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 cruci-ferins 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
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 expressed in 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 (TCCAGT) 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 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 paralogs 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 (TGTAAG) 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-kDa 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, W64Aa2, 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 RNasy 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 MgCl₂, 50 ng/µl poly 2’-deoxyinosinic-2’-deoxycytidylic acid (dT·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 3SS-O2 and the 3SS-PBF, 3SS-OHP1, and 3SS-OHP2 plasmids were prepared by cloning the coding sequences into the pRI101 vector containing the 3SS promoter by using enzyme digestion. *Agrobacterium* (strain GV3101) was transformed with the effector and reporter constructs.

For analyses of pLL00R constructs in *Nicotiana benthamiana*, overnight cultures of *Agrobacterium* were collected by centrifugation and resuspended in double-distilled H₂O (ddH₂O) liquid medium (OD₆₀₀ ≈ 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/CaCl₂ method. Twelve hours after transformation, total protoplast protein extract was analyzed
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.

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.

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-zeins, 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-zeins 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-zeins, 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
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 PbP RNAi; 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 fusion 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 leaf 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...
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 can bind to the z1A promoter and function synergistically. 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. 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. Our genetic data indicated that OHP1 and OHP2 are also involved in transcriptional regulation of the 19-kDa α-zein

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.
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 ACAT 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.

**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 triple 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.

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

| Reporters | +35S-O2 | +35S-OHP1 | +35S-OHP2 |
|-----------|--------|----------|---------|
| 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 |
| 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.
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 O2 and OHP complex, respectively, and the mProbe indicates mutated probe. We identified ACAT motifs in the z1B and z1D promoters as specific binding sites for O2 and OHPs by using EMSA assays. Although 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 OHPI and between O2 and OHPI2 is only 38%, in contrast to the 88% identity between OHPI and OHPI2. 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 OHPI2 (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 PbfrRNAi; 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 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 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 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, PbfrRNAi, and PbfrRNAi; 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 (OHPI and OHPI2), 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.
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 (Miclau et al. 2011).

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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 (TGTAAG) 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.
| Name          | Sequence (from 5' to 3')                                      | Usage       |
|---------------|---------------------------------------------------------------|-------------|
| z1AqF         | GCTCCTTGCTTTCTCTGCAA                                         | Quantitative RT-PCR |
| z1AqR         | GGTAACGTGCTGTAATAGGGCTGATG                                    |             |
| z1BqF         | CCCGCCCTATCTTTGTGCA                                          |             |
| z1BqR         | TCAGTGCCGCAATTGGTTA                                          |             |
| z1DqF         | GCACAACAACTAAACAAACA                                         |             |
| z1DqR         | AATGTTAGTGTTGTGTTG                                          |             |
| z1CqF         | TCCACATGGCTCACTTGT                                           |             |
| z1CqR         | GTTGTGTAAGACGCTGCCCCCA                                       |             |
| UbiquitiqF    | CTGGTGCCTCTCCCATATG                                          |             |
| UbiquitinR    | CAACACTGACACGACTCATGACA                                       |             |
| 35SO2oeF      | cccgggATGGAGCACGCTACATCTCAATG                                 | Effect construction |
| 35SO2oeR      | ggtaccCTAATACATGTCATGTGATATGGCC                              |             |
| 35S SOHP1F    | lcatATGGAGGCGGCTTCTCTCG                                       |             |
| 35S SOHP1R    | ggtaccCTAGGAGGTGACCCGATG                                      |             |
| 35S SOHP2F    | lcatATGGAGGCGGCTTCTCTCCA                                      |             |
| 35S SOHP2R    | ggtaccCTAGGAGGTGACCCGATG                                      |             |
| 35S PBFoeF    | ggtaccTTATTTGCTCCTGTTGTTGTGTTGTTG                            |             |
| 35S PBFoeR    | ggtaccTTATTTGCTCCTGTTGTTGTGTTGTTG                            |             |
| O2CDsF        | catatgCGCCGACGACCCATCAT                                       |             |
| O2CDsR        | tctagaATACATGTCATGTGATATGGCC                                  |             |
| 35SOHP1F      | lcatATGGAGGCGGCTTCTCTCG                                       |             |
| 35SOHP1R      | ggtaccCTAGGAGGTGACCCGATG                                      |             |
| 35SOHP2F      | lcatATGGAGGCGGCTTCTCTCCA                                      |             |
| 35SOHP2R      | ggtaccCTAGGAGGTGACCCGATG                                      |             |
| PBFCDsF       | ggtaccCTAGGAGGTGACCCGATG                                      |             |
| PBFCDsR       | ggtaccCTAGGAGGTGACCCGATG                                      |             |
| pz1AF         | aagcttTAAAATGCAACTAAAAAGTACCTAA                               | Reporter construction |
| pz1AR         | ggtaccTGTGGTACACTATGTGTTGTGTTCTA                              |             |
| pz1BF         | aagcttAATGTCCTAAATTTCTAACAACG                                 |             |
| pz1BR         | ggtaccTGTGGTACACTATGTGTTGTGTTACTG                             |             |
| pz1DF         | aagcttTGTGGTACACTATGTGTTGTGTTACTG                             |             |
| pz1DR         | ggtaccTGTGGTACACTATGTGTTGTGTTACTG                             |             |
| pz1CF         | aagcttGGATTTCAGTTAGCTCAGTTAGCTCAGTTAGCTCAG                   |             |
| pz1CR         | ggtaccGGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG  |             |
| z1AO2-likeF   | ATCAAGTTTGCTCTTTGTTACGTATGAAAAATCTATATAACTATACCCAAACG         | EMSA assay  |
| z1AO2-likeFM  | ATCAAGTTTGCTCTTTGTTACGTATGAAAAATCTATATAACTATACCCAAACG         |             |
| z1A P boxF    | GGCTACGTGTTAGTGTTAGTGATATGGGTATGGTACATCGACCCAT                |             |
| z1A P boxFM   | GGCTACGTGTTAGTGTTAGTGATATGGGTATGGTACATCGACCCAT                |             |
| z1B-P1F       | TATAGAGATTGTATTTCTACATATAATATAACTAACAAT                      |             |
| z1B-P2F | CAAAGCCAACCTAGATACCACATCCTTAGCTTATCTTA |
| z1B-P2FM | CAAAGCCAACCTAGATACCACCGCATCTCTAGGTTATCTTA |
| z1B-P3F | ACTCAATCTCACATCAAGCAAGGTCTAACATAGTGATAAA |
| z1B-P3FM | ACTCAATCTAAAGGACACAGGTCTAACAGGTGTATAA |
| z1B-P4F | TATTGAGACCAAAGCAACATAGAAGTGAATCCAGTA |
| z1D-P1 | TTTTGTCGAAATTTTTTCGACATATAAGGTAAGGATAAGAAA |
| z1D-P1M | TTTTGTCGAATTTGTTTTGTCGACAGGTAAGGTAAGGATAAGAAA |
| z1D-P2 | TGGTTGGTAAAGGAGAATTGTGTATACATAGCGCTATCAT |
| z1D-P3 | ACTAGCAACATACACAGTGAAGTACACTACATAGCTTGACAC |
| z1C O2-boxF | GAGATCTTTGCTGCTACTTACAGCTAGATAAAAAAGAATGCTATATA |
| z1C O2-boxFM | GAGATCTTTGCTGCTACTTACAGCTAGATAAAAAAGAATGCTATATA |
| z1C P-boxF | TCATGTTATATTTGGTCAGATACATGAAATGGTGAAGGAGACTTTGCGATGT |
| z1C P-boxFM | TCATGTTATATTTGGTCACATGAAATTTGGTGAAGGAGACTTTGCGATGT |
| Z1B-P2MF | ATGTCAGCCAGCAACTAGATACCaaggtCATCCT |
| Z1B-P2MR | ATTAGTAAGATAAGCTAGAGTCggTGGGT |
| Z1B-P3MF | TGCTAAACTCAATCTaagggACGCAAGGCTACAGGTTGT |
| Z1B-P3MR | AGGAGGATTTTACACccgTACGCTTCTGCTACcTGGAT |
| Z1D-P1MF | TTTTGTCGAAATTTTTTCGGAAGGTAAGGATAAGTG |
| Z1D-P1MR | TTTTTCTTACTCACTTTATcctcTGGCAAA |