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ORIGINAL ARTICLE

5-Azacytidine treatment and *TaPBF-D* over-expression increases glutenin accumulation within the wheat grain by hypomethylating the *Glu-1* promoters

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Abstract

Key message **5-azaC treatment and *TaPBF-D* over-expression decrease C-methylation status of three *Glu-1* gene promoters, and aid in enhancing the expression of the *Glu-1* genes.**

Abstract The wheat glutenins exert a strong influence over dough elasticity, but the regulation of their encoding genes has not been firmly established. Following treatment with 5-azacytidine (5-azaC), both the weight and glutenin content of the developing and mature grains were significantly increased. The abundance of transcript produced by the *Glu-1* genes (encoding high-molecular-weight glutenin subunits), as well as those encoding demethylases and transcriptional factors associated with prolamin synthesis was higher than in grain of non-treated plants. These grains also contained an enhanced content of the prolamin box binding factor (PBF) protein. Bisulfite sequencing indicated that the *Glu-1* promoters were less strongly C-methylated in the developing grain than in the flag leaf, while in the developing grain of 5-azaC treated plants, the C-methylation level was lower than in equivalent grains of non-treated plants. Both *Glu-1* transcript abundance and glutenin content were higher in the grain set by three independent over-expressors of the D genome homoeolog of *TaPBF* than in the grain set by wild type plants. When assessed 10 days after flowering, the *Glu-1* promoters' methylation level was lower in the developing grains set by the *TaPBF-D* over-expressor than in the wild type control. An electrophoretic mobility shift assay showed that PBF-D was able to bind in vitro to the P-box of *Glu-1By8* and *-1Dx2*, while a ChIP-qPCR analysis revealed that a lower level of C-methylation in the *Glu-1By8* and *-1Dx2* promoters improved the TaPBF binding. We suggest that promoter DNA C-methylation is a key determinant of *Glu-1* transcription.

Abbreviations

HMW-GS	High-molecular-weight glutenin subunit
LMW-GS	Low-molecular-weight glutenin subunit
PBF	Prolamin-box binding factor
SPA	Storage protein activator
GAMYB	GA-dependent MYB transcription factor
MET	Methyltransferase
CMT	Chromomethylase
DRM	Domains-rearranged methyltransferase
DNMT	DNA methyltransferase homologue
DME	Demethyltransferase
DML	DME-like genes

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Introduction

The end-use quality of bread wheat is largely determined by the flour's gluten fraction, which comprises gliadins and glutenins. The former are monomeric, non-aggregating proteins, while the latter form a complex aggregate composed of both high- and low-molecular weight glutenin subunits held together by intermolecular disulfide bonds. The glutenins account for 30–50% of the grain protein (Shewry et al. 2003). The high-molecular-weight glutenin subunits (HMW-GS) are encoded by a set of *Glu-1* homoeoloci, with each locus housing two genes, one of which encodes an *x*-type and the other a *y*-type subunit (Payne et al. 1987; Gao et al. 2010). The *Glu-1* genes

are specifically expressed in the developing endosperm (Shewry and Halford 2002), thanks to the activity of their promoter sequence (Lamacchia et al. 2001). HMW-glutenin synthesis is controlled at the transcriptional level by five *cis* elements within the *Glu-1* promoters in conjunction with a number of transcription factors (TFs) belonging to four distinct families (Shewry and Halford 2002; Ravel et al. 2014). More precisely, the GCN4 like-motif (GLM, 5′-ATGAG/CTCAT-3′) is bound by bZIP proteins that belong to the Opaque2, like SPA (Storage Protein Activator) (Ravel et al. 2014). The prolamin box (P-box, 5′-TGT AAAG-3′) is recognized by PBF (Prolamin-box binding factor), which is a DOF-type TF (Diaz et al. 2005). The promoter region of wheat *alpha*-gliadin or *LMW-GS* gene possessed typical P-box, while the *HMW-GS* promoter only contained P-like box, and its core motif was different from typical P-box (Dong et al. 2007). Others additional *cis* elements, MYB recognition sites (5′-AACAAC-3′), VP1 recognition sites, and basal promoter elements, are conserved in the promoters of seed storage protein genes (Guo et al. 2015). However, the details of how the genes' expression is regulated have not been fully established as yet (Guo et al. 2015).

Cytosine (C-) methylation regulates the expression of many eukaryotic genes (Steward et al. 2002; Vaillant and Paszkowski 2007). In *Arabidopsis thaliana*, CpG and CpNpG methylation is maintained largely through the activity of, respectively, the methyltransferase MET1 and the chromomethylase CMT3 (Cao and Jacobsen 2002), while de novo C-methylation is carried out by the enzymes Domains-Rearranged Methyltransferases, DRM1 and DRM2 (Henderson and Jacobsen 2007), with some contribution from MET1 and CMT3 (Gehring and Henikoff 2008). Over a third of the *A. thaliana* gene complement experiences a degree of C-methylation, which underlies their differential expression both spatially and temporally (Zilberman et al. 2007). With respect to the *Glu-1* gene family, Flavell and O'Dell (1990) noted that their overall C-methylation level is lower in the grain than elsewhere in the plant.

The cytosine analog 5-azacytidine (5-azaC) has been exploited as a demethylating agent in several different plant systems. The effects of 5-azaC treatment are wide ranging (Solís et al. 2015). According to Vanyushin (2005), exposure to 5-azaC can raise the glutenin content of the wheat grain by as much as 30%. Here, the effect of 5-azaC treatment on both grain glutenin content and the C-methylation level of the *Glu-1* promoter have been explored, along with an analysis of its influence over the transcription of three *Glu-1* members and key genes encoding methylases and relevant TFs. A comparison is also made of the size and number of protein bodies (PBs) present in the developing endosperm.

Materials and methods

Wheat materials and growth conditions

Grains of cv. Chinese Spring (CS) were germinated on moist filter paper for 2 days at 20 °C. The seedlings were transplanted into soil and grown in greenhouse at 21/18 °C (day/night) and 65% relative humidity under a short-day (8/16 h light/dark) photoperiod with light intensity of 120 µmol m^{-2} s^{-1} for 4 weeks. The plants were then switched to long-day conditions with a 16/8 h light/dark photoperiod and the same light intensity. For the 5-azaC treatment, CS plants at the flag leaf emergence stage (Zadoks et al. 1974) were irrigated five times with distilled water containing either 0, 25, 50 or 100 µM 5-azaC at 2-day intervals. Each treatment was replicated three times. Developing grains from the central portion of the ear were harvested 10, 15, 20, 25 and 30 days after flowering (DAF), and flag leaf samples were taken at 10 DAF. The samples were snap-frozen in liquid nitrogen and stored at − 80 °C until use.

Analysis of glutenin content, grain weight and the imaging of PBs

The grain morphology from seven different developmental stages (5, 10, 15, 20, 25, 30, and 45 DAF) was observed by stereo microscope, and dry weight was measured. The grains collected from different stages were put into a 65 °C oven and dried to constant weight. Thousand-kernel weight (TKW) was evaluated by weighing three samples of 100 kernels for each plants including control and 5-azaC treatment. Glutenin was extracted from mature grains and electrophoresed as described by Chen et al. (2007). A 2-D Quant Kit (GE Healthcare, Chicago, IL, USA) was used to determine the glutenin content. In this experiment, nine grains taken the middle six grains of three randomly chosen heads were divided into three groups, each group included three grains and each grain were analyzed including three technical repeats in 2-D quant assay. To image the PBs at various developmental stages of the immature grain, samples were sliced transversely into 1–2 mm pieces and fixed at 4 °C for 16 h in 4% (v/v) paraformaldehyde, 1% (v/v) glutaraldehyde dissolved in 50 mM potassium phosphate buffer (pH 6.8). The samples were then stained by immersion in 2% (w/v, 79 mM) OsO_4 dissolved in the same buffer. After rinsing three times in the buffer, the samples were dehydrated by passing through an ethanol series (10, 30, 50, 70, 90, 95 and 100%) with a 15 min immersion at each step, then were passed through an LR White resin (London Resin

Company Ltd, England) dissolved in ethanol series (20, 40, 60 and 80%) with a 2 h immersion at each step; finally the samples were left overnight in 100% LR white resin for 48 h with the resin exchanged every 12 h. The samples were placed in a plastic moulds and polymerized for 2 days at 55 °C, then sectioned by ultramicrotome (LKB Ultratome III, Stockholm, Sweden). The sections were overlaid on a copper grid coated with formvar membrane and carbon and post-stained for 5 min in 2.5% (w/v, 59 mM) uranyl acetate, followed by a 3 min immersion in 0.1% (w/v, 1 mM) lead citrate. The imaging was carried out using an H7500 transmission electron microscope (Hitachi, Tokyo, Japan) running at 80 kV.

Bisulfite sequencing

Genomic DNA was isolated following the Li and Dahiya (2002) method, and then processed using an EpiTect Bisulfite kit (Qiagen, Düsseldorf, Germany). MethPrimer software (Li and Dahiya 2002) was used to identify CG islands and to design relevant primers (Table S1). The resulting PCR products were inserted into the pEASY-T Vector (TransGen, Beijing, China). At least 30 clones per insert were processed for sequencing. Sequences for which the cytosine transformation efficiency was < 97% were removed using Biq Analyzer software (Bock et al. 2005). The ratio of C-methylation recorded at each CG, CNG and CNN site was calculated and transformed into a percentage using CyMATE software (http://www.gmi.oeaw.ac.at/research-groups/cymate/cymate/). PLACE software (http://www.dna.affrc.go.jp/PLACE/signalscan) was used to identify conserved motifs (Table S2) in the target promoter regions.

Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from immature grains or leaf material using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using an M-MLV reverse transcriptase kit (Invitrogen). The set of qPCR primers was designed using Primer Premier v5.0 (Table S3). The qPCRs were implemented using the iCycler iQTM real-time PCR detection system (Bio-Rad, Hercules, CA, USA), following the manufacturer's protocol. The wheat *ACTIN* gene (GenBank: GQ339780) was used as the reference sequence. Each 10 µL reaction contained 1 µL diluted cDNA (2000 ng/µL), 5 µL 2× Power SYBR Premix Ex Taq II (Takara, Dalian, China), 3 µL RNase free water and 0.5 µM of each primer. The data were analyzed using Bio-Rad CFX Manager software. Each reaction was repeated three times for each of three independent biological samples.

Antibodies against TaPBF and Western blotting

A monoclonal antibody recognizing the CS TaPBF-D protein (GenBank: CAA09976) was developed by Abmart (Shanghai, China). This antibody could also recognize TaPBF-A and -B, because peptide used to prepare antibody was same among TaPBF-A, -B and -D. The western blotting procedure followed Chen et al. (2014) with minor modifications. Proteins (10 µL) were separated on SDS-PAGE, with a 10% gel, and transferred to a polyvinylidene difluoride membrane (Millipore, Massachusetts, USA), which was blocked with 5% fat-free milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h, incubated with antibody diluted 1:2000, washed three times for 5 min each with TBST and incubated for 1 h with secondary antibody diluted 1:5000. The membrane was washed five times for 8 min each with TBST before the next step. Specific protein bands were visualized with Immobilon Western Chemiluminescent horseradish peroxidase substrate (http://www.millipore.com).

The construction of TaPBF-D transgenic wheat

To generate a *TaPBF-D* over-expressor, the coding sequence was amplified from cDNA extracted from immature grain of cv. CS, using the sense primer, 5′-AAGCTTATGGAGGAA GTGTTTCCGTCAAA-3′, and antisense primer, 5′-GAG CTCTTACATCAGGGAGCTGCTGTTGAG-3′, then ligated into the pGA3626 vector to place it under the control of the *Ubi* promoter (Kim et al. 2009). Introduced restriction site is *Hin*dIII for sense primer and *Sac*I for antisense primer, respectively. The resulting cassette was transformed into cv. Jimai22 using the shoot apical meristem method (Zhao et al. 2006). The stable integration of *TaPBF-D* was confirmed by PCR using either genomic DNA (including partial vector and *TaPBF-D*, Fig. S1) or cDNA as the template and Western blotting.

Electrophoretic mobility shift assay (EMSA)

TaPBF-D was expressed in *E. coli* by constructing a transgene containing *TaPBF-D* cDNA cloned into pGEX6P-1 (obtained from Taiyong Quan, Shandong University, Jinan, China), which produces a translational fusion to GST, using the sense primer, 5′-CTGATATCATGGAGG AAGTGTTTCCGTC-3′, and antisense primer, 5′-CTC TCGAGCATCAGGGAGCTGCTGTTGAG-3′. Introduced restriction site is *Eco*RV for sense primer and *Xho*I for antisense primer, respectively. The expression of the recombinant protein was induced by the addition to the bacterial growth medium of 0.1 mM IPTG dissolved in Luria–Bertani buffer, and an overnight incubation at 16 °C. The cells were harvested, washed and resuspended in 30 mL phosphate buffered saline. PMSF (final concentration 1 mM) was

added to inhibit protease activity, and the cells were then sonicated for 1 h with incubation on ice between repetitions (100 W, ultrasound working 2 s, interval 5 s, and then ultrasound working 30 s, repeated 3–4 times) and centrifuged (13,000*g*, 45 min). The supernatant was filtered through a 0.22 μm membrane, then mixed with 200 μL GST MAG agarose beads (Novagen, http://www.novagen.com) and rocked overnight at 4 °C. The GST beads were rinsed seven times with PBS, and the fusion protein eluted and enriched from the beads by immersion for > 7 h in 50 mM Tris–HCl (pH 8.0) containing 10 mM reduced glutathione (Fig. S2). The protein concentration of the eluant was determined spectrophotometrically. EMSAs were carried out according to the manufacturer's instruction (Dig Gel Shift Kit, 2nd Generation, Roche, Germany). The probes were prepared by cooling double-stranded oligonucleotides from 100 °C to room temperature in annealing buffer. The resulting products were 5′-end labeled with biotin. DNA binding reactions were performed in a 20 μL mixture of 100 mM Tris–HCl, 500 mM KCl, 10 mM DTT (pH 7.5), 2.5% (v/v) glycerol, 0.2 mM EDTA, 50 ng/μL poly(dI–dC). After holding at room temperature for 20 min, the samples were electrophoresed through a 6% native polyacrylamide gel, electrophoretically transferred to a nylon membrane and detection of the biotin-labeled DNA was performed according to the manufacturer's instructions (Dig Gel Shift Kit, 2nd Generation, Roche, Germany).

Chromatin immunoprecipitation-quantitative real-time PCR (ChIP-qPCR)

Immature grains were harvested at 5, 10, 15, 20, 25 and 30 DAF, cut into pieces of ~ 0.2 cm^3, fixed by immersion under vacuum for 15 min in 1% (v/v) formaldehyde and neutralized by adding 0.125 M glycine. ChIP was performed according to the manufacturer's instruction (EpiQuik™ Plant ChIP kit, Epigentek, USA), with three technical times for each of three independent biological samples. The immunoprecipitated and purified DNAs (three biological replicates) were used as the template for a series of qPCRs based on primers to specifically amplify the target sequences (Table S4). Fold enrichment was normalized to the quantity of input chromatin and then the control gene (wheat *ACTIN*, GenBank: GQ339780), which is indicated in the figure legends, and expressed in the form mean ± standard error of the mean (SEM).

Statistical analysis

Quantitative data including three biological and three technical replicates respectively, are presented in the form of mean ± SD. Means were compared using the Student's two-tailed *t* test.

Results

Effect of the 5-azaC treatment on the weight and glutenin content of the grain and the size and number of PBs

The 25 μM 5-azaC treatment had no perceptible effect on the C-methylation status of the *Glu-1* gene promoter sequences, while the 100 μM treatment compromised plant growth too strongly (data not shown); thus, plants exposed to the 50 μM 5-azaC were compared with non-treated ones (CS). Compared with CS, the grain plumpness from the mature kernels and developing grain was slightly increased under 5-azaC treatment (Fig. 1a, b). The TKW of the 5-azaC treatment was significantly higher than non-treated in grain development stages after 20 DAF, increased by 23.6% at 45 DAF (Fig. 1c). According to the 2-D Quant kit analysis, the effect of the 5-azaC treatment was to increase the glutenin content by 14.2% (Fig. 1d), which was borne out by the SDS-PAGE profiles (Fig. 1e). Both the number and size of the PBs harbored by the developing grains were also raised by the treatment (Fig. S3a, b). A larger number of small (< 2 μm diameter) PBs were formed in the non-treated plants' developing grains sampled at 10 DAF, but the treated plants' grains harbored a higher number of PBs of diameter 2–6 μm (Fig. S3c). At 15 DAF, the number of PBs of diameter 2–4 μm was greater in the treated plants' grains, and the same was true for PBs of diameter > 6 μm at 20 DAF (Fig. S3d, e).

The 5-azaC treatment down-regulated genes encoding *methylase* and up-regulated those encoding related *TFs* and *Glu-1*

When the transcriptional response of certain methylase and demethylase genes was examined by qPCR, the genes encoding methylases, *TaMET2a*, *TaMET2b*, *TaMET*3 and *TaCMT*, were shown to be down-regulated by the 5-azaC treatment (Fig. 2a). In contrast, the genes encoding demethylases, *TaDNMT*, *TaDRM*, *TaDME* and *TaDML*, were up-regulated (Fig. 2b). The abundance of related TFs transcript *TaPBF* was significantly increased, particularly apparent during the period 10–20 DAF (Fig. 2c), which was consistent with changes in protein levels as tested by Western blotting (Fig. 2d). The expression of *TaSPA* was also significantly increased during the period 10–20 DAF (Fig. 2e). The A genome homologue of *TaGAMYB* (*TaGAMYB-A*) was up-regulated over the period 10–15 DAF, as was *TaGAMYB-B* over the period 15–20 DAF; *TaGAMYB-D* was not responsive (Fig. 2f). The *Glu-1* genes, *1Dx2*, *1Dy12*, and *1By8,* but not *1Bx7*, responded to the treatment with increases in transcript levels (Fig. 2g).

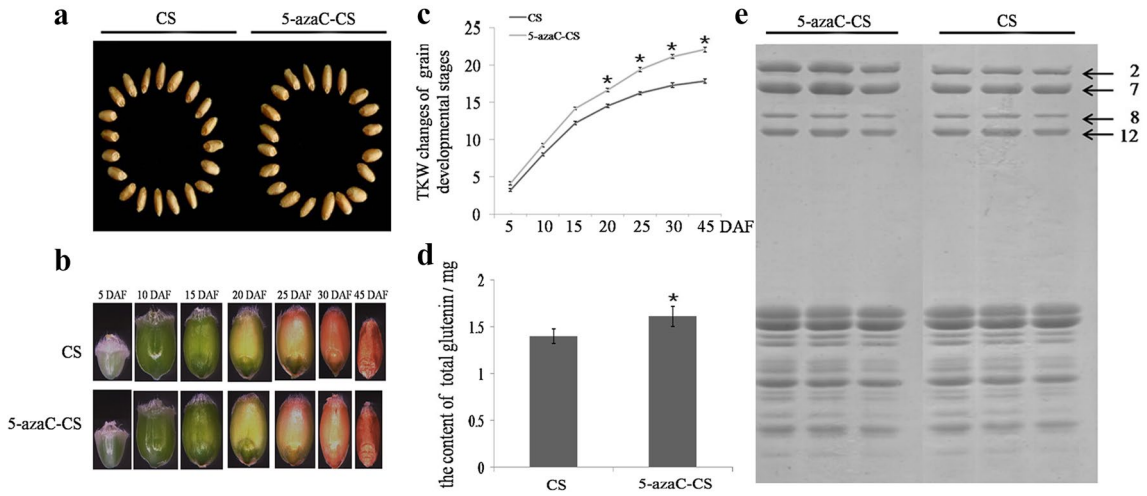

Fig. 1 The weight and glutenin content of grains set by plants treated with 50 μM 5-azaC. **a** Observation of surface of the mature kernels from non-treated (CS) and treated (5-azaC-CS) plants. **b** The appearance of the developing grain between 5 and 45 DAF. **c** The TKW of developing grain between 5 and 45 DAF. **d** glutenin content of grain set by non-treated (CS) and treated (5-azaC-CS) plants. Whiskers indicate a confidence interval based on the Student's *t* test ($P < 0.05$, $n = 3$); asterisks indicate values which differ significantly between grain set by non-treated and treated plants. **e** The high-molecular-weight glutenin subunit profile of grain set by non-treated (CS) and treated (5-azaC-CS) plants. 2, 1Dx2 subunit; 7, 1Bx7 subunit; 8, 1By8 subunit; 12, 1Dy12 subunit

The methylation status of the *Glu-1* promoter sequences

Bisulfite sequencing was used to track the C-methylation status of the promoters of *Glu-1Bx7* (nucleotide positions − 849 to − 90), *-1By8* (− 789 to − 5) and *-1Dx2* (− 789 to − 105) (Fig. 3a, Fig. S4). The efficiency of the sodium bisulfite treatment in converting non-methylated C to T was close to 100% (Fig. 3a, Fig. S4). The scan involved 499 C nucleotides, of which 324 were methylated. In DNA recovered from the flag leaf, the level of C-methylation was greater than in DNA recovered from developing grains (Fig. 3b). According to PLACE analysis, 114 of the methylation sites lay within a conserved motif. Of these, 25.3% were methylated in the flag leaf DNA, but only 8.8% in the DNA isolated from developing grain. The cytosines lying at positions − 789 and − 785 of the *Glu-1Bx7* promoter were methylated in the flag leaf DNA but not in developing grain DNA (Fig. 3a). The C-methylation response to the 5-azaC treatment was stronger in the developing grain than in the flag leaf. The treatment resulted in a reduction in C-methylation at CG sites outside of the conserved motifs, but a only modest reduction at CHG and CHH sites (Fig. 3c, d). As an example, C-methylation in the *-1Bx7* promoter sequence between positions − 561 and − 90 was decreased by the treatment in the DNA recovered from developing grain, especially in the period 10–30 DAF (Fig. S4a). In the *-1Bx8* (positions − 789 to − 364) and the *-1Dx2* (positions − 789 to − 462) promoter sequences, the treatment also reduced the level of C-methylation (Fig. 3b, c).

The effect of over-expressing *TaPBF-D*

A total of 3 independent stable transgenic lines over-expressing *TaPBF-D* were successfully generated (Fig. 4a, Fig. S1), which expressed higher TaPBF-D compared with wild type (CK) (Fig. 4b, c). The glutenin content of their grains was higher by 11–15% than that of the wild type grains (Fig. 4d, e). Two OE lines were selected further analysis. Compared to wild type, a qPCR-based analysis showed that the transcript abundance of *TaSPA* and the *demethylase* genes were up-regulated at 15–20 DAF in transgenic lines, while the transcription of *methylase* genes was mostly the same (Fig. S5). In addition, the transcription of *TaGaMYB* genes was not significantly different between wild and transgenic lines (Fig. S5a). When bisulfite sequencing was applied to the three *Glu-1* promoter sequences, it was apparent that the over-expression of *TaPBF-D* resulted in a reduction in the level of methylation at CG sites outside of the conserved motifs, but only a modest reduction in the CHG and CHH sites (Fig. 4f, Fig. S6).

Hypomethylation of the *Glu-1* promoter sequences aids the binding of related TFs

An analysis of the *Glu-1* upstream sequences showed that each gene harbored one P-like box within 1000 nt of the transcription start codon (Table S5). The potential binding activity of these P-boxes was tested using EMSA based on a synthetic oligonucleotide P-box and recombinant TaPBF-D (Fig. S2). A DNA–protein complex was formed

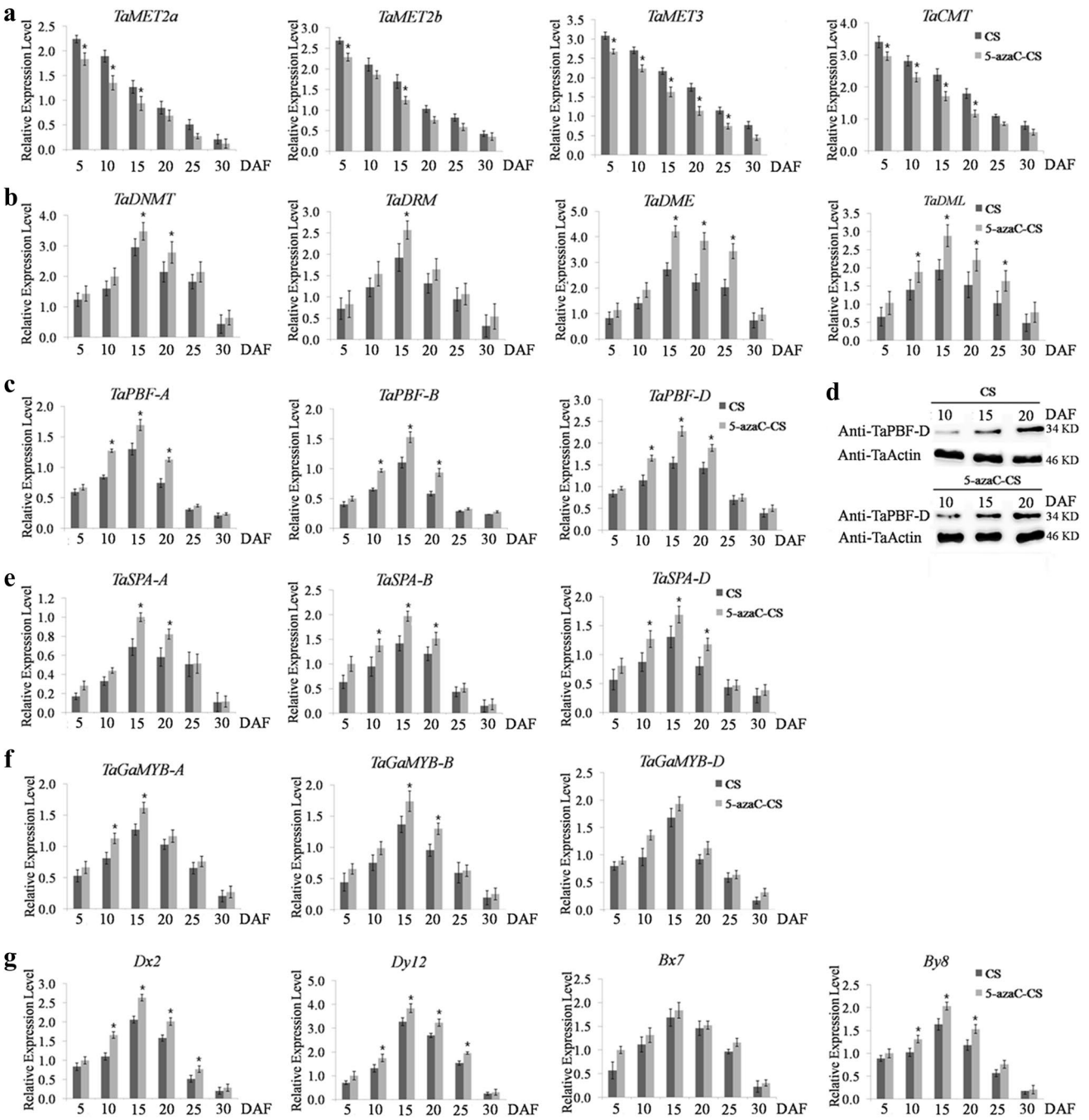

Fig. 2 Transcription and expression of key genes during grain development of non-treated (CS) and treated (5-azaC-CS) plants. **a** Genes encoding methylases. **b** Genes encoding demethylases. **c** *TaPBF* (*A*, *B*, and *D*). **d** Western blot analysis of TaPBF. **e** *TaSPA* (*A*, *B*, and *D*). **f** *TaGaMYB* (*A*, *B*, and *D*). **g** *Glu-1* in the developing grain set by non-treated (CS) and treated (5-azaC-CS) plants. Whiskers indicate the confidence interval based on a Student's *t* test ($P < 0.05$, $n = 3$); asterisks indicate values which differ significantly between grain set by non-treated and treated plants

with the P-box motif for the *WT* probes, which have been derived from the native promoter of *alpha-Gli* gene (Accession number: pW1215) containing the TGTAAA G motif, while a band shift was detected when either the *Glu-1By8* or the *-1Dx2* probes were used (Fig. 5a, lane 4, 5, 6). Furthermore, the WT probes had the strongest binding ability with recombinant TaPBF-D (lane 4), followed by *1Dx2* (lane 6), and the binding ability of *1By8* was the weakest (lane 5) (Fig. 5a). The difference of TaPBF-D binding ability among the three probes was directly related

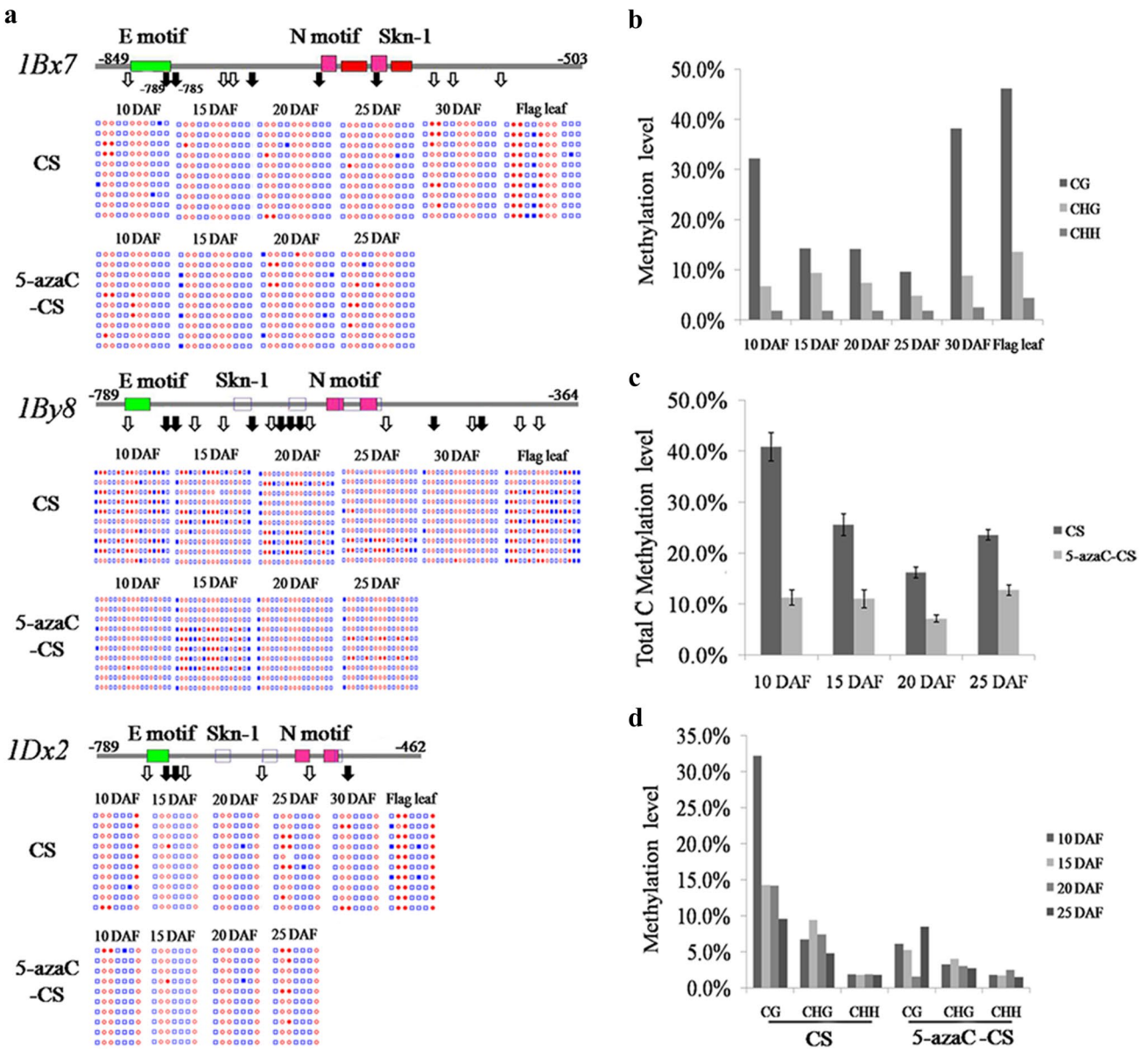

Fig. 3 Variation in C-methylation level in the *Glu-1* promoter sequences. **a** Bisulfite sequencing of the *Glu-1* promoter in DNA recovered from developing grain and leaf of CS. Upper panel: *Glu-1Bx7* (from positions − 849 to − 503); Middle panel: *Glu-1By8* (from positions − 789 to − 364); Lower panel: *Glu-1Dx2* (from positions − 789 to − 462). The two types of cytosine residues, CG (red circles), CHG (blue square) are shown in the map. Filled and empty circles or squares denote methylated and unmethylated cytosines, respectively. The arrows indicate the location of different methylation types: CHG (empty arrows); CG (filled arrows). − 789 and − 785 bp indicate the difference in methylation status at the same base position in grain and flag leaf. **b** C-methylation levels in CG, CHG and CHH sites in the developing grain and leaf of CS. **c** Global C-methylation levels in developing grain set by non-treated (CS) and treated (5-azaC-CS) plants. **d** The effect of 5-azaC treatment on the C-methylation level at CG, CHG and CHH sites in CS developing grain

to bases deficiency of P-like box in the Glu-*1By8* and the *-1Dx2* probes. The data was interpreted as meaning that TaPBF bound in vitro to the P-box motif. ChIP-qPCR was then performed to test the extent to which the C-methylation level of the promoter affected TF binding. Enrichment of TaPBF-D was observed in the case of the *Glu-1By8* and *-1Dx2* promoters, particularly in 15 DAF grains (Fig. 5b, c). This time coincided with the strongest level of *Glu-1* transcription (Fig. 2g). Compared to untreated wheat, in 5-azaC-CS, significantly high levels were detected at four different endosperm development stages, 10–25 DAF (Fig. 5b, c). Taking these results together, it was concluded that TaPBF-D could bind the P-like box motif of the *Glu-1By8* and *-1Dx2* promoters, and the low methylation level of the promoter could enhance the binding ability of TaPBF-D to P-like box.

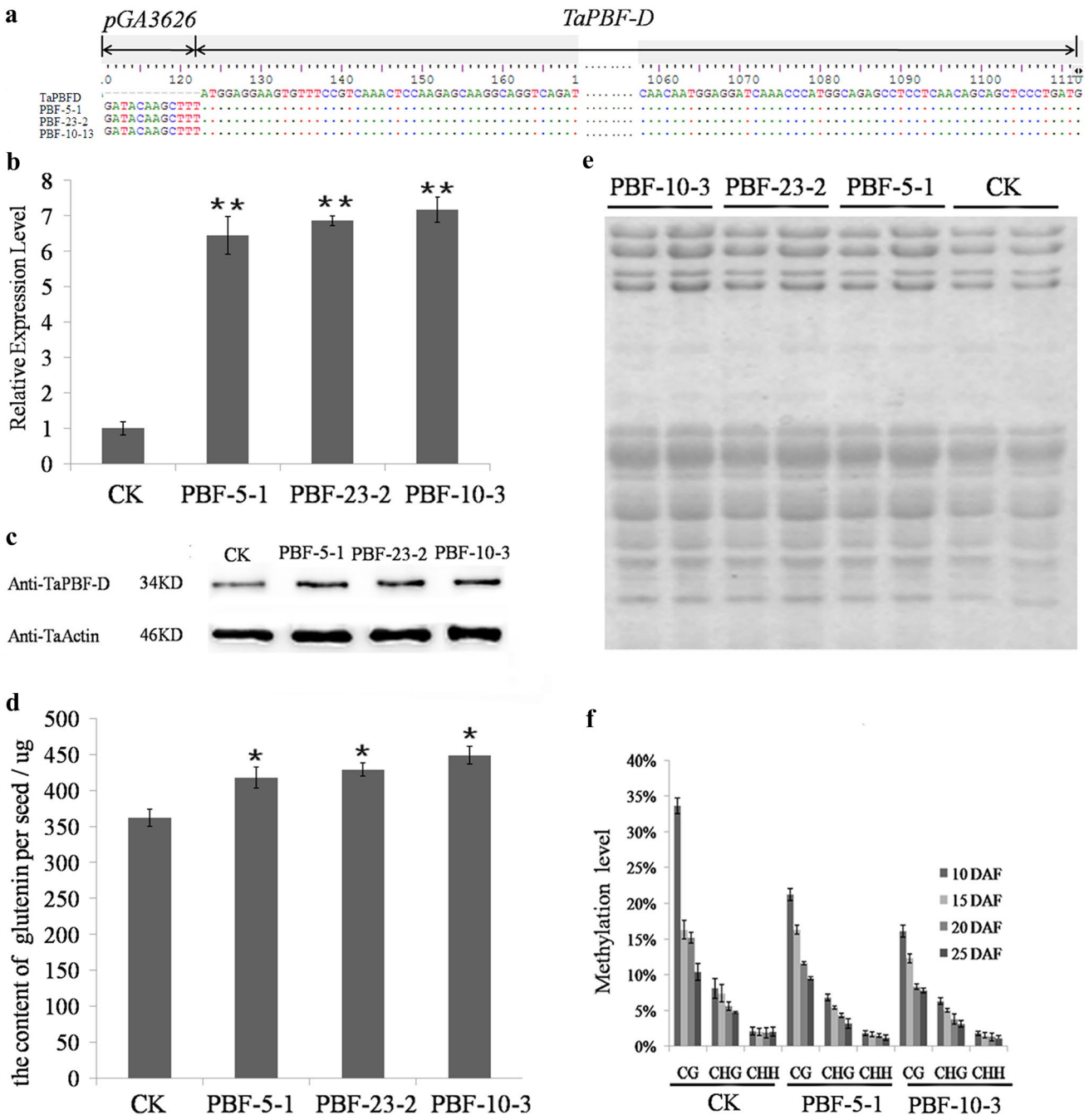

Fig. 4 Validation of the *TaPBF-D* over-expressor line and the accumulation of glutenin in its developing grain. **a** Partial sequence of the vector and *TaPBF-D*. **b** The transcription of *TaPBF* in wild type and the over-expressor lines. **c** The expression of *TaPBF* in wild type and the *TaPBF-D* over-expressor lines. **d** The glutenin content in the grain set by wild type and the *TaPBF-D* over-expressor lines. **e** SDS-PAGE profiles showing the glutenin subunit composition of wild type and the *TaPBF-D* over-expressor lines. **f** The C-methylation levels at CG, CHG and CHH sites in the *Glu-1* promoters of wild type and the *TaPBF-D* over-expressor lines. Whiskers indicate the confidence interval based on a Student's *t* test ($P < 0.05$, $n = 3$); asterisks indicate values which differ significantly between grain set by wild type and the *TaPBF-D* over-expressor line

Discussion

Treatment with 5-azaC induces some demethylation of genomic DNA through its inhibition of methyltransferase activity (Stresemann and Lyko 2008). Phenotypic changes induced by 5-azaC treatment have been documented in a number of plant species (Burn et al. 1993; Vyskot et al. 1995; Sano et al. 1990). The alteration of flowering time

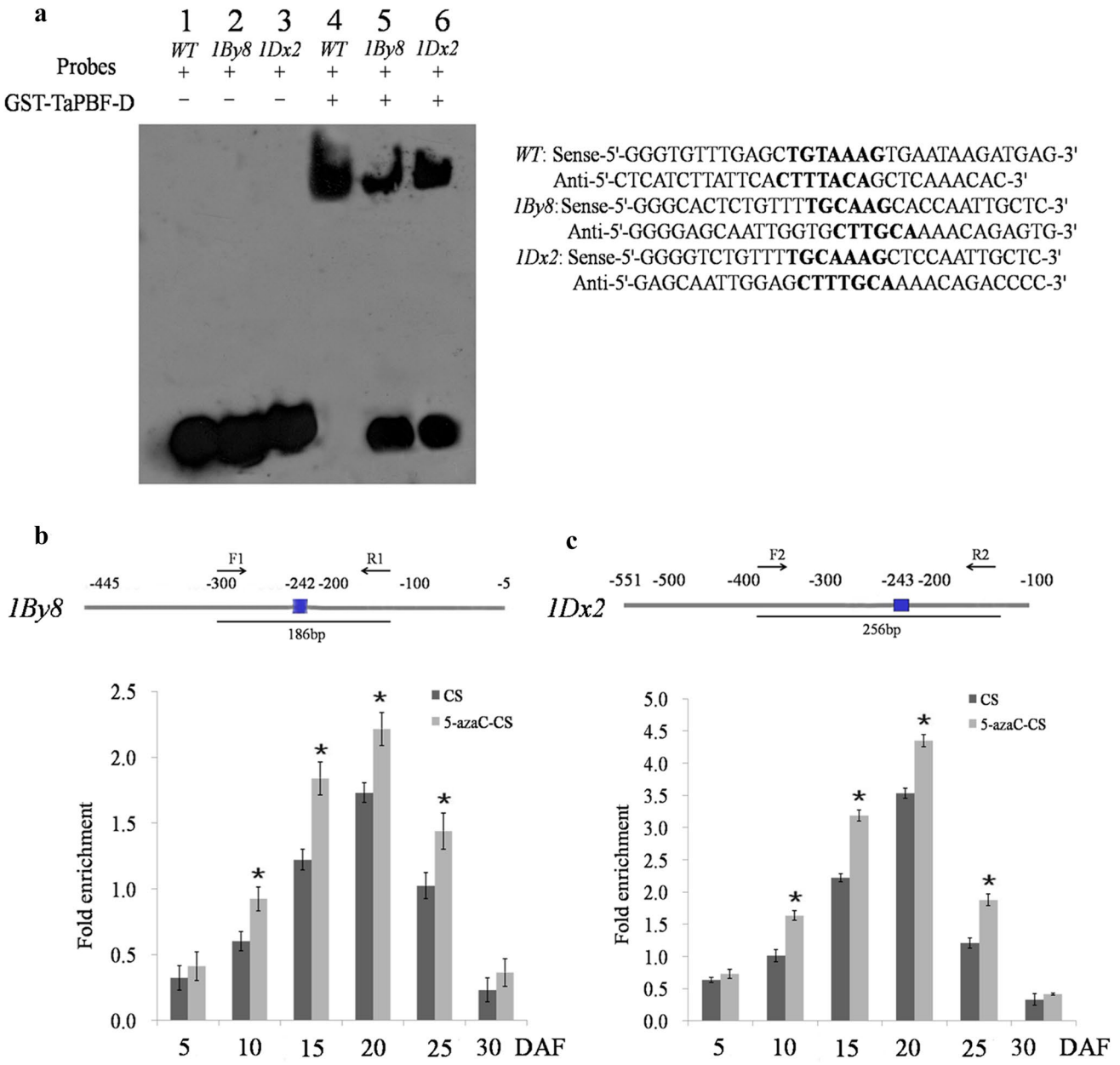

Fig. 5 Hypomethylation in the *Glu-1* promoter sequence aids the binding of TaPBF. **a** EMSA based on recombinant TaPBF-D protein fused to GST, in conjunction with biotin-labeled probes derived from the *Glu-1* promoters, supplied in equimolar concentration. The level of C- methylation in the P-like box present in the promoters of **b** *Glu-1By8* and **c** *-1Dx2* during the development of grain set by non-treated (CS) and treated (5-azaC-CS) plants. F1 + R1 and F2 + R2 indicate primer pairs used to amplify, respectively, the *Glu-1By8* and *-1Dx2* promoter sequences. The fold-enrichment value (*y*-axis) was normalized to input and a non-target control gene wheat *ACTIN* (GenBank: GQ339780); values given in the form mean ± SEM ($n = 3$). The asterisks represent significant difference determined by Student's *t* test at $P < 0.05$, respectively

has been the major focus: the treatment typically brings flowering forward (Fieldes and Amyot 1999; Fieldes et al. 2005; Brown et al. 2008; Kondo et al. 2007; Iwase et al. 2010). Here, the intention was to characterize the effect of 5-azaC treatment on the accumulation of dry matter in the grain, and specifically, the grains' glutenin content.

The expression of the *Glu-1* genes occurs primarily at the transcriptional level, involving *cis*-acting motifs in the promoter sequences and *trans*-acting TFs (Shewry and Halford 2002). The specificity and levels of expression of various subunits are based on the interaction of *cis*-elements and related TFs, some of these TFs can directly regulate the

expression of HMW-GS, or through interactions between protein and protein to form large complex to regulate the expression of the seed storage protein (Ravel et al. 2014). The present experiments confirmed that the over-expression of *TaPBF-D* stimulated the accumulation of glutenin (Fig. 4), along with an up-regulation of *TaSPA* (Fig. S5a); the implication is that TaSPA, in co-operation with TaPBF, regulates the *Glu-1* genes. Diaz et al. (2002) have noted that in the developing barley endosperm, the product of the TF GAMYB interacts (at least in vivo) with BPBF (Barley PBF), an endosperm-specific DNA-binding one zinc finger transcriptional activator of the *Glu-1* homolog *Hor2*. In maize, Hwang et al. (2004) have suggested that the maize O_2 and PBF proteins can act separately or additively as stimulators of heterologous storage protein promoters in the developing rice endosperm.

Epigenetic modifications, including C-methylation and histone modification, are an important means by which gene expression in eukaryotes is regulated and the structure and transcriptional competence of chromatin are determined (Vaillant and Paszkowski 2007). Histone modifications modulate chromatin structure and transcription (Shahbazian and Grunstein 2007). The *DME* gene encodes a 5-methylcytosine DNA glycosylase responsible for the transcriptional de-repression of genes encoding the monomeric gliadins and the low molecular weight glutenin subunits via the active demethylation of their promoters (Wen et al. 2012). In the maize endosperm, DNA methylation modulates O_2 activity in vivo (Hwang et al. 2004). During the pre-storage phase, methionine synthase and *S*-adenosylmethionine synthase genes are expressed at a high level: S-adenosylmethionine is thought to be required for various methylation processes. On the other hand, the expression of genes encoding prolamins is repressed due to CpG methylation (Wen et al. 2012). The present data indicate that the expression of *Glu-1* is also influenced by promoter C-methylation (Figs. 2, 3). Here, the treatment reduced methylase expression and increased that of demethylase (Fig. 2a, b), which resulted in a hypomethylation of the *Glu-1* gene promoter sequence (Fig. 3, Fig. S4) and further led to increased expression of three of the four *Glu-1* genes (Fig. 2g). A reduction in the level of C-methylation appeared to promote the expression of *TaPBF* and *TaSPA* (Fig. 2c, e), which enhanced *Glu-1* expression and thus the accumulation of glutenin (Figs. 1d, 2g). The increased level of transcription of genes encoding demethylases observed in the *TaPBF-D* over-expressor was accompanied by small but not significant decreases in the level of transcripts of genes encoding methylases (Fig. S5b, c); the implication is that the consequence of over-expressing *TaPBF-D* may be to enhance the recruitment of demethylases acting on the *Glu-1* promoter sequences. A further feature of the *TaPBF-D* over-expressor was that *TaSPA* was up-regulated (Fig. S5a). Working in barley, Rubio-Somoza et al. (2006) were able to show that a MYB TF was able to bind to the promoter region of an endosperm-specific TF gene, and that in vitro at least, the product of another *myb* gene binds to BLZ and PBF to form a ternary complex. An investigation of the potential of maize O_2 and PBF to act as transcriptional activators of the rice glutelin gene *Gt1* showed that either of these proteins on their own was able to enhance the level of *Gt1* transcription, while mutated forms of each protein defective with respect to DNA binding could not (Hwang et al. 2004). When both genes were introduced simultaneously, there was an additive effect on the activation of the *Gt1* promoter. Guo et al. (2015) have suggested that the wheat myb product TaGAMYBb both activates the expression of *Glu-1* by binding to its promoter, and interacts with the wheat histone acetyltransferase TaGCN5 recruited by TaGAMYB to establish the histone acetylation of *Glu-1*. The results of EMSA and ChIP also suggest that the binding between TFs and motifs could promote the expression of *Glu-1* (Fig. 5). The global level of C- methylation in the *TaPBF-D* over-expressor was lower than in the wild type (Fig. 4f). A hypo-methylated promoter sequence may favor the formation of the transcriptional complexes required to drive the synthesis of gluten.

The effect of 5-azaC treatment on the C-methylation status of the three *Glu-1* gene promoters, along with the impact on the transcriptome of over-expressing *TaPBF-D*, has provided a more nuanced picture of how the *Glu-1* genes are regulated. Notably, the enhanced accumulation of HMW-GS and the reduced level of C-methylation within the *Glu-1* promoter brought about by *TaPBF-D* over-expression illustrate a relationship between the C-methylation status of the *Glu-1* promoter P-like box and the quantity of TaPBF protein; this implies that the interaction between TaPBF and the *Glu-1* promoter is involved in modulating the expression of the *Glu-1* genes.

Author contribution statement FC designed the research. FC and JZ wrote the paper. JZ, LF JY, and YZ performed the experiments. FC, JZ, and GX contributed to data analysis. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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