of GUS activity

A ~2.5 kb fragment of the *WPBF* promoter region was amplified by PCR, using the sense primer, 5′-AAA CTGCAGCCATCTTTTCTATATGTTGGC-3′, and antisense primer, 5′-CGCGGATCCTGCTATGGGA-TAGGGTGAAG-3′, digested and cloned into the linear pCAMBIA1391Z.

Seeds of Arabidopsis (ecotype Col-0) were sown on half-strength Murashige and Skoog (MS) plates (Murashige and Skoog 1962) supplemented with 3% sucrose, pH 5.8, and 0.8% (w/v) agar in Petri dishes. Plants were grown in a growth chamber (16 h/8 h of light and darkness at 23°C).Transformation of Arabidopsis was performed by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). The T2 progenies were analyzed for β-glucuronidase expression.

Histochemical staining and microscopic analysis were performed as described previously (Jefferson et al. 1987). The assays were analyzed in at least three independent transformants of T2 progeny. 3- and 7-day old plantlets, 8-week old seeds, stem, siliques and inflorescence were examined under a light microscope.

Subcellular localization of GFP-WPBF and GPF-TaQM fusion proteins

The *WPBF* and *TaQM* coding region were amplified and fused in-frame into the 3' end of GFP sequence of pEGAD. Onion (*Allium cepa*) transformation was carried out by the Helium Biolistic gene transformation system (PDS-1000/He, BioRad, Hercules, CA, USA). Inner epidermal layers were peeled and placed inside half strength MS medium as described previously (Varagona et al. 1992). Plasmids DNA was delivery by particle bombardment (Klein et al. 1987). Epidermal cells were cultured at 22°C in darkness for 20 h, and then the tissue was viewed and imaged using argon laser (488 nm) for green fluorescence by an Axioskop confocal microscopy (Carl Zeiss).

Yeast two-hybrid screen

Yeast two-hybrid library was constructed and screened according to the manufacture's instruction (BD Matchmaker^{TM} Library Construction & Screening Kit). pGBKT7 was used to generate in-frame fusions with WPBFBD (the Dof domain of WPBF) as the bait. pGADT7-Rec was used to construct the root cDNA library of 3338 wheat. ds cDNA, pGADT7-Rec and pGBKT7WPBFBD were cotransformed into yeast strain AH109 cells using PEG/LiAc method. The cotransformation mixture was spread on QDO plates (QDO: SD/–Ade/–His/–Leu/–Trp, the high stringency screen for decreasing the false positive), and incubated at 30°C until colonies appeared. The positive clones were retested on the QDO plates containing X-α-Gal. Under this procedure, the $\approx 5 \times 10^5$ clones screen generated 32 primary positives. The library cDNA Insert of positive clones were rescued by plasmids isolation and aligned by the BLAST program.

In vitro interaction between WPBF and *TaQM*

WPBF cDNA was cloned in-frame into GST-fusion pGEX-4T-1 vector (Amersham Pharmacia), the cDNA of *TaQM* was inserted in-frame into His-tagged pET-28a vector (Novagen), and then expressed in *E. coli* BL21 cells. The GST-WPBF and GST-WPBFBD fusion proteins were immobilized on glutathione-agarose columns (Amersham Pharmacia) and incubated with the TaQM-His bacterial sonic supernatant protein overnight at 4°C, columns were washed three times. The proteins were resolved by

SDS-PAGE and transferred to nitrate cellulose membranes (Amersham Pharmacia). The target proteins were detected by Western blot using monoclonal anti-His antibody (Huamei, China). As a negative control, GST protein interacting with TaQM-His, was conducted to exclude any non-specific action.

The pull-down assay was repeated but following a different order. The TaQM-His protein firstly was immobilized on the nickel chelate columns (Novagen), and then equal amounts of cell lysate containing GST-WPBF, GST-WPBF-BD or control GST proteins were injected into different columns, washed three times and resolved by SDS-PAGE. Western blot detection was carried out using monoclonal anti-GST antibody (Amersham Pharmacia).

Bimolecular fluorescence complementation (BiFC)

The interaction between WPBF and TaQM proteins in onion cells was confirmed using bimolecular fluorescence complementation. The GFP gene of pEGAD was split into two non-overlapping fragments by a PCR technique, as previously described (Hu et al. 2002, Diaz et al. 2005). The N-terminal region of the GFP protein (nt 1–465) encoded the first 155 amino acid residues of the GFP protein, and the C-terminal region (nt 466–717) included the 84 C-terminal residues. These fragments were cloned into the pEYFP plasmid (Clontech) under the control of Lac promoter by replacement of the YFP gene and generated pNGFP and pCGFP. The coding regions of *WPBF* and *TaQM* were amplified and fused in-frame into the N-terminal of pNGFP and pCGFP, generating the following constructions: *Lac::WPBF-NGFP*, *Lac::WPBF-CGFP*, *Lac::TaQM-NGFP* and *Lac::TaQM-CGFP*. Inner epidermal layers of onions were prepared and co-bombarded, as described earlier, using the appropriate plasmid combination indicated in each case. The fluorescence emission was observed after 24 h of incubation at 22°C in dark, under the microscope, as described before.

Electrophoretic mobility shift assays

Proteins produced in bacteria were purified as described above. The biotin end-labeled probe derived from the *Gli* promoter containing the putative consensus PB (*WT*) and its mutated sequences (*mt*) was designed as described (Mena et al. 1998). EMSAs were carried out according to the manufacturer's instruction (Pierce, USA).

WT:5′-BIOTIN-GTGTTTGAGCTGTAAAGTGA-ATAAGATGAG-3′
mt: 5′-BIOTIN -GTGTTTGAGCTGTAgAcTGAAT-AAGATGAG -3′

Transient expression assays in BY-2 protoplast cells

The effector constructs were generated by introduced the whole *WPBF* and *TaQM* cDNA to the downstream of the CaMV35S promoter. The reporter vectors pGli and pGli* contain the 530 bp native promoter of *Gli* and its mutated version, respectively, fused to the luciferase reporter. Plasmid DNA was introduced by the PEG method (Hartmann et al. 1998; Mathur and Koncz 1998) to tobacco BY-2 protoplast cells cultured for 24 h after transfer to a fresh medium. Luciferase activity was assayed using luciferase assay systems (Promega) according to the manufacturer's instructions. Luciferase luminescence was measured using Microlumat LB 96P (Berthold). *Renilla* luciferase gene under the control of the CaMV35S was used as the internal control. Relative luciferase activities were the averages with SD of three independent experiments, after normalization with the internal control.

Results

Cloning of WPBF full-length cDNA and its promoter sequence

One cDNA fragment, homologous to *PBF* (GenBank accession number: AY496057.2), was previously isolated from the roots at jointing stage using the differential display reverse transcription PCR method (data not shown). In this study, the full-length cDNA sequence of *WPBF* was isolated by 3′- and 5′-RACE (rapid amplification of cDNA ends) from root. After cloning and sequencing, the 5′- and 3′ untranslated regions (UTR) were identified. The deduced amino acid sequence of the WPBF contains 330 residues with a conserved Dof domain at its N terminus.

Subsequently, inverse PCR (IPCR) was used to isolate the promoter region of *WPBF*. After PCR, several bands with different sizes were obtained and sequenced. BLAST searches identified a known sequence of AF385139 that has been isolated previously; thereafter, we cloned the putative promoter region of *WPBF*, and validated it using the specific-primers. The transcription-related elements or motif were identified, including TATA-box (–950), the transcriptional start

site (–920 bp), but no CAAT box was found in the sequence.

Expression profiles of WPBF

Previous study suggested that expression of *WPBF* is correlated with the expression of endosperm-specific genes during seed development (Mena et al. 1998). To explore the possible roles of *WPBF*, we investigated its expression during seed development by semi-quantitative RT-PCR. Meanwhile, the expression patterns of wheat *Gli* (U51306), the putative target genes of WPBF, were also examined. Total RNA was extracted from developing seed of 8–25 days after pollination (DAP). As shown in Fig. 1A, the *WPBF* mRNA were detected at 8 DAP, approached the peak at 15–20 DAP, and subsequently decreased at 25 DAP. The expression of *Gli* was abundant in developing seeds after 15 DAP (Fig. 1A). It was found that by adding 2 × cDNA levels template, the expression of WPBF, TaQM and gliadin increased about two folds (Fig. 1A).

Furthermore, the expression of *WPBF* was analysed in root, leaves and seed during germination. The expression of *WPBF* was firstly detected at 8 h after imbibitions, and the expression showed no increase at 16 and 24 h of imbibition (Fig. 1B). *WPBF* expression was observed at 48, 72 and 96 h after germination in both roots and leaves (Fig. 1C). In addition, the transcripts of *WPBF* were present in the etiolated seedlings that were exposed to dark for 6 days (Fig. 1D). The abundance of *WPBF* mRNA in the dark treatment was slightly higher than in the light. At vigorous tillering stage, *WPBF* is expressed in both root and leaves, but not in stem (Fig. 1E). The expression of *WPBF* can be detected in flag leaves, but not in root and stem of heading stage (Fig. 1F). The expression patterns of *WPBF* observed in this study were different from that of *BPBF* in barley, which was constrained to the endosperm.

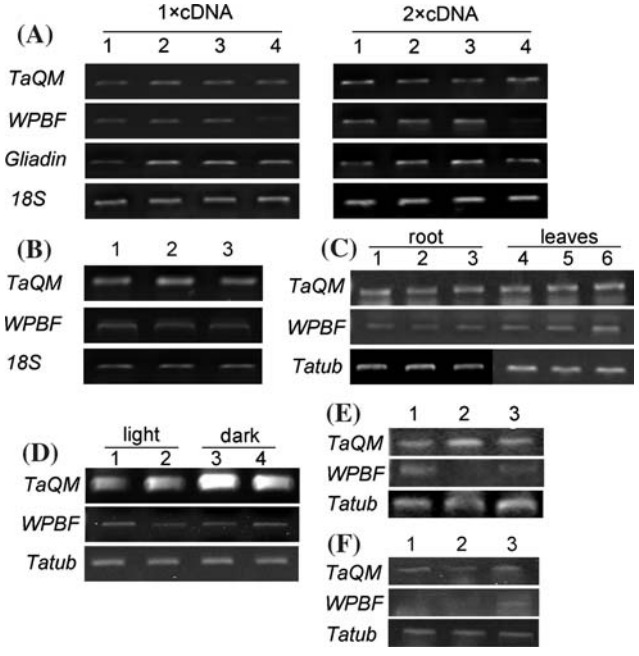

Fig. 1 Expression patterns of *WPBF*, *Ta-gliadin* and *TaQM*. A 18S ribsomomal RNA (for **A** and **B**) and a *tubulin* (for **C**–**F**) was used as the internal control, respectively. (**A**) RT-PCR analysis of *WPBF* expression in wheat developing seeds. Lanes 1–4 represent developing wheat seeds of 8, 15, 20, and 25 DAP (day after pollination). 1 × cDNA and 2 × cDNA indicates the quantity of template used in semi-RT-PCR. (**B**) Lanes 1–3 correspond to imbibed seed of 8, 16 and 24 h, respectively. (**C**) Root (lanes 1–3) and leaves (lanes 4–6) at 48, 72 and 96 h after germination. (**D**) Root (lanes 1–3) and leaves (lanes 2–4) of 6-day old seedlings grown in light or dark under the same temperature and humidity. (**E**) Lanes 1–3 represent root, stem and leaves of vigorous tillering stage, respectively. (**F**) Lanes 1–3 represent root and stem of heading stage and flag leaves, respectively

GUS localization of WPBF promoter in transgenic Arabidopsis

To investigate the expression pattern of *WPBF* in vivo, a construct containing the *GUS* reporter gene under the control of the *WPBF* promoter was used to transform the *Arabidopsis* plants. At least 10 independent transgenic lines were analyzed. As shown in Fig. 2, the GUS-specific staining was observed in 3 and 7 day-old plantlets, the vascular system of hypocotyls, mature region of primary root, cotyledon, leaf, stem, seedpod and flower. In the roots, GUS staining was observed only in mature region but not in the root tips and elongation regions. GUS staining was also detected in stem, the funiculus connecting the placenta to the ovule, endosperm and embryo of the developing seeds, and the seedpod. Furthermore, the staining was displayed in the stamen and pistil of flower (Fig. 2). It is noticeable that the persistent staining was observed in the vascular system, suggesting that *WPBF* might function in vascular system during plant growth and development.

Screening of potential proteins interacted with WPBF by yeast two-hybrid

In order to explore new mediators that can interact with WPBF protein, we performed a yeast two-hybrid screening using the binding domain of WPBF as bait to screen the wheat root cDNA library. Since the whole protein activates transcription in yeast, we chose the

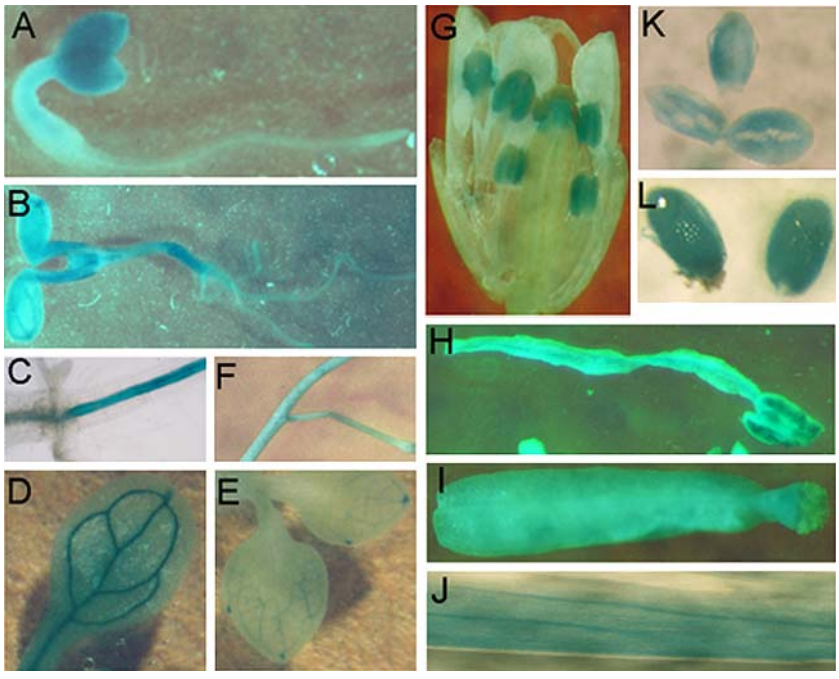

Fig. 2 GUS localization of *WPBF* promoter. (**A**–**L**) show the activities of *WPBF::GUS* in 3 and 7 day-old plantlets, root, cotyledon, leaf, stem, flower, pistil, stamen, seedpod, embryo and seeds, respectively

binding domain of WPBF (WPBFBD) as bait (data not shown). After screening of $\approx 5 \times 10^5$ clones on QDO conditional medium containing X-α-gal, the positive blue colonies were picked up and the prey plasmid were rescued from each of them. The isolated plasmids were again transformed separately into yeast AH109 cells with bait construct together. After this procedure, we obtained 32 positive transformants. Nucleotide sequencing and BLAST analysis showed that, among the positive tansformants, two of the 32 cDNA inserts were identical to each other, and encoding a QM protein that has been deposited in GenBank database and designated *TaQM* (CK167290). One of 30 cDNAs is high molecular weight glutenin sequence, and the others are rRNA sequence (data not shown).

TaQM protein individually was incapable of transcription activation in yeast cells. The 660 bp cDNA of *TaQM* contain an open reading frame of 219 residues with predicted molecular weight of 24.7 kDa. TaQM, the homolog of ribosomal protein L10, is a member of the QM family, which was originally identified as a putative tumor suppressor gene from a Wilms' tumor cell lines and has been isolated from all kinds of species of fungal, plant and animal kingdoms (Mills et al. 1999). QM family proteins are highly conserved in charged amino acids, including one basic and one acidic alpha-helices (Farmer et al. 1994).

RT-PCR analysis showed that *TaQM* was constitutively expressed in all wheat tissues, including developing and germinating seeds, root and leaves at seedling stage, etiolated seedlings, stem and flag leaves (Fig. 1A–F).

In vitro interaction between *TaQM* and WPBF

To further examine the interaction between *WPBF* and *TaQM*, GST and His pull-down assays were performed. For this purpose, the fusion proteins of GST-WPBF and GST-WPBFBD expressed in bacteria were immobilized on glutathione-agarose columns, and assayed for the ability to pull-down the recombinant TaQM-His. Subsequently, the pull-down assay was analyzed by immunoblotting with anti-His antibodies. The results showed that both GST-WPBF and GST-WPBFBD interacted with TaQM-His, but not with GST alone (Fig. 3A). Additionally, an inverse pull-down assay was also carried out by immunoblotting with anti-GST antibodies. When the GST-WPBF, GST-WPBFBD and GST were added to immobilized TaQM-His columns, and the results also showed that GST-WPBF and GST-WPBFBD interacted with TaQM-His (Fig. 3B). All the data indicated that WPBF interacts specifically with TaQM in vitro.

In vivo interaction of WPBF with *TaQM*

To detect whether *WPBF* interacts with *TaQM* in plant nuclei, BiFC approach was applied. Two constructs were made and designated as pNGFP and pCGFP,

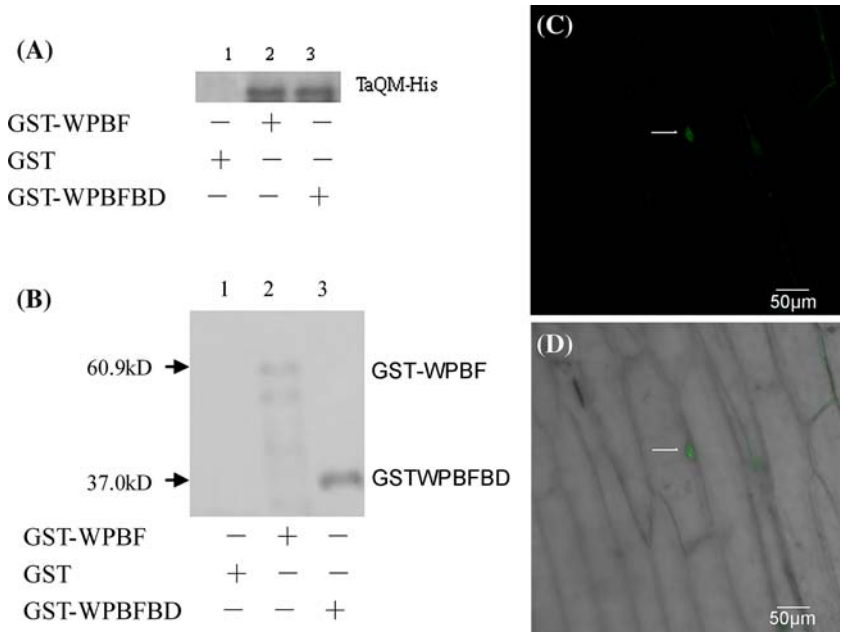

Fig. 3 Interaction analysis of *WPBF* and *TaQM* by pull-down. (**A**) GST pull-down analysis. The sonicated supernatant of *TaQM*-His fusion raw protein was flowed through the columns where the GST-WPBF, GST-WPBFBD or GST bound firstly on resin. The bound *TaQM* was eluted together with GST-WPBF or GST-WPBFBD. The samples were resolved by SDS-PAGE, transferred to NC, and determined by immunoblot using mouse anti-His antibody and goat anti-mouse IgG-HRP. *TaQM*-His was effectively pulled by GST-WPBF and GST-WPBFBD but not with GST alone. Approximately 10 μg of raw protein was loaded for each sample. 1, GST; 2, GST-WPBF; 3, GST-WPBF-BD. The bands show the *TaQM*-His. (**B**) His pull-down assay. The sonicated supernatant of GST-WPBF, GST-WPBFBD or GST raw protein was flowed through the Ni-columns where the *TaQM*-His was bound firstly on resin. The bound GST-WPBF, GST-WPBFBD or GST was eluted together with *TaQM*. The samples were resolved by SDS-PAGE, transferred to NC, and determined by immunoblot using anti-GST-HRP antibody. GST-WPBF and GST-WPBFBD was effectively pulled by TaQM-His except for GST. Approximately 10 μg of raw protein was loaded for each sample. 1, GST; 2, GST-WPBF; 3, GST-WPBF-BD. The bands show the GST-WPBF AND GST-WPBFBD, respectively. (**C**) In vivo interaction assay of *TaQM* and *WPBF* by bimolecular fluorescence complementary. *Lac::WPBF-CGFP* and *Lac::TaQM-NGFP* or *Lac::TaQM-CGFP* and *Lac::WPBF-NGFP* were transformed into onion epidermal cells in equal amount. After incubation at 22°C for 24 h, reconstituted fluorescence was observed under a Zeiss Axiophot microscope under fluorescence. (**D**) Overlay of fluorescence image and light image. Arrows indicate the fluorescence

respectively, which contained the N-region and C-region of the GFP (the N-terminal 155 amino acid residues, or the C-terminal 84 residues), respectively, and then *WPBF* and *TaQM* were inserted in-frame into these two vectors, in all combinations as described earlier (Diaz et al. 2005). GFP fluorophore would be recovered when two interacting proteins brought these two fragments together. After co-bombardment into onion epidermal cells, we observed the recovered GFP fluorescence in the nucleus, which is in agreement with the subcellular localization of *WPBF* and *TaQM*. Fluorescence was observed in the combination of pWPBF-CGFP/pTaQM-NGFP or pTaQM-CGFP/pWPBF-NGFP. We observed green fluorescence in the nuclei from the merged pictures (Fig. 3D). No fluorescence was detected in the tissue bombarded with either pNGFP or pCGFP (data not shown).

Subcellular localization of WPBF and *TaQM*

To characterize the interaction, the subcellular localization of the *WPBF* and *TaQM* proteins in vivo, a transient expression system based on onion epidermal cells was deployed. Two chimeric vectors, *35S::GFP-WPBF* and *35S::GFP-TaQM*, were constructed by in-frame fusion to the 3′ terminus of Green Fluorescent Protein (GFP) encoding gene in the pEGAD binary vector, under the control of 35S promoter. At 20 h after bombardment, the subcellular location of the GFP was visualized under both bright-field and fluorescent (BP 450/90 FT 510 LP520 Zeiss filter Zeiss, USA) microscope. As expected, the onion inner epidermal cells expressing both *GFP-WPBF* and *GFP-TaQM* proteins showed green fluorescence in the nucleus exclusively (Fig. 4B, C), whereas the control,

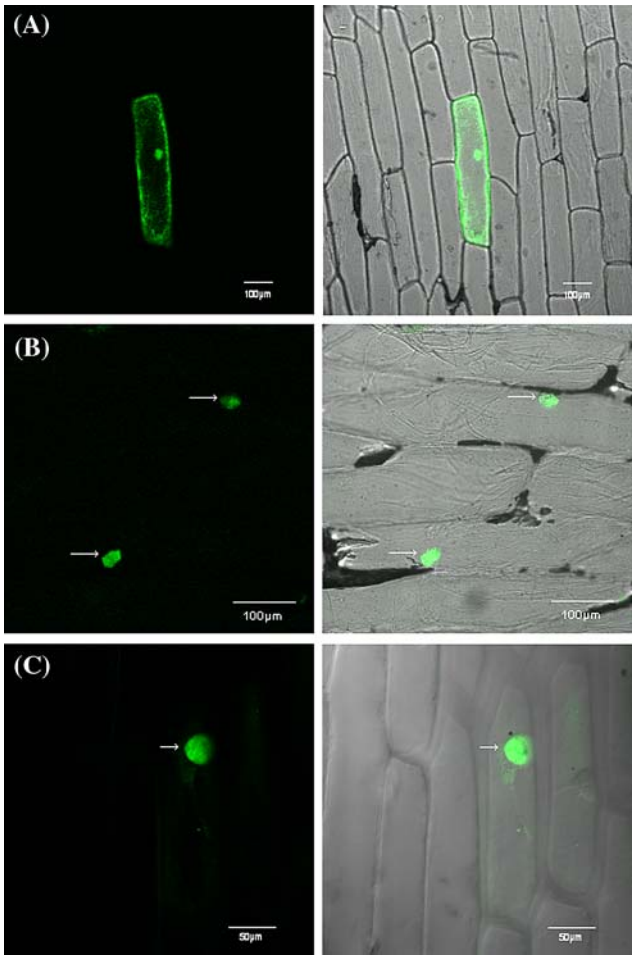

Fig. 4 Subcellular localization of the *WPBF* and *TaQM* in onion cells. Onion epidermal cells were bombarded with *35S::GFP-WPBF*, *35S::GFP-TaQM* and *35S::GFP*, respectively. After Incubation at 22°C for 20 h, the transformed cells were observed under a Zeiss Axiophot microscope. The fluorescent (left) and merged pictures (right) are shown. Arrows show the nucleus. (**A**) *35S::GFP* (**B**) *35S::GFP-WPBF* (**C**) *35S::GFP-TaQM*

35S::GFP construct displayed fluorescence in the cytoplasm and nucleus (Fig. 4A).

WPBF binds to the promoter of *Gli* gene

To determine whether WPBF can specifically bind to the *Gli* promoter, EMSAs were conducted. The fusion protein of GST-WPBF was expressed in bacteria and purified from the sonicated supernatant through GST-affinity chromatography. The purified fusion protein was mixed and incubated with two 30 bp biotin end-labelled oligonucleotide probes; one of them derived from the native promoter of *Gli* gene containing the TGTAAAG motif (*WT*), the other was its mutated version containing the TGTAgAc sequence (*mt*) (Mena et al. 1998). As shown in Fig. 5, the mobility of the *WT* probe was shifted only when incubated with

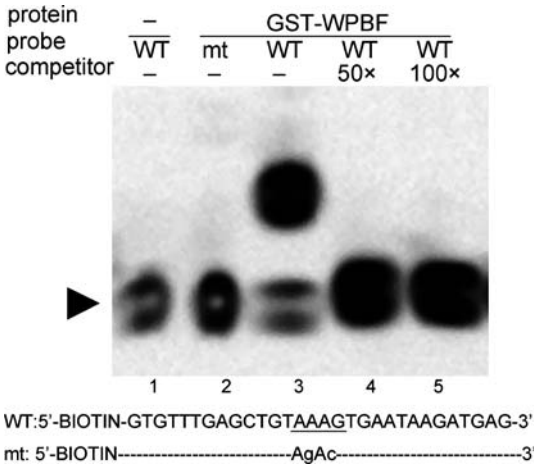

Fig. 5 Electrophoretic mobility shift assays (EMSA) of the recombinant WPBF protein fused to GST, and the TGTAAAGT motif of the *alpha-gliadin* gene promoter. EMSAs with the 30 bp biotin end-labelled wild-type (WT) and mutant (mt) oligonucleotides probes are presented at the bottom of the panel, identical residues are indicated by dashes (-) and the two mutated bases are shown by lower case letters. The P-box is underlined. Competition experiment was performed using excessive amounts (50 ×) unlabelled probe. Arrowheads show the position of the free probes

the GST-WPBF protein (lane 3) and not with a GST control protein (lane 1). This binding was effectively competed by a 50-fold molar excess of unlabelled WT probe (lane 4). The mutated *mt* probe could not form a protein-DNA complex with GST-WPBF protein (lane 2).

Transcriptional activity of WPBF

As described above, WPBF can bind to the *Gli* promoter in vitro. To further determine whether WPBF can regulate expression of the *Gli* promoter in vivo, the transient expression assays was explored. For this purpose, the full-length of *WPBF* and *TaQM* cDNA were placed under the control of CaMV35S promoter as effectors (Fig. 6A). The reporter vectors, pGli and pGli*, were constructed by introducing a 530 bp native promoter of *Gli* gene and its mutated version in pyrimidine sequence (AAAG → AgAc), respectively. The tobacco BY-2 protoplast cells were cotransfected by PEG-mediated method with the reporters alone or in combination with the effectors at a 1:1 molar ratio, and all sets contain *Renilla* luciferase gene as internal control. When BY-2 protoplasts were cotransfected with the pGli construct and the WPBF effector, a 6-fold increase in relative LUC activity was observed as compared with the activity measured when the pGli reporter was transfected alone (Fig. 6B). The combination of *WPBF* and *TaQM* proteins showed a higher

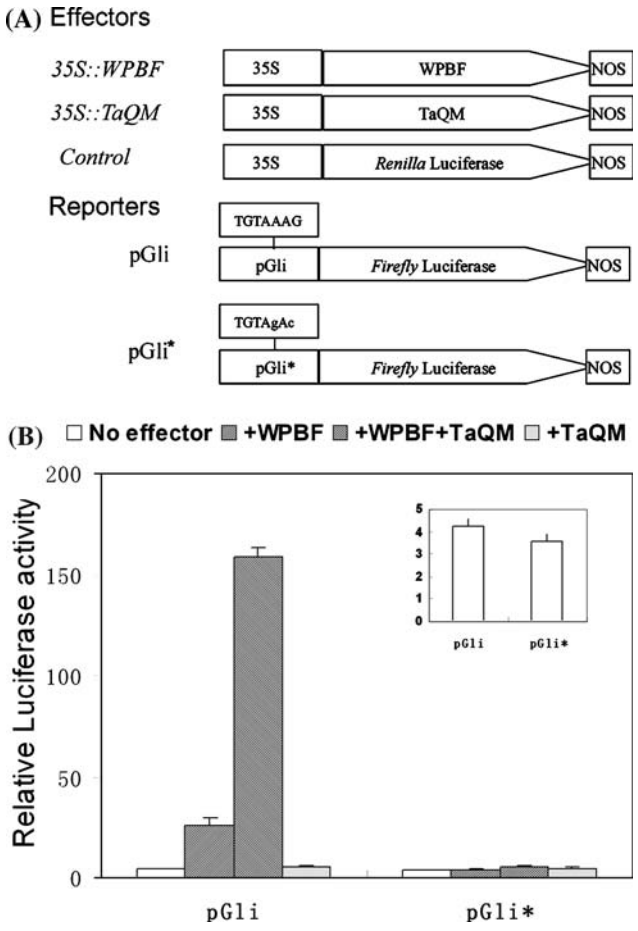

Fig. 6 Transient expression assays of the *alpha-gliadin* gene promoter by WBPF and *TaQM*. (**A**) Diagram of the reporter and effector constructs used in the BY-2 cell cotransfection assays. In the effector constructs, the complete *WPBF* and *TaQM* coding sequences were driven by the CaMV35S promoter. The reporter constructs contain 530 bp upstream sequence of the *alpha-gliadin* gene (pGli) or its mutated version (pGli*) that control the luciferase gene expression. The internal control construct contains the *Renilla* luciferase gene driven by the CaMV35S promoter. All the constructs have a nopaline synthase (Nos) sequences as transcription terminator. (**B**) Relative LUC activities of transfected BY-2 protoplasts. The reporters and effectors were cotransfected into BY-2 protoplasts with the indicated combinations (reporter : effector : internal control = 10:10:1 molar ratio). The values are the averages with SD of three independent experiments, after normalization with the internal control

transactivation effect than *WPBF* alone (Fig. 6B), but *TaQM* individually failed to activate luciferase reporter gene (Fig. 6B). When the mutated pGli* construct was used as reporter, only the background LUC activity was obtained from the transfected protoplast cells with *WPBF* or *TaQM* alone, or together (Fig. 6B). These results showed that WPBF regulate the *trans*-activation of the *Gli* gene promoter in BY-2 protoplast cells. Furthermore, the interaction of *TaQM* and *WPBF* had an additive effect in regulating the transcription of the *Gli* gene.

Discussion

WPBF is expressed in various tissues of wheat

WPBF, firstly isolated as a homologue of *BPBF*, was presumed to function during seed development. In the previous studies, it was found that the expression of *PBF* was constrained to the endosperm in barley (Mena et al. 1998), and the expression of maize *PBF* was also limited to the endosperm (Vicente-Carbajosa et al. 1997).

In our previous study, *WPBF* was identified from the roots at jointing stage using the differential display reverse transcription PCR method and was upregulated in wheat hybrid as compared to its parental inbred lines (Wang, et al. 2006). The fact that *WPBF* was expressed in the root attracted us to investigate its expression completely. Subsequently, the expression pattern was assayed by RT-PCR in the wheat tissues, including root, leaves and seeds. We observed that *WPBF was* constitutively expressed in all the tissues tested, which is quite different from that of the *PBFs* in barley and maize, where the expression was constrained to the endosperm.

To further determine the expression pattern, the promoter of *WPBF* was isolated and fused to GUS reporter and transformed into Arabidopsis (Col-0). GUS-specific staining showed the activity of *WPBF* in the vascular system and the embryo, which was in agreement with RT-PCR results, suggesting that *WPBF* was expressed throughout the whole life cycle, which is quite different from other endosperm-specific *PBFs*. The different expression patterns of *WPBF*, *BPBF* and maize *PBF* most likely resulted from the divergence of the promoter region between *WPBF* and other *PBFs*.

Dof domain of WPBF interacts with *TaQM*

With the exception of PB, there are several different *cis*-elements in the promoters of the storage protein genes, for instance, GLM (GCN4-like motif) and Myb-binding motif, can be recognized by O2 and MYB transcription factors, respectively, which can interact with Dof proteins to activate or repress expression of the target gene (Diaz et al. 2002; Diaz et al. 2005; Vicente-Carbajosa et al. 1997). WPBF might also

interact with MYB or O2 proteins to regulate the development or germination of seeds, but this needs further investigation.

An attempt was made to find the possible interaction partner of *WPBF* in wheat roots using yeast two-hybrid system. *TaQM*, a potential partner, was isolated from the root cDNA library at vigorous tillering stage. *TaQM* is a member of the QM family, which was originally identified from human as a putative tumor suppressor gene from a Wilms' tumor cell lines (Dowdy et al. 1991). In recent years, several *QM* genes have been identified from yeast, mouse, rat, chicken, Arabidopsis and rice. Sequence comparison shows that the homologues of QM are highly conserved throughout eukaryotes evolution (Dick et al. 1997; Kim et al. 1995; Lillico et al. 2002; Marty et al. 1993; Mills et al. 1999; Nguyen-Yue et al. 1997; Rivera-Madrid et al. 1993). Previous studies show that QM could interact with c-Jun, c-Yes and other Src family members by the bZIP or SH2 domain (Anderson et al. 1990; Kay et al., 2000; Shima et al. 2001). Here, we report that *TaQM* can interact with the Dof domain of WPBF. In addition, in vitro interaction of WPBF and *TaQM* was confirmed by pull-down assay (Fig. 3A, B). Subcellular localization performed in mammalian and yeast cells showed that QM was localized to the rough endoplasmic reticulum of cytoplasm or perinuclear region, but not in the nucleus (Dick et al. 1997; Eisinger et al. 1997; Loftus et al. 1997; Nguyen et al. 1998). In contrast, GFP-TaQM protein was localized in the nucleus of onion epidermal cells but not in the cytoplasm, while the WPBF protein was also localized in the nucleus (Fig. 4B, C). Furthermore, the interaction between *WPBF* and *TaQM* was also confirmed by BiFC experiment (Fig. 3C, D). The results of localization studies further provided the evidence that they are associated with each other, and might finally form a complex to regulate the target gene expression. Interestingly, the interaction between *WPBF* and *TaQM* appeared not to be affected by mutation in the Dof domain of WPBF, where Ala was replaced by Cys^{41} (data not shown). There has been evidence that a protein kinase inhibitor activate ZmDof1, suggesting that Dof proteins might be regulated by protein phosphorylation–dephosphorylation (Yanagisawa 2002). Furthermore, we also found a protein kinase C phosphorylation site in Dof domain (75–77aa, SLR) of WPBF by bioinformatics analysis.

Expression of *Gli* gene was *trans*-activated by WPBF

The putative target sites of WPBF in the promoter regions of *Gli* and *HMW-glutenin* genes, found by sequences alignment, was used to perform EMSAs. WPBF can bind specifically to the *WT* probe from the native promoter of *Gli* gene in wheat (Fig. 5), whereas the interaction was abolished when the mutated version (*mt*) was used. These results were consistent with the previous studies (Mena et al. 1998; Vicente-Carbajosa et al. 1997), suggesting that WPBF might also function during seed development.

To further investigate the function of *WPBF* in the *trans*-activation of *Gli* gene in vivo, a transient expression experiments by cotransfection BY-2 protoplast cells was explored. Though the experiment system was different, the results obtained were consistent with previous studies (Mena et al. 1998; Diaz et al. 2005). Here, the promoter sequence of *Gli* gene was fused to the upstream of *luciferase* gene as reporter (pGli). When the effector containing the full-length *WPBF* cDNA (*35S::WPBF*) was cotransfected into BY-2 protoplasts along with pGli, the transactivation was six-fold higher than that of the reporter alone (Fig. 6B). Moreover, a six-fold higher LUC activity was also observed through the cotransfection with *35S::WPBF*, *35S::TaQM* and pGLi than that of the combination of *35S::WPBF* and pGLi (Fig. 6B). In contrast, when the effector of *35S::WPBF* was cotransfected with the mutated reporter of pGli* together, no higher LUC activity was detected. These results indicated that an additive effect exist in the combination of WPBF and TaQM over the expression of *Gli* gene. This phenomena was also observed in BPBF and GAMYB, SAD and BPBF, and PBF (maize) and O2 (Diaz et al. 2002, 2005; Vicente-Carbajosa et al. 1997). The effector of *35S::TaQM* individually could not exert upon the promoter of *Gli* gene, suggesting that TaQM could not bind the DNA sequence.

In conclusion, our data provide evidence that *WPBF* interacts with *TaQM*, both in vivo and in vitro, and the expression of the *Gli* gene was related to the expression and interaction of *WPBF* and *TaQM*. However, the functions of *WPBF* in the root and leave development are still unknown. Hence, more works are needed to understand the roles of *WPBF* during wheat growth and development.

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