Rapid Divergence of Prolamin Gene Promoters of Maize After Gene Amplification and Dispersal

Yongrui Wu and Joachim Messing[1]

Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ 08854

ABSTRACT Seeds have evolved to accommodate complicated processes like senescence, dormancy, and germination. Central to these is the storage of carbohydrates and proteins derived from sugars and amino acids synthesized during photosynthesis. In the grasses, the bulk of amino acids is stored in the prolamin superfamily that specifically accumulates in seed endosperm during senescence. Their promoters contain a conserved *cis*-element, called prolamin-box (P-box), recognized by the *trans*-activator P-box binding factor (PBF). Because of the lack of null mutants in all grass species, its physiological role in storage–protein gene expression has been elusive. In contrast, a null mutant of another endosperm-specific *trans*-activator Opaque2 (O2) has been shown to be required for the transcriptional activation of subsets of this superfamily by binding to the O2 box. Here, we used RNAi to knockdown *Pbf* expression and found that only 27-kDa γ- and 22-kDa α-zein gene expression were affected, whereas the level of other zeins remained unchanged. Still, transgenic seeds had an opaque seed phenotype. Combination of *PbfRNAi* and *o2* resulted in further reduction of α-zein expression. We also tested the interaction of promoters and constitutively expressed PBF and O2. Whereas transgenic promoters could be activated, endogenous promoters appeared to be not accessible to transcriptional activation, presumably due to differential chromatin states. Although analysis of the methylation of binding sites of PBF and O2 correlated with the expression of endogenous 22-kDa α-zein promoters, a different mechanism seems to apply to the 27-kDa γ-zein promoter, which does not undergo methylation changes.

ENDOSPERM is a terminal organ, which stores proteins and carbohydrates to nourish the embryo when it germinates and early seedlings when they grow. In *Zea mays*, the major storage proteins are alcohol-soluble prolamins called zeins, which are exclusively expressed in endosperm starting about 10 days after pollination (DAP). Transcription of the zein-gene superfamily is characteristic for three outstanding features: tissue specificity, developmental timing, and its strength. Maize seeds contain ∼10% proteins and ∼70% of them are storage proteins, of which 70% are zeins. Therefore, the zein promoters are probably among the strongest promoters in maize endosperm. Indeed, zein cDNA sequences accounted for nearly 50% of the cDNAs in a nonnormalized endosperm cDNA library, of which 30 and

15% are from α- and γ-zein genes, respectively (Hunter *et al.* 2002). Zeins are divided into four classes, *i.e.*, α (19 and 22 kDa), γ (50, 27, and 16 kDa), β (15 kDa), and δ (18 and 10 kDa). In the maize B73 genome, there are 26 and 16 gene copies for 19- and 22-kDa α-zeins, respectively, whereas other sizes are only single-copy genes (Song and Messing 2003; Xu and Messing 2008; Miclaus *et al.* 2011).

Prolamin gene expression is synchronized during seed development. Many promoter regions retained a conserved 7-bp DNA *cis*-acting element (5′-TGTAAAG-3′), called the prolamin box (P-box), approximately 300 nucleotides upstream of the start codon (Figure 1) (Vicente-Carbajosa *et al.* 1997; Wang and Messing 1998; Xu and Messing 2009). The P-box binding factor (PBF) interacts with the P-box as an endosperm-specific transcriptional activator that belongs to the Dof class of plant zinc-finger DNA-binding proteins. Indeed, PBF was expressed about 10 DAP concomitant with activation of all zein genes, as expected for the spatial and temporal regulation of zein genes by a common transcriptional activator (Vicente-Carbajosa *et al.* 1997).

Yet, another endosperm-specific transcription factor Opaque 2 (O2) co-activates transcription of a subset of zein and

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P27 GCAAAGAAACACGGTGAGTCATGCCGAGATCATACTCATCTGATATACATGCTTACAGCT -371
P22 GGATTTCAGTTAGTCTCAGTCTTGTAGTATTTGTTCCTTCCTTGTCAAATCACTTCTCAT -373


P27 CACAAGACATTACAAACAACTCATATTGCATTACAAAGATCGTTTCATGAAAAATAAAAT -311
P22 CTAAAAACTATGCTTGTTTAACCATCAGAACATAACTACAACAATATCCATTTATAAAGG -313

                                P-Box(-282~-276)
P27 AGGCCGGACAGGACAAAAATCCTTGACGTGTAAAGTAAATTTACAACAAAAAAAAGCCAT -251
P22 CTTCGATAGCAAACTTTACATATTCATATCATGTTAATGTTGTCACATGTGTAAAGGTGA -253
                    O2-like Box(-227~-217)  P-Box(-263~-257)
P27 ATGTCAAGCTAAATCTAATTCGTTTTACGTAGATCAACAACCTGTAGAAGGCAACAAAAC -191
P22 AGAGATCTTGCATGTCATTCCACGTAGATAAAAAGAATGCATATATAAAAATGGCACATT -193
            O2 Box(-234~-224)
P27 TGAGCCACGCAGAAGTACAGAATGATTCCAGATGAACCATCGACGTGCTACGTAAAGAGA -131
P22 TTCTTGTAGGTAGTGGAAAGTATCTTTCCAGCTAAGACCATATAATCCGATAAAGGTGAT -133


P27 GTGACGAGTCATATACATTTGGCAAGAAACCATGAAGCTGCCTACAGCCGTCTCGGTGGC -71
P22 AACTAAATGTCGAAATCGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGAAA -73
        CAAT Box(-65~-60)                TATA Box(-34~-28)
P27 ATAAGACACAAGAAATTGTGTTAATTAATCAAAGCTATAAATAACGCTCGCATGCCTGT -11
P22 AAAAAGACAAAATCCAAAAAATTTATATGAGATCTCACCTATATAAATAGCGCCCAAATC -13
        +1 Transcription Start Site  TATA Box(-33~-25)
P27 GCACTTCTCCATCACCACCACTGGGTCTTCAGACCATTAGCTTTATCTACTCCAGAGCGC +50
P22 AGTAGTTAATCCATCACCCATAATATTTTGAGCATTCAGAAACACACCAAGCGAAGCGCA +48


P27 AGAAGAACCCGATCGACACCATG +73
P22 CTAGCAACGACCAAACAACAATG +71
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Figure 1 Alignment of the 22-kDa α-zein and 27-kDa γ-zein promoters. The P-box, O2 (or O2-like), CAAT, TATA boxes, transcription start site, and start codon are highlighted. Their positions relative to transcription start site are indicated.

many other genes in maize endosperm (Hunter *et al.* 2002). In the zein-gene superfamily, O2 had been reported to regulate only 22-kDa α- and β-zein genes (Cord Neto *et al.* 1995; Schmidt *et al.* 1992). In the promoters of the 22-kDa α-zein gene copies, O2 recognizes the O2 box (5′-TCCACGTAGAT-3′), in which "ACGT" is the core element (Figure 1). In nonendosperm tissues, the promoters of the 22-kDa α-zein genes are highly methylated, especially at the "C" in the core motif, whereas in endosperm tissue the promoters are almost free from methylation (Locatelli *et al.* 2009).

P- and O2-boxes are separated by only 20 bp and it has been suggested that PBF functions in recruiting class-specific transcriptional activators like O2 for gene expression (Vicente-Carbajosa *et al.* 1997; Wang and Messing 1998). Although the 27-kDa γ-zein gene is not regulated by O2, an O2-like box (5′-TTTACGTAGAT-3′) was also found in its −300 element position (Figure 1). When this motif 5′-T<u>TT</u>ACGTAGAT-3′ was modified to the conventional O2 box 5′-T<u>CC</u>ACGTAGAT-3′ by site-directed mutagenesis, the binding affinity of O2 was dramatically increased *in vitro* and the transcription activity was significantly elevated compared to the wild-type promoter (Ueda *et al.* 1992). Interestingly, overexpression of maize PBF or O2 alone could increase the expression of rice and wheat storage protein genes and cotransfection of them resulted in an additive increase (Hwang *et al.* 2004).

However, because of the lack of a *Pbf* null mutant in any grass species, the extent of regulation of gene expression by PBF during seed development has remained unclear. Furthermore, as all zein-gene genomic sequences were available online, it was found that not all their promoters contain a P-box. To address the divergence of promoter regions of prolamin genes, we used RNA interference and constitutive overexpression of PBF and O2 in maize to study promoters of each class of zeins, where each combination of

trans- and *cis*-acting elements was unique, demonstrating the divergence of gene regulation after amplification.

Materials and Methods

Plasmid construction, plant transformation

The method for making RNAi constructs have been described elsewhere (Wu and Messing 2010). The primers for amplification of the inverted *Pbf* cDNA fragments are listed in supporting information, Table S1. All primers for construction of pTF102-P4, pTF102-P5, and pTF102-P9 are listed in Table S1 and indicated in simplified diagrams (Figure S5). The binary vector pTF102 was used to build constructs for transformation and a series of intermediate constructs was also built with pTF102. The construct pTF102-P4 (P27::GFP::Tdzs10) contains a transcriptional fusion of the GFP gene with the 27-kDa γ-zein promoter (1067 bp) and the termination and polyadenylation signals 961 bp downstream from the stop codon of the 10-kDa δ-zein gene (Wu and Messing 2009). The constructs pTF102-P5 (P27::GFP::Tdzs10-P35S::PBF::T35S) and pTF102-P9 (P27::GFP::Tdzs10-P35S::PBF::T35S- P35S::O2::T35S) were derived from pTF102-P35SPBF and pTF102-P35SO2 (Figure S5A). The full-length coding sequences of *Pbf* and *O2* genes were amplified from B73 endosperm cDNAs and cloned into the T-easy vector (Promega) for sequence verification. The CaMV 35S promoter (P35S) was amplified from the pTF102 vector with two pairs of primers to simultaneously introduce different restriction enzyme cutting sites (Figure S5A and Table S1). For inserting the fragment *Bam*H1-P35S::PBF::T35S-*Avr*II*Hin*dIII into pTF102-P4 downstream of Tdzs10, pTF102-P4-*Bam*H1*Hin*dIII was created by simple replacement of a partial Tdzs10 with the same sequence except for a *Bam*H1 next to the *Hin*dIII by PCR (Figure S5B).

To construct pTF102-P9, the 27-kDa γ-zein promoter in pTF102-P4-*Bam*H1*Hin*dIII was replaced with a 22-kDa α-zein promoter (1237 bp) to create an intermediate construct, pTF102-P7. Another intermediate construct pTF102-P8 was created in the same way as pTF102-P5. Then, pTF102-P8 was digested with *Avr*II and *Hin*dIII and ligated with the fragment *Avr*II-P35S::O2::T35S-*Hin*dIII to form the final product of pTF102-P9 (Figure S5C).

Hi-II B and A lines were grown in the greenhouse at the Waksman Institute, Rutgers University. For each construct, ∼200 immature embryos at ∼10 DAP from the crosses of B × A were dissected for transformation. All the subsequent steps were performed according to known protocols (Frame *et al.* 2002).

Generation of the double mutant of PbfRNAi and o2

The *PbfRNAi* gene could be specifically amplified with a primer pair in the GFP coding and T35S regions as described recently (Wu and Messing 2010). Heterozygous and homozygous *o2* genotypes could be identified with an *O2* polymorphic marker umc1066 (http://www.maizegdb.org/cgi-bin/displayssrrecord.cgi?id=193779).

Fluorescence dissection microscopy

Immature embryos 24 hr after infection, callus, root, leaf, and seeds were monitored for GFP expression with fluorescence dissection microscope (Leica MZ16 F).

RT–PCR and SDS–PAGE

Total RNA was extracted by using TRIzol reagent (Invitrogen). A total of 5 μg RNA was digested with DNase I (Invitrogen) and then reverse transcribed. All primers for RT–PCR were listed in Table S1. The procedures for zein extraction and SDS-PAGE analysis have been described in detail elsewhere (Wu and Messing 2012).

DNA extraction and DNA methylation analysis

Genomic DNA was extracted by the cetyltrimethyl-ammonium bromide (CTAB) method. For bisulfite sequencing, genomic DNA was extracted from the leaves punched from three to five individuals for each event. A total of 2 μg DNA was subject to bisulfite treatment (Invitrogen) and 2 μl of the eluted material was applied for PCR with 55 cycles. All primers for bisulfite PCR were listed in Table S1. The common forward primer p22bisF and the specific reverse one α22bisR or GFPbisR were used to amplify the endogenous or transgenic 22-kDa α-zein promoters. Likewise, the p27bisF and the specific reverse primer γ27bisR or GFPbisR were used to amplify the endogenous or transgenic 27-kDa γ-zein promoter; the p35SbisF and the specific reverse primer PbfbisR or BarbisR were used to amplify the CaMV 35S promoter driving the *Pbf* or *bar* gene coding sequence. The PCR product was cloned into T-easy vector (Promega) and a total of 96 colonies for each sample were selected for sequencing with the ABI 3730XL sequencer in our

laboratory. The sequences were aligned and the percentage of cytosine methylation was calculated by the program of DNASTAR SeqMan.

The restriction enzyme *Hyp*CH4IV cuts "ACGT" and is blocked by cytosine methylation. The leaf and endosperm genomic DNAs (1 μg of each) from P22 and P9-1 were digested with *Hyp*CH4IV overnight. Fifty nanograms of the digested DNA was applied for PCR amplification with 30 cycles. The primer pair p22F1 and GFPR2 was used to specifically amplify the transgenic 22-kDa α-zein promoter (Table S1).

Results

Effect of PbfRNAi on the expression of the zein-gene superfamily

Pbf was cloned by amplification with primers based on the conservation of the Dof domain between *Arabidopsis* and maize (Vicente-Carbajosa *et al.* 1997). Because no null mutant has been found in any grass species it has been suggested that PBF could be a master regulator of endosperm development and its absence would result in a defective kernel mutant (*dek*). If this were to be the case, quantitative reduction of PBF should still permit us to identify target genes without preventing seed development. Therefore, we constructed an RNAi transgene for *Pbf* with the strong endosperm-specific 27-kDa γ-zein promoter (Figure 2A) and recovered three independent events. For each transgenic event, individual clones (*PbfRNAi/-*; *O2/O2*) were self-crossed or pollinated with W64A*o2* (*o2* mutant in inbred W64A background). To evaluate the knockdown effect, six immature kernels for each event at 18 DAP were dissected from the cross *PbfRNAi/-*; *O2/O2* × −/−; *o2/o2*. All progeny were heterozygous for the *o2* mutant with a recessive phenotype, which would not affect zein expression, whereas half of the kernels from the crosses would inherit the dominant *PbfRNAi* transgene. Therefore, one-half of the progeny should have the genotype *PbfRNAi/*−; +/*o2*, and the other −/−; +/*o2*. The embryo and endosperm were separated individually. The embryos were saved to extract genomic DNA for transgene genotyping by PCR. Progeny from crosses of *PbfRNAi/*−; *O2/O2* × −/−; *o2/o2* inheriting and not inheriting the *PbfRNAi* construct were combined to extract RNA, respectively. As expected, the transcripts for *Pbf* were dramatically reduced in the three *PbfRNAi* events compared to the normal genotype (Figure 2B).

Self- and outcrossed ears of all three events segregated vitreous and opaque kernels with ratio of 1:3 and 1:1, respectively. One vitreous and three opaque kernels from events 1 and 2 were sliced in half and shown in Figure 2C. Sliced seeds were used to extract zein proteins individually and analyzed by SDS–PAGE (Figure 2D). Only the levels of 27-kDa γ- and 22-kDa α-zeins were significantly reduced in opaque seeds, whereas other zeins had no noticeable reduction (Figure 2D).
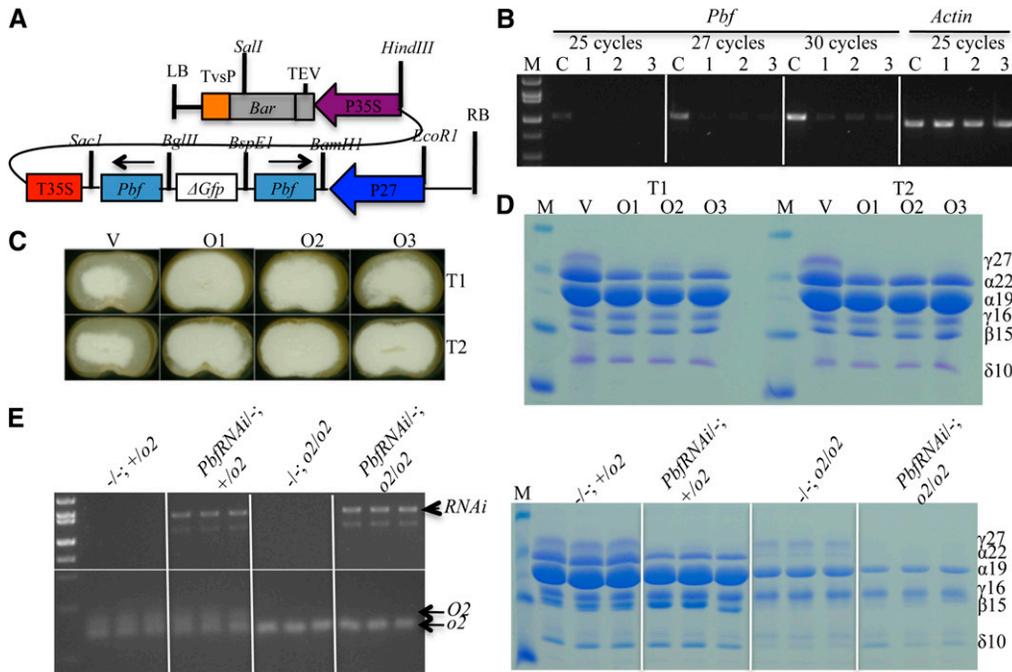
Figure 2 Silencing of *Pbf* and the expression of all zeins. (A) *PbfRNAi* construct. (B) RT–PCR analysis of the expression of *Pbf* in the three *PbfRNAi* events. The *Actin* gene was used as control. (C) *PbfRNAi* seed phenotype. V, vitreous kernel, not inheriting the *PbfRNAi* construct; O1–O3, opaque kernels, inheriting the *PbfRNAi* construct. (D) Zein protein accumulation pattern in *PbfRNAi* seeds. (E) Zein protein accumulation pattern in double mutant of *PbfRNAi* and *o2*. Left, genotyping of the progeny seeds with PCR; right, SDS-PAGE analysis of zein accumulation in the genotyped seeds from the left.

Because all genomic zein-gene sequences were now available online, the *cis*-elements of their promoters could be aligned for sequence homology. Indeed, in the −300 regions, the sequences of *cis*-acting elements in different zein genes varied considerably (Figure S1). Although the 27-kDa γ- and 22-kDa α-zein genes are the most diverged prolamin genes, one being the oldest and one being the youngest (Xu and Messing 2009), their promoters have been quite conserved in that they both contain the P-, O2-, or O2-like boxes. However, only a subset of the 22-kDa α-zein genes are regulated by O2 (Song *et al.* 2001). The 27-kDa and 16-kDa γ-zein genes originated from a common progenitor by allotetraploidization and share high DNA and protein sequence similarity (Xu and Messing 2008). However, the P- and O2-like boxes are missing in the 16-kDa γ-zein promoter due to a deletion (Figure S2). The 18- and 10-kDa δ-zein genes also lack a P-box in their −300 regions, consistent with their unchanged expression in *PbfRNAi*. Interestingly, the regulation of the 19-kDa α-zein genes is also decreased in the *o2* mutant despite the absence of an O2 box and the lack of O2 binding to their promoters (Schmidt *et al.* 1992). On the other hand, a P-box is present in the 19-kDa α- and 15-kDa β-zein promoters, but knockdown of PBF does not reduce their expression (Figure S1 and Figure 2D). One possibility could be that even small amounts of PBF are still sufficient for expression of 19-kDa α- and 15-kDa β-zeins in the presence of O2 because the RNAi line is not a null mutant. We also cannot exclude the possibility that the 19-kDa α- and 15-kDa β-zeins are regulated by other redundant Dof-containing transcription factors. Clearly, the *in-vitro* binding studies cannot be confirmed *in vivo*, indicating interactions of several factors in addition to those that have been described.

Zein accumulation in the double mutant of PbfRNAi and o2

Double mutant of *PbfRNAi* and *o2* was generated by backcross of *PbfRNAi/−*; *+/o2* to W64A*o2*. Kernels were collected at 36 DAP. The embryos were separated from single kernels for genotyping. We expected four genotypes among the backcrossed progeny, heterozygous *o2* without *PbfRNAi* (−/−; *+/o2*) and with *PbfRNAi* (*PbfRNAi/−*; *+/o2*), and homozygous *o2* without *PbfRNAi* (−/−; *o2/o2*) and with *PbfRNAi* (*PbfRNAi/−*; *o2/o2*). Three kernels for each genotype were identified for zein accumulation analysis (Figure 2E, left, see *Materials and Methods* for details). Kernels with genotype −/−; *+/o2* showed the normal accumulation pattern of all zein proteins. In *PbfRNAi/−*; *+/o2*, kernels had significant reduced accumulation in 27-kDa γ- and 22-kDa α-zeins as observed in Figure 2D, whereas in −/−; *o2/o2* kernels had dramatically decreased levels of the 22-kDa, 19-kDa α-, and 15-kDa β-zeins (Figure 2E, right).

The expression of the 10-kDa δ-zein was always lower in *o2* than normal seeds, but so far there is no evidence that it was directly regulated by O2. On the other hand, O2 regulates many genes and its mutant has pleiotropic effects on endosperm development (Hunter *et al.* 2002). Interestingly, the 10-kDa δ-zein is expressed at higher levels in the double mutant than in *o2*, which is consistent with our recent finding that balancing of sulfur storage can occur between the cysteine-rich γ- and β-zeins and the methionine-rich 10-kDa δ-zein. In the biosynthesis of methionine it receives the sulfur moiety from cysteine. When cysteines are not incorporated into the cysteine-rich γ- and β-zeins in the double mutant, biosynthesis of methionine can progress and be incorporated into the methionine-rich 10-kDa δ-zein (Figure 2E),
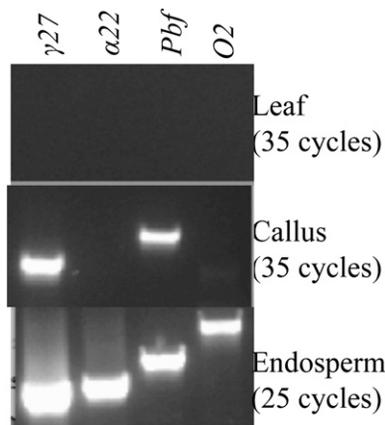
Figure 3 RT–PCR analysis of the expression of the 22-kDa α-zein, 27-kDa γ-zein, *Pbf* and *O2* genes in endosperm, callus, and leaf from a regenerated control plant. PCR cycles were 25 for endosperm and 35 for callus and leaf. Top, leaf; middle, callus; bottom, endosperm.

which synthesis is probably regulated at the translational level (Wu *et al.* 2012).

The levels of 22-kDa α-zeins in −/−; *o2/o2* were more decreased than in *PbfRNAi/−*; +/*o2*, but were still detectable in SDS–PAGE. In the double mutant of *PbfRNAi/−*; *o2/o2*, the effect on α-zein expression is additive (Figure 2E, right). The expression of 22-kDa α-zeins was no longer visible in SDS–PAGE. Although the accumulation of 19-kDa α-zeins was not affected by *PbfRNAi* alone, their expression was significantly lower in *PbfRNAi/−*; *o2/o2* than in −/−; *o2/o2*. In all genotypes, the 16-kDa γ-zein expressed evenly, indicating that it has evolved to be subject to a new regulation.

Epigenetic states of transgenic and endogenous 27-kDa γ-zein promoters

Besides endosperm-specific transactivators, differential epigenetic modification between endosperm and nonendosperm tissues plays an important role in endosperm-specific expression of zein genes (Bianchi and Viotti 1988). Because of their strict tissue specificity, transgenes driven by any zein gene promoter are expected to be expressed only in endosperm. However, a positive callus generated from immature embryos transformed with the construct pTF102-P4 (abbreviated as P4), in which the GFP marker gene was driven by the promoter of the 27-kDa γ-zein gene, was GFP positive, when analyzed under a fluorescence dissection microscope. Indeed, RT–PCR and Western blot analysis showed that also the endogenous 27-kDa γ-zein gene was expressed (Wu and Messing 2009). Furthermore, RT–PCR analysis showed that the *Pbf* gene was also transcribed (Figure 3), consistent with the fact that the 27-kDa γ-zein gene was *trans*-activated by PBF (Figure 2, D and E). However, the 22-kDa α-zein and *O2* genes were not activated (Figure 3).

Because the 27-kDa γ-zein and *Pbf* genes were not expressed in nonendosperm tissues, we expected that their epigenetic states would change upon regeneration of plants

from tissue culture cells. Indeed, both 27-kDa γ-zein and *Pbf* genes became inactivated in leaf after regeneration. Lack of expression of the 27-kDa γ-zein gene in regenerated plants could be due to the absence of *Pbf* gene expression or change of its epigenetic status of its promoter or both. However, DNA methylation analysis by bisulfite sequencing showed that the 421-bp fragment upstream of the transcription start site, composed of P-, O2-like, and TATA boxes with 90 cytosines in the 27-kDa γ-zein gene promoter (Figure S3), was hypomethylated throughout development in endosperm, callus, or leaf tissue (Figure 4A). If a change in its epigenetic state occurred, it would not correlate with DNA methylation. In contrast, the 22-kDa α-zein promoter regions, comprising 263-bp upstream of the transcription start site including the P-, O2-, CAAT-, and TATA boxes with 42 cytosines in this region (Figure S4), cytosines were highly methylated in the CG and CHG positions in leaf, but not in endosperm tissue. Some CG sites including the core ACGT motif were also methylated in callus, but fewer sites than in leaf (Figure 4B), indicating that callus-induced demethylation is different from developmentally induced demethylation of the 22-kDa α-zein genes in endosperm. In this respect, one must keep in mind that callus generated from immature embryos is a dedifferentiated tissue induced by the culturing conditions. It has been shown that under those conditions, transposons become demethylated and activated (Peschke *et al.* 1987; Huang *et al.* 2009; Ngezahayo *et al.* 2009). Therefore, a decrease in DNA methylation of 22-kDa α-zein promoters in callus is probably caused by tissue-culture-induced global demethylation (Kaeppler and Phillips 1993).

If no DNA methylation change of the 27-kDa γ-zein promoter occurs during plant development, constitutive expression of PBF should turn on the expression of the 27-kDa γ-zein gene in leaf tissue. Therefore, we constructed pTF102-P5 (abbreviated as P5) with PBF under the control of the CaMV 35S promoter (Figure 5A and Figure S5, A and B). About 200 10-DAP-old immature embryos were transformed with P4 and P5 constructs (Figure 5A). About 70% of the embryos in the two groups showed already transient expression of GFP 24 hr after infection (Figure 5B). After two rounds of bialaphos selection, independent calli for P4 (P4-1 and P4-2) and P5 (P5-1 to P5-4) grew and all strongly expressed GFP as shown with representative samples P4-1 and P5-1 (Figure 5B), consistent with our previous results (Wu and Messing 2009). When plants had been regenerated on regeneration II medium, a piece of root tissue for each event was examined for GFP expression. Whereas three events from P5 (P5-1, P5-2, and P5-3) strongly expressed GFP, this was not the case for P5-4, although the two P4 events did (Figure 5B). As expected, expression of the 27-kDa γ-zein gene itself was not detected in the two P4 transgenic plant leaves, neither in the four events of P5 (Figure 5C), indicating that the endogenous 27-kDa γ-zein promoter could not be activated by PBF alone in leaf. In contrast, the ectopic copy of the 27-kDa γ-zein promoter
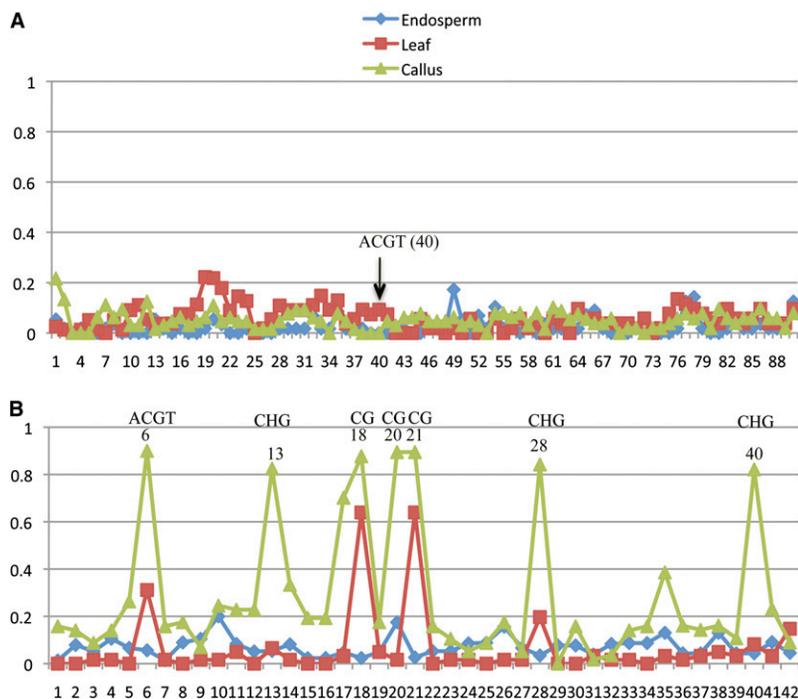
Figure 4 DNA methylation in the 27-kDa γ- and 22-kDa α-zein promoters in callus, leaf, and endosperm. The (A) 421-bp (27-kDa γ-zein promoter) and (B) 263-bp (22-kDa α-zein promoters) fragments immediately adjacent to transcription start site covering P-box, O2-like, and TATA boxes containing 90 and 42 cytosines, respectively, were analyzed by bisulfite sequencing. The cytosine in the O2-box core motif at position 40 (γ27-zein promoter) and 6 (α-zein promoter) is indicated. Other symmetric cytosines (CG and CHG) in the 22-kDa α-zein promoters are also indicated.

driving GFP expression was turned on in P4 and P5 transgenic plants with higher levels in P5-1, P5-2, and P5-3 than in P4-1 and P4-2 (Figure 5B), indicating that expression of PBF was sufficient to enhance a transgenic 27-kDa γ-zein promoter, but not the endogenous promoter. An exception was P5-4. RT–PCR results showed that the *Pbf* and *Gfp* genes were not expressed in this event (Figure 5C). Interestingly, the expression of the *Bar* gene in P5-4 was also significantly reduced compared to the other three events. Furthermore, although GFP expression of P5-4 was not detected in leaves, it was strongly expressed in the seed like the other three P5 and two P4 transgenic events, indicating that P5-4 underwent an epigenetic phase change whereas the other events did not. Indeed, the two 35S promoters each driving the *Bar* and *Pbf* genes were highly methylated in P5-4 not only in leaf (Figure S6, A and B) but also in endosperm (Figure S6C), whereas no significant methylation changes occurred in the transgenic 27-kDa γ-zein promoter relative to the endogenous gene (Figure S6D).

Reactivation of P5-4 in mop1 null mutant

Inactivation of the CaMV35S promoter in transgenic plants has been described previously (Meyer *et al.* 1994; Park *et al.* 1996). A maize mutant with a lesion in *Mop1*, encoding a RNA-dependent RNA Polymerase 2 (RDR2), has been reported to suppress gene expression via a siRNA-mediated mechanism in transgene-induced silencing (McGinnis *et al.* 2006). To investigate whether the epigenetic state of P5-4 could be reversed in the *mop1* mutant, the F1 of the P5-4 event and *mop1* was backcrossed with *mop1*. Since P5-4 could express GFP in seeds, GFP-positive seeds were easily scored under a fluorescence dissection microscope as shown

by arrows (Figure 6A). Twenty-four GFP-positive seeds were grown, about half of which should be homozygous for the *mop1* mutant allele. Homozygous *mop1* mutant could easily be recognized by strong pigmentation of anthocyanin in the plant stem and tassel (Alleman *et al.* 2006). Moreover, the mutant allele used in this work resulted from a *Mu* insertion in its fourth exon (Alleman *et al.* 2006). Therefore, a homozygous mutant could be identified using PCR amplification with specific primer pairs. Genotyping was performed at flowering time. Indeed, 12 plants that were strongly pigmented proved to be homozygous *mop1* mutants (Figure 6B). Furthermore, bisulfite sequencing showed that methylation of the two 35S promoters in plants homozygous for the *mop1* allele was dramatically reduced compared to nonmutated *Mop1* (Figure S6, A and B), whereas the upstream transgenic 27-kDa promoter was hypomethylated both in *Mop1-P5-4* and *mop1-P5-4* (Figure S6D). Consistent with this result, the expression of *Bar* and *Pbf* genes was dramatically increased (Figure 6C). As a consequence of reactivation of the ectopic *Pbf* gene, the transgenic 27-kDa γ-zein promoter driving GFP gene was *trans*-activated (Figure 6, B–D). However, transcripts of the endogenous 27-kDa γ-zein could not be detected (Figure 6C), indicating that the endogenous copy is under a specific developmental control.

Basal transcription of transgenic 22-kDa α-zein promoter in leaf

As noted above, there seems to be differences in the epigenetic state of the 27-kDa γ-zein and 22-kDa α-zein genes (Figure 4). The 22-kDa α-zein genes could not be activated in callus (Figure 3) (Wu and Messing 2009), but weak expression had been observed when a zein genomic DNA
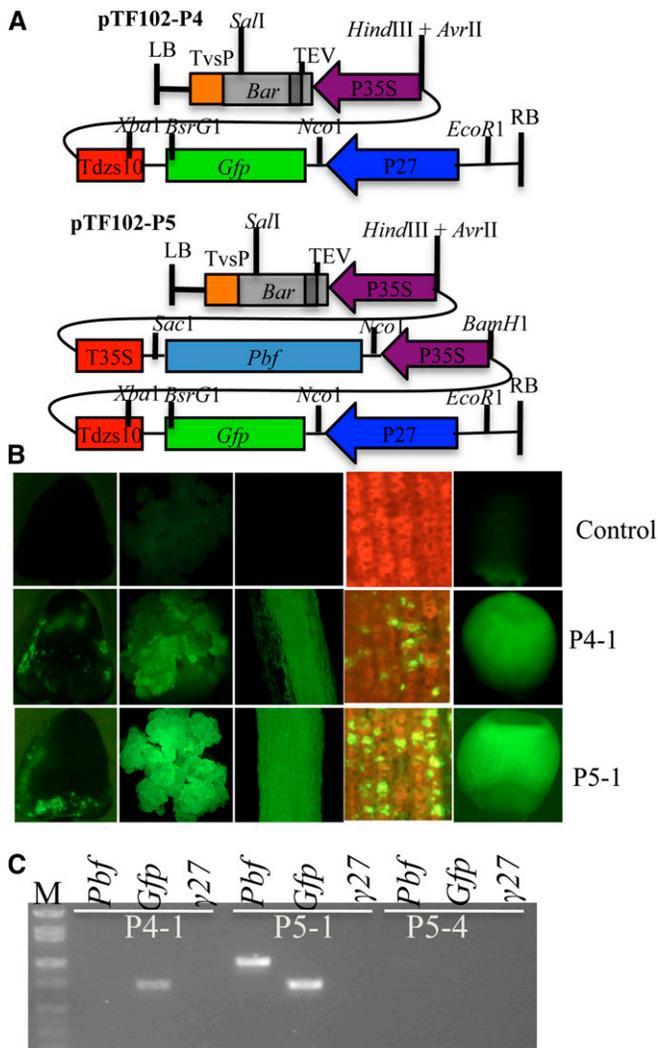
Figure 5 Ectopic activation of the transgenic 27-kDa γ-zein promoter. (A) Structures of constructs pTF102-P4 and -P5. (B) GFP signal under fluorescence dissection microscope in immature embryo 24 hr after cocultivation, callus, regenerated root, and adult leaf. Top, nontransgenic control; middle, P4-1; bottom, P5-1. (C) RT–PCR analysis of the expression of the *Pbf*, *Gfp*, and the 27-kDa γ-zein genes in P4-1, P5-1, and P5-4. The expression of the *Gfp* gene was detected in P4-1 and P5-1. In P5-4, no visible expression of the *Pbf* and *Gfp* genes was detected. M, DNA markers from top to bottom being 2, 1.55, 1.4, 1, 0.75, 0.5, 0.4, and 0.3 kb.

fragment Z4 comprising the entire length of a 22-kDa α-zein gene (Lewis *et al.* 1981; Hu *et al.* 1982) was transferred to sunflower callus (Matzke *et al.* 1984). However, different from the 27-kDa γ-zein gene, whose promoter was not significantly differentially methylated in callus, leaf, and endosperm (Figure 4A), the promoters of the 22-kDa α-zein genes were mildly methylated in callus and highly in leaf and demethylated only in endosperm (Figure 4B). Perhaps there are two classes of promoters, those that undergo developmental methylation changes (22-kDa α-zein) and those that do not (CaMV35S). When pAHC25-P22 (abbreviated as P22), a construct with the 22-kDa α-zein *Zp22/6* promoter from BSSS53 driving GFP expression from
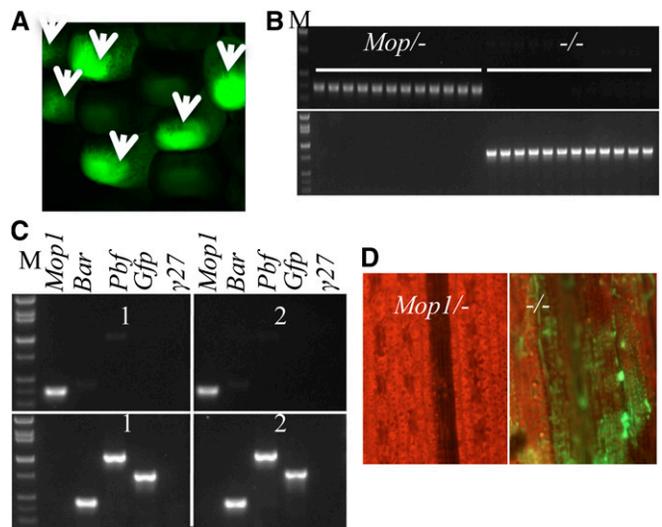
Figure 6 Reactivation of P5-4 in *mop1* mutant. (A) Normal expression of GFP in P5-4 seed (section of an ear). (B) Correlation of GFP expression with *mop1* zygosity. Top, zygosity test of the *mop1* mutant allele in the BC1 population; bottom, RT–PCR analysis of GFP expression of the genotyped plants as shown in the top. (C) Silencing and reactivation of CaMV 35S promoters in progenies with *Mop1/-* and *mop1* genotypes. For each genotype, two individuals were chosen for further analysis of *Pbf* and *O2* expression. Top, genotype *Mop1/+*; bottom, *mop1*. (D) GFP expression observed under fluorescence dissection microscope; left, genotype *Mop1/+*; right, *mop1*. M, DNA markers from top to bottom being 2, 1.55, 1.4, 1, 0.75, 0.5, 0.4, and 0.3 kb.

an earlier particle bombardment-mediated transformation event (Figure 7A), was followed during plant development, fluorescent GFP signal was observed only in seeds (Figure 7B). Bisulfite sequencing analysis showed that the 263-bp fragment upstream of the transcription start site comprising the P-, O2-, CAAT-, and TATA boxes in the transgenic and endogenous 22-kDa α-zein promoters were highly methylated in leaf tissue (Figure 7C). As those in the endogenous α-zein promoters, cytosines were mainly methylated in the CG and CHG positions (Figure 7C, Figure 4B, and Figure S4). Still, low levels of GFP transcript levels could be detected by RT–PCR in leaf in the absence of the transcriptional activator O2, whereas no transcript of the endogenous 22-kDa α-zein genes was detectable (Figure 7D). Similarly, although expression of 22-kDa α-zein genes is dramatically reduced in the endosperm of the *o2* mutant, their transcripts could still be detected by RT–PCR. The basal transcription of the GFP gene in leaf driven by the highly methylated 22-kDa α-zein promoter could also be confirmed with two other independent events generated with *Agrobacterium*-mediated transformation (data not shown).

Transgenic 22-kDa α-zein promoter in the presence of PBF and O2 in leaf

If transcription was not completely suppressed by DNA methylation, could transcription be promoted in the presence of PBF and O2? In the new construct pTF102-P9 (abbreviated as P9) (Figure 8A), O2 and PBF are co-
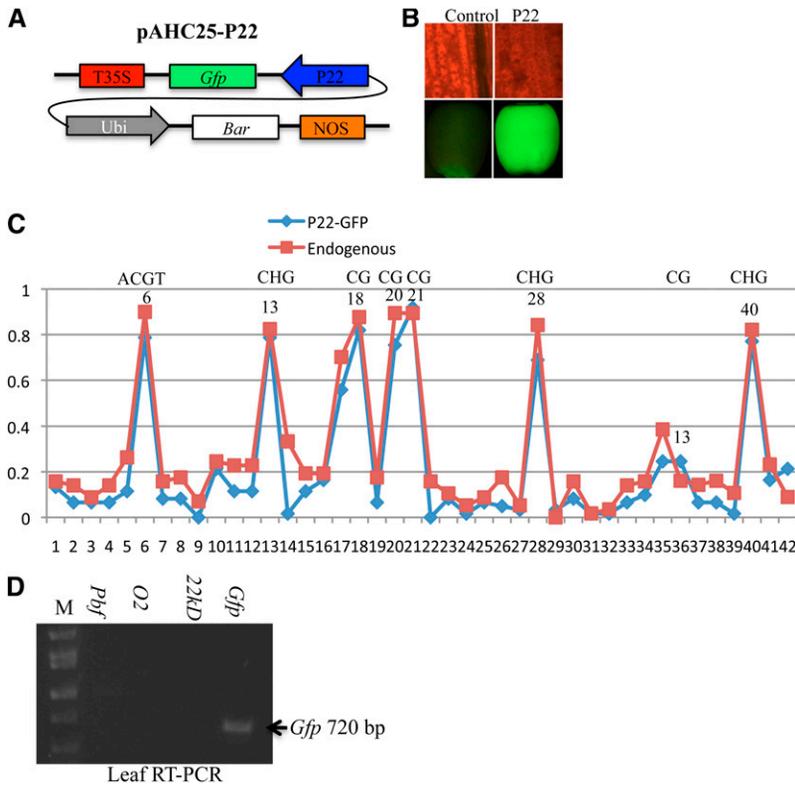
Figure 7 Genetic behavior of the transgenic 22-kDa α-zein promoter in leaf and endosperm. (A) Structure of construct pAH25-P22. (B) GFP fluorescent signal absent in leaf (top) but strongly expressed in seed (bottom). (C) DNA methylation of the transgenic and endogenous 22-kDa α-zein promoters in leaf. Blue and red curves indicate the transgenic and endogenous 22-kDa α-zein promoters, respectively; the cytosine in the O2-box core motif at position 6 is indicated. Other symmetric cytosines (CG and CHG) are also indicated. (D) Weak expression of the transgenic 22-kDa α-zein promoter in leaf. M, DNA markers from top to bottom being 2, 1.55, 1.4, 1, 0.75, and 0.5 kb.

expressed by two separate CaMV 35S promoters and the GFP gene is driven by the *Zp22/6* promoter (Figure S5C). Two hundred immature embryos were infected with *Agrobacterium* bearing this construct. Like P4 and P5 (Figure 4B), ∼70% embryos were monitored for transient GFP expression under a fluorescence dissection microscope (Figure 8B). Three growing calli had strong GFP expression. The event P9-1 was shown as an example in Figure 8B. Although the methylation level of the 22-kDa α-zein promoters is reduced in callus (Figure 4B), they were not expressed in the presence of PBF and O2, while the expression of the 27-kDa γ- and 15-kDa β-zein genes was significantly elevated compared to nontransgenic callus (Figure 8C). The regenerated

root and later the adult leaf also continued to express GFP at high levels. RT–PCR analysis was performed to check expression in leaf of regenerated plants. The *O2*, *Pbf*, and *Zp22/6*-GFP genes were all strongly expressed in the three independent events as shown for the event P9-1 (Figure 8D) compared to low levels of expression in the absence of *O2* and *Pbf* as shown in Figure 7D. Because of Mendelian segregation, progeny kernels inheriting the construct are entirely GFP positive. The GFP signal could even be observed in the maternal glume tissue, but not in the pericarp of progenies not inheriting the construct (Figure 8E).

Nevertheless, similarly to the endogenous promoters of the 22-kDa α-zein genes, the cytosines in CG and CHG
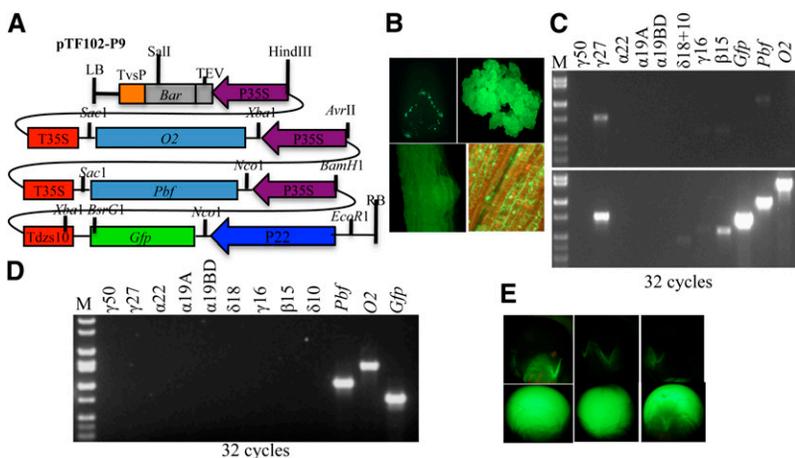
Figure 8 *trans*-activation of the transgenic 22-kDa α-zein promoter by overexpression of PBF and O2 in leaf. (A) Structure of construct pTF102-P9. (B) Ectopic expression of GFP in immature embryo, callus, root, and leaf; (C and D) RT–PCR analysis of construct P9 and zein genes in callus (C) and leaf (D). In C, the top and bottom are the nontransgenic control and transgenic calli, respectively. M, DNA markers from top to bottom are 4, 3, 2, 1.55, 1.4, 1, 0.75, 0.5, 0.4, and 0.3 kb. (E) GFP expression in progeny seeds not inheriting (top) or inheriting (bottom) the construct.
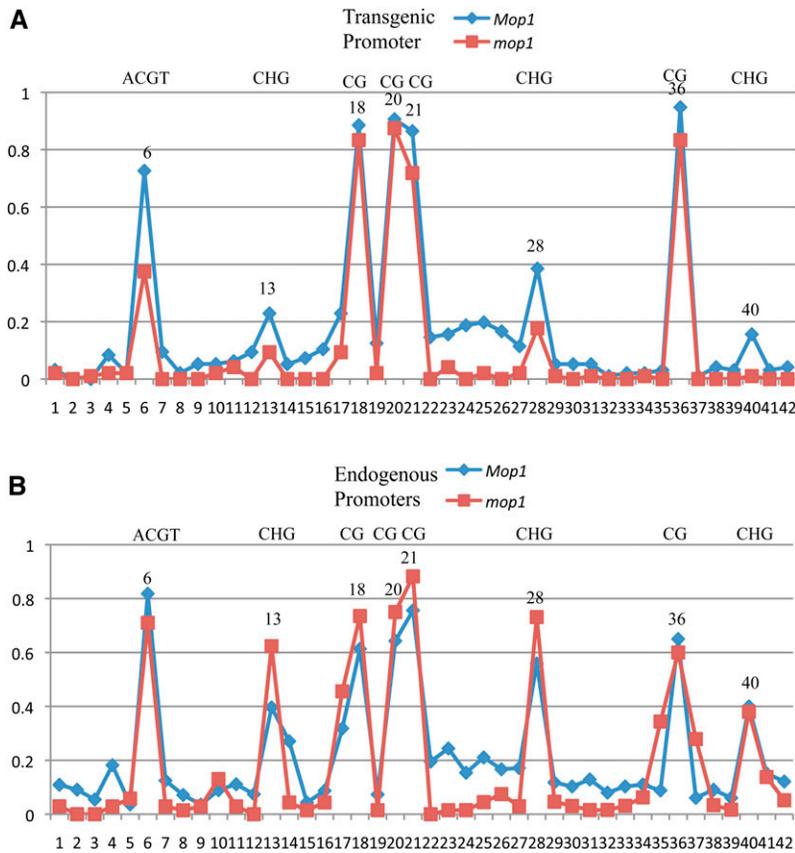
Figure 9 DNA methylation of the transgenic and endogenous 22-kDa α-zein promoters of pTF102-P9-1 in *Mop1* wild-type and *mop1* null mutant. (A) Transgenic 22-kDa α-zein promoter. (B) Endogenous 22-kDa α-zein promoters. The cytosine in the O2-box core motif at position 6 is marked. Other symmetric cytosines (CG and CHG) are also indicated.

positions were highly methylated (Figure 9). The restriction enzyme *Hyp*CH4IV recognizes the ACGT target sequence, but is blocked by cytosine methylation. Because the ACGT element is unique in the interval from the P-box to the ATG start codon in the 22-kDa α-zein promoter (Figure S4), a methylation-sensitive PCR assay could be performed. Indeed, a primer pair as shown in Figure 10 could specifically amplify the transgenic 22-kDa α-zein promoter comprising the *Hyp*CH4IV recognition site ACGT. This specific fragment could be both amplified from undigested leaf and endosperm genomic DNA of P22 and P9-1. When DNA was digested with *Hyp*CH4IV, the fragment could only be amplified from leaf genomic DNA, in which *Hyp*CH4IV digestion was blocked by cytosine methylation (Figure 10), indicating that DNA methylation/demethylation of the transgenic and endogenous promoters were coordinatedly regulated by a common set of factors. Still, tissue-specific transcription was restricted to the endogenous promoter.

Because transcriptional silencing of the CaMV35S promoter could be reversed in the *mop1* mutant concurrent with the removal of methylation (Figures 6C, Figure S6, A and B) one would wonder whether methylation of the 22-kDa α-zein promoters also required MOP1 function. Interestingly, whereas cytosines at non-CG sites were generally lower in methylation in the *mop1* mutant than in wild type, those at CG sites were still highly methylated in transgenic or endogenous promoters (Figure 9), indicating that maintenance of symmetric methylation in 22-kDa α-zein promoters does not require MOP1 function in contrast to the 35S promoter, in which methylation in all contexts was lost in the *mop1* mutant (Figure S6, A and B). Still, the reduction in non-CG methylation and constitutive expression of PBF and O2 in leaves did not activate the endogenous 22-kDa α-zein promoters (Figure 11A).
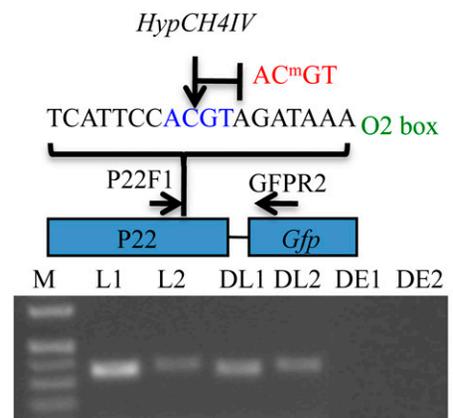
Figure 10 Detection of methylation and demethylation of the transgenic 22-kDa α-zein promoter in leaf and endosperm by methylation sensitive enzyme *Hyp*CH4IV. M, DNA markers from top to bottom are 0.75, 0.5, 0.4, and 0.3 kb.
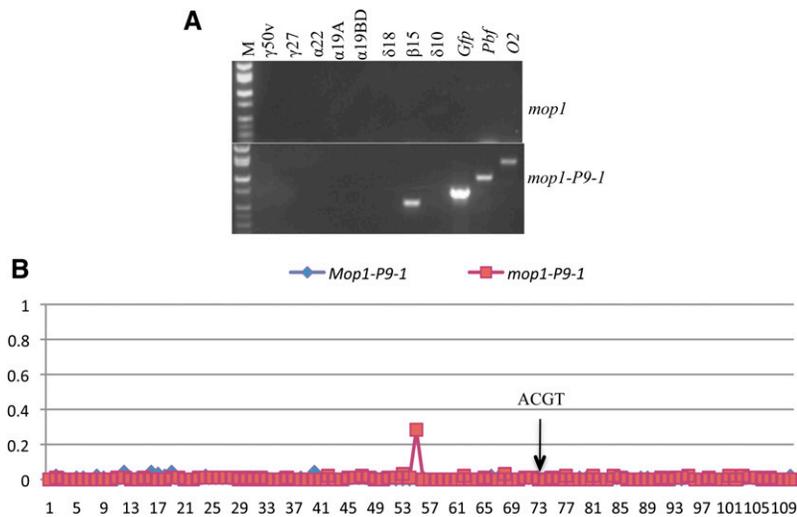
Figure 11 Activation of β-zein gene in *mop1* mutant with overexpression of PBF and O2. (A) RT–PCR analysis of the expression of zein genes in *mop1* (top) and *mop1-P9*-1 (bottom); M, DNA markers from top to bottom are 2, 1.55, 1.4, 1, 0.75, 0.5, 0.4, and 0.3 kb. (B) DNA methylation of β-zein promoter in *Mop1-P9-1* and *mop1-P9-1*. The cytosine in the O2-box core motif at position 73 is marked.

Activation of β-zein in mop1 in the presence of PBF and O2

Although the O2 box is located 124 bp distal to the P-box of the 15-kDa β-zein (Figure S7), it is also regulated by O2 (Cord Neto *et al.* 1995). An elite hybrid cDNA database also suggested expression in leaf (accession no. BM337640) (Swanson-Wagner *et al.* 2006), but we could not detect its expression in leaf of the Hi-II B × A hybrid even in the presence of PBF and O2 (Figure 8D). However, when constitutive expression of O2 and PBF in the *mop1* mutant was used to investigate expression of the 15-kDa β-zein gene by RT–PCR, it was expressed (Figure 11A), whereas other zein genes were not. Moreover, bisulfite sequencing results showed that the 532-bp fragment upstream of the start codon, comprising the P-, O2-, and TATA boxes with 110 cytosines, was hypomethylated in every context including the ACGT motif regardless whether MOP1 was present or not (Figures 11B and Figure S7), suggesting that DNA methylation of the target gene is not essential for RDR2's mode of function. Still, there could be an element further upstream of the 532 bp that was responsive to *mop1* and served as an enhancer for the expression of the 15-kDa β-zein.

Discussion

We have investigated here the genetic interactions of two transcription factors, O2 and PBF, with promoters of the prolamin gene family in maize. Because no null mutant was available, we used RNAi to knock down the expression of PBF. We examined tissue specificity of transgenic and endogenous zein gene expression by constitutively expressing PBF and O2. Because of differences in their capacity to respond to PBF and O2, we also examined the methylation status of target promoters during plant development. As methylation changes in target promoter regions appear to be critical for transcriptional activation of promoters, we also examined whether methylation changes were controlled by RDR2, which was facilitated through the maize *mop1* mutation.

Endosperm-specific transcriptional factors

O2 is endosperm-specifically expressed about 10 DAP, containing the basic leucine-zipper (bZIP) DNA binding motif (Schmidt *et al.* 1992). O2 forms a homodimer that transactivates transcription of many genes in maize endosperm (Hunter *et al.* 2002). However, O2 target elements are highly variable among different grass species. In the zein-gene superfamily, only a subset of α- and the β-zein genes are regulated by O2. In the *o2* mutant, γ-zeins are not affected, suggesting that O2 is not the common factor, regulating all zein-gene expression.

Compared to O2 target elements, the PBF target element, the P-box, is conserved across the grass family (Xu and Messing 2009). In addition, PBF is also expressed in endosperm from around 10 DAP, concomitant with *trans*-activation of all zein genes, leading to the hypothesis that the spatial and temporal regulation of zein genes might be synchronized by PBF. However, when all zein-gene genomic sequences are compared, some of them like the 16-kDa γ- and two δ-zein genes do not have a P-box in their −300 promoter regions (Figure S1 and Figure S2). Moreover, in *PbfRNAi*, only the synthesis of the 27-kDa γ- and 22-kDa α-zeins are dramatically reduced, while other zeins appear to be insensitive to the reduction of PBF (Figure 2D). Although the 19-kDa α-zein promoters do have a P-box (Figure S1), their expression is not significantly affected in contrast to the 22-kDa α-zeins. Similarly, the 19-kDa α-zeins are not reduced as much as the 22-kDa α-zeins in *o2* mutant, indicating that besides γ-zeins, the two subclasses of α-zeins have also undergone divergent gene regulation. Expression of PBF and O2 failed to *trans*-activate any of the endogenous zein genes in leaf, but did enhance the expression of transgenic 27-kDa γ- and 22-kDa α-zein promoters, which

Table 1 Summary of *cis*- and *trans*-regulations of zein genes

Zein genes	*Cis*-elements		Gene expression[a]		
	P-box	O2 or O2-like box	*PbfRNAi*	*o2*	*PbfRNAi/−; o2/o2*
γ-27	+	+	Reduced	Normal	Reduced
α-22	+	+	Reduced	Reduced	Additive effect
α-19	+	−	Normal	Reduced	Additive effect
γ-16	−	−	Normal	Normal	Normal
β-15	+	+	Normal	Reduced	Reduced
δ-10	−	−	Normal	Reduced	Reduced

[a] Gene expression in mutants compared to normal background.

have already basal transcription in nonendosperm tissues (Figures 5–8).

In summary, the expression of all zein genes except the 16-kDa γ-zein was affected either by *PbfRNAi* (27-kDa γ- and 22-kDa α-zein genes) or *o2* (α-, β-, and δ-zein genes), and the additive effect of the double mutant was observed only on the expression of 19- and 22-kDa α-zeins (Table 1).

Developmental change of epigenetic state of zein promoters

We have investigated the interplay between *trans*-acting factors and their target genes by varying the timing of the expression of the *trans*-acting factors. The interesting aspect of this approach is that we can unveil epigenetic properties of developmental regulated genes. Because it had been shown that the 22-kDa α-zein genes are methylated in leaf tissue and demethylated in endosperm, we could investigate whether such methylation would be sufficient to block the *trans*-acting factors in their function, even if expressed at sufficient levels. We also investigated how in the same circumstances the same promoters would behave when inserted into the genome by transformation. Indeed, when *Pbf* and *O2* are expressed in leaf, the *de novo* methylated transgenic 22-kDa promoter could be activated whereas the endogenous methylated ones could not. Therefore, *trans*-activation is not blocked or completely blocked by any methylation of the transgenic promoter.

We also took advantage of the RDR2 mutation in maize (*mop1*) that blocks the siRNA-directed DNA methylation and confirmed the canonical belief that transgenic promoters could be silenced through this pathway. Although the 22-kDa promoters undergo methylation changes correlating with their tissue-specific expression in endosperm (Figure 4B), we could corroborate that the methylation changes for tissue specificity were different from those caused by RDR2, like silencing of the 35S promoter (Figure 4B, Figure 9, and Figure S6, A–C). Although both of the transgenic 22-kDa and 35S promoters had been subjected to *de novo* methylation, they underwent different developmental changes. Methylation of the former was sustained without RDR2 (Figure 9A) and only developmentally demethylated in endosperm (Figure 10), whereas the latter could not be demethylated in endosperm (Figure S6C) and re-

quired RDR2 for maintenance of methylation (Figure S6, A and B). In contrast to the 22-kDa α-zein genes, the endogenous 27-kDa γ-zein promoter would not change its methylation status regardless whether it was expressed or not (Figure 4A and Figure S6D).

In the presence of PBF and O2 and in the absence of MOP1, the only transactivated zein gene in leaf is β-zein (Figure 11A). Although the ectopic expression of β-zein was not correlated with any methylation changes, there could be an enhancer element regulated by MOP1 further upstream in the promoter region of the β-zein gene.

Coordinated regulation of DNA methylation in transgenic and endogenous 22-kDa α-zein promoters

The transgenic 22-kDa α-zein promoter was methylated in regenerated plants regardless whether it was transformed by *Agrobacterium* or particle bombardment (Figures 7C and 9A). The *de novo* DNA methylation of cytosines in all sequence contexts requires DRM1 and DRM2, which is mediated by siRNAs (Cao and Jacobsen 2002; Chan *et al.* 2005), a process termed RNA-directed DNA methylation (RdDM). In flowering plants, the most abundant siRNAs originate from transposon loci and DNA repeats. The siRNA precursor transcripts are transcribed by DNA-dependent RNA polymerase IV (Erhard *et al.* 2009; Hale *et al.* 2009) and then serve as the template for an RNA-dependent RNA polymerase, RDR2, to generate double-stranded RNAs (dsRNAs), which are further processed by Dicer-like 3 (DCL3) into 24-nt siRNAs. Besides direction of *de novo* DNA methylation in heterochromatic regions, RDR2 is also required for transcriptional silencing of transgenes. The lack of MOP1 (RDR2 in maize) can reverse silencing of the 35S promoter by removal of methylation in all contexts (Figure S6, A and B) (McGinnis *et al.* 2006). Although a decrease of overall non-CG methylation was observed in the *mop1-P9-1* event for both endogenous and transgenic 22-kDa α-zein promoters, the methylation in CG context was still high (Figure 9). In contrast to fungi and animals, in which methylation is predominantly in the CG sequence context, plants have 5-methylcytosine at CG, CHG, and even asymmetric CNN sites. In *A. thaliana*, maintenance and *de novo* CG methylation depends on MET1, a homolog of the mammalian DNA methyltransferase 1, whereas non-CG methylation is regulated by two members of DOMAINS REARRANGED

METHYLTRANSFERASE (DRM1 and DRM2) and CHROMO-METHYLASE3 (CMT3) (Chan *et al.* 2005). It seems that the symmetric methylation in the 22-kDa α-zein promoters can be maintained in a DNA replication-coupled way by MET1.

Still, both the endogenous and the transgenic zein promoters were specifically demethylated in endosperm (Figures 10 and 4B), indicating that they were subject to synchronized epigenetic regulation different from a siRNA-mediated mechanism. However, due to lack of mutants that affect demethylation of the 22-kDa α-zein promoters in endosperm and methylation in leaf, it is not clear whether the switch of the methylation status is sufficient to mediate the specific expression of the 22-kDa α-zein genes. In addition, O2 regulates many genes in endosperm (Hunter *et al.* 2002) and can autoregulate the expression of its own promoter by recognition of its target sequence also comprising an ACGT core element. Like the 22-kDa α-zein promoters, *O2* promoter was less methylated in endosperm than in leaf. However, comparison of the methylation patterns in endosperm and leaf revealed that endosperm-specific expression of the *O2* gene was not mediated by demethylation of a specific cytosine. Further, gel-shift experiments showed that O2 binding activity was not impaired by CpG methylation (Rossi *et al.* 1997). Although the methylation in ACGT was shown to reduce the O2 affinity for the 22-kDa promoter, a detectable binding activity was still observed (Sturaro and Viotti 2001), indicating that DNA methylation alone was not sufficient to completely silence the transcription of the 22-kDa α-zein genes. On the other hand, when methylation levels were reduced in callus, expression of the 22-kDa α-zeins was not detected in the presence of PBF and O2 (Figure 8C).

These results indicate a rapid divergence of prolamin promoter structures and corresponding DNA binding properties probably due to amplification and dispersal of gene copies into a different chromosomal epigenetic context, which is consistent with our previous observations (Miclaus *et al.* 2011).

Acknowledgments

Literature Cited

Alleman, M., L. Sidorenko, K. McGinnis, V. Seshadri, J. E. Dorweiler *et al.*, 2006 An RNA-dependent RNA polymerase is required for paramutation in maize. Nature 442: 295–298.

Bianchi, M. W., and A. Viotti, 1988 DNA methylation and tissue-specific transcription of the storage protein genes of maize. Plant Mol. Biol. 11: 203–214.

Cao, X., and S. E. Jacobsen, 2002 Role of the Arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing. Curr. Biol. 12: 1138–1144.

Chan, S. W., I. R. Henderson, and S. E. Jacobsen, 2005 Gardening the genome: DNA methylation in *Arabidopsis thaliana*. Nat. Rev. Genet. 6: 351–360.

Cord Neto, G., J. A. Yunes, M. J. da Silva, A. L. Vettore, P. Arruda *et al.*, 1995 The involvement of Opaque 2 on beta-prolamin gene regulation in maize and Coix suggests a more general role for this transcriptional activator. Plant Mol. Biol. 27: 1015–1029.

Erhard, K. F. Jr., J. L. Stonaker, S. E. Parkinson, J. P. Lim, C. J. Hale *et al.*, 2009 RNA polymerase IV functions in paramutation in *Zea mays*. Science 323: 1201–1205.

Frame, B. R., H. Shou, R. K. Chikwamba, Z. Zhang, C. Xiang *et al.*, 2002 Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. 129: 13–22.

Hale, C. J., K. F. Erhard Jr., D. Lisch, and J. B. Hollick, 2009 Production and processing of siRNA precursor transcripts from the highly repetitive maize genome. PLoS Genet. 5: e1000598.

Hu, N. T., M. A. Peifer, G. Heidecker, J. Messing, and I. Rubenstein, 1982 Primary structure of a genomic zein sequence of maize. EMBO J. 1: 1337–1342.

Huang, J., K. Zhang, Y. Shen, Z. Huang, M. Li *et al.*, 2009 Identification of a high frequency transposon induced by tissue culture, nDaiZ, a member of the hAT family in rice. Genomics 93: 274–281.

Hunter, B. G., M. K. Beatty, G. W. Singletary, B. R. Hamaker, B. P. Dilkes *et al.*, 2002 Maize opaque endosperm mutations create extensive changes in patterns of gene expression. Plant Cell 14: 2591–2612.

Hwang, Y. S., P. Ciceri, R. L. Parsons, S. P. Moose, R. J. Schmidt *et al.*, 2004 The maize O2 and PBF proteins act additively to promote transcription from storage protein gene promoters in rice endosperm cells. Plant Cell Physiol. 45: 1509–1518.

Kaeppler, S. M., and R. L. Phillips, 1993 Tissue culture-induced DNA methylation variation in maize. Proc. Natl. Acad. Sci. USA 90: 8773–8776.

Lewis, E. D., G. Hagen, J. I. Mullins, P. N. Mascia, W. D. Park *et al.*, 1981 Cloned genomic segments of *Zea mays* homologous to zein mRNAs. Gene 14: 205–215.

Locatelli, S., P. Piatti, M. Motto, and V. Rossi, 2009 Chromatin and DNA modifications in the Opaque2-mediated regulation of gene transcription during maize endosperm development. Plant Cell 21: 1410–1427.

Matzke, M. A., M. Susani, A. N. Binns, E. D. Lewis, I. Rubenstein *et al.*, 1984 Transcription of a zein gene introduced into sunflower using a Ti plasmid vector. EMBO J. 3: 1525–1531.

McGinnis, K. M., C. Springer, Y. Lin, C. C. Carey, and V. Chandler, 2006 Transcriptionally silenced transgenes in maize are activated by three mutations defective in paramutation. Genetics 173: 1637–1647.

Meyer, P., I. Niedenhof, and M. ten Lohuis, 1994 Evidence for cytosine methylation of non-symmetrical sequences in transgenic *Petunia hybrida*. EMBO J. 13: 2084–2088.

Miclaus, M., J. H. Xu, and J. Messing, 2011 Differential gene expression and epiregulation of alpha zein gene copies in maize haplotypes. PLoS Genet. 7: e1002131.

Ngezahayo, F., C. Xu, H. Wang, L. Jiang, J. Pang *et al.*, 2009 Tissue culture-induced transpositional activity of mPing is correlated with cytosine methylation in rice. BMC Plant Biol. 9: 91.

Park, Y. D., I. Papp, E. A. Moscone, V. A. Iglesias, H. Vaucheret *et al.*, 1996 Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically

heritable alterations in methylation and gene activity. Plant J. 9: 183–194.

Peschke, V. M., R. L. Phillips, and B. G. Gengenbach, 1987 Discovery of transposable element activity among progeny of tissue culture–derived maize plants. Science 238: 804–807.

Rossi, V., M. Motto, and L. Pellegrini, 1997 Analysis of the methylation pattern of the maize opaque-2 (O2) promoter and in vitro binding studies indicate that the O2 B-Zip protein and other endosperm factors can bind to methylated target sequences. J. Biol. Chem. 272: 13758–13765.

Schmidt, R. J., M. Ketudat, M. J. Aukerman, and G. Hoschek, 1992 Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. Plant Cell 4: 689–700.

Song, R., and J. Messing, 2003 Gene expression of a gene family in maize based on noncollinear haplotypes. Proc. Natl. Acad. Sci. USA 100: 9055–9060.

Song, R., V. Llaca, E. Linton, and J. Messing, 2001 Sequence, regulation, and evolution of the maize 22-kD alpha zein gene family. Genome Res. 11: 1817–1825.

Sturaro, M., and A. Viotti, 2001 Methylation of the Opaque2 box in zein genes is parent-dependent and affects O2 DNA binding activity in vitro. Plant Mol. Biol. 46: 549–560.

Swanson-Wagner, R. A., Y. Jia, R. DeCook, L. A. Borsuk, D. Nettleton *et al.*, 2006 All possible modes of gene action are observed in a global comparison of gene expression in a maize F1 hybrid and its inbred parents. Proc. Natl. Acad. Sci. USA 103: 6805–6810.

Ueda, T., W. Waverczak, K. Ward, N. Sher, M. Ketudat *et al.*, 1992 Mutations of the 22- and 27-kD zein promoters affect transactivation by the Opaque-2 protein. Plant Cell 4: 701–709.

Vicente-Carbajosa, J., S. P. Moose, R. L. Parsons, and R. J. Schmidt, 1997 A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. Proc. Natl. Acad. Sci. USA 94: 7685–7690.

Wang, Z., and J. Messing, 1998 Modulation of gene expression by DNA-protein and protein-protein interactions in the promoter region of the zein multigene family. Gene 223: 333–345.

Wu, Y., and J. Messing, 2009 Tissue-specificity of storage protein genes has evolved with younger gene copies. Maydica 54: 409–415.

Wu, Y., and J. Messing, 2010 RNA interference-mediated change in protein body morphology and seed opacity through loss of different zein proteins. Plant Physiol. 153: 337–347.

Wu, Y., and J. Messing, 2012 RNA interference can rebalance the nitrogen sink of maize seeds without losing hard endosperm. PLoS ONE 7: e32850.

Wu, Y., W. Wang, and J. Messing, 2012 Balancing of sulfur storage in maize seed. BMC Plant Biol. 12: 77.

Xu, J. H., and J. Messing, 2008 Organization of the prolamin gene family provides insight into the evolution of the maize genome and gene duplications in grass species. Proc. Natl. Acad. Sci. USA 105: 14330–14335.

Xu, J. H., and J. Messing, 2009 Amplification of prolamin storage protein genes in different subfamilies of the Poaceae. Theor. Appl. Genet. 119: 1397–1412.

Communicating editor: S. Poethig

GENETICS

Rapid Divergence of Prolamin Gene Promoters of Maize After Gene Amplification and Dispersal

Yongrui Wu and Joachim Messing
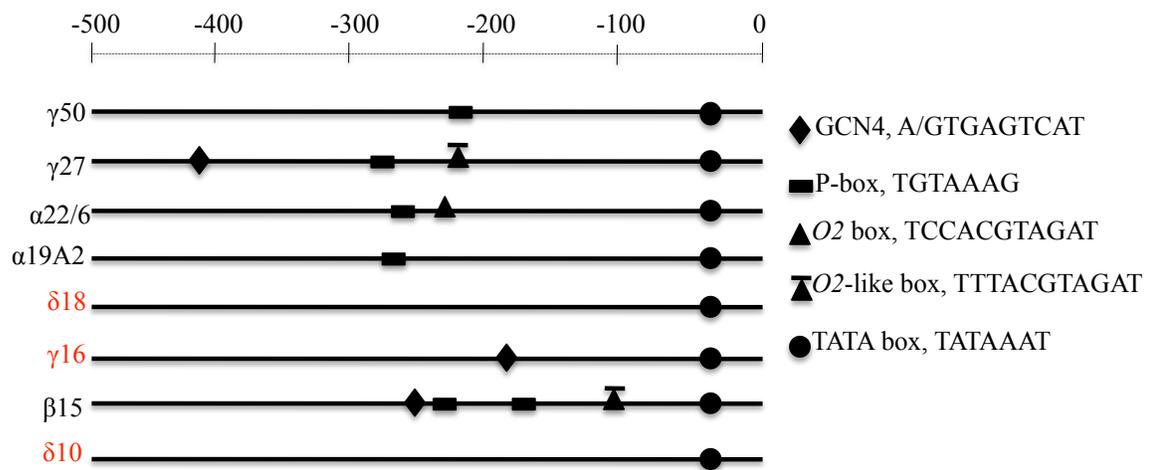
Figure S1 Cis-elements in zein-gene promoters.

Y. Wu and J. Messing

```
ZM27   --AAAAATAA-AATAGGCCGGACAG--GACAAAAATCCTTGACGTGTAAAGTAAATTTAC -267
SB27   GTAAAAACAA-AGTAGGCCGGAGAGAGGACAATAATCCTTGACGTGTAAAGTGAATTTAC -250
ZM16   --AAAAGCAAGAGTAGGACGGAGAG--GAAAATAATCCTTGACG---------ACGTGC -183
          ****   ** * **** **** **   ** ** **********       *  * *

ZM27   AACAAAAAAAAGCCATATGTCAAGCTAAATCTAATTCGTTTTACGTAGAT--CAACAACC -209
SB27   AA--------AGCCATATATCAATTTATATCTAATTCGTTTCATGTAGATATCAACAACC -198
ZM16   A------------CATGAGCCA-------------------------------------- -173
       *            ***     **

ZM27   TGTAGAAGGCAACAAAACTGAGCCACGCAGAAGTACAGAATGATTCCAGATGAACCATCG -149
SB27   TGTAAAAGGCAACAAA-TTGAGCCACGCAAAATTACA-AGTGAGTCCAAATAAACCCTC- -141
ZM16   ---------------------CACGCAAGAGTACTGAATAAATCCAGATGAACCCTCC -136
                            ******   * ***   * * * **** ** **** **

ZM27   ACGTGCTACGTAAAGA-GAGTGACGAGTCATATACATTTGGCAAGAAACCATGAAGCTGC -90
SB27   ACATGCTACATAAAAGTGAATGATGAGTCATGTATATCTGGCAAGAAACTGTAGA---AG -84
ZM16   AAAAG-TGAATGAGAT-GAGTCATG-----TATACATTTGGCAAGAAACCGTAGA---AG -86
       *   * *   * *   ** * *       * ** ** **********   *   *

ZM27   CTACAGCCGTCTCGGTGGCATAAGAACACAAGAAATTGTGTTAATTAATCAAAGCTATAA -30
SB27   CTACAGTCATC--GGTAGCAAAGAAACACAAGAAAATGTGCTAATAA----AAGCTATAA -30
ZM16   CTACCGCCATC--GATTTCATCATAGAAGAAGAAATTGTGGTAATCGG--AAAGCTATAA -30
       **** * * **   * *  **     *  * ****** **** ****       *********
                                          +1 Transcription Start
ZM27   ATAACGCTCGCATGCCTGTGCACTTCTCCATCACCACCACTGGGTCT---TCAGACCATT 28
SB27   ATAACCCTCGTACGCCTATGCACATCTCCATCACCACCACTGGTCTTCATTCAGCCTATT 31
ZM16   ATAACCGTCGTATGCCTATGCACTTCTCCACCACCACCACTGGACGTCAGCCTAGCAATT 31
```

Figure S2 Alignment of the 27- and 16-kDa γ-zein and 27-kDa γ-kafirin promoters.

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```
1     2                       3       4   5                   6
C  A  C  G  G  T  G  A  G  T  C  A  T  G  C  C  G  A  G  A  T  C  A  T
   7     8           9                 10          11             12
A  C  T  C  A  T  C  T  G  A  T  A  T  A  C  A  T  G  C  T  T  A  C  A
   13    14    15          16          17          18          19
G  C  T  C  A  C  A  A  G  A  C  A  T  T  A  C  A  A  A  C  A  A  C  T
20          21          22                      23
C  A  T  A  T  T  G  C  A  T  T  A  C  A  A  A  G  A  T  C  G  T  T  T
24                                        25 26             27
C  A  T  G  A  A  A  A  A  T  A  A  A  A  T  A  G  G  C  C  G  G  A  C
         28                   29 30             31
A  G  G  A  C  A  A  A  A  A  T  C  C  T  T  G  A  C  G  T  G  T  A  A
                  32          33                               34
A  G  T  A  A  A  T  T  T  A  C  A  A  C  A  A  A  A  A  A  A  A  A  G  C
35                   36          37                38             39
C  A  T  A  T  G  T  C  A  A  G  C  T  A  A  A  T  C  T  A  A  T  T  C
                     40             41       42          43 44
G  T  T  T  T  A  C  G  T  A  G  A  T  C  A  A  C  A  A  C  C  T  G  T
                     45             46             47       48 49    50
A  G  A  A  G  G  C  A  A  C  A  A  A  A  C  T  G  A  G  C  C  A  C  G
51                         52                         53 54
C  A  G  A  A  G  T  A  C  A  G  A  A  T  G  A  T  T  C  C  A  G  A  T
            55 56          57          58          59          60
G  A  A  C  C  A  T  C  G  A  C  G  T  G  C  T  A  C  G  T  A  A  A  G
                           61          62                63
A  G  A  G  T  G  A  C  G  A  G  T  C  A  T  A  T  A  C  A  T  T  T  G
   64                      65 66                67       68 69       70
G  C  A  A  G  A  A  A  C  C  A  T  G  A  A  G  C  T  G  C  C  T  A  C
      71 72          73          74             75                76
A  G  C  C  G  T  C  T  C  G  G  T  G  G  C  A  T  A  A  G  A  A  C  A
77                                                          78
C  A  A  G  A  A  A  T  T  G  T  G  T  T  A  A  T  T  A  A  T  C  A  A
      79                            80    81    82    83          84 85
A  G  C  T  A  T  A  A  A  T  A  A  C  G  C  T  C  G  C  A  T  G  C  C
         86       87          88    89 90
T  G  T  G  C  A  C  T  T  C  T  C  C  A  (Transcription Start Site)
```

Figure S3 Cytosines in the 27-kDa γ-zein promoter. The 421-bp fragment immediately upstream of the transcription start site contains 90 cytosines, covering the P-box, O2-like box and TATA box. P-box, "ACGT" core motif and TATA box are shaded. The cytosine positions indicated in Figs. 4a and S6d are numbered in the sequence.

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```
                                    1     2     3     4
Zp22/6      TGTGTAAAGGTGAAGAGATCTTGCATGTCATTC
azs22.11    TGTGTAAAGGTGAAGAGATGATGCATGTCAT-C
azs22.12    TGTGTAAAGGTGAAGAGATGATGCATGTCAT-C
azs22.13    TGTGCAAAGGTGAAGAGATCATGCATGTCATTC
azs22.4     TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.1     TGTGTAAAGGTGAAGCGATCATGCATGTCATTC
azs22.3     TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.5     TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.19    TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.7     TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.8     TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.2     TGTGTAAAGGTGAAGCGATCATGCATGTCATTC
azs22.20    TGTGTAAAGGTGAAGAGATCATGCATTTCATTC
azs22.6     TGTTTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.9     TGTGTAAAGGTGAAGAGATCATGCATGTCATTC
azs22.16    TGTGTAAAGGTGAAGAGATCATGCATGTCATTC

            5 6             7              8 9    10
Zp22/6      CACGTAGAT-AAAAAGAATGCATATATAAAAATGGCACATTTTCTTGTAGGTAGTGGAAA
azs22.11    CAAGTATATGAAAAT-AATTCCTATA-GAAAATGACACTTTTTCTTATAGGTAGTGGAAA
azs22.12    CAAGTATATGAAAAG-AATTCCTATA-GAAAATGACAATTTTTCTTGTAGGTAATGGAAA
azs22.13    CTCATGTATGGAAAG-AATTCCAATA-GAAAATGACGAATTTTCCTGTAGGTAGTGAAAA
azs22.4     CACGTAGATGAAAA--AATTCCTATATAAAAATGACACCTTTTCTTGTAGGTAGTGGAAA
azs22.1     CACGTAGATGAAAAAAAATTCCTATATAAAAATGACAATTTTTCTTGTAGGTAGTGGAAA
azs22.3     CACGTAGATGAAAAG-AATTCCTATATAAAA-TGACACCTTTTCTTGTAGGTAGTGGAAA
azs22.5     CACGTAGATGAAAAG-AATTCCTATATAAAA-TGACACCTTTTCTTGTAGGTAGTGGAAA
azs22.19    CACGTAGATGAAAAG-AATTCCTATATAAAA-TGACACCTTTTCTTGTAGGTAGTGGAAA
azs22.7     CACGTAGATGAAAAG-AATTCCTATATAAAA-TGACACCTTTTCTTGTAGGTAGTGGAAA
azs22.8     CACGTAGATGAAAAG-AATTCCTATATAAAA-TGACACCTTTTCTTGTAGGTAGTGGAAA
azs22.2     CACATAGATTAAAAA-AAATCCTATATAAAAATGACAATTTTTCTTGTAGGTAGTGGAAA
azs22.20    CACGTAGATAAAAAG-AGTTCCTATATAAGAATGGCACATTTTCTTGTAGGTAGTGGAAA
azs22.6     CACGTAGATGAAAAG-AATTCCTATATAAAA-TGACACATTTTCTTGTAGGTAGTGGAAA
azs22.9     CACGTAGATAAAAAG-AATGCCTATATAAAAATGGCACATTTTCTTGTAGGTAGTGGAAA
azs22.16    CACATAAATGAAAAG-AATTCCTATATAAAAATGACATGTTTTGTTGTAGGTAGTGGAAA

            11   1213 14    1516          1718          19        20
Zp22/6      GTATCTTTCCAGCTAAGACC----ATATAATCCGATAAAGGTGATAACTAAATGTCGAAA
azs22.11    CTATCTTTCTA-------------TATAATCCGATGAAACCGATAACTAAATGTCGAAA
azs22.12    CTAGCTTTCCAGCAAAGACCA----TATAATCTGATGAAACTGATAACCAAATGTCGAAA
azs22.13    CTATCTTTATAGCAAAGTCCA----TATAATCATATGAAACTGATGACTAAATGTCAAAA
azs22.4     GTACCTTTCCAGCAAAGATCATATATATAATCCGATAAAGCTGAAAACTAAATGTCGAAA
azs22.1     CTATCTTTCTAGCAAAGACCATATATATAATCCGATAAAGCCGATAACTAAATGTCGAAA
azs22.3     GTATCTTTCCAGCAAAGACCATATA----ATCCGATAAAGTTGATAACTAAATGTCAAAA
azs22.5     GTATCTTTCCAGCAAAGACCATATA----ATCCAATAAAGTTGATAACTAAATGTCAAAA
azs22.19    GTATCTTTCCAGCAAAGACCATATA----ATCCGATAAAGTTGATAACTAAATGTCAAAA
azs22.7     GTATCTTTCCAGCAAAGACCATATA----ATCCGATAAAGTGATAACTAAATGTCAAAA
azs22.8     GTATCTTTCCAGCAAATACCATATA----ATCCGATAAAGCGGATAACTAAATGTCAAAA
azs22.2     GTATCTTTCCAGCAAAGACTATATATATAATCCGATAAAGCCGATAACTAAATGTCAAAA
azs22.20    GTATCTTTCCAGCAAATACCATATA----ATCCGATAAAGATGATAACTAAATGTCGAAA
azs22.6     GTATCTTTCCAGCAAAGACTATA------ATCCGATAAAGTTGATAACTAAATGTCGAAA
azs22.9     GTATCTTTCCAGCAAAGACCATATA----ATCCGATAAAGCTGATAACTAAATGTCGAAA
azs22.16    TTATCTTTCCAGCAAAGACCATATA----ATCCGATAAAGCTGATAACTAAATGTCAAAA

            21      2223  24 25  2627  28                          29
Zp22/6      TCGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGG--AAAAAAAAGACAAAAT
azs22.11    TTGAGTAGGTGCCATATCATCGATAGCTTATCTATTGTTTGGC--AAAAA--GACAAAAT
azs22.12    TTGAGTAGGTGCCATATCATTGATAGCTTATCTATTGTTTGGC--AAAAA--GATAAAAT
azs22.13    TTGAGTAGGTGCCATATCATCTATAGCTTATCCATTGTTGGGA--AAAAA--TACAAAAT
azs22.4     TCGAGTAGATGCCATATCATCTATACCTTATCTCTTGTTTGGA--AAAAA--GACAAAAC
azs22.1     TCGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGG---AAAAA--GACAAAAT
azs22.3     TAGAGTAGATGCCATATCATATATACCTTATCTGTTGTTTGGA--AAAAAAAGACTAAAT
azs22.5     TAGAGTAGATGCCATATCATTTATACCTTATCTGTTGTTTGGA--AAAAAA-GACTAAAT
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azs22.19        TAGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGA--AAAA---GACTAAAT
azs22.7         TAGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGA--AAAA---GACTAAAT
azs22.8         TCGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGA--AAAAA--GACAAAAT
azs22.2         TCGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGA--AAAAA--GACAAAAT
azs22.20        TCGAGTAGATGCCATATCATCTATATCTTATCTGTTGTTTGGA--AAAAAA-GACAAAAT
azs22.6         AAGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGA--AAAAAA-GACTAACT
azs22.9         TCGAGTAGATGCCATATCATCTATACCTTATCTGTTGTTTGGAAAAAAAAAAAGACAAAAT
azs22.16        TCGAGTAAGTGCCATATCATCTATATCTTATCTGTTGTTTGGA—-AAAA---GACAAAAT


                3031                     32333435          36373839  40
Zp22/6          CCAAAAAA-TTTATAT----GAGATCTCACCTATATAAATAGCGCCCAAATCAGTAGTTA
azs22.11        CCAAATATATATATAT----GAGATCTCACATGTATGAATATCTCCCAAATCAGTAGTTA
azs22.12        CCAAATATATATATATATGAGATCTCACCTATATAAATATCTCCCAAATCAGTAGTTA
azs22.13        CCAAAAA-ATATATAT----GAGACCTCACCTGTATATATAACCCCCAAATCAGTAGTTA
azs22.4         CAAAAAA--TATTTAT----GAGGTCTCACTTGTATAAATAGCTCCCAAATCAGTAGTTA
azs22.1         CCAAAAA-ATATATAT----GAGATCTCACTTGTATGAATAGCTCCCAAATCAGTAATTA
azs22.3         CCCAAAA-ATATATAT----GAGATCTCACTTGTATAAATAGCTCCCAAATTAGTAGTTA
azs22.5         CC--AAA-ATATATAT----GACATCTCACTTGTATAAATAGCTCCCAAATCAGCAGTTA
azs22.19        CCAAAAA-ATATATAT----GAGATCTCACTTGTATAAATAGCTCCCAAATCAGTAGTTA
azs22.7         CCAAAAATATATATAT----AAGATCTCACTTGTATAAATAGCTCCCAAATTAGTAGTTA
azs22.8         CCAAAAA-ATATATTT----GAGACCTCACCTGTATAAATAGCTCCCAAATCAGTAGTTA
azs22.2         CCAAAAAAATATATAT----GAGATCTCACTTGTATAAATAGCTCCCAAATCAGTAGTTA
azs22.20        CCAACAA-AAATATTC----GAGACCTCACCTATATAAATAGCTCCCATATCAGTAGTTA
azs22.6         ACAAAAA-ATATATAT----GAGATCTCACTTGTATAAATAGCTCCCAAATCAGTAGTTA
azs22.9         CCAAAAAAAAATATAA----GAGATCTCACCTATATAAATAGCTCTGAAATCAGTAGTTA
azs22.16        CCAAAAAAAAATATAT----GAGATCTCACATGTATAAATAGCTCCCAAATCAGTAGTTA


                4142
Zp22/6          ATCCA
azs22.11        ATTCA
azs22.12        ATCCA
azs22.13        ATTCA
azs22.4         ATCCA
azs22.1         ATCCG
azs22.3         A-CCA
azs22.5         A-CCA
azs22.19        A-CCA
azs22.7         A-CCA
azs22.8         ATCCA
azs22.2         ATCCA
azs22.20        ATCCA
azs22.6         A-CCA
azs22.9         ATCCA
azs22.16        ATACA
```

Figure S4   The alignment of the 22-kDa α-zein promoters from B73 and the copy Zp22/6 from BSSS53. The cytosine positions indicated in Figs. 4b, 7c and 9 are marked in the corresponding places; P-box and "ACGT" core motif are shaded.
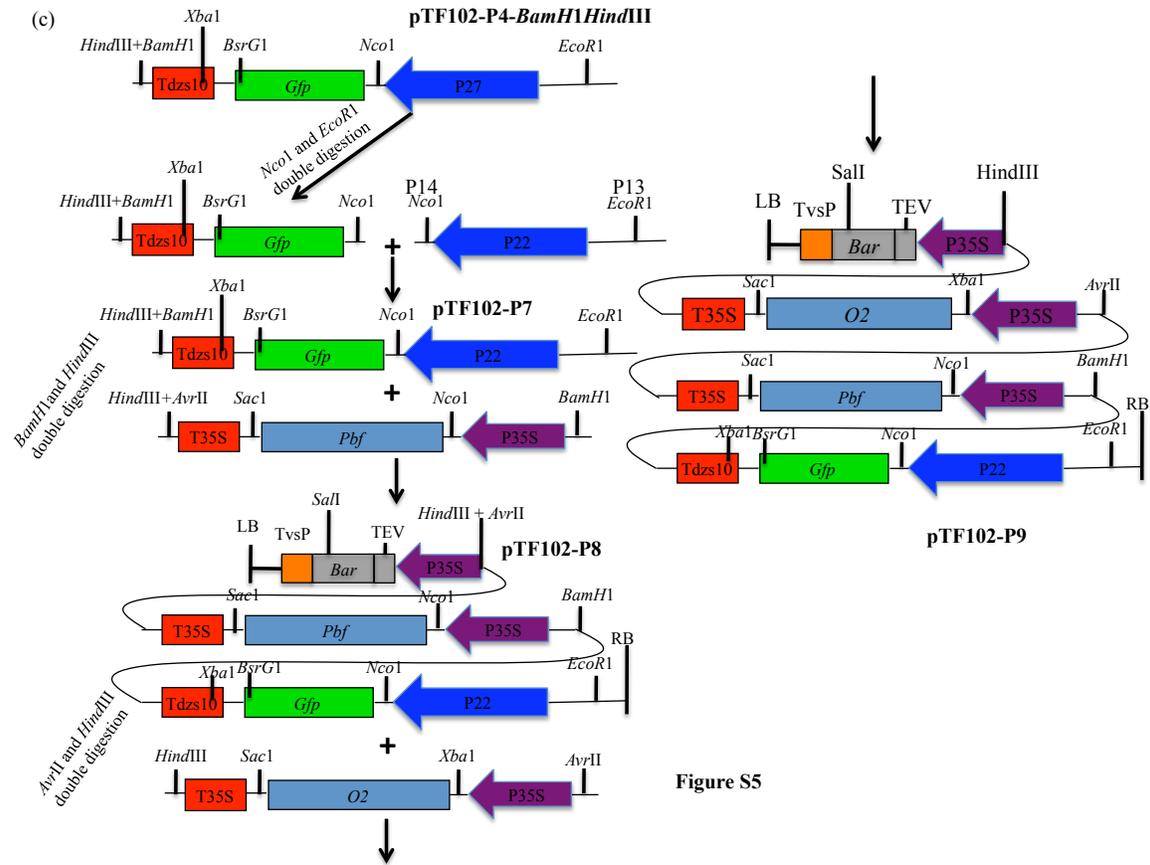
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(a)

(b)

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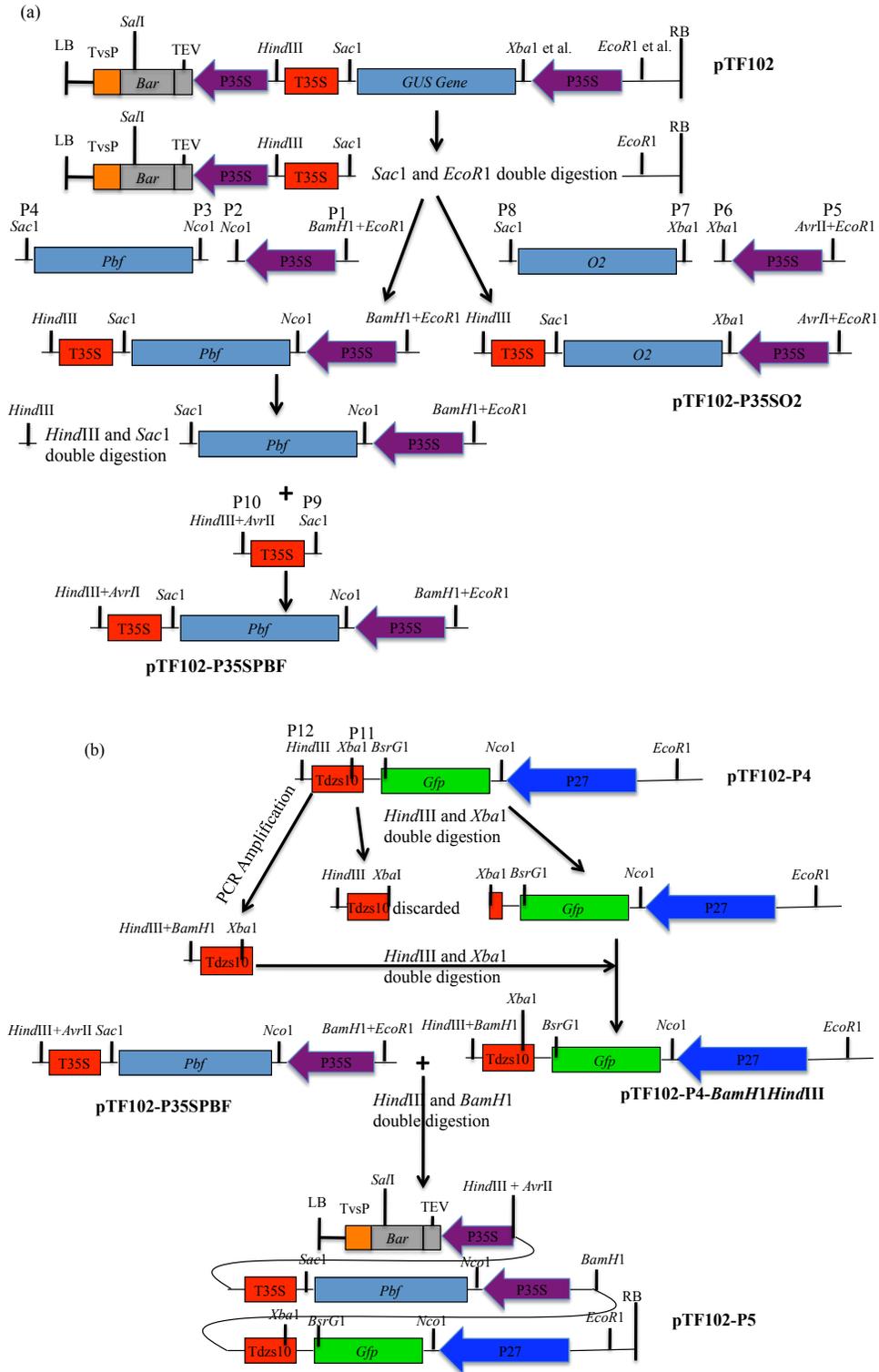
Figure S5 Strategies of plasmid construction. (a) P35SPBF and P35SO2 cassettes. (b) pTF102-P5. (c) pTF102-P9. Primers P1-P14 used for plasmid construction are indicated in the corresponding step.

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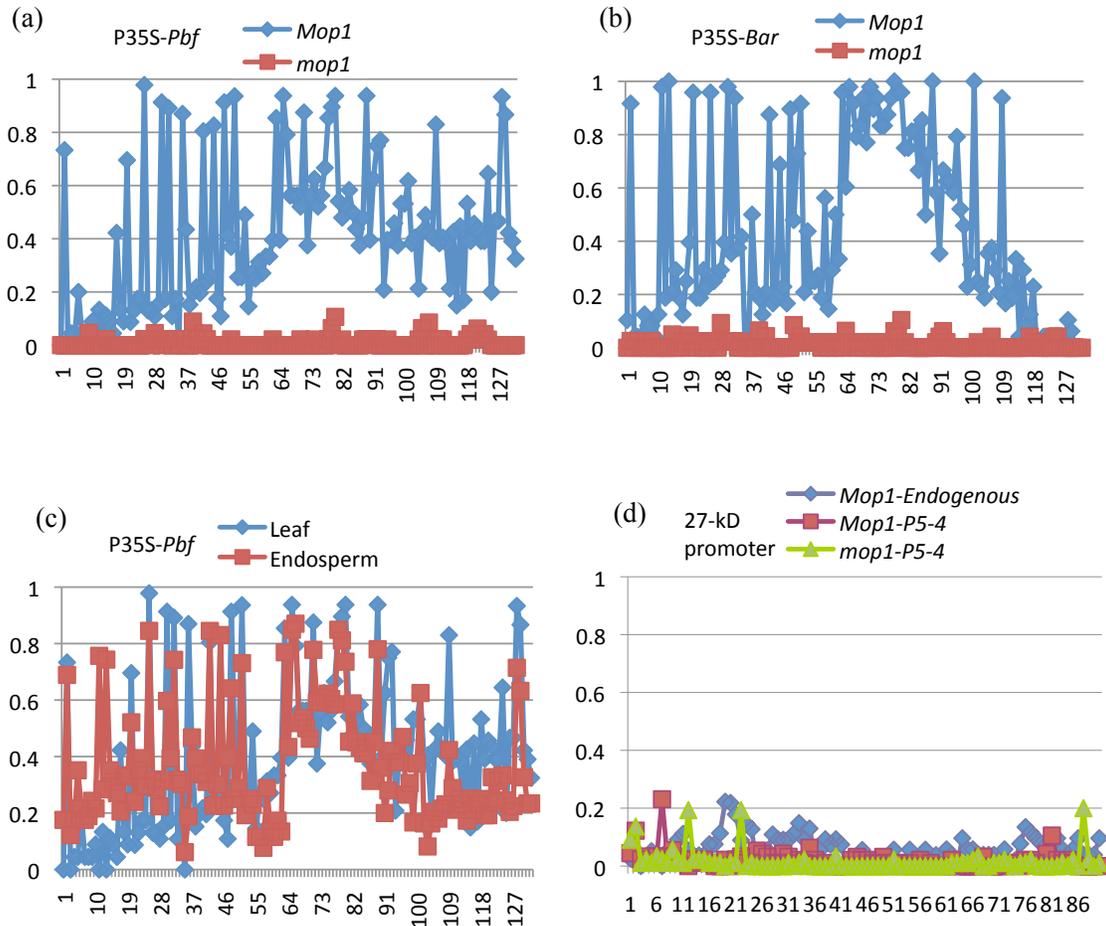**Figure S6** DNA methylation of 35S and transgenic 27-kDa γ-zein promoters in *Mop1-P5-4* and *mop1-P5-4*. (a) The 35S promoter driving the *Pbf* gene in *Mop1-P5-4 and mop1-P5-4*. (b) The 35S promoter driving the selective marker *Bar* gene in *Mop1-P5-4 and mop1-P5-4*. (c) The 35S promoter driving the *Pbf* gene in *Mop1-P5-4* leaf and endosperm. (d) The transgenic 27-kDa γ-zein promoter driving the *Gfp* gene.

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```
G A G C A A T G T A T T G G T T G T G A G T T T
      1       2       3       4     5
T T G A C G G C G C T C A C A G T G A T A G A T
        6     6   6   6   6                    
                        7 8     9 10 11 12
T T G T T A T C T A T A T G C C A G C C C C A G
13            14      15 16      17            18
C A T A T T C A T C C T T G T G C T G T G G G C
19          19      20 21      22              
G T C T A G A G G A C C G A C A A T A T A T A T
      19          23    23  24                  
A T T T T T A A A A C A A A T T C G T G A A G A
    25    26    27        28      29        30
A C A T C A C A A G T T A T G C A T G C A A A C
    31  32          33        34    35
T G C T C A A G T C A T G T G G A T C C A A G G
36    37 38        39      40        41  42    43
C A T C C T A A C A A C T A G C A C A G C A T T
44      45            46        47      48
A C A A C A A A A T A T T G G T G T A T A T G T
  46 47      48
G C C T A C A A T G A A G T G A A A G G T G A T
  49    49
G A G T C A T G G T G A T G T G T A A A G A G G
50        51                  52    52    54      55
C A T T A C A A A G T T A G C T T C A C A A G C
          55          56        57    58 59 60
G T A T G A A T T C A T T G A C A A C C C T T G
    61              62            62
A C A T G T A A A G T T G A T T C A T A T G T A
  63                        64        65
T A A G A A A G C T T A A T G A T C T A T C T G
          66 67        68 69        70
T A A A T C C A A A T C C A T G T A C T A T G T
        71 72  73      74        75      76  77      78
T T C C A C G T C A T G C A A C G C A A C A T T
79 80  73      81 82    83                      84
C C A A A A C C A T G G G T T G C A A G A T G C
85          86        87 88          89
T G C A G A A T G C A A G C C A T G G A T C A T
90            91        92    93 94 95    96
C T A T A A A T G G C T A G C T C C C A C A T A
97        98      99        100    101  102 103
T G A A C T A G T C T C T A T C A T C A T C C A
  104 105      106    107        108    109
A T C C A G A T C A G C A A A G C G G C A G T G
110
C G T A G A G A G G A T C G T C G A A C A G A A

C A G C **A T G**
```

Figure S7 Cytosines in β-zein promoter. The 559-bp fragment upstream of the start codon contains 110 cytosines, covering the P-box, O2 box and TATA box. P-box, "ACGT" core motif and TATA box are shaded. The cytosine positions indicated in Fig. 11b are numbered in the sequence.

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Table S1 List of primers

Primer	Sequence	Note
pbfBamH1	5'-AATTGGATCCACTAACCTTATTGTCCCTTG-3'	
pbfBspE1	5'-TATATCCGGAATGGACATGATCTCCGGCAG-3'	
pbfBblII	5'-AATAAGATCTATGGACATGATCTCCGGCAG-3'	*Pbf* RNAi construct
pbfSac1	5'-AATTGAGCTCACTAACCTTATTGTCCCTTG-3'	
Umc1066F	5'-ATGGAGCACGTCATCTCAATGG-3'	
Umc1066R	5'-AGCAGCAGCAACGTCTATGACACT-3'	*O2* polymorphic markers
P1	5'- TATATGAATTCAAGGATCCGCATGCCGGTCAACATGGTG -3'	P35S forward, *EcoR*1 and *BamH*1 introduced
P2	5'- AAATCCATGGTAGATCCCCCGTTCGTAAATG -3'	P35S reverse, *Nco*1 introduced
P3	5'- ATTACCATGGACATGATCTCCGGCAG -3'	*Pbf* CDS forward, *Nco*1 introduced
P4	5'- AATTGAGCTCACTAACCTTATTGTCCCTTG -3'	*Pbf* CDS reverse, *Sac*1 introduced
P5	5'- TATATGAATTCAACCTAGGGCATGCCGGTCAACATGGTG -3'	P35S forward, *EcoR*1 and *Avr*II introduced
P6	5'- AAATTCTAGAGGTAGATCCCCCGTTCGTAAATG -3'	P35S reverse, *Xba*1 introduced
P7	5'- ATTATCTAGACCATGGAGCACGTCATCTCAATG -3'	*O2* CDS forward, *Xba*1 introduced
P8	5'- TAATGAGCTCCTTATTCAGCGACGCCTG -3'	*O2* CDS reverse, *Sac*1 introduced
P9	5'-TATAGAGCTCGGCGCAAAAATCACCAGTC-3'	T35S forward, *Sac*1 introduced
P10	5'- ATTAAGCTTAATTCCTAGGTGCAGGTCACTGGATTTTGG -3'	T35S reverse, *Hind*III and *Avr*II introduced
P11	5'- AAAGCTGTACAAGTAAATAGAAATATTTGTGTTGTATCG -3'	Terminator of 10-kDa δ-zein gene, forward
P12	5'- ATCAAGCTTACTAGTGGATCCTACCAGCTGAGAATTAGGAG -3'	Terminator of 10-kDa δ-zein gene, reverse, *Hind*III and *BamH*1 introduced
P13	5'- ATTTGAATTCGTCCACGCGCAAATAGACC -3'	22-kDa zein promoter forward, *EcoR*1 introduced
P14	5'- TAATCCATGGTTGTTTGGTCGTTGCTAGTG -3'	22-kDa zein promoter

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		reverse, *Nco*1 introduced
γ50F	5'-ATGAAGCTGGTGCTTGTGGTTC-3'	For γ50 RT-PCR
γ50R	5'-TAATGTCATTGCTGCTGCATGG-3'	
γ27F	5'-ATGAGGGTGTTGCTCGTTGC-3'	For γ27 RT-PCR
γ27R	5'-ACTCAACTAGCTAGCTAGCC-3'	
α22F1	5'-ACACCATATGTTCATTATTCCACAATGCTCA-3'	For α22 RT-PCR
α22R1	5'-TTAAGGATCCTATATAATCTAAAAGATGGCA-3'	
α19AF	CTCTTAa/gATTAGTAGCTAATAt/cATC	For α19A RT-PCR
α19AR	CTGGGAAGCCACAAACATCA	
α19BDF	ATTAGTCGGTAATCCATCAACC	For α19B and D RT-PCR
α19BDR	CTAGAAGATGGCACCACCAATG	
δ18F	5'-CGCCATGGCAGCCAAGATG-3'	For δ18 RT-PCR
δ18R	5'-ATGCCGACTTCATTATTGGG-3'	
γ16F	5'-TCGACACCATGAAGGTGCTG-3'	For γ16 RT-PCR
γ16R	5'-TGGTGATGGGTGACACTACG-3'	
β15F	5'-AGGATCGTCGAACAGAACAGC-3'	For β15 RT-PCR
β15R	5'-AGATGGATAGAGGAGATTTCCC-3'	
δ10F	5'-ATACTCTAGGAAGCAAGGAC-3'	For δ10 RT-PCR
δ10R	5'-TAAGAACATGGGTGGAATCG-3'	
δ18-10F	5'-CGCCATGGCAGCCAAGATG-3'	Common primer pair for δ18 and δ10 RT-PCR
δ18-10R	5'-TATCTAGAATGCAGCACCAAC-3'	
PbfF	5'-ATGGACATGATCTCCGGCAG-3'	For *Pbf* RT-PCR
PbfR	5'-ACTAACCTTATTGTCCCTTG-3'	
O2F	5'-ATGGAGCACGTCATCTCAATG-3'	For *O2* RT-PCR
O2R	5'-CCTTATTCAGCGACGCCTG-3'	
GFPF1	5'-ATGGTGAGCAAGGGCGAGG-3'	For *Gfp* RT-PCR
GFPR1	5'-TTACTTGTACAGCTCGTCC-3'	
P22F1	5'-ACATGTGTAAAGGTGAAGAG-3'	
GFPR2	5'- GTGAGCAAGGGCGAGGAGCT-3'	
P22bisulF	5'-ATATGTGTAAAGGTGAAGAG-3'	
α22bisulR	5'-CTAATATCTTAATAACCATT -3'	
GFPbisulR	5'-AACTCCTCACCCTTACTCAC-3'	
P27bisulF	5'-GAAATATGGTGAGTTATGTTGAG-3'	
γ27bisulR	5'-AAAACAACAAACAACACCCTC-3'	
P35SbisulF	5'-ATGTTGGTTAATATGGTGGAG-3'	
BarbisulR	5'-ACCACCAACATATCCACCTC-3'	
PbfbisulR	5'-CTTTAAATCCCTAACTTCCTCC-3'	

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β15bisulF   5'- GAGTAATGTATTGGTTGTGAG -3'

β15bisulR   5'- CTATTCAACAATCCTCTCTAC -3'

Restriction enzyme cutting sites are colored in red.