

# Mutations of the 22- and 27-kD Zein Promoters Affect Transactivation by the Opaque-2 Protein

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By utilizing a homologous transient expression system, we have demonstrated that the *Opaque-2* (*O2*) gene product *O2* confers positive *trans*-regulation on a 22-kD zein promoter. This *trans*-acting function of the *O2* protein is mediated by its sequence-specific binding to a *cis* element (the *O2* target site) present in the 22-kD zein promoter. A multimer of a 32-bp promoter fragment containing this *O2* target site confers transactivation by *O2*. A single nucleotide substitution in the *O2* target sequence not only abolishes *O2* binding *in vitro*, but also its response to transactivation by *O2* *in vivo*. We have also demonstrated that an amino acid domain including the contiguous basic region and the heptameric leucine repeat is essential for the *trans*-acting function of the *O2* protein. Similar but not identical *O2* target sequence motifs can be found in the promoters of zein genes of different molecular weight classes. Conversion of such a motif in the 27-kD zein promoter to an exact *O2* target sequence by site-directed mutagenesis was sufficient to increase the binding affinity of the *O2* protein *in vitro* and to confer transactivation by *O2* *in vivo*.

## INTRODUCTION

Zeins, the major storage proteins in maize, constitute a group of alcohol-soluble proteins that are coordinately synthesized in the endosperm of maize kernels. Zeins are divided into several classes and subclasses, according to their structural similarities or their molecular weights as determined by SDS-PAGE. The  $\alpha$ -class zeins, including 22- and 19-kD zeins, are the most abundant of the zeins, and they are encoded by a large multigene family of 50 to 100 members (Hagen and Rubenstein, 1981; Burr et al., 1982). The  $\beta$ -class zein (15-kD zein), the  $\gamma$ -class zein (16- and 27-kD zeins), and the  $\delta$ -class zein (10-kD zein), on the other hand, are encoded by genes present in a few copies (Kiriwara et al., 1988).

Studies on genetic mutations that affect the accumulation of zein proteins have indicated the presence of several regulatory mechanisms controlling the expression of specific members of the zein multigene family (reviewed by Motto et al., 1989). One of these mutations, *opaque-2* (*o2*), causes a significant reduction (50 to 70%) in zein content (Mertz et al., 1964; Tsai et al., 1978). In plants homozygous for *o2*, the synthesis of the  $\alpha$ -class zeins, particularly of the 22-kD zeins, is primarily reduced (Jones et al., 1977; Burr and Burr, 1982). This reduction is at least in part due to a decreased transcription rate (Kodrzycki et al., 1989). Thus, the *O2* locus has been proposed to encode a transcriptional activator of the 22-kD zein genes.

The *O2* gene has been isolated by transposon tagging (Schmidt et al., 1987; Motto et al., 1988), and *O2* cDNA has recently been cloned and sequenced (Hartings et al., 1989; Schmidt et al., 1990). The primary amino acid sequence deduced from the *O2* cDNA suggests that the *O2* gene encodes a DNA binding protein belonging to the "leucine zipper" (bZIP) family. It contains the contiguous basic region and a heptameric leucine repeat that are proposed to play roles as a DNA contact surface of the DNA binding protein and a dimerization interface, respectively (Landschulz et al., 1988; Vinson et al., 1989). Binding of the *O2* protein to the 5' flanking regions of 22-kD zein genes has been demonstrated (Schmidt et al., 1990). The *O2* protein has also been shown to bind to a specific sequence in the promoter of the *b-32* gene, a maize albumin gene that is also under the regulation of the *O2* locus, and to transactivate the promoter (Lohmer et al., 1991). However, the *O2* binding sequence recently identified in a 22-kD zein promoter (Schmidt et al., 1992) differs from that reported for the *b-32* promoter.

By utilizing two types of maize transient expression systems, we have demonstrated that the *O2* protein confers positive *trans*-regulation on the 22-kD zein gene through its interaction with the *O2* binding sequence identified in the 22-kD zein promoter. We have also demonstrated that a zein gene belonging to a different class can be rendered susceptible to transactivation by *O2* through a one-step mutagenesis of its promoter sequence.

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## RESULTS

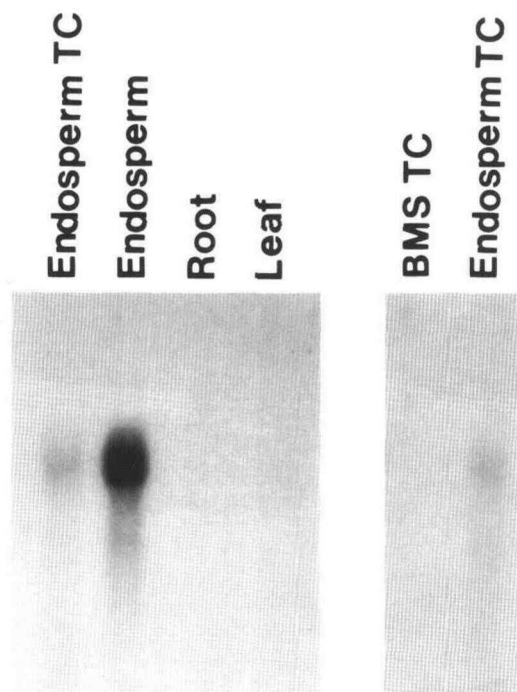
Tissue-Specific Expression of the *O2* Gene in Maize Endosperm Cell Culture

We have previously shown that the suspension culture derived from endosperm tissue of a maize inbred line, A636, which has the normal *O2* gene, maintains endosperm-specific expression of zein genes (Ueda and Messing, 1991). RNA gel blot analysis of the total RNA isolated from this endosperm culture has also revealed a significant level of *O2* gene expression indicating the maintenance of the endosperm-specific expression of the *O2* gene in this culture, as shown in Figure 1. As was previously shown for the zein genes (Ueda and Messing, 1991), the level of the *O2* transcript is much lower in the endosperm culture than in endosperm of developing (16 days after pollination) kernels. However, the expression of the *O2* gene in cultured maize cells is specific to the origin of explants because no *O2* transcripts were detected in leaf tissue-derived suspension culture of a maize cultivar, Black Mexican Sweet (BMS), at the corresponding input RNA levels (Figure 1). Thus, to examine the potential role of the *O2* protein in the tissue- and multigene member-specific regulations of zein genes, we have utilized these two types of maize suspension cultures as transient expression systems. The A636 endosperm cell culture serves as a homologous cell system, whereas the BMS cell culture serves as a heterologous cell system.

A Short Upstream Sequence Containing the *O2* Binding Sequence Confers the Transactivation by Transiently Coexpressed *O2* Protein

The *O2* binding sequence recently identified in the 22-kD zein gene promoter (Schmidt et al., 1992) consists of an imperfect palindromic sequence, 5'-TCCACGTTAGA-3', located at position -295 to -286 with respect to the initiation codon, as shown in Table 1. We have examined whether this *O2* target sequence alone is sufficient to respond to the transactivation by *O2* in vivo. Our functional analysis involved the electroporation of A636 endosperm or BMS protoplasts with two kinds of plasmids: the "reporter" plasmid expressing the chloramphenicol acetyltransferase (*CAT*) reporter gene under the regulation of a synthetic promoter containing the zein promoter sequence, and the "effector" plasmid expressing the intact or modified *O2* gene. Transactivation of the synthetic promoter function by the coexpressed *O2* protein was assayed by monitoring the level of *CAT* activity present in the transiently transformed protoplasts.

For the construction of the reporter plasmid, we have generated two pentamers of the 32-bp promoter sequences containing the *O2* binding motif from the 22-kD zein 22Z-4 or *pML1* gene (Table 1) multimerized in head-to-tail orientation. These pentamer sequences were fused, in the correct



**Figure 1.** Tissue-Specific Expression of the *O2* Gene in Maize Tissues and Cultured Cells.

The *O2* transcript levels present in the maize plant tissues and cultured cells were examined by RNA gel blot analysis. Total RNA was isolated from A636 endosperm (at 16 days after pollination), root, and leaf tissues of A636 maize plants, as well as from A636 endosperm culture (endosperm TC) and leaf tissue-derived BMS culture (BMS TC). Five micrograms of RNA sample from each tissue was fractionated in a formaldehyde-agarose gel, transferred onto a filter, and hybridized to a  $^{32}$ P-labeled *O2* cDNA probe.

orientation, 5' to a truncated (-90) cauliflower mosaic virus 35S (CaMV 35S) promoter, pCR, as shown in Figure 2A. The 32-bp sequence from the *pML1* promoter differs from that of the 22Z-4 promoter by two nucleotides, one of which is located within the *O2* binding sequence (Table 1). These synthetic promoters and the pCR were fused to the *CAT* gene, equipped with the CaMV 35S terminator (35ST) (Figure 2A). To overexpress *O2* proteins in maize protoplasts, an *O2* cDNA clone (Schmidt et al., 1990) was placed under the regulation of the CaMV 35S promoter and its terminator in pFF19 plasmid (Timmermans et al., 1990) (pFFO2+ in Figure 2C). As a negative control, pFF19 plasmid lacking the *O2* cDNA insert was used (Figure 2C). In addition, an internal deletion clone of the *O2* cDNA, lacking a 279-bp sequence of the major *O2* transcript that encodes a part of the *O2* protein including the bZIP motif (Schmidt et al., 1990), was also inserted into the pFF19 plasmid (pFFO2m in Figure 2C).

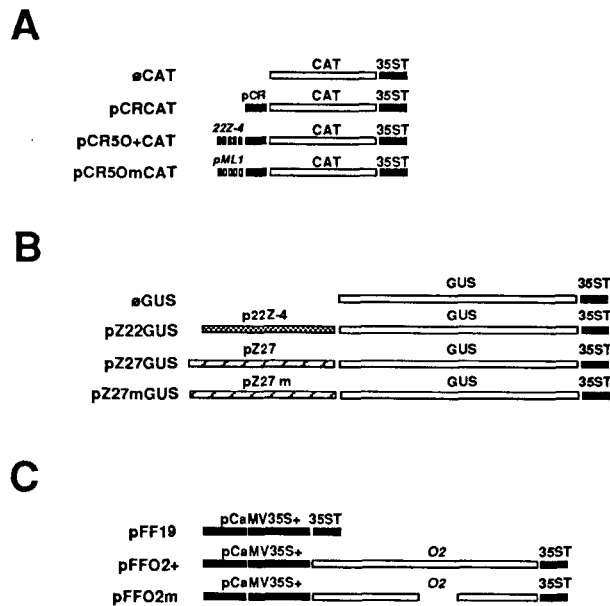
Upon cotransformation with pFF19, a residual level of *CAT* activity was derived from the truncated CaMV 35S promoter in pCRCAT in the transiently transformed protoplasts of both

**Table 1.** Conservation of a Sequence Resembling the O<sub>2</sub> Target Site among Different Zein Promoters

Gene	Nucleotide Sequence <sup>a</sup>	Location <sup>b</sup>	Reference
22 kD (22Z-4)	TGCATGTCAT <u>TCCACGTAGA</u> TGAAAAGAATTC	-295/-286	Schmidt et al. (1992)
22 kD ( <i>pML1</i> )	TGCATGTCAT <u>TCCACaTAGA</u> TtAAAAGAATTC	-305/-296	Langridge and Feix (1983)
19 kD	acCATtgtca cCCAtGTAtt TGgAcAaAATaC	-298/-289	Pedersen et al. (1982)
27 kD	TctAatTCgT TttACGTAGA TcAAcAaccTgt	-297/-288	Geraghty (1985)
27 kD*	TctAatTCgT <u>TCCACGTAGA</u> TcAAcAaccTgt	-297/-288	
10 kD	cGtATacaAg TCCAaGTAtt aagtatccgatC	-464/-455	Kirihara et al. (1988)

<sup>a</sup> The 32-base sequences surrounding the O<sub>2</sub> binding motif from various zein genes are aligned; the O<sub>2</sub> binding sequence in the 22Z-4 gene is underlined; the two nucleotide changes made by site-directed mutagenesis in the modified 27-kD promoter (27 kD\*) are also underlined.

<sup>b</sup> The location of the decanucleotide sequence is indicated with respect to the initiation codon of the gene.

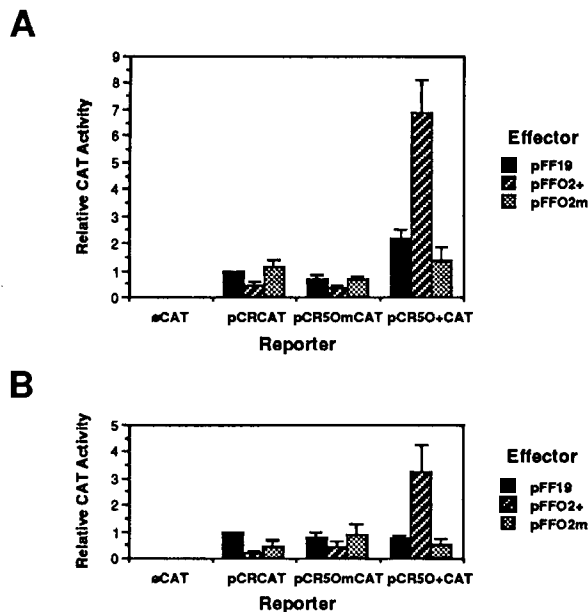
**Figure 2.** Schematic Representations of Reporter and Effector Constructs.

**(A)** The chimeric CAT reporter constructs used to analyze the interaction between the O<sub>2</sub> protein and the O<sub>2</sub> target motifs from two 22-kD promoters:  $\phi$ -CAT, the promoterless CAT reporter gene equipped with the CaMV 35S terminator sequence (35ST); pCRCAT, a truncated (-90) CaMV 35S promoter (pCR) fused to the CAT-35ST construct; pCR50+CAT, a synthetic promoter consisting of pCR and a 5-mer of a 32-bp 22Z-4 promoter upstream sequence fused to the CAT-35ST construct; pCR50mCAT, a synthetic promoter consisting of pCR and a 5-mer of a 32-bp *pML1* promoter upstream sequence fused to the CAT-35ST construct.

**(B)** The chimeric GUS reporter constructs used to analyze the interaction between the O<sub>2</sub> protein and the zein promoters:  $\phi$ -GUS, the promoterless GUS reporter gene equipped with 35ST; pZ22GUS, GUS-35ST placed under the control of a 0.9-kb 22Z-4 zein promoter; pZ27GUS, GUS-35ST placed under the control of a 1.1-kb 27-kD zein promoter; pZ27mGUS, GUS-35ST placed under the control of the 1.1-kb 27-kD zein promoter with the restored O<sub>2</sub> target sequence.

cell types, as shown in Figure 3. The cotransformation with pFFO2+ resulted in the reduction of the residual CAT expression from pCRCAT, whereas the cotransformation with pFFO2m caused no or less reduction (Figure 3). When cotransformed with pFF19, the CAT activity derived from the synthetic promoter containing the pentamer of the 22Z-4 sequence in pCR50+CAT was significantly higher than that from pCRCAT in the transiently transformed endosperm protoplasts, but not in the BMS protoplasts. However, a reproducibly high level of enhancement in the synthetic 22Z-4 promoter activity was observed in both protoplast types when pFFO2+ was coelectroporated (Figure 3). The levels of enhancement of the synthetic 22Z-4 promoter activity were about threefold in the transiently transformed endosperm protoplasts and about 4.5-fold in the BMS protoplasts, relative to their promoter activities derived in response to cotransformation with pFF19. These levels of enhancement are expected to be higher in reality because cotransformation with pFFO2+ tends to reduce the basal promoter activity of the truncated CaMV 35S promoter. The enhancement of the synthetic promoter activity was clearly dependent on the integrity of the coexpressed O<sub>2</sub> gene because it was absent when pFFO2m was coelectroporated. Furthermore, the transactivation of the synthetic promoter by the coexpressed intact O<sub>2</sub> protein is specific to the zein promoter sequence placed in the synthetic promoter. Under the corresponding transformation conditions, the synthetic promoter containing the pentamer of the *pML1* sequence failed to undergo transactivation by the coexpressed intact O<sub>2</sub> protein; rather, its activity was reduced in a manner similar to that observed in pCRCAT (Figure 3). As was observed for the synthetic 22Z-4 promoter, coexpression of the modified O<sub>2</sub> protein had little effect on the activity of this synthetic promoter.

**(C)** The effector constructs: pFF19, a negative control effector plasmid; pFFO2+, the full-length O<sub>2</sub> cDNA placed under the control of the CaMV 35S promoter with a duplicated enhancer (pCaMV35S+) and 35ST; pFFO2m, the O<sub>2</sub> cDNA with the internal deletion placed under the control of the pCaMV35S+ and 35ST.



**Figure 3.** Sequence-Specific Transactivation of Synthetic Promoters Containing the 22-kD Zein Promoter Fragments by the O<sub>2</sub> Protein.

**(A)** Relative CAT activities derived from the CAT reporter constructs upon cotransformation with the effector constructs in the transiently transformed endosperm protoplasts.

**(B)** Relative CAT activities observed in the transiently transformed BMS protoplasts.

The coelectroporated effector plasmid is indicated in the right margins. The average value of data from five independent experiments is shown for each chimeric construct in the histogram with the standard deviation from the mean indicated by an error bar. In each experiment, 25  $\mu$ g of CAT reporter plasmid and 50  $\mu$ g of effector plasmid were coelectroporated into the protoplasts. The level of CAT activity derived from the  $\phi$ -CAT construct was subtracted from those obtained from other CAT reporter constructs for each effector plasmid in order to correct for nonspecific CAT activity. In both histograms, the CAT activities derived from CAT reporter constructs in conjunction with different effector plasmids were standardized to that obtained with pCRCAT coelectroporated with pFF19 plasmid.

### Transactivation of a Whole Zein Promoter by the O<sub>2</sub> Protein

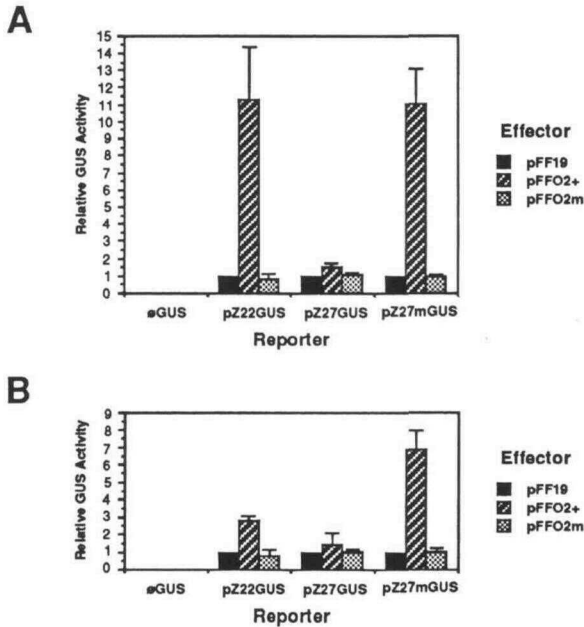
In the preceding analysis, we have demonstrated that the pentamer of a 32-bp 22Z-4 promoter sequence containing the O<sub>2</sub> target sequence is sufficient to respond to the transactivation by the coexpressed intact O<sub>2</sub> protein. However, promoter function of eukaryotic genes is generally accomplished through several *cis* and *trans* interactions. The observation of the binding of a nuclear factor to the prolamin box sequence located in the vicinity of the O<sub>2</sub> target site (Maier et al., 1987) suggests the presence of such multiple regulatory mechanisms for zein gene expression. Thus, we have further examined the *trans*-acting function of the O<sub>2</sub> protein on the whole 22Z-4 zein

promoter. In our analysis, we have also included the promoter of a 27-kD zein gene that is not presumably under the regulation of the O<sub>2</sub> locus as a control. The 0.9- and 1.1-kb 5' flanking sequences of the 22-kD 22Z-4 and the 27-kD zein genes, respectively, were fused to the  $\beta$ -glucuronidase (*GUS*) reporter gene equipped with the CaMV 35S terminator (Figure 2B). Using electroporation, we have cotransformed maize protoplasts with the zein promoter-*GUS* reporter constructs and the O<sub>2</sub> expression effector constructs (Figure 2C), as performed for the chimeric CAT constructs described previously.

In the transiently transformed endosperm protoplasts, the level of the *GUS* expression derived from the 0.9-kb 22Z-4 promoter was low when the control effector plasmid, pFF19, was coelectroporated. However, when the intact O<sub>2</sub> gene was coexpressed from the pFFO2+ plasmid, a dramatic enhancement (about 11-fold) in the level of *GUS* gene expression was observed, as shown in Figure 4A. On the other hand, the coexpression of the intact O<sub>2</sub> gene resulted in only a slight enhancement of the 1.1-kb 27-kD zein promoter activity (Figure 4A). Coexpression of the modified O<sub>2</sub> gene from the pFFO2m plasmid had little effect on both the 22Z-4 and the 27-kD zein promoter activities (Figure 4A). Similarly, in the transiently transformed BMS protoplasts, cotransformation with pFFO2+ enhanced the 22Z-4 promoter activity but to a lesser extent (about threefold) than in endosperm protoplasts (Figure 4B). As observed in the transiently transformed endosperm protoplasts, the 27-kD zein promoter activity was not significantly influenced by the coexpression of the intact O<sub>2</sub> gene in BMS protoplasts. The transactivation of the 22-kD 22Z-4 promoter function was again absent when pFFO2m was coelectroporated (Figure 4B). These results demonstrate that the *trans*-acting function of the O<sub>2</sub> protein on the zein promoter is gene specific, which is in agreement with the phenotype in the *o2* mutation.

### Two Nucleotide Substitutions in the O<sub>2</sub>-Like Target Sequence Result in the Increased O<sub>2</sub> Binding to the 27-kD Zein Promoter

The previous *in vitro* binding study with the *pML1* promoter has demonstrated that the ACGT core motif in the O<sub>2</sub> binding sequence is essential for the sequence-specific binding of the O<sub>2</sub> protein (Schmidt et al., 1992). A single nucleotide substitution in the ACGT core motif in the *pML1* promoter (Table 1) abolishes high-affinity binding of the O<sub>2</sub> protein *in vitro* (Schmidt et al., 1992) and consequently its response to transactivation by O<sub>2</sub> *in vivo* (Figure 3). We have found an O<sub>2</sub>-like binding motif in the 27-kD zein promoter located also at about -300 from the initiation codon. It is identical to the O<sub>2</sub> binding sequence of the 22Z-4 gene except that the two nucleotides immediately preceding the ACGT core motif are substituted from CC to TT (Table 1). However, despite the presence of an intact ACGT core sequence in the O<sub>2</sub>-like binding motif, the 27-kD zein promoter failed to respond well to transactivation



**Figure 4.** Transactivation of Whole Zein Promoter by the O2 Protein.

**(A)** Relative GUS activities derived from the *GUS* reporter constructs upon cotransformation with the effector constructs in the transiently transformed endosperm protoplasts.

**(B)** Relative GUS activities observed in the transiently transformed BMS protoplasts.

The coelectroporated effector plasmid is indicated in the right margins. The average value of data from five independent experiments is shown for each chimeric construct in the histogram with the standard deviation from the mean indicated by an error bar. In each experiment, 100 µg of *GUS* reporter plasmid and 50 µg of effector plasmid were coelectroporated into the protoplasts. The level of GUS activity derived from the ø-*GUS* construct was subtracted from those from other reporter constructs for each effector plasmid. For each zein promoter-*GUS* construct, the GUS activity was standardized to that obtained from cotransformation with the pFF19 plasmid.

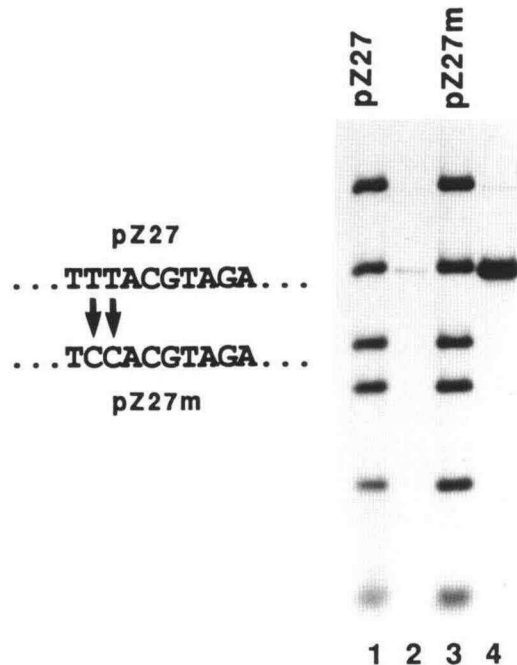
by O2 (Figure 4). This may be due to low-affinity binding of the O2 protein to the sequence as a result of the two nucleotide substitutions.

Thus, we have investigated the binding affinity of the O2 protein to the O2-like binding motif of the 27-kD zein promoter. A 252-bp upstream sequence of the 27-kD zein promoter containing the O2 binding motif was tested for binding by O2 using the *in vitro* assay described in Aukerman et al. (1991). In addition, we have restored an intact O2 binding sequence in this 252-bp fragment by changing the two successive nucleotides preceding the ACGT core motif from TT to CC by site-directed mutagenesis (Table 1). As shown in Figure 5, whereas the O2 fusion protein exhibited a low-affinity binding to the original 252-bp 27-kD zein promoter fragment, it showed remarkably high-affinity binding to the mutated fragment containing the sequence match to the O2 target site. The binding affinity of

the O2 protein to the mutated fragment was increased by about 10-fold as compared with that of the nonmutated original fragment. This result clearly demonstrates that not only the ACGT core motif but also its flanking nucleotide sequence in the O2 target sequence is essential for the high-affinity binding of the O2 protein.

**Coexpressed O2 Protein Transactivates the 27-kD Zein Promoter Containing an Intact O2 Binding Motif**

We have shown that the establishment of an intact O2 target sequence in the 27-kD zein promoter results in an increased binding affinity of the O2 protein. Subsequently, we have examined whether this restored O2 binding sequence would



**Figure 5.** Establishment of an Intact O2 Binding Motif from an O2-Like Target Sequence Results in a High Binding Affinity of the O2 Protein to a Heterologous Zein Promoter.

The pUC119 plasmids containing the 252-bp 27-kD zein promoter sequence with the original (pZ27) or mutated (pZ27m) O2 binding motif were digested with *Hin*I, end labeled with <sup>32</sup>P-dATP by the Klenow fragment of DNA polymerase I, and incubated with immunoselected β-galactosidase-O2 fusion protein (Schmidt et al., 1990). Lanes 1 and 3 show the end-labeled *Hin*I restriction fragments of the plasmids containing the pZ27 and pZ27m O2 binding motifs, respectively, used in the binding assays. Lanes 2 and 4 show the restriction fragments containing the pZ27 and pZ27m O2 binding motifs that were selectively bound by the O2 fusion protein, respectively. The two nucleotide substitutions made in the O2-like binding motif of the 27-kD promoter fragment (from TT in the original promoter sequence [pZ27] to CC in the mutated promoter sequence [pZ27m]) are shown in the left margin.

render the 27-kD zein promoter highly responsive to transactivation by O2 in vivo. For this analysis, we have placed the intact 1.1-kb promoter with the mutated O2 binding sequence (pZ27m) in the *GUS* expression cassette (pZ27mGUS in Figure 2B) and compared its response to transactivation by O2 with that of the nonmutated promoter (pZ27 in Figure 2B) in our transient expression systems. As shown in Figure 4, the two nucleotide changes made in the O2 binding motif clearly resulted in a dramatic enhancement in O2-dependent transactivation of the 1.1-kb 27-kD zein promoter in both endosperm and BMS protoplasts. This enhancement appears to be about twofold stronger in endosperm protoplasts than in BMS protoplasts. Whereas in endosperm protoplasts the levels of the O2-dependent transactivation of the 22Z-4 and pZ27m promoters appear to be comparable, they differ significantly in BMS protoplasts where the level of transactivation of the pZ27m promoter is much higher. These results show that the promoter of a distantly related zein gene that is not normally under the regulation of the O2 locus can be rendered responsive to transactivation by O2 by a single-step mutagenesis of its O2 binding motif.

## DISCUSSION

The O2 locus in maize encodes a bZIP factor (Hartings et al., 1989; Schmidt et al., 1990) that regulates the transcription of the 22-kD zein genes (Kodrzycki et al., 1989). If the O2 protein is the predominant transcription factor limiting the multigene member-specific expression of the 22-kD zein genes, the overexpression of the O2 proteins in maize endosperm cells would be expected to enhance the transcription of the 22-kD zein genes but not that of other zein genes. By utilizing the suspension culture-derived maize endosperm protoplasts as a homologous transient expression system, we have demonstrated that the transiently coexpressed O2 protein transactivates the 0.9-kb 22Z-4 zein promoter, but does not significantly affect the activity of the 1.1-kb 27-kD zein promoter. Furthermore, coexpression of the O2 protein in *trans* also confers transactivation of the 22Z-4 promoter in the heterologous BMS protoplasts although the level of transactivation is significantly lower than that seen in the endosperm protoplasts. These results suggest that the endosperm-specific expression of the 22-kD zein gene may be controlled, in part, by the tissue-specific expression of the O2 gene. The lower extent of transactivation of the 22Z-4 promoter in the BMS protoplasts than in the endosperm protoplasts may further suggest the involvement of additional *trans*-acting mechanisms in the regulation of 22-kD zein genes in maize endosperm cells.

The O2 target sequence, 5'-TCCACGTAGA-3', recently identified in the 22Z-4 promoter (Schmidt et al., 1992) plays an essential role in the *trans*-acting function of the O2 protein. A 32-bp 22Z-4 promoter sequence containing the O2 target sequence can respond to transcriptional transactivation by the coexpressed O2 protein in both endosperm and BMS protoplasts. However, multimerization of this 32-bp sequence is

required for a high response level to transactivation by O2. Our titration experiment indicates that to achieve a corresponding transactivation level with a synthetic promoter containing one copy of the O2 binding sequence, a much larger amount of the O2 expression effector plasmid is required (data not shown). This may be due to the titration of transiently overexpressed O2 proteins by the O2 binding sequence present in the endogenous 22-kD zein genes, because there are about 20 gene members in their multigene family (Heidecker and Messing, 1986). The multimerization of the O2 binding sites would enhance the chance of O2 binding to the target sequences in the synthetic promoter. Alternatively, the physical distance of the O2 binding sequence relative to other *cis* sequences such as the TATA box may be critical for the binding and/or the transactivation mechanism of O2 in vivo.

Like many other plant bZIP factors characterized so far, the O2 protein recognizes a promoter *cis* sequence containing the ACGT core sequence. The ACGT core sequence has been shown to be essential for the DNA binding and/or the *trans*-acting function of GBF (Giuliano et al., 1988), EmBP-1 (Guilinan et al., 1990), and TAF-1 (Oeda et al., 1991). Similarly, the ACGT core sequence present in the O2 target site is essential for the high-affinity binding of the O2 protein and, consequently, for the promoter response to transactivation by O2. A single nucleotide substitution in the ACGT core sequence as it occurs in the promoter of another 22-kD gene, *pML1*, abolishes not only the O2 binding in vitro (Schmidt et al., 1992), but also its response to transactivation by O2 in vivo. It is clear that not all members of the 22-kD zein multigene family contain an intact O2 binding sequence (Schmidt et al., 1992). This heterogeneity in the promoter sequence among different members of 22-kD zein genes probably reflects the fact that many members of this gene family are pseudogenes. *pML1* not only lacks the O2 target site but also contains a stop codon interrupting the open reading frame. In contrast, 22Z-4 has both the O2 target site and an intact open reading frame (Schmidt et al., 1992).

Interestingly, nucleotide sequences similar to the O2 binding motif can be found in the promoters of various zein genes other than the 22-kD zein genes that are not presumably under the regulation of the O2 locus (Table 1). However, nucleotide substitutions are often found within the ACGT core motif in their sequences. As inferred from the finding with the *pML1* promoter, the O2 protein, at least in the homodimeric form, is expected to have a low or no binding affinity to these sequences. In the case of the 27-kD zein promoter, the O2-like binding sequence contains an intact ACGT core motif, but two nucleotide differences relative to the O2 target site of the 22Z-4 promoter occur in the sequence immediately preceding it (Table 1). However, the O2 protein exhibits a low binding affinity to this O2-like binding sequence and a low level of transactivation on the 27-kD promoter. The establishment of an intact O2-target site in the 27-kD zein promoter by site-directed mutagenesis of the two nucleotides drastically enhances the binding affinity of the O2 protein to the promoter in vitro and, consequently, renders it responsive to transactivation by O2 in vivo. Thus, both the ACGT core motif and its flanking sequence in

the O2 target site are essential for the high-affinity binding of the O2 protein. Similar results have been obtained for the TAF-1 (Oeda et al., 1991) and the HBP-1 factors (Tabata et al., 1991). The introduction of a novel regulatory *cis* element in the heterologous zein promoter does not seem to interfere with its function, suggesting the *trans*-acting function of the O2 protein is independent and additive. Whether or not the conserved O2 binding motif present in the heterologous zein promoters plays a role in member-specific regulation of their genes through interaction with other forms of leucine zipper factors remains to be elucidated.

Finally, the contiguous basic region and the heptameric leucine repeat present in the bZIP factor play essential roles in DNA binding and protein dimerization, respectively. Amino acid substitutions in the basic region have been shown to inactivate the functions of C/EBP (Landschulz et al., 1989) and CYS3 proteins (Fu et al., 1989). Similarly, a single amino acid substitution in the basic region drastically reduces the DNA binding affinity of the O2 protein as well as its *trans*-acting function (Aukerman et al., 1991). In our work, we have demonstrated that the internal domain in the O2 protein including the bZIP motif is essential for the *trans*-acting function of the O2 protein, further strengthening the idea that the O2 protein functions as a bZIP transcription factor.

## METHODS

### RNA Gel Blot Analysis

Total RNA was isolated from plant tissues and cultured cells of maize inbred line A636 according to the procedure described by Das et al. (1990). RNA gel blot analysis was performed according to the procedure described by Cruz-Alvarez et al. (1991). Five micrograms of total RNA isolated from each maize tissue and cell culture was analyzed for the presence of *Opaque-2* (O2) transcripts. The O2 gene probe used was a deletion clone of O2 cDNA in which a portion of the untranslated sequence containing the three upstream ATGs had been deleted (Varagona et al., 1991). It was labeled with <sup>32</sup>P-dCTP by nick-translation (Rigby et al., 1977).

### Construction of Chimeric CAT "Reporter" Plasmids

Construction of the control plasmid,  $\phi$ -CAT (Figure 2A), containing the coding sequence of the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene fused to the terminator sequence of the cauliflower mosaic virus 35S (CaMV 35S) gene, has been described previously (Ueda and Messing, 1991). A truncated CaMV 35S promoter (pCR) (spanning to -90 with respect to the transcription start site) (Figure 2A) was isolated from pFF19 plasmid (Timmermans et al., 1990), and cloned into the pBluescript SK+ (Stratagene) vector. A 32-bp NsiI-EcoRI fragment containing the previously identified O2 binding sequence (Schmidt et al., 1992) was isolated from the 22-kD 22Z-4 promoter subclone. Similarly, the corresponding 32-bp NsiI-EcoRI region was isolated from the promoter of another highly homologous 22-kD zein gene clone, *pML1*. These 32-bp NsiI-EcoRI fragments of the 22Z-4 and *pML1* promoters were subsequently cloned into the PstI-EcoRI sites of the pBluescript SK+ vector. Each of these two fragments was multimer-

ized in head-to-tail orientation in the vector to generate pentamers. The monomers as well as pentamers of the 32-bp fragments from the 22Z-4 and *pML1* promoters were isolated and fused 5' to the truncated CaMV 35S gene promoter in the correct orientation. Subsequently, these fusion promoters and the truncated CaMV 35S gene promoter were cloned into the CAT expression vector equipped with the CaMV 35S terminator sequence (35ST) (pCR5O+CAT and pCR5OmCAT in Figure 2A).

### Construction of the Zein Promoter-GUS "Reporter" Plasmids

The cloning of the 1.1-kb 5' flanking sequence of a 27-kD zein gene (spanning from -1042 to +61 with respect to the transcription start site) into pUC119 plasmid vector has been described previously (Ueda and Messing, 1991). A 0.9-kb 5' flanking sequence of the 22Z-4 gene (spanning from -918 to -1 with respect to the ATG initiation codon) (Schmidt et al., 1992) was isolated from a genomic subclone, and it was also cloned into pUC119 plasmid vector. These 5' flanking sequences of the two zein genes were cut out at the HindIII and XbaI sites in the polylinker and cloned into the corresponding sites in pFF19G (Timmermans et al., 1990). This cloning has resulted in the fusion of the 5' flanking sequences to the  $\beta$ -glucuronidase (GUS) reporter gene equipped with the CaMV 35S terminator sequence (35ST) via replacement of the CaMV 35S promoter (pZ22GUS and pZ27GUS in Figure 2B). As a negative control, a promoterless GUS construct,  $\phi$ -GUS, was constructed by deleting the CaMV 35S promoter from pFF19G (Figure 2B).

### Construction of O2 Expression "Effector" Plasmids

Synthesis and cloning of a full-length O2 cDNA1-4 have been described previously (Schmidt et al., 1990). This cDNA clone contains three short open reading frames in the untranslated leader. For the construction of a deletion clone lacking the sequence encoded for the region including the basic amino acid region and leucine repeat, an internal 279-bp sequence spanning from +572 to +850 with respect to the ATG initiation codon of the major open reading frame was deleted from the full-length O2 cDNA clones. This internal deletion was achieved by fusing the two fragments of the cDNA, one containing the upstream domain (from +573 to the 5' end) and the other containing the downstream domain (from +851 to the 3' end) in the pBluescript SK+ vector. While keeping the codons in the 3' domain in frame with those in the 5' domain, this fusion has introduced three nucleotides from the polylinker at the junction, which, in turn, will create two novel codons (for alanine and leucine) within the mutant cDNAs. The full-length as well as the deletion clone of O2 cDNAs were placed under the regulation of the CaMV 35S promoter (with duplicated enhancer elements) and terminator in pFF19 vector (pFFO2+ and pFFO2m in Figure 2C).

### Site-Directed Mutagenesis

To convert the two nucleotides TT to CC in the O2-like binding motif of the 27-kD zein promoter to establish an intact O2 binding sequence (Table 1), site-directed mutagenesis was performed according to the procedures described by Zoller and Smith (1984) and Kunkel (1985). The 1.1-kb 27-kD zein promoter clone and a subclone of a 252-bp upstream sequence, both cloned in the pUC119 vector, were used for the mutagenesis. The 252-bp upstream sequence spans from -415



to -164 with respect to the transcription start site of a 27-kD zein (gene A) promoter (Geraghty, 1985; Das and Messing, 1987). It was isolated by HinfI digestion of the 1.1-kb promoter fragment, and cloned into the HincII site in pUC119 vector after the protruding HinfI ends had been made blunt by the Klenow fragment of DNA polymerase I. Single-stranded DNA was prepared according to the procedure described by Vieira and Messing (1987) with the addition of uridine as described by Kunkel (1985), using the CJ-236 strain of *Escherichia coli* and the bacteriophage M13KO7. The following 30-base oligonucleotide with the desired two nucleotide mutations was synthesized for site-directed mutagenesis: 5'-TGT TGATCTACGTGGAACGAAT TAGATTTA-3'. After annealing of the oligonucleotide to single-stranded DNA and T4 DNA polymerase reaction, the mutated clone was amplified in the JV-30 strain of *E. coli*. The nucleotide sequence of the mutated clone was subsequently confirmed by the dideoxy sequencing method. The mutated 1.1-kb 27-kD zein promoter was fused to the *GUS* reporter with 35ST in the pFF19G vector (pZ27mGUS in Figure 2B) as described above.

### Isolation of Protoplasts from Maize Cell Cultures

The establishment of maize (*Zea mays* inbred line A636) endosperm cell suspension cultures and isolation of protoplasts from these cultures have been described previously (Ueda and Messing, 1991). Isolation of protoplasts from leaf tissue-derived suspension cultures of a maize cultivar, Black Mexican Sweet (BMS), was carried out in the following manner. Approximately 50 mL of suspension cultures containing 3-mL packed volume of BMS cells was taken out on the second day after subculture, and cells were collected by centrifugation in a sterile 50-mL disposable tube. The suspension cells were mixed with 30 mL of enzyme solution, containing 1% (w/v) cellulysin (Calbiochem, San Diego, CA), 0.5% (w/v) Rhozyme HP150 (Genecor, San Francisco, CA), 0.02% (w/v) pectolyase (Sigma), 0.5% (w/v) bovine serum albumin, and 0.5  $\mu$ L/mL of  $\beta$ -mercaptoethanol in the protoplast isolation solution (50 mM  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 12 mM sodium acetate, and 0.25 M D-mannitol, pH 5.8). The digestion was carried out for 3 to 5 hr at room temperature in the dark with gentle shaking (50 rpm) on a horizontal shaker. Isolated protoplasts were purified by passing the digestion mixture through a sterile stainless steel sieve (a 74- $\mu$ m mesh) (Bellco Glass, Vineland, NJ). Purified protoplasts were washed twice with the protoplast isolation solution, and protoplast yield was determined with a hemacytometer.

### Electroporation of Maize Protoplasts

Electroporation and subsequent culture of A636 endosperm protoplasts were carried out according to the procedure described by Ueda and Messing (1991). Electroporation of BMS was performed as follows. Isolated BMS protoplasts were washed once with electroporation solution (0.15 mM  $\text{KH}_2\text{PO}_4$ , 0.81 mM  $\text{Na}_2\text{HPO}_4$ , 128 mM NaCl, 4 mM  $\text{CaCl}_2$ , and 0.2 M D-mannitol, pH 7.2). They were resuspended in the above solution at a final density of 2 to 4 million protoplasts per mL. Upon mixing plasmid DNA harboring the chimeric gene construct with protoplasts, electroporation was carried out at 150 V and 1450  $\mu$ F. The electroporated BMS protoplasts were cultured in 10 mL of culture medium; eight volumes of BMS suspension culture medium; Murashige and Skoog salts (Murashige and Skoog, 1962), 200 mg/L *myo*-inositol, 130 mg/L asparagine, 1.3 mg/L niacin, 0.25 mg/L thiamine HCl, 0.25 mg/L pyridoxine HCl, 0.25 mg/L calcium pantothenate, 20 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8, supplemented with 2.5% (v/v) coconut water (GIBCO), 0.3 M D-mannitol, and two

volumes of conditioned medium. The conditioned medium consisted of the filtrate of culture medium taken from BMS suspension culture that had been grown for 2 days. The electroporated protoplasts were cultured at 25°C in the dark for 2 days. The amount of plasmid DNA used in electroporation of A636 endosperm and BMS protoplasts is described in the figure legends.

### Chloramphenicol Acetyltransferase and $\beta$ -Glucuronidase Enzyme Assays

At the end of a 44- and 48-hr culture period, cellular protein extracts were prepared from electroporated protoplasts and assayed for the chloramphenicol acetyltransferase (CAT) or  $\beta$ -glucuronidase (GUS) enzyme activity as described by Ueda and Messing (1991). The CAT activity was quantitated by measuring in a scintillation counter the radioactivity of silica gel spots containing the  $^{14}\text{C}$ -labeled chloramphenicol and its acetylated forms. GUS activity was determined fluorometrically, using 4-methylumbelliferyl glucuronide as a substrate. Fluorescence was measured with a fluorometer (model No. TKO100, Hoefer, San Francisco, CA) with excitation at 365 nm and emission at 460 nm.

### In Vitro DNA Binding Assay

Binding of O2 proteins to the 252-bp upstream fragment (spanning from -415 to -164 with respect to the transcription start site) of the 27-kD zein promoter was assessed by the in vitro DNA binding assay according to the procedure described by Schmidt et al. (1990) and Aukerman et al. (1991). For the assay, the pUC119 plasmid containing the 27-kD zein upstream fragment was digested with HinfI and labeled with  $^{32}\text{P}$ -dATP by the Klenow fragment of DNA polymerase I. The promoter subclones containing the original and mutated O2-binding motif were tested.

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