Divergent Transactivation of Maize Storage Protein Zein Genes by the Transcription Factors Opaque2 and OHPs

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ABSTRACT Maize transcription factors (TFs) opaque2 (O2) and the O2 heterodimerizing proteins (OHP1 and OHP2) originated from an ancient segmental duplication. The 22-kDa (z1C) and 19-kDa (z1A, z1B, and z1D) α-zeins are the most abundant storage proteins in maize endosperm. O2 is known to regulate α-zein gene expression, but its target motifs in the 19-kDa α-zein gene promoters have not been identified. The mechanisms underlying the regulation of α-zein genes by these TFs are also not well understood. In this study, we found that the O2 binding motifs in the α-zein gene promoters are quite flexible, with ACGT being present in the *z1C* and *z1A* promoters and a variant, ACAT, being present in the *z1B* and *z1D* promoters. OHPs recognized and transactivated all of the α-zein promoters, although to much lower levels than did O2. In the presence of O2, the suppression of OHPs did not cause a significant reduction in the transcription of α-zein genes, but in the absence of O2, OHPs were critical for the expression of residual levels of α-zeins. These findings demonstrated that O2 is the primary TF and that OHPs function as minor TFs in this process. This relationship is the converse of that involved in 27-kDa γ-zein gene regulation, indicating that the specificities of O2 and the OHPs for regulating zein genes diverged after gene duplication. The prolamine-box binding factor by itself has limited transactivation activity, but it promotes the binding of O2 to O2 motifs, resulting in the synergistic transactivation of α-zein genes.

KEYWORDS maize; endosperm; storage proteins; transcription factors; subfunctionalization

SEEDS are products of the advanced evolution that propagate life. Seed storage reserves are synthesized during seed filling, and these mainly consist of starch, oil, and protein. Starch and oil provide energy, and protein offers sulfur and nitrogen, which support seed germination and early seedling development. Storage proteins are also a staple protein source for humans and livestock.

In angiosperms, storage protein genes appear to be regulated by a common transcription factor (TF) family, *i.e.*, the basic leucine zipper (bZIP) family. In *Arabidopsis*, the main storage protein genes encoding 2S albumins and 12S cruciferins are transcriptionally regulated by bZIP TFs (AtbZIP10 and AtbZIP25) (Lara *et al.* 2003) and B3 TFs [ABSCISIC ACID INSENSITIVE3 (AtABI3), FUSCA3 (AtFUS3), and LEAFY COTYLEDON2 (AtLEC2)] (Giraudat *et al.* 1992; Bäumlein *et al.* 1994; Parcy *et al.* 1994; Stone *et al.* 2001). In cereals (maize, rice, wheat, and barley), endosperm storage protein genes are also regulated by two main TF families: bZIP TFs and DNA-binding with one finger (Dof) family TFs (Schmidt *et al.* 1992; Albani *et al.* 1997; Vicente-Carbajosa *et al.* 1997; Conlan *et al.* 1999; Onate *et al.* 1999; Onodera *et al.* 2001; Mena *et al.* 2002; Yamamoto *et al.* 2006; Kawakatsu *et al.* 2009; Wu and Messing 2012; Zhang *et al.* 2015). In maize, the main storage proteins in endosperm are called zeins, and these are classified into four families including α (19 and 22 kDa), γ (50, 27, and 16 kDa), β (15 kDa), and δ (18 and 10 kDa) (Esen 1987; Coleman and Larkins 1999). Opaque2 (O2) was the first cloned bZIP TF involved in the regulation of storage protein genes in plants (Schmidt *et al.* 1987). The bZIPs identified to date that regulate storage protein genes in

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barley (BLZ2), wheat (SPA), rice (RISBZ1), and *Arabidopsis* (AtbZIP10 and AtbZIP25) are all O2 homologs, indicating that O2 family genes are functionally conserved during evolution.

The 19- and 22-kDa α-zeins are encoded by a large multigene family and account for 60–70% of the total zein fraction (Thompson and Larkins 1994). The α-zein sequences are the most abundant transcripts in seeds, and intact copies of most of the α-zein genes are ranked among the 100 most highly expressed genes in maize endosperm (Hunter *et al.* 2002; Chen *et al.* 2014). The huge abundance of α-zeins in maize seeds is largely attributed to their extremely robust transcription. O2 regulates the expression of the 22-kDa α-zein and β-zein genes by recognizing the O2 box (TCCACGT) in the gene promoters (Schmidt *et al.* 1992; Neto *et al.* 1995). A recent study demonstrated that the transcription of all zein genes, except for the 16-kDa γ-zein gene, is affected by the *o2* mutation (Li *et al.* 2015). The protein and RNA transcript levels of the 19-kDa α-zeins, which can be further divided into *z1A*, *z1B*, and *z1D* (Thompson and Larkins 1994; Coleman and Larkins 1999), are decreased in *o2*, and the levels of the 22-kDa α-zeins (also called *z1C*) are much lower. The promoter activity of the *z1A* gene has been studied, and the results have suggested that all elements required for its maximum transcription are located in the first 483 bp preceding the start codon (Roussell *et al.* 1988; Thompson *et al.* 1990). However, the promoters of *z1A* and the other two 19-kDa α-zein genes (*z1B* and *z1D*) do not contain a canonical O2 box, and the motifs for O2 recognition in 19-kDa α-zein gene promoters have not been precisely characterized. In addition, *o2* mutants have higher levels of the remaining 19-kDa α-zeins than of the 22-kDa α-zeins, indicating that O2 is not the only bZIP TF that can activate 19-kDa α-zein transcription.

In maize, O2 heterodimerizing protein 1 (OHP1) is a homolog to O2, and the *Ohp1* gene originated from a segmental duplication before the split of maize and rice (Supplemental Material, Figure S1) (Pysh *et al.* 1993; Xu and Messing 2008). OHP1 and OHP2 are two paralogues that resulted from the allotetraploidization of two maize progenitors (Xu and Messing 2008), and their protein sequences are 88% identical (Pysh *et al.* 1993). Most analyzed inbred lines contain only one copy of *Ohp1* and *Ohp2* each, while a few bear duplications at the *Ohp1* locus, resulting in two or three copies of *Ohp1* (Pysh and Schmidt 1996). The sequences of *Ohp1* and the duplicated *Ohp1b* are nearly identical (Pysh and Schmidt 1996). After segmental duplication during the speciation of the grass family, O2 and the OHPs (OHP1 and OHP2) appear to have undergone subfunctionalization. Evidence for this arises from their divergent expression patterns, which indicate that O2 is specifically expressed in endosperm and that OHPs are generally expressed in all examined tissues (Pysh *et al.* 1993; Zhang *et al.* 2015). Although OHPs can form heterodimers with O2 and can recognize the O2 box (Pysh *et al.* 1993), the protein and transcript levels of the 27-kDa γ-zein but not the α-zeins were dramatically reduced in *OhpRNAi* seeds (Zhang *et al.* 2015).

The prolamin-box binding factor (PBF) belongs to the Dof class of plant zinc-finger DNA-binding proteins (Vicente-Carbajosa *et al.* 1997; Wang *et al.* 1998). PBF recognizes the P box, a conserved 7-bp (TGTAAAG) DNA *cis*-element (Vicente-Carbajosa *et al.* 1997). The P box has been found in most grass storage protein gene promoters, including maize zein promoters. *PbfRNAi* resulted in less accumulation of the 27-kDa γ-zeins and 22-kDa α-zeins, confirming a regulatory role for PBF in zein gene expression (Wu and Messing 2012; Zhang *et al.* 2015). PBF can interact with O2, and the combination of *PbfRNAi* and *o2* led to a further reduction of 19- and 22-kD α-zeins compared with those in the single mutants (Wu and Messing 2012; Zhang *et al.* 2015). In *o2* mutants, 22-kDa α-zeins were detectable by SDS-PAGE, but in the double mutant *PbfRNAi;o2*, they could not be detected (Wu and Messing 2012; Zhang *et al.* 2015). In addition, in *PbfRNAi;o2*, the 19-kDa α-zein proteins were present in barely detectable amounts, in contrast to the significant levels of these proteins in *o2* mutants (Zhang *et al.* 2015). However, the molecular basis of this synergism of O2 and PBF in the transcriptional activation of α-zein genes remains unknown. In this study, we performed detailed genetic and biochemical experiments, and found that O2 is the major TF in α-zein gene expression and that OHPs are minor TFs in this process, which is the converse of the relationship of these TFs in 27-kDa γ-zein gene regulation. Using *in vitro* electrophoretic mobility shift assay (EMSA) experiments, we identified the O2- and OHP-binding motifs in the 19-kDa α-zein promoters. The mechanism by which O2 and PBF synergistically activate α-zein gene expression was also studied and is discussed.

Materials and Methods

Plant materials and protein extraction

The maize genetic materials for W64A, W64A*o2*, *PbfRNAi*, *ohpRNAi*, *ohpRNAi*;*o2* and *PbfRNAi;o2* have been described elsewhere (Zhang *et al.* 2015). Immature kernels at different developmental stages were harvested and ground into a fine powder. Next, 100 mg of the flour was mixed and vortexed with 1 ml of zein extraction buffer [70% ethanol, 2% 2-mercaptoethanol (vol/vol), 3.75 mM sodium borate (pH 10), and 0.3% SDS] in a 2-ml tube. The mixture was incubated at room temperature for 2 hr and then centrifuged at 13,000 rpm (Eppendorf) for 15 min. Then, 100 μl of the supernatant was transferred to a new tube with 10 μl of 10% SDS. The mixture was dried using a Concentrator plus (Eppendorf) and then dissolved in 100 μl of distilled water. Two microliters of each sample was separated in a 15% SDS-PAGE gel.

Real-time PCR

Endosperm powder ground in liquid nitrogen was suspended in 300 μl of RNA extraction buffer (50 mM Tris pH 8.0, 150 mM LiCl, 5 mM EDTA pH 8.0, and 1% SDS). After three extractions by 1:1 phenol-chloroform, total RNA was

extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) and purified with the RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA (5 μg) was used for reverse transcription with Superscript III First Strand Kit (Invitrogen). The resulting complementary DNA (cDNA) was diluted to 10 ng/μl and real-time PCR was performed with SYBR Green (TAKARA) on a Bio-Rad CFX-96. The comparative CT method (ΔΔCT method) was employed to quantify gene expression. The maize *Ubiquitin* gene was used as an internal control. All primers are listed in Table S1.

Recombinant protein purification

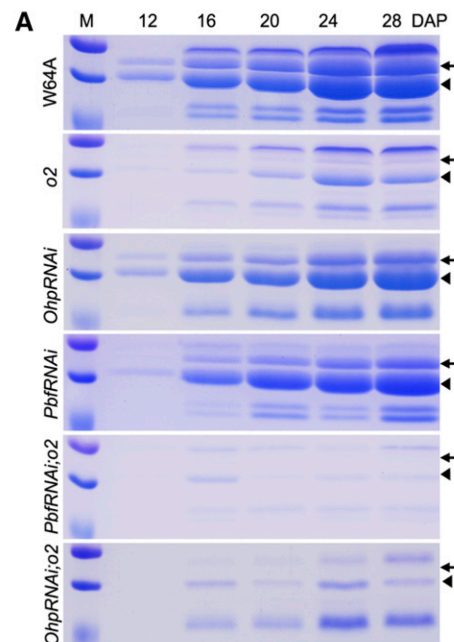
To produce the His-fused proteins, the full-length coding sequences were amplified and inserted into cloning sites in the pCold-TF expression vector (TAKARA) and the proteins were then expressed in *Escherichia coli* DE3 (BL21) cells. The cells were collected and sonicated, and the supernatants containing the corresponding fusion proteins were purified with NI-HA beads (QIAGEN). The eluted proteins were analyzed on a 10% SDS-PAGE gel.

EMSA

The oligonucleotide probes corresponding to the native and mutated sequences were synthesized by Sangon Biotech (Shanghai, China). The labeling process was performed using a 3′ biotin end DNA Labeling kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instruction. Double-strand biotin-labeled or competitive probes were produced by annealing the labeled or unlabeled sequences, respectively. DNA binding reactions were performed in a final concentration of 1× binding buffer, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly 2′-deoxyinosinic-2′-deoxycytidylic acid (dI•dC) sodium salt, and 0.05% NP-40 at room temperature for 30 min. The reactions were resolved on 6% polyacrylamide gels in 0.5× Tris/borate/EDTA (TBE) buffer. Subsequent detection was performed using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific). Luminescence was visualized using a Tanon-5200 Chemiluminescent Imaging system (Tanon Science and Technology, Shanghai, China).

Luciferase assay

The reporter plasmids (pLL00R and pGreen-0800) were generated by inserting the promoters of the 22- and 19-kDa α-zein genes (*z1C*, *z1A*, *z1B*, and *z1D*, each from the first 500 bp upstream of the start codon) into the cloning site upstream of the firefly luciferase (LUC) coding sequence. For the mutation of *z1B* promoter, three ACAT motifs in P2 and P3 probes were mutated to AAGG by using the PCR method and validated by sequencing. Similarly, the ACAT motif in the P1 probe of *z1D* promoter was also mutated to AAGG. The effector plasmid 35S-O2 and the 35S-PBF, 35S-OHP1, and 35S-OHP2 plasmids were prepared by cloning the coding sequences into the pRI101 vector containing the 35S promoter by using enzyme digestion. *Agrobacterium* (strain GV3101) was transformed with the effector and reporter constructs.



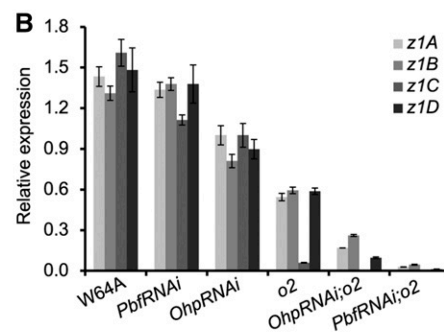


Figure 1 Expression analysis of α-zeins. (A) SDS-PAGE analysis of zein proteins in W64A, *o2*, *OhpRNAi*, *PbfRNAi*, *PbfRNAi;o2*, and *OhpRNAi;o2* endosperm from 12–28 DAP. Arrows and arrowheads indicate 22- and 19-kDa α-zeins, respectively. M, protein markers from top to bottom correspond to 25, 20, and 15 kDa. (B) Quantitative RT-PCR analysis of *z1A*, *z1B*, *z1C*, and *z1D* gene expression in W64A, *PbfRNAi*, *OhpRNAi*, *o2*, *OhpRNAi;o2*, and *PbfRNAi;o2* endosperm at 20 DAP. All expression levels were normalized to *Ubiquitin*. Three replicates for each sample were evaluated and are presented as the means ± SD.

For analyses of pLL00R constructs in *Nicotiana benthamiana*, overnight cultures of *Agrobacterium* were collected by centrifugation and resuspended in double-distilled H_2O (ddH_2O) liquid medium (OD_{600} ≈0.8). The reporter and effector strains were mixed at a 1:2 ratio before leaf infiltration. For the combination of O2 and PBF, a volume ratio of 1:1:1 (reporter:O2:PBF) was used. The leaves of *N. benthamiana* were infiltrated with the mixture, and the assays were performed 48 hr after infiltration. LUC activity was detected as described by Zhang *et al.* (2015). For each reporter, more than five independent experiments were performed.

For quantitative LUC activity analysis, *Arabidopsis* protoplasts were transformed with all indicated combinations of plastids using the PEG/$CaCl_2$ method. Twelve hours after transformation, total protoplast protein extract was analyzed

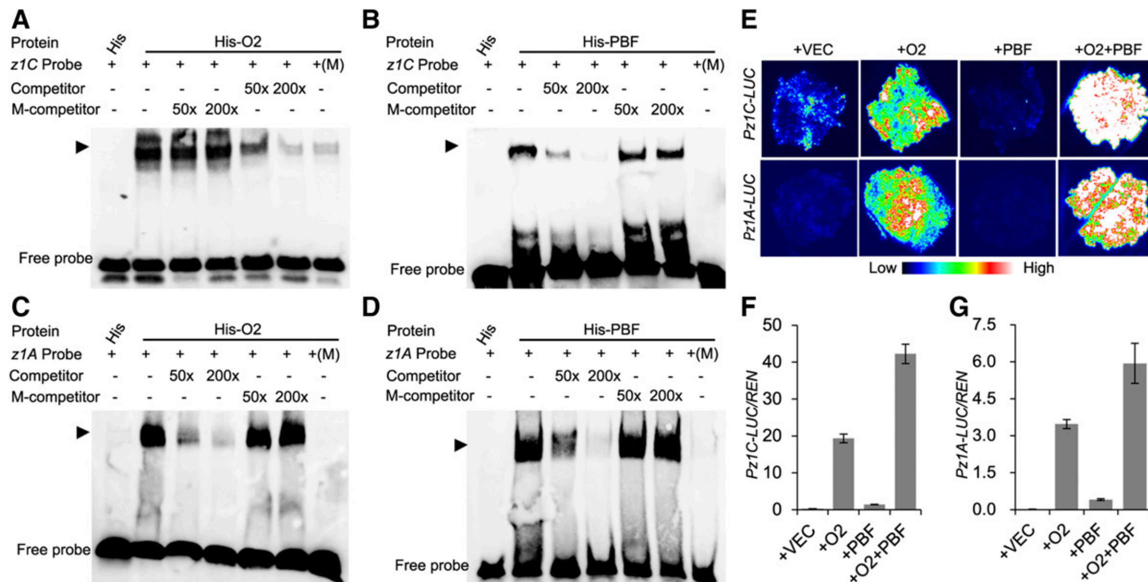


Figure 2 O2 and PBF directly bind to the *z1C* and *z1A* promoters. (A) EMSA showing that the His-O2 protein binds to the O2 box in the 22-kDa (*z1C*) promoter. (B) EMSA showing that the His-PBF protein binds to the P box in the *z1C* promoter. (C) EMSA showing that O2 binds to the O2-like box in the 19-kDa (*z1A*) promoter. (D) EMSA showing that PBF binds to the P box in the *z1A* promoter. Biotin-labeled probes were incubated with the corresponding recombinant proteins and were separated on a native acrylamide gel. Numbers indicate concentration of unlabeled competitor. M, mutated probe, in which the ACGT (O2) or TGTAAAG (PBF) core binding site was replaced. Arrowheads indicate the bound probes. (E) Transient activation of the *z1C* or *z1A* promoter fused LUC reporter gene. *N. benthamiana* leaves were infiltrated with LUC reporter and the indicated effectors. Five independent experiments were performed and a representative leaf is shown. Fluorescence signal intensities represent the levels of transactivation. (F and G) Quantitative transient activation of *Pz1C-LUC* (F) and *Pz1A-LUC* (G) expression. *Arabidopsis* protoplasts were transformed with the *Pz1C-LUC* or *Pz1A-LUC* reporter and indicated effectors. The 35S-REN plastid was used as an internal control. Quantification was obtained for the LUC activity divided by the activity of REN. Error bars represent the SD of three technical replicates. The experiment was repeated three times with similar results.

using a luminometer (Promega 20/20), and a commercial LUC analysis kit according to the manufacturer's instructions (Promega, Madison, WI). At least three biological replicates were measured for each experiment.

Phylogenetic analysis

For the phylogenetic analysis, the protein sequences of O2 and OHP orthologs from common dicot and monocot plants were obtained from National Center for Biotechnology Information (NCBI) BLAST results (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were aligned using MEGA 6.0 software, and a neighbor-joining phylogenetic tree was constructed using MEGA 6.0 software according to the default settings (Tamura *et al.* 2013).

Accession numbers

The maize genome locus identifiers for the genes mentioned in this article are listed as follows: 19-kDa *z1A*, GRMZM2G008913; *z1B*, AF546188.1_FG005; and *z1D*, AF546187.1_FG007; 22-kDa *z1C*, GRMZM2G160739; 27-kDa γ-zein, GRMZM2G138727; *Ohp1*, GRMZM2G016150; *Ohp2*, GRMZM2G007063; *O2*, GRMZM2G015534; and *Pbf*, GRMZM2G146283.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Expression analysis of 19- and 22-kDa α-zein genes in various TF mutants

To compare the expression patterns of 19- and 22-kDa α-zeins in wild type (WT W64A) and various mutants in the W64A background (*o2*, *PbfRNAi*, *PbfRNAi;o2*, *OhpRNAi*, and *OhpRNAi;o2*), we examined protein levels in the developing endosperms from 12 to 28 days after pollination (DAP). Overall, the accumulation of 22- and 19-kDa α-zein proteins, compared with that in the WT, was affected to a greater extent in *o2* than in the other two single mutants (*PbfRNAi* and *OhpRNAi*) (Figure 1A), indicating that O2 is the major TF for α-zein gene expression. We also conducted transcript analysis of α-zein gene expression in these mutants at 20 DAP by using quantitative RT-PCR. In *o2*, the expression levels of both 19- and 22-kDa α-zein genes were markedly lower than in the WT, but the protein and RNA transcript levels of the 19-kDa α-zeins remained significantly higher than those of 22-kDa α-zeins (Figure 1, A and B). In *PbfRNAi*, 22-kDa α-zein gene expression was moderately affected at both the protein and transcript levels (Figure 1, A and B), while for 19-kDa α-zein genes, there was only a mild decrease in transcript levels, but not protein content, for all *z1A*, *z1B*, and *z1D* subfamilies (Figure 1, A and B). In the *OhpRNAi* mutant, the RNA transcript levels of the 22- and 19-kDa α-zein genes were lower than that in the WT, but the

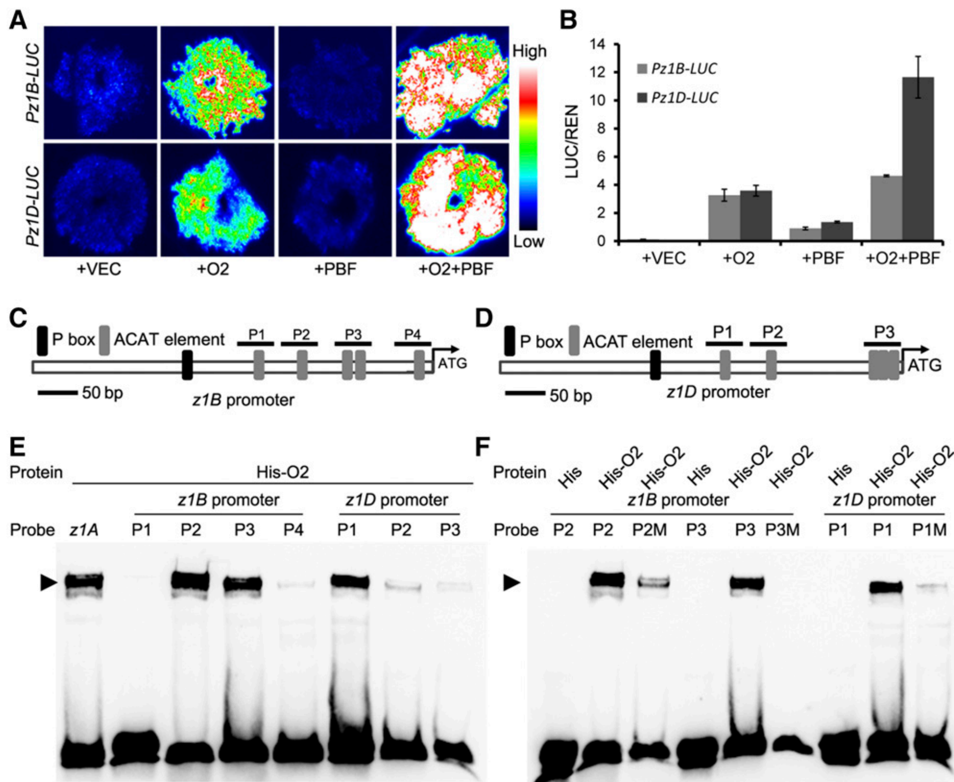


Figure 3 O2 binds to and activates the *z1B* and *z1D* promoters. (A) Transient activation of the *z1B* or *z1D* promoter fused to LUC in *N. benthamiana* leaves. Representative images are shown for five independent experiments. (B) Quantitative transient activation assay in *Arabidopsis* protoplasts. Error bars represent the SD of three technical replicates from one of three biological repeats. (C) Schematic representation of the 500-bp *z1B* promoter. Four probes (P1–P4) containing the five ACAT motifs are indicated. (D) Schematic representation of the 500-bp *z1D* promoter. Three probes (P1–P3) containing the five ACAT motifs are indicated. (E) EMSA showing that the His-O2 protein binds to the P2 and P3 probes from the *z1B* promoter and to the P1 probe from the *z1D* promoter. (F) EMSA showing the binding specificity of His-O2. P2M, P3M, and P1M indicate mutated probes, in which the ACAT motifs were substituted with AAGG.

protein levels did not differ, indicating that this decrease in transcription was insufficient to lead to a reduction in translation (Figure 1, A and B). In the double mutants *PbfRNAi;o2* and *OhpRNAi;o2*, the proteins and transcripts corresponding to both the 19- and 22-kDa α-zein genes were present at barely detectable levels in contrast to the levels in the corresponding single mutants (Figure 1). Taken together, these results indicated that O2 and PBF play a synergistic role in 19- and 22-kDa α-zein gene expression and that O2 and OHPs exhibit partially redundant functions in the regulation of α-zein genes.

O2 and PBF can bind to the z1A, z1B, and z1D promoters

O2 was recently shown to recognize the 19-kDa α-zein gene promoters *in vivo* in chromatin immunoprecipitation PCR (ChIP-PCR) experiments (Li *et al.* 2015), but the regulatory *cis*-motifs of these genes have not yet been identified. A previous study showed that the 483-bp fragment upstream of the start codon in the *z1A* promoter contains all elements required for maximum transcriptional activity (Roussell *et al.* 1988; Thompson *et al.* 1990). We aligned the 500-bp promoters of the 11 intact copies of *z1A*, *z1B*, and *z1D* (Chen *et al.* 2014) in the B73 genome and discovered that they all contain a conserved P box (Figure S2). However, an O2-like box (TTACGT) containing the ACGT core element was discovered only in the *z1A* promoters (Figure S2). To verify that the *z1A* promoters can be recognized by PBF and O2, we first expressed and purified His-O2 and His-PBF fused proteins in *E. coli* (Figure S3) and then tested the quality of the two proteins on the well-defined 22-kDa α-zein (*z1C*) promoters (Schmidt *et al.* 1992; Vicente-Carbajosa *et al.* 1997). EMSA showed that the His-O2 protein, but not the His protein, recognized the O2 box (CCACGT) in the *z1C* promoter, and the signals gradually reduced with the addition of unlabeled competitor probes (Figure 2A). A mutated probe failed to be recognized by O2 and did not compete with the WT probe (Figure 2A), suggesting that O2 specifically binds to the O2 box. A similar EMSA experiment showed that PBF specifically recognized the P box in the 22-kDa α-zein promoter (Figure 2B). Next, we tested the *z1A* promoter and found that O2 specifically bound to the O2-like box and that PBF specifically bound to the P box (Figure 2, C and D).

To test whether the interaction of O2 and PBF can synergistically activate the *z1A* promoter, we performed a transient transcription assay. The *z1C* promoter was used as a positive control. The *z1C* and *z1A* promoters (500 bp in length) were fused to the LUC coding sequence, to generate the reporter vectors, Pz1C-LUC and Pz1A-LUC, respectively (Figure S4A). The effectors were 35S-O2 and 35S-PBF, which are driven by the cauliflower mosaic virus 35S promoter (Figure S4B). Coexpression of Pz1C-LUC or Pz1A-LUC with 35S-O2 in tobacco leave resulted in an apparent enhancement of LUC activity compared with the negative vector control (Figure 2E), while no visible increase in LUC activity was observed when Pz1C-LUC or Pz1A-LUC was co-injected with 35S-PBF. However, the LUC signals were substantially increased when each vector was cotransfected with 35S-PBF plus 35S-O2 (Figure 2E). We also used a dual-LUC quantitative plastid containing the same Pz1C-LUC or Pz1A-LUC cassette and a second cassette carrying the Renilla LUC (REN) reporter gene

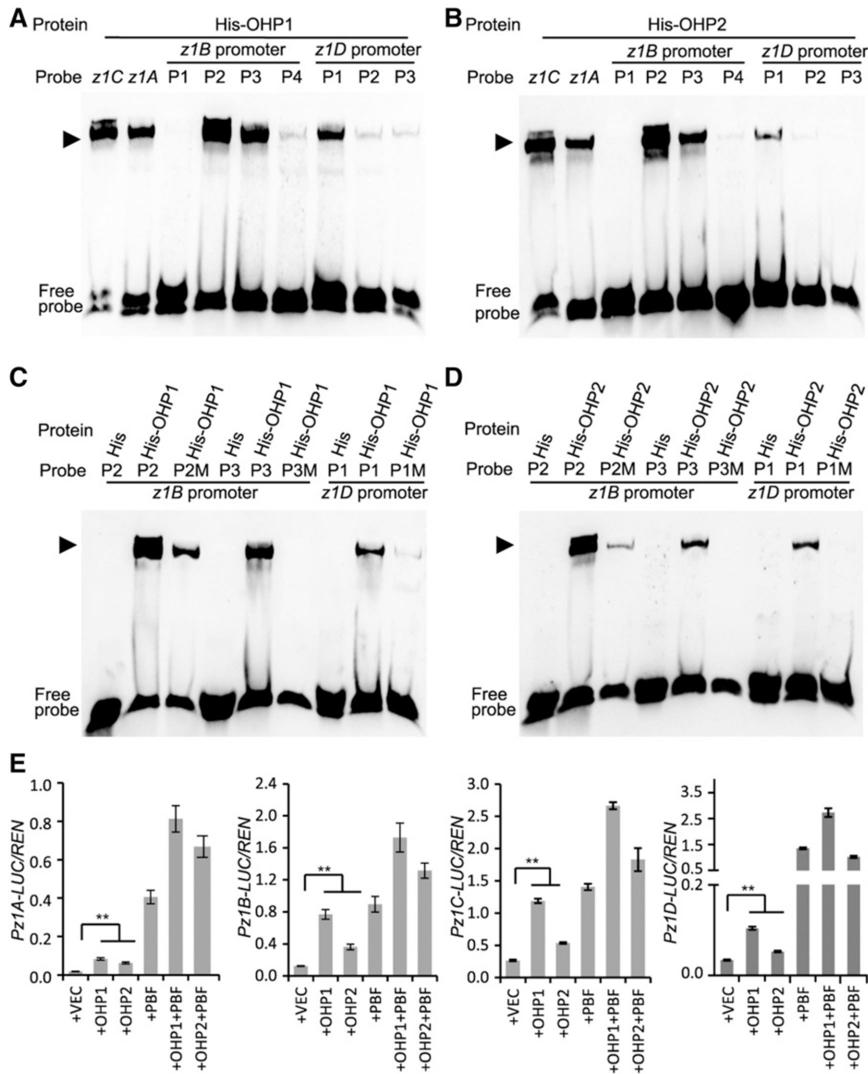


Figure 4 OHP1 and OHP2 bind to the *z1A*, *z1B*, and *z1D* promoters. (A and B) EMSA showing that the His-OHP1 (A) and His-OHP2 (B) proteins bind to the P2 and P3 probes from the *z1B* promoter and to the P1 probe from the *z1D* promoter. (C and D) EMSA showing the binding specificities of His-OHP1 (C) and His-OHP2 (D) for the P2 and P3 probes from the *z1B* promoter and for the P1 probe from the *z1D* promoter. Both the WT and mutated probes are described above. (E) Quantitative transactivation of the *z1A*, *z1B*, *z1C*, and *z1D* promoters fused with LUC by OHPs and PBF. Data represent the means ± SD of three technical replicates. Each experiment was repeated three times with similar results. ** $P < 0.01$, Student's *t*-test.

driven by the 35S promoter (35S-REN) (Figure S4A). *Arabidopsis* leaf protoplasts were transiently transformed and quantitative LUC activities were analyzed. Consistent with the results obtained using tobacco leaves, O2 or the combination of O2 and PBF significantly promoted *LUC* expression (Figure 2, F and G). These results suggest that O2 and PBF can bind to the *z1A* promoter and function synergistically.

Although no canonical ACGT motif was found in the *z1B* and *z1D* promoters, the genetic data encouraged us to test whether O2 and PBF are able to activate the expression of these genes (Figure 1). We fused the first 500 bp of the promoter of *z1B* or *z1D* to the *LUC* gene and performed a transient expression analysis as described above. *LUC* expression was clearly activated by O2 and by the O2/PBF complex (Figure 3, A and B), indicating that O2 can directly recognize a motif in the *z1B* and *z1D* promoters. By sequence alignment, a sequence that differs from the ACGT core element by only one nucleotide substitution, ACAT, was identified in the *z1B* and *z1D* promoters in a similar position to the ACGT core element (Figure S2). Next, we searched the *z1B* and *z1D* promoter sequence and found that both contain five ACAT motifs between the P box and the start codon (Figure 3, C and D). To test whether the ACAT motifs can be recognized by O2, we generated four probes containing the ACAT motif corresponding to different positions in the *z1B* promoter and three such probes for the *z1D* promoter (Figure 3, C and D). The EMSA results revealed that O2 displayed biased binding to two sites in the *z1B* promoter and one site in the *z1D* promoter (Figure 3E). The binding affinity of O2 was markedly lower or completely abolished when the ACAT motif was mutated into AAGG (Figure 3F).

Taken together, these data demonstrated that PBF regulates the expression of all 19-kDa α-zein genes by targeting the conserved P box and that O2 activates the expression of these genes by recognizing the TTACGT motif in the *z1A* promoter and a variant motif, ACAT, in the *z1B* and *z1D* promoters.

OHP1 and OHP2 recognize the z1A, z1B, and z1D promoters

Our genetic data indicated that OHP1 and OHP2 are also involved in transcriptional regulation of the 19-kDa α-zein

genes (Figure 1). To verify the direct interaction between OHPs and the 19-kDa α-zein promoters, His-fused OHP1 and His-fused OHP2 proteins were purified (Figure S3), and EMSAs were again performed. Because O2 and OHPs belong to the same bZIP family, the probes generated for O2 were used here. Both OHP1 and OHP2 also specifically bound to the ACGT element in the *z1C* and *z1A* promoters (the first two lanes in Figure 4, A and B) and to the same regions in the *z1B* and *z1D* promoters as were recognized by O2 (Figure 4, A and B). When the ACAT motif was mutated to AAGG, the binding affinities of OHP1 and OHP2 were markedly lower or completely abolished (Figure 4, C and D).

To compare the capacity of OHPs and O2 to activate the expression of the 22- and 19-kDa α-zein genes, we performed a quantitative transient activation assay with the dual-LUC system. Both OHP1 and OHP2 activated the expression of the reporter gene driven by the *z1C*, *z1A*, *z1B*, or *z1D* promoter. However, the largest increase in OHP1 activation of the *z1B* promoter was ~5.3-fold and the smallest increase was observed for *z1D*, the promoter activated by OHP2, at ~1.5-fold (Table 1). In contrast, O2 activated the transcription of all α-zein gene promoters by 22-fold (*z1B*) to 191-fold (*z1A*) (Table 1), consistent with the genetic observations suggesting that O2 is the major TF and that OHPs are minor TFs for the α-zein gene expression (Figure 1). Interestingly, OHP1 appeared to induce greater activation of each α-zein promoter than did OHP2 (Table 1). Furthermore, coexpression of OHP1 and PBF had a stronger synergistic effect than did OHP2 and PBF on the expression levels of all the reporter genes (Figure 4E). To confirm that the identified ACAT motifs in *z1B* and *z1D* promoters are critical for transactivation by O2 and OHPs, we mutated the ACAT from P2 and P3 probes in *z1B* and the ACAT from P1 probe in *z1D* promoters and then fused these promoters to LUC and performed transient transcription assay. The results showed that the enhancement by O2 and OHPs on *Pz1B-LUC* and *Pz1D-LUC* expression was significantly reduced when the ACAT motifs were mutated (Table 1), suggesting that O2 and OHPs can directly bind to the selected ACAT motifs and exert transactivation. Taken together, these results indicate that OHP proteins also directly recognize the 19-kDa α-zein gene promoters and interact with PBF to regulate the expression of these genes, although this activation is much weaker than that by O2.

Table 1 Fold enhancement in *LUC* gene expression driven by α-zein promoters

Reporters		Effectors		
		+35S-O2	+35S-OHP1	+35S-OHP2
19 kDa	*Pz1A-LUC*	191.1 ± 22.6	4.58 ± 0.12	3.43 ± 0.12
	Pz1B-LUC	22.08 ± 0.69	5.30 ± 0.36	2.51 ± 0.06
	Pz1BM-LUC	9.42 ± 0.85	1.24 ± 0.04	0.61 ± 0.02
	Pz1D-LUC	41.32 ± 0.88	2.60 ± 0.27	1.54 ± 0.11
	Pz1DM-LUC	28.70 ± 0.78	0.97 ± 0.04	0.50 ± 0.06
22 kDa	*Pz1C-LUC*	72.8 ± 3.8	4.47 ± 0.13	2.02 ± 0.04

The fold changes were calculated from the ratio of the effector to the vector control in the dual-LUC experimental system. The SD was calculated from three technical replicates. Each experiment was repeated three times with similar results. *Pz1BM-LUC* and *Pz1DM-LUC* indicated mutated promoters.

PBF and O2 cooperatively promote each other's binding to the recognition sites

To investigate the underlying molecular mechanism of the synergistic action of PBF and O2 on α-zein gene expression, we used the *z1A* and *z1C* promoters. Because the binding affinity of a TF for its target strongly affects the activation of gene expression, we examined whether the protein–protein interaction between O2 and PBF mutually affects their binding to the respective O2 and P boxes by using EMSAs. We had already shown that His-O2 has a strong binding affinity for the O2 box in the *z1A* and *z1C* promoters (Figure 2, A and C), and in the presence of His-PBF protein, O2 had a significantly stronger binding affinity (Figure 5A). In addition to the band representing the O2 box probe bound by His-O2, a higher molecular weight band representing the probe bound by the His-O2 and His-PBF complex was observed. Furthermore, the signal intensity of the triplex complex was proportional to the His-PBF concentration, indicating that the enhancement was specific to the protein–protein interaction (Figure 5A). Unexpectedly, the His-PBF protein also shows weak binding affinity for the O2 box, and the His-O2 had only weak affinity for the mutated O2-like box in *z1A* in the presence of His-PBF (Figure 5A, left). These data suggested that PBF can efficiently facilitate O2's binding to its target motifs, even in some circumstances when a couple of tolerable substitutions occurred in the promoter. Likewise, we examined the effect of His-O2 on His-PBF binding to the P box. Similar experiments were designed, and the binding of His-PBF to the P box in the *z1A* (Figure 5B, left) and *z1C* (Figure 5B, right) promoters was also clearly increased by the addition of His-O2.

Discussion

Maize α-zeins are the most abundant endosperm storage proteins, and this abundance has largely ascribed to the high transcription levels of these genes (Thompson and Larkins 1994; Chen *et al.* 2014). Although transcriptional regulation of the 19-kDa α-zein genes (Langridge and Feix 1983; Maier *et al.* 1988; Roussell *et al.* 1988; Schwall and Feix 1988; Quayle *et al.* 1989; Maier *et al.* 1990; Thompson *et al.* 1990) and the 22-kDa α-zein genes (Schmidt *et al.* 1990, 1992; Vicente-Carbajosa *et al.* 1997; Wu and Messing 2012; Zhang *et al.* 2015) has been studied for decades, many facets of the underlying mechanism remain unclear, especially for the 19-kDa α-zein genes. Here, we precisely defined the regulatory motifs in the promoters of the *z1A*, *z1B*, and *z1D* gene subfamily and demonstrated that OHPs and O2 have partially redundant functions in the regulation of 19- and 22-kDa α-zein genes. The molecular basis of the synergistic effect of O2 and PBF on α-zein transcription was also investigated.

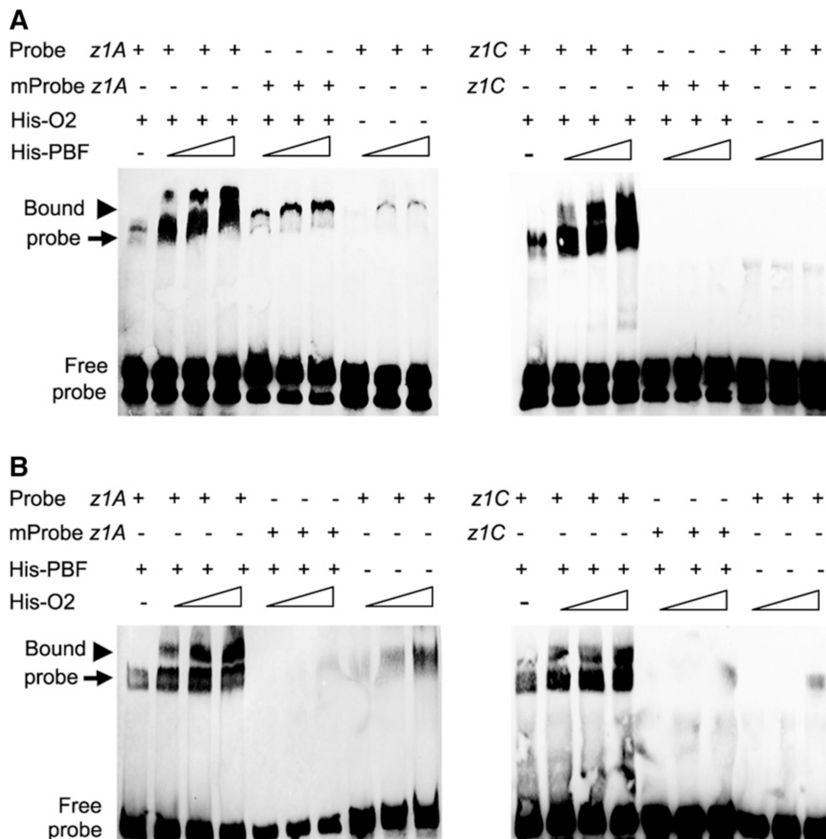


Figure 5 O2 and PBF mutually increase each other's binding affinity. (A) EMSA showing that PBF enhances the binding affinity of O2 for the O2-like box from the 19-kDa *z1A* promoter (left) and for the O2 box from the 22-kDa *z1C* promoter (right). Arrows and arrowheads indicate probes bound by O2 and the O2 and PBF complex, respectively, and the mProbe indicates mutated probe. (B) EMSA showing that O2 enhances the binding affinity of PBF for the P box. (Left) The P box from the 19-kDa *z1A* promoter. (Right) The P box from the 22-kDa *z1C* promoter. The lower bound band in each panel represents binding by PBF and the upper bound band represents binding by the O2 and PBF complex.

Flexible O2 recognition motifs in 19-kDa α-zein gene promoters

Plant TFs usually recognize secondary motifs in DNA, which in some cases are unrelated to the primary binding elements (Shoji *et al.* 2013; Franco-Zorrilla *et al.* 2014). Although the canonical ACGT element, recognized by bZIP TFs (Franco-Zorrilla *et al.* 2014) was not found within the first 500 bp of the *z1B* and *z1D* promoters relative to the start codon, O2 and OHPs activated the reporter constructs driven by the *z1B* and *z1D* promoters. We identified ACAT motifs in the *z1B* and *z1D* promoters as specific binding sites for O2 and OHPs by using EMSA assays. Athough five ACAT motifs are present in the *z1B* and *z1D* promoters, only the P2 and P3 sites in the *z1B* promoter and P1 site in the *z1D* promoter were selectively recognized by O2 and OHPs (Figure 3, E and F and Figure 4, A–D). A possible explanation for this phenomenon is that the flanking sequences of the ACAT core element can also contribute to DNA and protein interactions, potentially by affecting binding stability (Afek *et al.* 2014).

In addition to the O2 box (also called Z3) (TCCACGTAGAT), it has been reported that O2 binds to two other sites, *i.e.*, the Z1 (TCACATGTGT) and Z2 (TCATGCATGT) elements in the 22-kDa α-zein gene promoter. O2 can also recognize the B1–B5 DNA probes corresponding to the *b-32* promoter. The ACAT motifs have previously been found to be present in the Z1, B3, and B4 probe regions (Muth *et al.* 1996; Li *et al.* 2015). However, although neither the ACAT nor the ACGT motif was found in the Z2, B1, and B2 probes, O2 still bound to these sequences *in vitro* (Li *et al.* 2015). Additionally, O2 has been found to bind to the GCN4-like motif in a wheat glutenin promoter and to activate the *LMWGID1* gene in plant and yeast cells (Holdsworth *et al.* 1995), suggesting that O2 can recognize more than one site in different promoters and that these O2 targets are much less conserved than the P box that is recognized by PBF. Taken together, these observations suggest that O2 binding sites are quite flexible, and one could imagine that O2 can directly regulate extensive gene expression not only in zein protein synthesis, but also in other pathways (Hunter *et al.* 2002; Li *et al.* 2015).

Subfunctionalization of O2 and OHPs

The OHPs and O2, both of which encode bZIP family TFs, are expressed in endosperm cells and interact with PBF, although OHPs are also expressed in other tissues (Pysh *et al.* 1993; Zhang *et al.* 2015). Our EMSA experiments showed that both O2 and OHPs bound to all α- zein promoters. Like OHPs, O2 bound to the 27-kDa γ-zein promoter (Li *et al.* 2015). In this study, we observed a functional divergence between O2 and OHPs in the regulation of zein gene expression. A comparison of their abilities to activate the α-zein gene promoters revealed that O2 has a much stronger activation power than OHPs (Table 1). In the presence of O2, the transcript levels of all α-zein genes were affected, but the protein levels showed no obvious decline in the *OhpRNAi* mutant (Figure 1 and Figure 6A). However, in the double mutant of *OhpRNAi*;*o2*,

the transcript and protein levels of α-zeins were markedly lower than those in the *o2* single mutant (Figure 1 and Figure 6A), indicating that in the absence of O2, OHP expression appears to be critical to maintain the residual α-zein accumulation. On the basis of these experiments, we propose a model showing that O2 and OHPs have partial functional redundancy (Figure 6B). In the regulation of α-zein expression, O2 is the major TF and the OHPs are the minor TFs, although both interact with PBF and exert synergistic activation. The converse case is observed for the regulation of 27-kDa γ-zein expression. The expression level of the 27-kDa γ-zein transcript was only mildly decreased in the *o2* mutant, but the protein content had no apparent reduction. In contrast, the transcript and protein levels of the 27-kDa γ-zein were markedly lower in *OhpRNAi* compared with that in WT (Figure 6A) (Zhang *et al.* 2015).These data indicated that O2 is the minor TF and that the OHPs are the major TFs for 27-kDa γ-zein expression (Figure 6B).

Homologous gene duplicates usually produce only one functional protein because one gene copy is silenced (Walsh 1995). However, in some cases, both copies retain their function. For example, the maize *r* and *b* genes, which encode two bHLH TFs, originated from the tetraploidization. R regulates anthocyanin synthesis in the aleurone, anther, and coleoptile, while B controls pigmentation in leaves, sheaths, and tassels (Swigonova *et al.* 2005). The high similarity of the R and B proteins and their functional substitution in seeds indicate that the different expression patterns of the two genes determine the subfunctionalization (Swigonova *et al.* 2005). The *O2* and *Ohp* genes were duplicated ~56 MYA, before the split of rice and maize 50 MYA and *Ohp1* and *Ohp2* were from the tetraploidization of maize similarly to the *r* and *b* genes (Xu and Messing 2008). After the ancient gene duplication, the *O2* and *Ohp* genes diverged not only in sequence but also in expression pattern. The protein sequence identity between O2 and OHP1 and between O2 and OHP2 is only 38%, in contrast to the 88% identity between OHP1 and OHP2. O2 is an endosperm-specific TF, while OHPs are expressed in all tissues that have been examined (Zhang *et al.* 2015). Here, we showed that O2 and OHPs display subfunctionalization in the regulation of zein gene expression, likely caused by the divergent protein sequences rather than their expression patterns. Interestingly, OHP1 appeared to more strongly activate α-zein promoters than did OHP2 (Table 1), suggesting that the two homologous duplicates have started to undergo further functional divergence.

Mechanism of the synergistic action of O2 and PBF on α-zein gene expression

O2 and PBF are the two major TFs involved in α-zein gene expression. In the double mutant *PbfRNAi;o2*, both the transcript and protein levels of the 22- and 19-kDa α-zein genes were reduced to barely detectable levels. The zein content of the double mutant was 89% lower than in the WT (Zhang *et al.* 2015). Transient activation experiments showed that

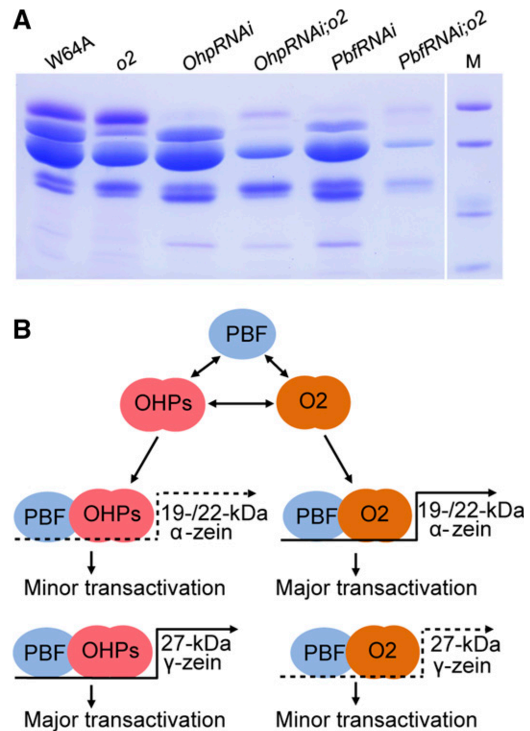


Figure 6 The proposed model for the transcriptional regulation of the 19- and 22-kDa α-zein genes and the 27-kDa γ-zein gene. (A) SDS-PAGE analysis of zein proteins from the mature endosperm of W64A, o2, OhpRNAi, OhpRNAi;o2, PbfRNAi, and PbfRNAi;o2. M, protein markers from top to bottom correspond to 25, 20, and 15 kDa. (B) Model for 19- and 22-kDa α-zein gene and 27-kDa γ-zein gene regulation. PBF, OHPs (OHP1 and OHP2), and O2 interact with each other. On the basis of our genetic and molecular analyses, OHPs interacting with PBF function as the major TFs for 27-kDa γ-zein gene expression and as the minor TFs for α-zein gene expression. Conversely, O2 interacting with PBF plays a major role in α-zein gene expression and a minor role in 27-kDa γ-zein gene expression.

the reporter gene driven by the *z1A*, *z1B*, *z1D*, or *z1C* promoter was strongly activated when 35S-O2 was co-injected (Figure 2, Figure 3, and Figure 4). Although PBF alone did not have an overt effect on reporter expression, the maximum activation mediated by O2 depended on its protein–protein interaction with PBF, which synergistically amplified transcription (Figure 2, E–H and Figure 3, A and B). The mechanism underlying this synergistic activation has not been understood. Our EMSA assay showed that PBF promoted O2 binding to the ACGT motif in the *z1A* and *z1C* promoters, and that O2, in turn, facilitated PBF binding to the P box. These data indicate that O2 and PBF can mutually promote one another's binding to their corresponding DNA elements. An interaction between two proteins that promotes the binding of one protein to its target DNA element has also been observed for rice ERF3, which interacts with WOX11 and promotes WOX11 binding to the RR2 promoter (Zhao *et al.* 2015). Here, we propose that the physical interaction between O2 and PBF and their mutual recruitment may generate an amplification cascade reaction, like a rolling snowball effect, by which more O2 or PBF could be recruited to the

promoters for binding than when each TF is present alone, leading to an exponential increase in transcription strength. This effect may contribute to the dominance of the 19- and 22-kDa α-zein transcripts (Chen *et al.* 2014) and proteins in maize endosperm (Thompson and Larkins 1994; Hunter *et al.* 2002), in addition to the gene copy numbers (Miclaus *et al.* 2011).

Acknowledgments

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Literature Cited

Afek, A., J. L. Schipper, J. Horton, R. Gordan, and D. B. Lukatsky, 2014 Protein-DNA binding in the absence of specific base-pair recognition. Proc. Natl. Acad. Sci. USA 111: 17140–17145.

Albani, D., M. C. Hammond-Kosack, C. Smith, S. Conlan, V. Colot *et al.*, 1997 The wheat transcriptional activator SPA: a seed-specific bZIP protein that recognizes the GCN4-like motif in the bifactorial endosperm box of prolamin genes. Plant Cell 9: 171–184.

Bäumlein, H., S. Miséra, H. Luerßen, K. Kölle, C. Horstmann *et al.*, 1994 The FUS3 gene of Arabidopsis thaliana is a regulator of gene expression during late embryogenesis. Plant J. 6: 379–387.

Chen, J., B. Zeng, M. Zhang, S. Xie, G. Wang *et al.*, 2014 Dynamic transcriptome landscape of maize embryo and endosperm development. Plant Physiol. 166: 252–264.

Coleman, C., and B. Larkins, 1999 The prolamins of maize, pp. 109–139 in *Seed Proteins*, edited by P. Shewry, and R. Casey. Springer, Dordrecht, The Netherlands.

Conlan, R. S., M. Hammond-Kosack, and M. Bevan, 1999 Transcription activation mediated by the bZIP factor SPA on the endosperm box is modulated by ESBF-1 in vitro. Plant J. 19: 173–181.

Esen, A., 1987 A proposed nomenclature for the alcohol-soluble proteins (zeins) of maize (Zea mays L.). J. Cereal Sci. 5: 117–128.

Franco-Zorrilla, J. M., I. Lopez-Vidriero, J. L. Carrasco, M. Godoy, P. Vera *et al.*, 2014 DNA-binding specificities of plant transcription factors and their potential to define target genes. Proc. Natl. Acad. Sci. USA 111: 2367–2372.

Giraudat, J., B. M. Hauge, C. Valon, J. Smalle, F. Parcy *et al.*, 1992 Isolation of the Arabidopsis ABI3 gene by positional cloning. Plant Cell 4: 1251–1261.

Holdsworth, M. J., J. Munoz-Blanco, M. Hammond-Kosack, V. Colot, W. Schuch *et al.*, 1995 The maize transcription factor Opaque-2 activates a wheat glutenin promoter in plant and yeast cells. Plant Mol. Biol. 29: 711–720.

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.

Kawakatsu, T., M. P. Yamamoto, S. M. Touno, H. Yasuda, and F. Takaiwa, 2009 Compensation and interaction between RISBZ1 and RPBF during grain filling in rice. Plant J. 59: 908–920.

Langridge, P., and G. Feix, 1983 A zein gene of maize is transcribed from two widely separated promoter regions. Cell 34: 1015–1022.

Lara, P., L. Onate-Sanchez, Z. Abraham, C. Ferrandiz, I. Diaz *et al.*, 2003 Synergistic activation of seed storage protein gene expression in Arabidopsis by ABI3 and two bZIPs related to OPAQUE2. J. Biol. Chem. 278: 21003–21011.

Li, C., Z. Qiao, W. Qi, Q. Wang, Y. Yuan *et al.*, 2015 Genome-wide characterization of cis-acting DNA targets reveals the transcriptional regulatory framework of opaque2 in maize. Plant Cell 27: 532–545.

Maier, U.-G., J. W. S. Brown, L. M. Schmitz, M. Schwall, G. Dietrich *et al.*, 1988 Mapping of tissue-dependent and independent protein binding sites to the 5′ upstream region of a zein gene. Molecular and General Genetics 212: 241–245.

Maier, U. G., K. D. Grasser, M. M. Haass, and G. Feix, 1990 Multiple proteins bind to the P2 promoter region of the zein gene pMS1 of maize. Mol. Gen. Genet. 221: 164–170.

Mena, M., F. J. Cejudo, I. Isabel-Lamoneda, and P. Carbonero, 2002 A role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in barley aleurone. Plant Physiol. 130: 111–119.

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.

Muth, J. R., M. Muller, S. Lohmer, F. Salamini, and R. D. Thompson, 1996 The role of multiple binding sites in the activation of zein gene expression by Opaque-2. Mol. Gen. Genet. 252: 723–732.

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

Onate, L., J. Vicente-Carbajosa, P. Lara, I. Diaz, and P. Carbonero, 1999 Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in vivo and activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. J. Biol. Chem. 274: 9175–9182.

Onodera, Y., A. Suzuki, C. Y. Wu, H. Washida, and F. Takaiwa, 2001 A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. J. Biol. Chem. 276: 14139–14152.

Parcy, F., C. Valon, M. Raynal, P. Gaubier-Comella, M. Delseny *et al.*, 1994 Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. Plant Cell 6: 1567–1582.

Pysh, L. D., M. J. Aukerman, and R. J. Schmidt, 1993 OHP1: a maize basic domain/leucine zipper protein that interacts with opaque2. Plant Cell 5: 227–236.

Pysh, L. D., and R. J. Schmidt, 1996 Characterization of the maize OHP1 gene: evidence of gene copy variability among inbreds. Gene 177: 203–208.

Quayle, T. J., J. W. Brown, and G. Feix, 1989 Analysis of distal flanking regions of maize 19-kDa zein genes. Gene 80: 249–258.

Roussell, D. L., R. S. Boston, P. B. Goldsbrough, and B. A. Larkins, 1988 Deletion of DNA sequences flanking an Mr 19,000 zein gene reduces its transcriptional activity in heterologous plant tissues. Mol. Gen. Genet. 211: 202–209.

Schmidt, R. J., F. A. Burr, and B. Burr, 1987 Transposon tagging and molecular analysis of the maize regulatory locus opaque-2. Science 238: 960–963.

Schmidt, R. J., F. A. Burr, M. J. Aukerman, and B. Burr, 1990 Maize regulatory gene opaque-2 encodes a protein with a "leucine-zipper" motif that binds to zein DNA. Proc. Natl. Acad. Sci. USA 87: 46–50.

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.

Schwall, M., and G. Feix, 1988 Zein promoter activity in transiently transformed protoplasts from maize. Plant Sci. 56: 161–166.

Shoji, T., M. Mishima, and T. Hashimoto, 2013 Divergent DNA-binding specificities of a group of ETHYLENE RESPONSE FACTOR transcription factors involved in plant defense. Plant Physiol. 162: 977–990.

Stone, S. L., L. W. Kwong, K. M. Yee, J. Pelletier, L. Lepiniec *et al.*, 2001 LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. USA 98: 11806–11811.

Swigonova, Z., J. L. Bennetzen, and J. Messing, 2005 Structure and evolution of the r/b chromosomal regions in rice, maize and sorghum. Genetics 169: 891–906.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar, 2013 MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Thompson, G., and B. Larkins, 1994 Characterization of zein genes and their regulation in maize endosperm, pp. 639–647 in *The Maize Handbook*, edited by M. Freeling, and V. Walbot. Springer-Verlag, New York.

Thompson, G. A., R. S. Boston, L. A. Lyznik, T. K. Hodges, and B. A. Larkins, 1990 Analysis of promoter activity from an alpha-zein gene 5′ flanking sequence in transient expression assays. Plant Mol. Biol. 15: 755–764.

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.

Walsh, J. B., 1995 How often do duplicated genes evolve new functions? Genetics 139: 421–428.

Wang, Z., T. Ueda, and J. Messing, 1998 Characterization of the maize prolamin box-binding factor-1 (PBF-1) and its role in the developmental regulation of the zein multigene family. Gene 223: 321–332.

Wu, Y., and J. Messing, 2012 Rapid divergence of prolamin gene promoters of maize after gene amplification and dispersal. Genetics 192: 507–519.

Xu, J. H., and J. Messing, 2008 Diverged copies of the seed regulatory Opaque-2 gene by a segmental duplication in the progenitor genome of rice, sorghum, and maize. Mol. Plant 1: 760–769.

Yamamoto, M. P., Y. Onodera, S. M. Touno, and F. Takaiwa, 2006 Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. Plant Physiol. 141: 1694–1707.

Zhang, Z., J. Yang, and Y. Wu, 2015 Transcriptional regulation of zein gene expression in maize through the additive and synergistic action of opaque2, prolamine-box binding factor, and O2 heterodimerizing proteins. Plant Cell 27: 1162–1172.

Zhao, Y., S. F. Cheng, Y. L. Song, Y. L. Huang, S. L. Zhou *et al.*, 2015 The interaction between rice ERF3 and WOX11 promotes crown root development by regulating gene expression involved in cytokinin signaling. Plant Cell 27: 2469–2483.

Communicating editor: J. A. Birchler

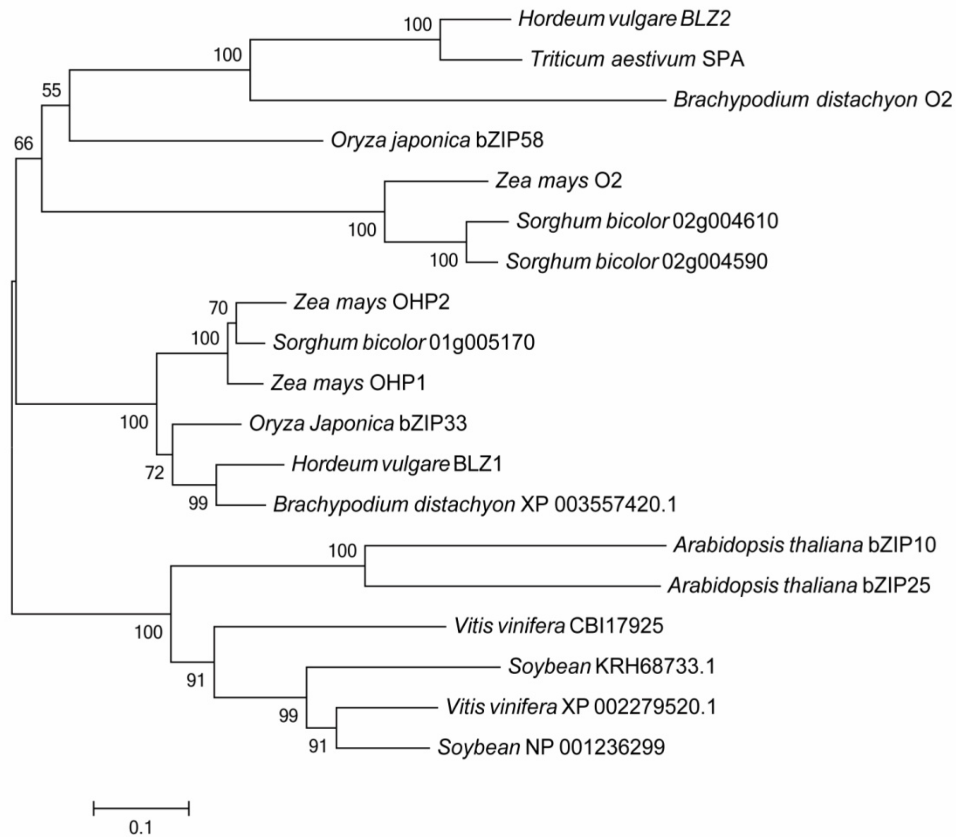

Figure S1. *Phylogenetic analysis of O2 and OHPs*. Neighbor-joining phylogenetic tree summarizing the evolutionary relationships of O2 and OHP-related proteins in common monocots and dicots. The proteins are named according to their gene names, NCBI accession numbers or annotated names. The numbers under the branches refer to the bootstrap values of the neighbor-joining phylogenetic tree. The length of the branches is proportional to the amino acid variation rates.

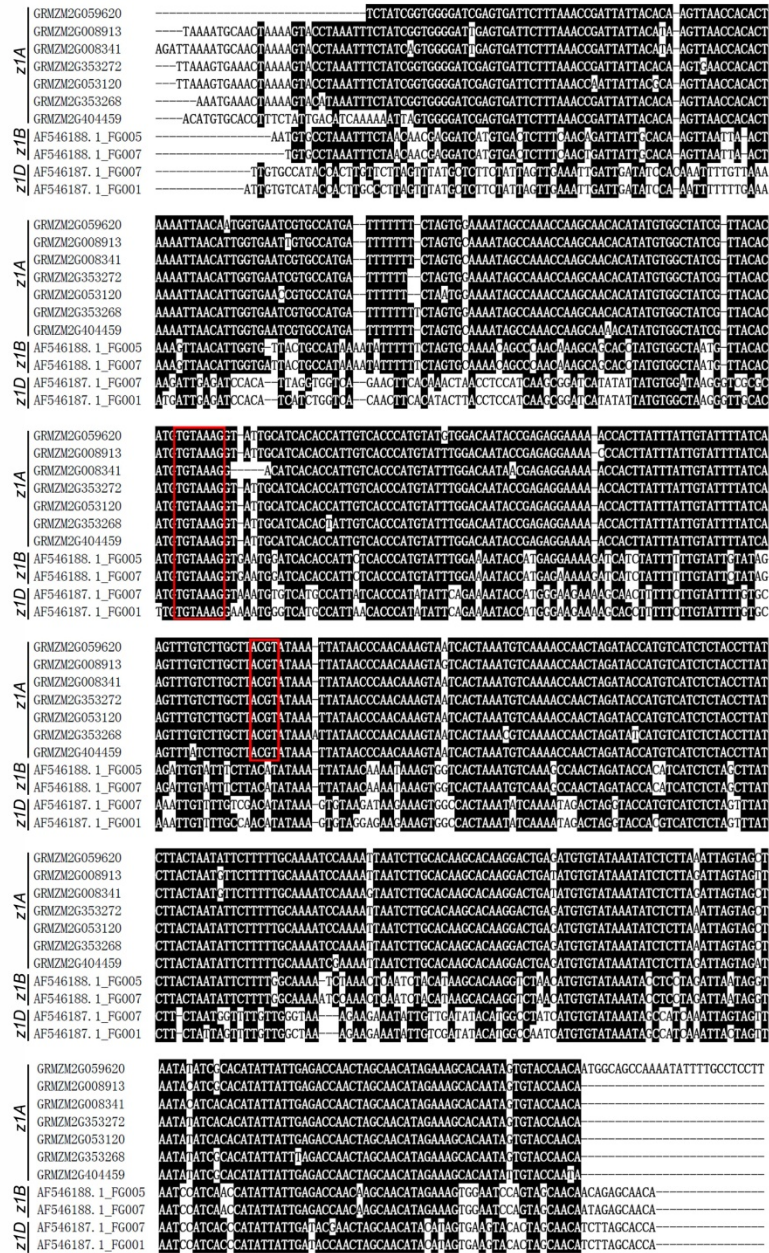

Figure S2. *Alignment of the intact 19-kD-zein gene promoters*. The sequences 500-bp upstream of the start codon was used for alignment. The P (TGTAAAG) and O2-like (TTACGT) boxes are indicated.

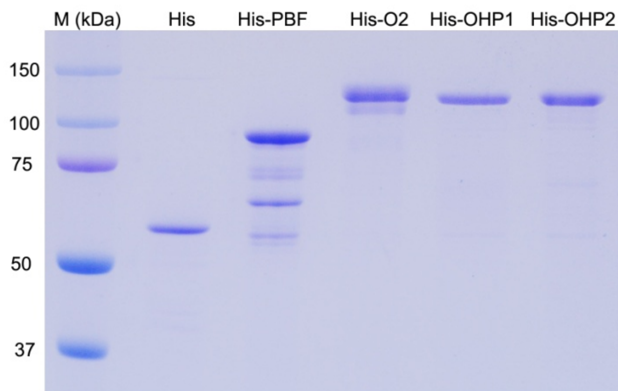

Figure S3. *SDS-PAGE analysis of the purified proteins*. The pCold-TF vector product (His) and pCold TF fusion protein (His-PBF/O2/OHP1/OHP2) were expressed in E. coli BL21 cells and purified. The purified proteins were analyzed via 10% SDS-PAGE and stained by Coomassie blue. M, protein marker.

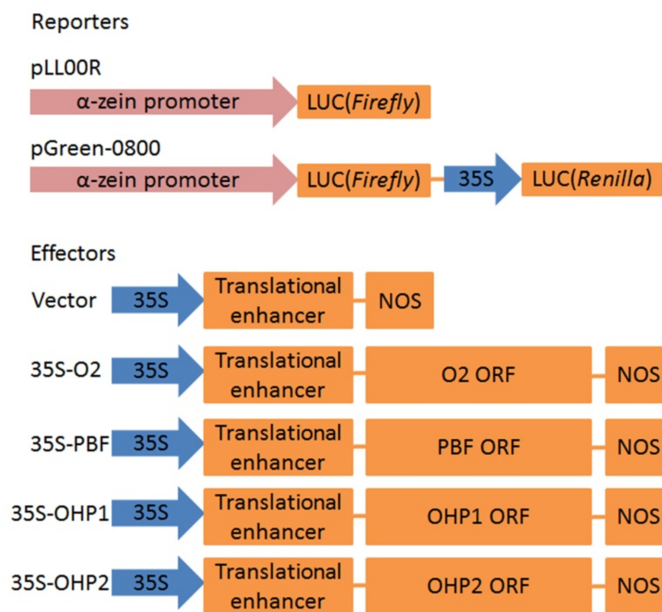

Figure S4. *Diagrams of reporter and effector constructs for transactivation experiments*. (A) Reporter constructs. (B) Effector constructs. 35S represents the CaMV 35S promoter. The translational enhancer is the 58-bp 5'- untranslated region (5'-UTR) of AtADH gene.

Table S1. Primers used in this study.

Name	Sequence (from 5'to 3')	Useage
z1AqF	GCTCCTTGGTCTTTCTGCAA	Quantitative RT-PCR
z1AqR	GGTAACTGCTGTAATAGGGCTGATG	
z1BqF	CCAGCCCTATCTTTGGTGCA	
z1BqR	TCAGTGCGGCCAATTGGTTA	
z1DqF	GCACAACAACTACAACAACA	
z1DqR	AATGGTAGTAGCTGTTGTGC	
z1CqF	TTCCACAATGCTCACTTGCT	
z1CqR	GTTGTTGTAAGACGCTCGCC	
UbiquitinqF	CTGGTGCCCTCTCCATATGG	
UbiquitinQr	CAACACTGACACGACTCATGACA	
35SO2oeF	cccgggATGGAGCACGTCATCTCAATG	Effector construction
35S O2oeR	ggtaccCTAATACATGTCCATGTGTATGGCC	
35SOHP1F	tcatATGGAGCGCGTCTTCTCCG	
35SOHP1R	ggatccCTAGGAGGTCGACCCCGATGA	
35SOHP2F	tcatATGGAGCGCGTCTTCTCCA	
35SOHP2R	ggatccCTAGGAGGTCGAGCCGGATGAA	
35S PBFoeF	gtcgacATGGACATGATCTCCGGCAG	
35S PBFoeR	ggatccTTATTGTCCCTTGTTGTTGTTGTTG	
O2CDsF	catatgCCGCCGACGACCCATCAT	Protein expression
O2CDsR	tctagaATACATGTCCATGTGTATGGCCCCA	
35SOHP1F	tcatATGGAGCGCGTCTTCTCCG	
35SOHP1R	ggatccCTAGGAGGTCGACCCCGATGA	
35SOHP2F	tcatATGGAGCGCGTCTTCTCCA	
35SOHP2R	ggatccCTAGGAGGTCGAGCCGGATGAA	
PBFCDsF	ggatccATGGACATGATCTCCGGCAG	
PBFCDsR	gtcgacTTATTGTCCCTTGTTGTTGTTGTTG	
pz1AF	aagcttTAAAATGCAACTAAAAGTACCTAA	Reporter construction
pz1AR	ggatccTGTTGGTACACTATTGTGCTTTCTA	
pz1BF	aagcttAATGTGCCTAAATTTCTAACAACG	
pz1BR	ggatccTGTTGCTCTGTTGTTGCTACTGG	
pz1DF	aagcttTTGTGCCATACCACTTGTTCTTAG	
pz1DR	ggatccTGGTGCTAAGATGTTGCTAGTG	
pz1CF	aagcttGGATTTCAGTTAGTCTCAGTCT	
pz1CR	ggatccGGTTGTTTGGTCGTTGCTAGTG	
z1AO2-likeF	ATCAAGTTTGTCTTGCTT**ACGT**ATAAATTATAACCCAACA	EMSA assay
z1AO2-likeFM	ATCAAGTTTGTCTTGCTT**AAGG**ATAAATTATAACCCAACA	
z1A P boxF	GGCTATCGTTACACATG**TGTAAAG**GTATTGCATCACACCATT	
z1A P boxFM	GGCTATCGTTACACATG**AAATTTG**GTATTGCATCACACCATT	
z1B-P1F	TATAGAGATTGTATTTCTT**ACAT**ATAAATTATAACAAAAT	

z1B-P2F	CAAAGCCAACTAGATACC**ACAT**CATCTCTAGCTTATCTTA	EMSA assay
z1B-P2FM	CAAAGCCAACTAGATACC**AAGG**CATCTCTAGCTTATCTTA	
z1B-P3F	AACTCAATCT**ACAT**AAGCACAAGGTCTA**ACAT**GTGTATAA	
z1B-P3FM	AACTCAATCT**AAGG**AAGCACAAGGTCTA**AAGG**GTGTATAA	
z1B-P4F	TATTGAGACCAACAAGCA**ACAT**AGAAAGTGGAATCCAGTA	
z1D-P1	TTTTGTGCAAATTGTTTTGTCG**ACAT**ATAAAGTGTAAGATAAGAAA	
z1D-P1M	TTTTGTGCAAATTGTTTTGTCG**AAGG**ATAAAGTGTAAGATAAGAAA	
z1D-P2	TGTTGGGTAAAGAAGAAATATTGTTGATAT**ACAT**GGCCTATCAT	
z1D-P3	ACTAGCA**ACATACAT**AGTGAAGTACACTAGCA**ACAT**CTTAGCAC	
z1C O2-boxF	GAGATCTTGCATGTCATTCC**ACGT**AGATAAAAAGAATGCATATATA	
z1C O2-boxFM	GAGATCTTGCATGTCATTCC**AAGG**AGATAAAAAGAATGCATATATA	
z1C P-boxF	TCATGTTAATGTTGTCACATG**TGTAAAG**GTGAAGAGATCTTGCATGT	
z1C P-boxFM	TCATGTTAATGTTGTCACATG**AAATTTG**GTGAAGAGATCTTGCATGT	
Z1B-P2MF	AATGTCAAAGCCAACTAGATACCaaggCATCTC	Promoter mutation
Z1B-P2MR	ATTAGTAAGATAAGCTAGAGATGccttGGTAT	
Z1B-P3MF	TGTCTAAACTCAATCTaaggAAGCACAAGGTCTAaaggGTGT	
Z1B-P3MR	AGGAGGTATTTATACACccttTAGACCTTGTGCTTccttAGAT	
Z1D-P1MF	TTTTGTGCAAATTGTTTTGTCGAAGGATAAAG	
Z1D-P1MR	CTTTCTTATCTTACACTTTATccttCGACAA	