The Plant Cell, Vol. 6, 237–249, February 1994 © 1994 American Society of Plant Physiologists

The Transcriptional Activator Opaque2 Recognizes Two Different Target Sequences in the 22-kD-like α-Prolamin Genes

José A. Yunes,[a] Germano Cord Neto,[a] Marcio J. da Silva,[a] Adilson Leite,[a] Laura M. M. Ottoboni,[a] and Paulo Arruda [a,b,1]

[a] Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, 13081, Campinas, SP, Brazil
[b] Departamento de Genética e Evolução, IB, Universidade Estadual de Campinas, 13081, Campinas, SP, Brazil

The maize Opaque2 (O2) protein is a "leucine zipper" DNA binding factor that interacts with the sequence TCCACGTAGA in the promoters of the 22-kD α-zein genes and activates its transcription. A completely different consensus sequence (GATGAPyPuTGPu) identified in *b-32*, a gene that encodes an abundant albumin that is also under control of the *O2* locus, can also be bound by the O2 protein. We showed that the gene encoding the 22-kD-like α-coixin, the α-prolamin of the maize-related grass *Coix*, can also be transactivated by the O2 protein. A binding assay in vitro and footprint analysis demonstrated that the GACATGTC sequence of the α-coixin promoter can be recognized and protected by the maize O2 protein. Employing transient expression experiments in immature maize endosperm and tobacco mesophyll protoplasts, we demonstrated that the O2 protein can activate expression of the β-glucuronidase reporter gene placed under the control of the 22-kD-like α-coixin promoter. We also demonstrated that a 22-kD-like α-coixin pseudogene promoter is transactivated by the maize O2 protein.

INTRODUCTION

Prolamins, the major storage proteins of cereals (Osborne and Mendel, 1914), have proved to be a useful model system to study the regulation of gene expression in plants. We are currently studying the prolamins from *Coix lacryma-jobi*, which belongs to the grass tribe Andropogoneae. Other members of this tribe include maize, *Tripsacum*, and sorghum. Comparisons between the genes from these related species have provided insights into the structure and evolution of the prolamin genes. The prolamins of *Coix* and maize, coixins and zeins, respectively, can be separated into polypeptides of distinct molecular mass by SDS-PAGE (Ottoboni et al., 1990) and grouped into four classes, α-, β-, γ-, and δ-prolamins (Esen, 1986; Leite et al., 1990). In maize and *Coix*, the α-prolamins account for more than 70% of total prolamins and are composed of polypeptides of 19- and 22-kD in maize (Burr et al., 1982) and 25- and 27-kD in *Coix* (Leite et al., 1990). These size class proteins are encoded in both cereals by multigene families (Hagen and Rubenstein, 1981; Ottoboni et al., 1993), and their expression is under strict tissue-specific and developmental control (Shotwell and Larkins, 1989; Targon et al., 1992).

Recently, we have shown that the 25-kD α-coixin genes share high homology with the 22-kD α-zein genes in respect to both protein structure and to the regulatory sequences present in their promoter regions (Ottoboni et al., 1993). For this reason, we designated the 25-kD α-coixin gene family as 22-kD-like α-coixins (Ottoboni et al., 1993).

Several mutations are known to affect the level of zeins in the maize endosperm (Motto et al., 1989). One of these, *opaque2* (*o2*), exerts its major effect on the level of the 22-kD α-zeins (Soave et al., 1976; Burr and Burr, 1982). A homozygous loss-of-function mutation at *O2* results in an overall reduction of 50 to 70% in zein content. This is a consequence of a lower rate of transcription, particularly from those genes encoding the 22-kD α-zeins (Kodrzycki et al., 1989).

Another endosperm protein, a 32-kD albumin designated b-32, whose expression is temporally and quantitatively coordinated with the deposition of zeins, is greatly affected by the homozygous o2 mutation. Such a mutation reduces the b-32 mRNA content to 5 to 10% of the wild-type level (Di Fonzo et al., 1986).

The *O2* gene has been cloned by transposon tagging (Schmidt et al., 1987; Motto et al., 1988) and shown to be a basic "leucine zipper" (bZIP) DNA binding protein (Hartings et al., 1989; Schmidt et al., 1990). Subsequent experiments have shown that the O2 protein is capable of transactivating *b-32* gene transcription in vivo by interacting with five putative

[1] To whom correspondence should be addressed at Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas–Unicamp, Cidade Universitária "Zeferino Vaz," Cx.P. 6109, 13081-970-Campinas-SP, Brasil.

binding sites containing the consensus sequence GATGAPyPuTGPu (Lohmer et al., 1991).

A quite different target sequence (TCCACGTAGA) has been shown to be the O2 binding site in the 22-kD α-zein promoter (Schmidt et al., 1992). This sequence was verified by mobility shift assays using the O2 protein from maize endosperm (Schmidt et al., 1992). Furthermore, when a multimer of this O2 target site is placed upstream of a minimal promoter, it mediates transactivation of the reporter gene by O2 in various transient expression systems (Schmidt et al., 1992; Ueda et al., 1992).

We have previously shown the presence of putative O2 boxes in the promoter region of the 22-kD–like α-prolamins and also found sequences homologous to the *O2* gene in *Coix* and sorghum (Ottoboni et al., 1993). In this study, we report the binding to and transactivation of the 22-kD–like α-coixin promoter by the maize O2 protein in both homologous and heterologous expression systems. Surprisingly, we found that the core of the α-coixin O2 target site (GACATGTC) shares more homology with the B1 and B4 O2 binding sites of the *b-32* gene than with that of the 22-kD α-zein gene. Moreover, the α-coixin O2 box does not contain the ACGT core, an essential condition for the high-affinity binding of the O2 protein (Schmidt et al., 1992; Izawa et al., 1993).

RESULTS

Maize O2 Protein Binds to the 22-kD–like α-Coixin Promoter

Sequence analysis of the 22-kD–like α-prolamin genes from maize, *Coix*, and sorghum revealed two homologous O2 target sites in their promoters (Ottoboni et al., 1993). The target sites are designated as O2a and O2b boxes in this study. In the *α-3B* α-coixin promoter, the first box, O2a, is located at position −301 with respect to the ATG, and the second, O2b, at position −281. However, both boxes showed significant discrepancy in relation to the O2a target sequence 5′-TCCACGTAGA-3′ present in the *22Z-4* 22-kD α-zein gene promoter (Schmidt et al., 1992). Interestingly, as can be seen in Figure 1, other 22-kD α-prolamin genes share such a discrepancy, including two recently isolated 22-kD α-zein genes (Thompson et al., 1992).

To determine whether the O2-like boxes of the 22-kD–like α-coixin promoter are recognized by the O2 protein, we conducted a DNA binding assay using the maize O2 protein in a β-galactosidase (β-Gal)::O2 fusion (Schmidt et al., 1990). As shown in Figure 2A (lane 2), a 260-bp Sau3AI restriction fragment that is immediately 3′ of the "prolamin-box" (Brown et al., 1986b) and contains the two related O2 boxes (Figure 2B) was bound by the O2 protein. Digestion of the Sau3AI fragment with EcoRI, which separates the two O2-like boxes (Figure 2B), showed that the resulting 225-bp fragment retains a strong binding capacity (Figure 2A, lane 4) and indicated that the O2

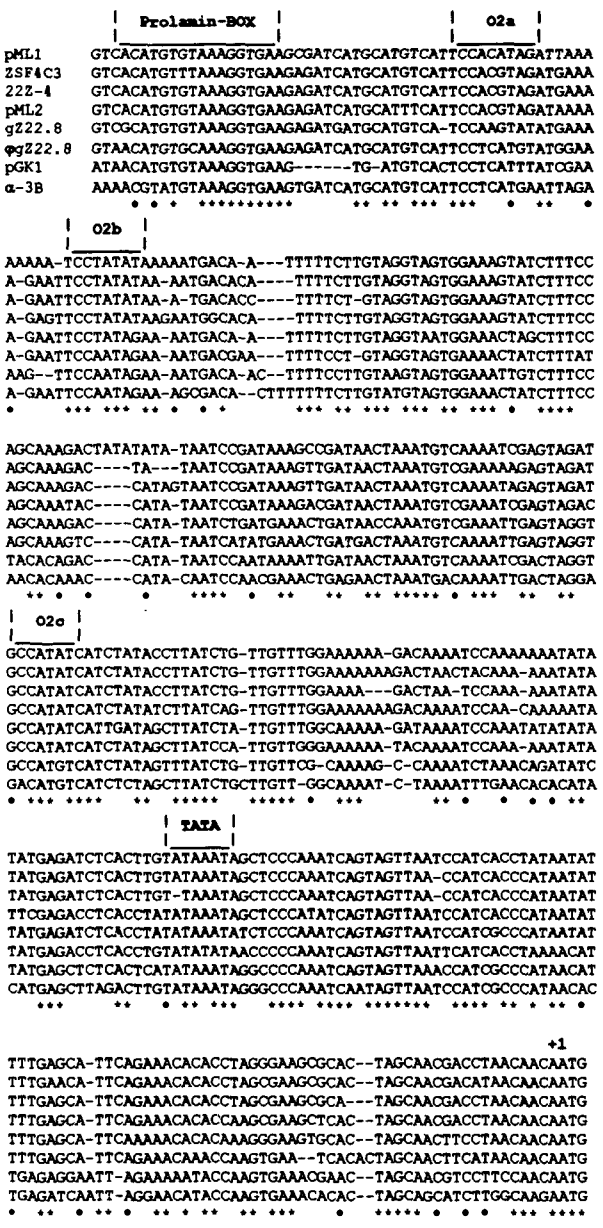

Figure 1. Sequence Alignment of 22-kD α-Prolamin Promoters.

The ∼350-bp promoter fragments extending from the ATG of six 22-kD α-zein clones are aligned. They include *pML1* and *pML2* (Wandelt and Feix, 1989), *ZSF4C3* (Liu and Rubenstein, 1992), *22Z-4* (Schmidt et al., 1992), and *gZ22.8* and *ψgZ22.8* (Thompson et al., 1992) from maize; the 22-kD–like α-kafirin clone *pGK1* (DeRose et al., 1989) from sorghum; and the *Coix* 22-kD–like α-coixin clone *α-3B* (Ottoboni et al., 1993). Dashes indicate deletions introduced to maximize the homology comparison. Identical nucleotides are marked by asterisks. The locations of the putative DNA regulatory sequences "prolamin-box;" O2 target sites O2a, O2b, and O2c; TATA box; and the translation start codon (+1) are indicated above the sequence data.

protein does not bind to the O2a box of the α-coixin promoter. Because in this EcoRI-Sau3AI digestion the O2a box remained in a 35-bp fragment that is too small to be retained by the β-Gal::O2 fusion, the binding was confirmed by digesting the α-coixin promoter fragment with EcoRI and XbaI. As shown in Figure 2A (lane 6), only the 310-bp fragment that contains the O2b (and O2c, see below) box (Figure 2B) was retained. The Sau3AI digestion of the 22-kD α-zein promoter (plasmid Pα-Z4103), which was included in the binding assay as a positive control, showed that the O2 protein binds to a 200-bp fragment that contains the O2a box (Figure 2A, lane 8), as was determined previously (Schmidt et al., 1992).

When the α-zein promoter was digested with EcoRI-Sau3AI, thus separating the O2 boxes present in the 200-bp Sau3AI fragment (Figure 2B), no binding was observed (Figure 2A, lane 10). This confirmed that the O2 protein does not recognize the O2b (and O2c, see below) in the α-zein promoter. When the α-zein promoter was digested with EcoRI-XbaI, only the 455-bp fragment containing the O2a box (Figure 2B) was retained (Figure 2A, lane 12). As shown in Figure 2A (lane 14), no binding was observed to the restriction fragments from the pBluescript KS+ vector included as a negative control.

The clone Pα-PCx103, containing the promoter of the 22-kD–like α-coixin pseudogene *α-3A* (Ottoboni et al., 1993), was also assayed for its retention by the O2 protein. Like the normal gene (*α-3B*), the 260-bp Sau3AI restriction fragment was bound by the O2 protein (Figure 2A, lane 16).

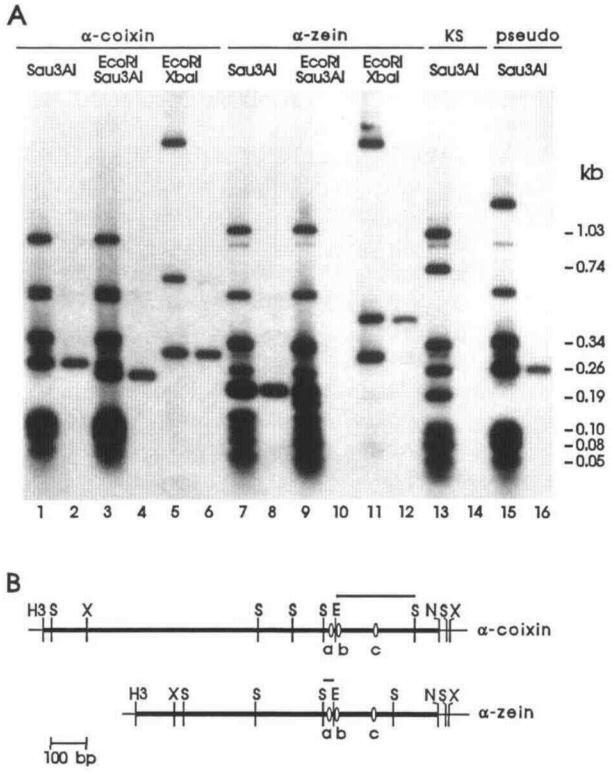

Figure 2. Selective Binding of the O2 Protein to Restriction Fragments from the 22-kD–like α-Coixin and 22-kD α-Zein Promoter Sequences.

(A) Plasmids containing the promoter sequence from α-zein *22Z-4* (Pα-Z4103), α-coixin *α-3B* (Pα-Cx103), α-coixin pseudogene (pseudo) *α-3A* (Pα-PCx103), and pBluescript KS+ (KS) vector were digested with Sau3AI, EcoRI and Sau3AI, or EcoRI and XbaI, end labeled, and incubated with the immunoselected β-Gal::O2 fusion protein. Odd-numbered lanes show the labeled restriction fragments that were incubated with the fusion protein. Even-numbered lanes show the fragments that were specifically retained by the β-Gal::O2 fusion. The lengths of the pBluescript KS+ Sau3AI fragments are given at right in kilobases.

(B) Partial restriction map of the promoter region from the 22-kD–like α-coixin *α-3B* and 22-kD α-zein *22Z-4* genes cloned in Pα-Cx103 and Pα-Z4103, respectively. The putative O2 target sites O2a, O2b, and O2c are represented by ovals. The solid lines above the map indicate the restriction fragment that was bound by the β-Gal::O2 fusion. Abbreviations for restriction sites are as follows: E, EcoRI; H3, HindIII; N, NcoI; S, Sau3AI; X, XbaI.

O2 Protein Recognizes the GACATGTC Sequence in the 22-kD α-Coixin Promoter

The site of interaction of β-Gal::O2 on the 260-bp α-coixin promoter fragment was mapped using DNase I footprint, as shown in Figure 3. The assay was performed on both strands by using increasing amounts of the protein A–Sepharose beads containing the immunoprecipitated β-Gal::O2 fusion (Schmidt et al., 1992). Surprisingly, the O2a and O2b boxes were not protected. Instead, a third region that we have designated O2c (Figure 1) was strongly footprinted.

As shown in Figure 3, the footprint observed in each strand produced an overlapping region spanning 23 nucleotides that is located at position −181 to −159 with respect to the ATG initiation codon. According to the assumption that bZIP proteins bind palindromic sequences, the region footprinted in *the α-coixin promoter encompasses the perfect palindrome* 5′-GACATGTC-3′. As shown in Table 1, the O2c target site of the α-coixin promoter, albeit completely different from that identified in its α-zein relative (Schmidt et al., 1992), has some homology with the O2 target sites B1 and B4 reported for the *b-32* gene (Lohmer et al., 1991).

The nucleotide positions of the α-coixin O2c box were numbered relative to the center of symmetry. Following the nomenclature used previously for the GCN4 binding site (Oliphant et al., 1989), the central two nucleotides A and T of the GACATGTC palindrome were designated as −0 and +0, respectively.

Among the 22-kD–like α-prolamin genes (Figure 1), the α-kafirin clone *pGK1* (DeRose et al., 1989) contains the most homologous O2c box, which differs from that found in the α-coixin promoter by an A-to-C replacement at position −2 (Table 1). Interestingly, the O2c box in the α-coixin pseudogene *α-3A* contains the same modification (Table 1) and is still recognized by the O2 protein (Figure 2A). Because the O2a site in the α-kafirin promoter is quite different from that of the *22Z-4*

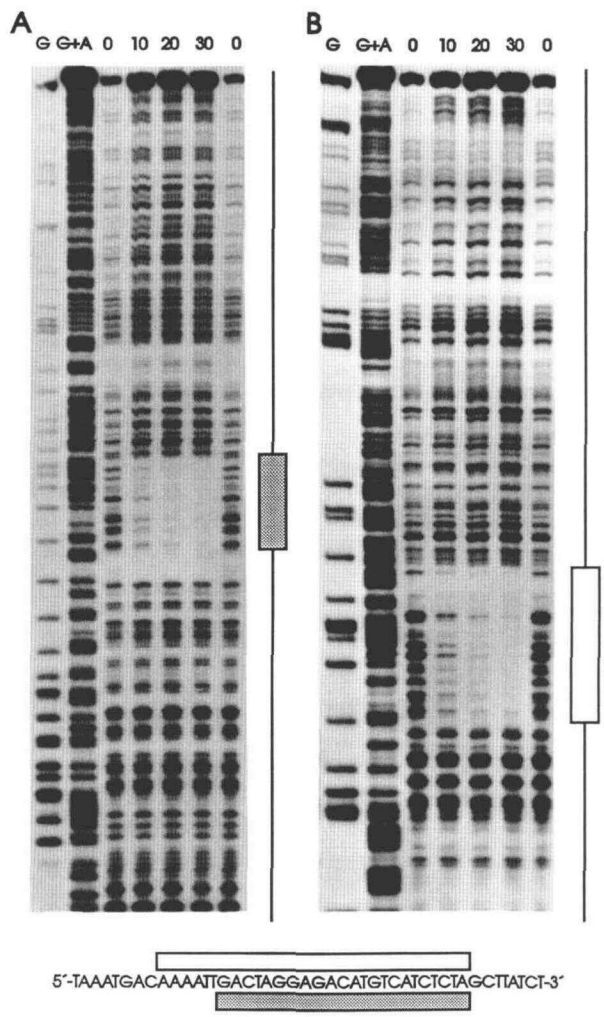

Figure 3. Footprint in Vitro of the O2 Fusion Protein on the 22-kD–like α-Coixin Promoter.

The 260-bp Sau3AI restriction fragment from the *α-3B* α-coixin promoter that was retained by the β-Gal::O2 fusion in the DNA binding assays was used for the footprint analysis. The lanes G and G+A in **(A)** and **(B)** are the Maxam and Gilbert sequencing ladders. The labeled α-coixin promoter fragment was incubated with DNase I without prior incubation with β-Gal::O2 (lanes 0) or with increasing amounts of the fusion protein (lanes 10, 20, and 30).

(A) Footprint analysis performed on the bottom strand.

(B) Footprint along the top strand.

The box beside each panel represents the region of footprint along box O2c. A portion of the sequence spanning box O2c is shown along the bottom of **(A)** and **(B)**. The protected nucleotides are indicated by the shaded and open boxes along the DNA strand given in single-letter code. Note that in **(A)**, the G and G+A sequencing ladders should be read C and C+T, respectively, when referring to the sequence shown along the bottom of the panels.

α-zein clone (Figure 1), we predicted that the O2c box is the preferred binding sequence in the *pGK1* α-kafirin clone.

In the *22Z-4* clone, as in all other 22-kD α-zein genes (Figure 1), the O2c box differs from that of the α-coixin by an A-to-C mutation and a G-to-A mutation at positions −2 and +1, respectively (Table 1). These alterations, as shown in Figure 2A (lane 10), were sufficient to prevent O2 binding to this box under conditions in which strong binding to the α-coixin box was apparent. The other differences in *22Z-4* at positions −4 and −5, although they improved either the similarity with boxes B1 and B4 of *b-32* or the symmetry with the nucleotides at positions +4 and +5, were not sufficient to restore the O2 binding affinity. Therefore, it appeared that within the 23–nucleotide footprinted sequence in the α-coixin promoter, the motif GACATGTC represents the main O2 binding requirement at the nucleotide level. Moreover, although it is not present in the B2, B3, or B5 O2 boxes of the *b-32* gene (Lohmer et al., 1991), the CATG core, as shown in Table 1, seems to be an important consensus motif.

To confirm the footprint result, we performed O2 DNA binding assays on plasmids that contained either the intact 285-bp EcoRI-NcoI α-coixin promoter fragment or an otherwise identical clone from which the sequence CAATAGA from the O2b box had been deleted. These plasmids, designated P285α-C and P285ΔO2b, respectively, are represented in Figure 4B. As shown in Figure 4A, a single restriction fragment of 225 bp in P285α-C (lane 4) and a fragment of 218 bp in P285ΔO2b (lane 6) were retained by the β-Gal::O2 fusion, confirming that the O2 binds to the O2c box and not to the O2b in the α-coixin promoter.

Transcriptional Activation of the 22-kD α-Coixin Promoter Is Mediated by the O2 Protein

Immature Endosperm

To determine whether the binding affinity in vitro of the α-coixin promoter reflected the situation in vivo, we conducted a histochemical spot-counting transient assay in situ. In this assay, immature maize endosperms were bombarded with DNA-

Table 1. Alignment of α-Coixin-like O2 Target Sites and Flanking Sequences

Box	Gene	DNA Sequence	Reference
B1	*b-32*	TGGGAT**GACATGGC**TAATATA	Lohmer et al. (1991)
B4	*b-32*	TATGAT**GACATGAC**-TAT-TA	Lohmer et al. (1991)
O2c	*α-3B*	CTAGGA**GACATGTC**ATCTCTA	This study
O2c	*α-3A*	CTAGGA**GCCATGTC**ATCTCTA	This study
O2c	*pGK1*	CTAGGT**GCCATGTC**ATCTATA	DeRose et al. (1989)
O2c	*22Z-4*	GTAGAT**GCCATATC**ATCTATA	Schmidt et al. (1992)

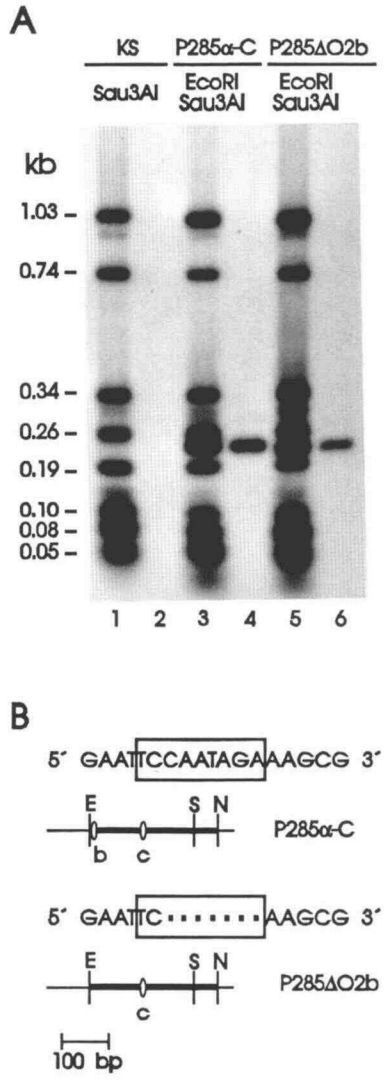

Figure 4. Binding of the O2 Protein to the Wild Type and Mutant 22-kD–like α-Coixin Promoter Fragment.

(A) A plasmid containing the wild 285-bp α-coixin promoter fragment (P285α-C), a mutant version in which the O2b box was deleted (P285ΔO2b), and the pBluescript KS+ (KS) vector was digested with EcoRI and Sau3AI or Sau3AI, end labeled, and incubated with the immunoselected β-Gal::O2 fusion protein. Odd-numbered lanes show the labeled restriction fragments that were incubated with the fusion protein. Even-numbered lanes show the fragments that were specifically retained by the β-Gal::O2 fusion. The lengths of the pBluescript KS+ Sau3AI fragments are given at left in kilobases.

(B) Partial restriction map of the subcloned promoter region from the α-coixin *α-3B* gene. The putative O2 target sites O2b and O2c are represented by ovals. The wild-type sequence spanning the O2b box (boxed sequence) is represented in single-letter code over its corresponding restriction map. The deleted nucleotides in the O2b box of P285ΔO2b (deleted version of boxed sequence shown above) are shown as points. Abbreviations for restriction sites are as follows: E, EcoRI; N, NcoI; S, Sau3AI.

coated microprojectiles. The success of this method in the analysis of promoter–regulatory protein interaction has been demonstrated previously (Oeda et al., 1991; Roth et al., 1991).

Figure 5 shows the different constructs used to analyze the role of the O2a and O2c boxes of α-coixin and α-zein promoters. Such constructs consist of reporter plasmids expressing the β-glucuronidase (*GUS*) gene under the control of the intact promoters (Pα-CxGUS and Pα-Z4GUS in Figure 5) or deleted versions containing only the fragment from the EcoRI site to the ATG codon (P285α-CGUS and P276α-Z4GUS in Figure 5). The deleted versions lacked the O2a box, the prolamin-box, and any other possible upstream *cis*-acting element. As a negative control, we used a plasmid in which the *GUS* gene was placed under the control of the α-coixin promoter fused in the inverted orientation (Pα-Cs[−]GUS in Figure 5). These constructs were delivered into immature maize endosperm alone or together with the effector plasmid (pRT101-O2) expressing the intact O2 protein from maize.

As shown in Figure 6, the intact α-coixin (bar A) and α-zein (bar C) promoters conditioned similar levels of activities. When the sequence upstream of the EcoRI site was deleted from both promoters, the activity was reduced ~50% for the α-zein (Figure 6, bar D), whereas for the α-coixin the activity was not significantly ($P < 0.05$) altered in relation to the intact promoter (Figure 6, bar B). These results agree with the DNA binding assay, indicating that the preferred O2 target site in the α-zein promoter is the O2a box, whereas in the α-coixin it is the O2c box. As expected, no activity was observed from the negative control construct (Figure 6, bar F).

When cotransformed with the effector plasmid pRT101-O2, a reproducible level of enhancement in GUS activity was observed for all the reporter plasmids (Figure 6, bars A+G to D+G), exempting the negative control (Figure 6, bar F+G). In addition, after cotransfection, blue spots were also observed in the pericarp, as shown in Figure 7 (compare Figures 7A, 7C, 7F, and 7H with 7B, 7D, 7G, and 7I, respectively), unequivocally indicating the occurrence of O2-mediated transactivation.

Strikingly, transactivation was also observed for the P276α-Z4GUS construct, suggesting that another sequence of the −276-bp α-zein promoter may also be recognized by the O2 protein in vivo. Because the −276-bp promoter lacks its preferred O2a target site in this construct, this enhancement of expression was expected to be lower than that observed for the intact α-zein *22Z-4* promoter (Pα-Z4GUS), which contains both O2 boxes. However, as can be seen in Figure 6 (bars C+G and D+G), both constructs reached the same level of expression. This could be explained by a condition in which a large amount of O2 protein, which is transiently expressed by pRT101-O2, increased the chance of O2 binding to the putatively less efficient O2 target site of the α-zein promoter.

Because the O2 protein is not able to transactivate the rice *actin1* promoter (data not shown), our results suggested a model in which the O2-mediated enhancement of transcription is a consequence of direct interaction with these prolamin gene promoters and not due to a general increase in transcription.

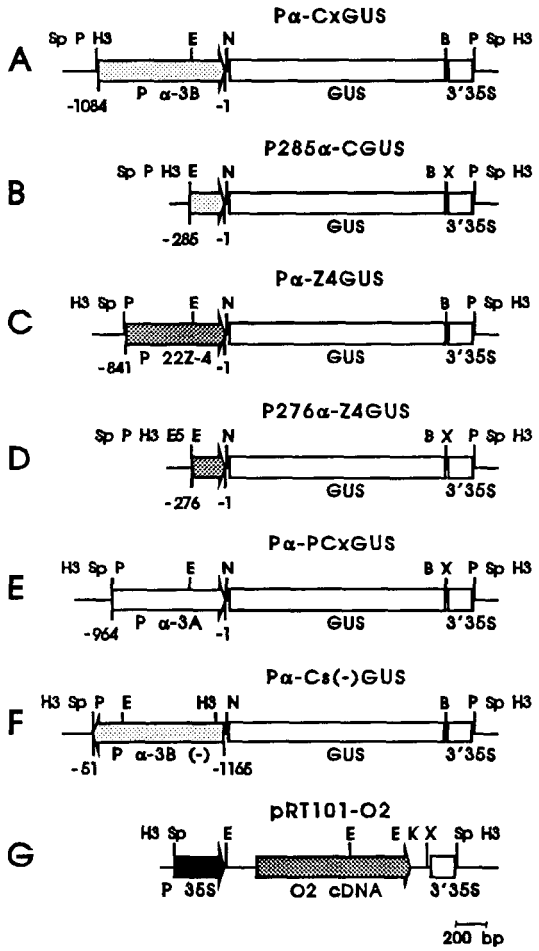

Figure 5. Schematic Representation of Chimeric *GUS* Constructs Used To Analyze the Interaction between the O2 Protein and the 22-kD–like α-Coixin and 22-kD α-Zein Promoters.

Distances are relative to the A residue of the initiator ATG codon. Unique restriction site enzymes are B, BamHI; E, EcoRI; E5, EcoRV; H3, HindIII; K, KpnI; N, NcoI; P, PstI; Sp, SphI; X, XbaI.

(A) Pα-CxGUS, the *GUS* + 35S poly(A) cassette under the control of a 1.08-kb *α-3B* coixin promoter.

(B) P285α-CGUS, the *GUS* + 35S poly(A) cassette under the control of a 285-bp *α-3B* coixin promoter fragment that contains only the putative *boxes O2b and O2c.*

(C) Pα-Z4GUS, the *GUS* + 35S poly(A) cassette under the control of a 0.84-kb *22Z-4* zein promoter.

(D) P276α-Z4GUS, *GUS* + 35S poly(A) cassette placed under the control of a 276-bp *22Z-4* zein promoter fragment that contains only the putative boxes O2b and O2c.

(E) Pα-PCxGUS, a 0.96-kb fragment of the *α-3A* coixin pseudogene promoter controlling *GUS* + 35S poly(A) reporter gene expression.

(F) Pα-Cs(−)GUS, a 1.1-kb *α-3B* coixin promoter in a reverse orientation driving *GUS* + 35S poly(A) reporter gene expression.

(G) The effector plasmid pRT101-O2 consisting of an *O2* cDNA from *clone O2cDNA1-4 under the control of the CaMV 35S promoter and* 35S poly(A) signal. The translated sequence of the *O2* cDNA is represented as a shaded arrowhead.

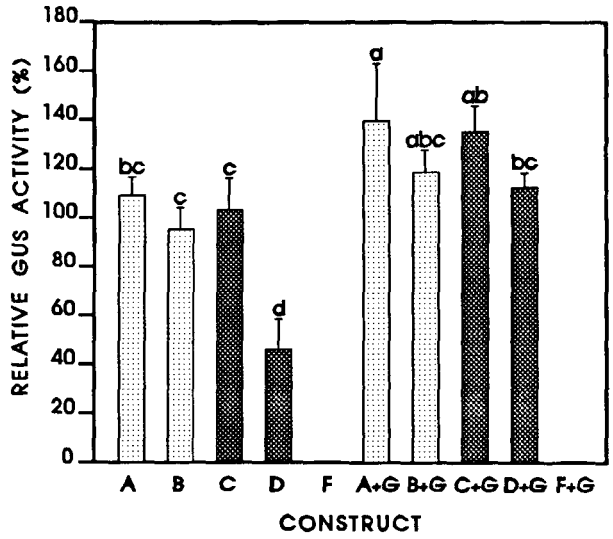

Figure 6. Transient Expression from the 22-kD–like α-Coixin and 22-kD α-Zein Whole and Truncated Promoters Vectors in Immature Maize Endosperm.

GUS activity was normalized according to the expression of the pACT1-F vector (100% activity equals average 673 [± 92] blue spots per plate). The bars correspond to activity of the construct with the same nomenclature as given in Figure 5. Bars A to D and F indicate activity of reporter constructs transfected alone, and bars A+G to F+G indicate activity upon cotransfection with the effector plasmid (Figure 5G) in a 1:2 molar concentration. The values represent the mean GUS activity, and standard deviation is represented by an error bar. Lowercase letters over the bars represent the significant differences ($P < 0.05$) as determined by analysis of variance and SNK multiple range tests (Steel and Torrie, 1980).

Tobacco Mesophyll Protoplasts

To ensure that the pRT101-O2 enhancement of expression from the α-coixin promoter is not a mere consequence of repressor titration by heterodimerization (Pysh et al., 1993), we conducted transient expression assays using the same set of constructs in tobacco mesophyll protoplasts. Our rationale was based on the assumption that tobacco does not contain an endosperm repressor. Moreover, by using tobacco protoplasts we eliminated the interference of both the endogenous O2 protein and other endosperm-specific *trans*-acting factors.

The GUS activity driven by the α-coixin and α-zein promoters was greatly enhanced upon cotransfection with the pRT101-O2 effector plasmid, as shown in Figure 8. The levels of activity from both α-coixin and α-zein intact promoters were statistically similar (Figure 8, bars A+G and C+G). Both truncated constructs were significant less efficiently transactivated by the O2 protein than the whole promoters (Figure 8, bars B+G and D+G), although the upstream deletion in the α-coixin promoter was less detrimental to the transactivation efficiency than the same deletion in the α-zein promoter. In fact, the level of

GUS activity obtained after cotransformation of the truncated α-coixin promoter was approximately twice that observed for the truncated α-zein. No activity was obtained when using the construct with the inverted α-coixin promoter (Figure 8, bars F and F+G).

These results confirmed those observed in the transient expression assay in immature endosperm. The O2a box accounted for most of the O2 transactivation of the α-zein promoter, whereas the O2c box accounted for most of the O2 transactivation of the α-coixin promoter. However, the following two questions can be raised. Why was the truncated α-zein promoter, which does not contain the O2a box, substantially transactivated? Why did the whole α-coixin promoter result in higher GUS activity when compared to the truncated version if the O2c box is already present in the deleted promoter? It appeared that in our transient expression assay, not only the preferred O2 target site but also other sequence motifs are involved in the transactivation of both promoters. Such sequences may represent alternative weakly O2 binding sites.

O2-Mediated Transcription of an 22-kD α-Coixin Pseudogene Promoter

The presence of in-frame stop codons is a common feature of multigene families containing tandemly repeated gene blocks. They are called pseudogenes, and they have been found in several cereal storage protein genes. The presence of such "premature" stop codons, however, does not imply that the gene is inactive. In fact, zein cDNA clones containing in-frame stop codons have been isolated, indicating that such pseudogenes may not be transcriptionally silent (Viotti et al., 1985). Recently, it was shown that a C-hordein pseudogene is transcriptionally active and that its amber codon can be partially suppressed during translation (Entwistle et al., 1991). In contrast, α-glutenin (Halford et al., 1989) and γ-gliadin (Rafalski, 1986) pseudogenes of wheat are reported to be transcriptionally silent.

A genomic clone containing three contiguous 22-kD–like α-coixin genes, two of which (*α-3A* and *α-3C*) harbor a

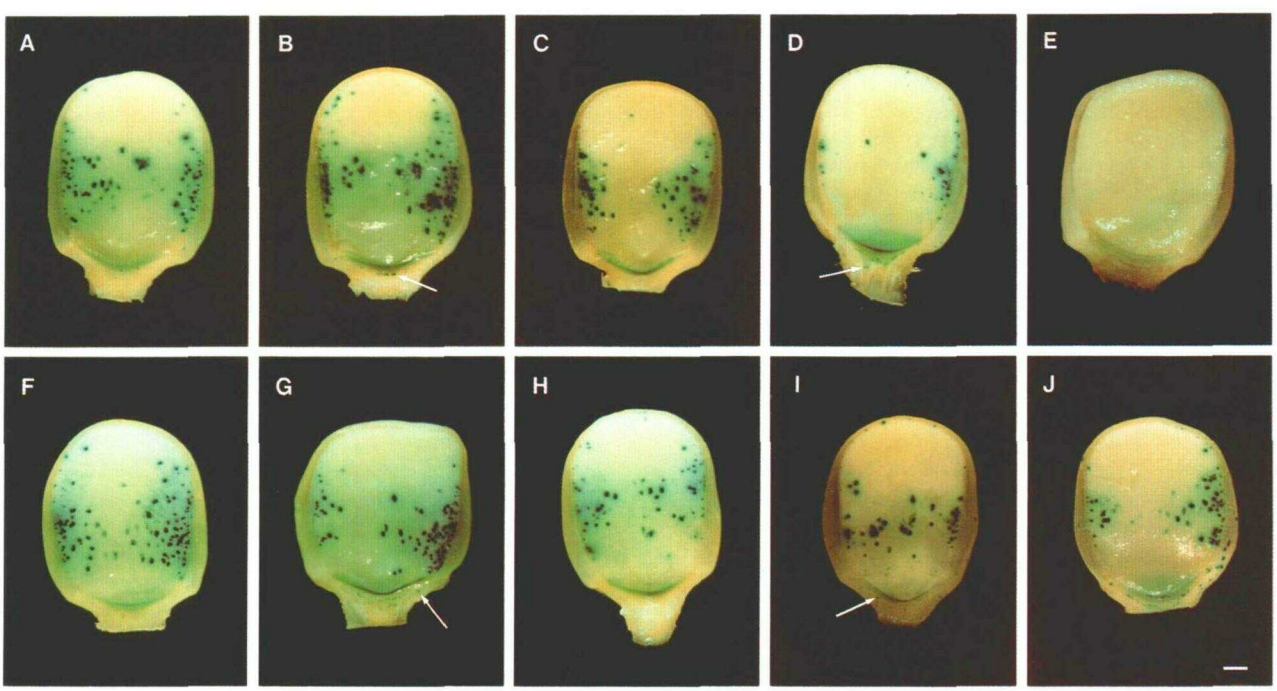

Figure 7. Spatial Distribution of GUS Activity in Immature Maize Endosperms Following Microprojectile Bombardment with the 22-kD–like α-Coixin and 22-kD α-Zein Constructs.

(A) and **(B)** Expression of the Pα-CxGUS (see Figure 5) reporter construct.
(C) and **(D)** Expression of the P285α-CGUS.
(E) Expression of Pα-Cs(−)GUS. The α-coixin promoter in a reverse orientation fails to drive GUS expression.
(F) and **(G)** Expression of Pα-Z4GUS.
(H) and **(I)** Expression of P276α-Z4GUS.
(J) Expression of pAct1-F (McElroy et al., 1990). The constitutive rice *actin1* promoter, as expected, showed nontissue-specific activity. Bar = 1 mm.
(B), **(D)**, **(G)**, and **(I)** Reporter construct expression upon cotransfection with the effector plasmid pRT101-O2. Note that in this case the pericarp showed blue spots (arrows).

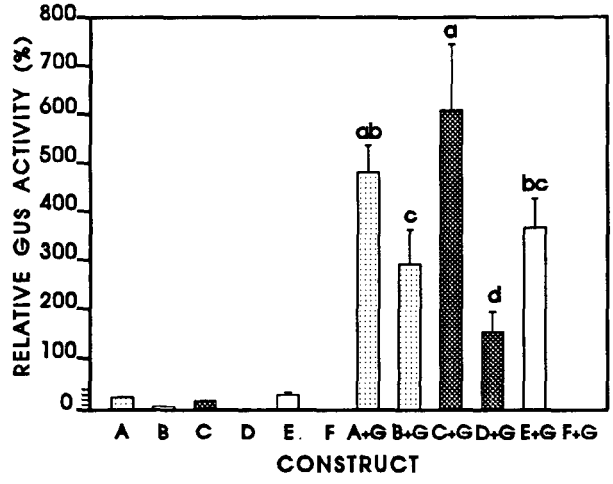

Figure 8. Transactivation of Whole and Truncated 22-kD–like α-Coixin and 22-kD α-Zein Promoters by O2 Protein in Tobacco Protoplasts.

GUS activity was normalized according to the expression derived from the pRT103GUS vector (average 165 nmol of 4-methylumbelliferone per minute per milligram of protein = 100%). The bars correspond to activity of the construct with the same nomenclature as given in Figure 5. Bars A to F represent the activity of reporter plasmids transfected alone. Bars A+G to F+G show activity upon cotransfection with the effector plasmid pRT101-O2 (see Figure 5). The values indicated show the average GUS activity and the standard deviation. Lowercase letters over the bars represent the significant differences ($P < 0.05$) between activities as determined by analysis of variance and SNK multiple range tests.

"premature" stop codon, was isolated from a *Coix* genomic library (Ottoboni et al., 1993). Because the promoter sequences of both pseudogenes are nearly identical to the normal *α-3B* gene and contain the putative O2c box, we fused the *α-3A* pseudogene promoter with the *GUS* gene (Pα-PCxGUS in Figure 5) and tested its propensity to be transactivated by the O2 protein. Transient assays in immature endosperm (data not shown) and tobacco mesophyll protoplasts (Figure 8, bars E and E+G) indicated that the *α-3A* pseudogene promoter drives the same level of expression and is transactivated in a similar manner with respect to the normal *α-3B* gene.

DISCUSSION

Comparisons of the 22-kD–like α-prolamin genes from maize, *Coix*, and sorghum have shown that they share highly conserved protein structure and putative *cis*-acting regulatory elements. The presence of *O2* homologous sequences in the *Coix* and sorghum genome (Ottoboni et al., 1993) indicates that the O2 protein may represent a conserved regulatory *trans*-acting factor. In fact, we have isolated an *O2*-like clone from a *Coix* cDNA library (A. Vettore, unpublished data). However, a perfect counterpart to the recently identified O2 target sequence 5′-TCCACGTAGA-3′ present in the 22-kD α-zein clone *22Z-4* (Schmidt et al., 1992) could not be found in the homologous α-prolamin promoters. When all of the 22-kD–like α-prolamin promoters were compared (Ottoboni et al., 1993), two homologous O2 putative target sites were found in each gene. In the α-coixin promoter, such target sites are represented by the sequences 5′-TCCTCATGAA-3′ (box O2a) and 5′-TCCAATAGA-3′ (box O2b), located at positions −301 to −291 and −281 to −273, respectively (Figure 1).

The Maize O2 Protein Recognizes a *b-32*-like Target Site in the 22-kD α-Coixin Promoter

In this study, we used DNA binding assays and DNase I footprint analysis to demonstrate that the maize O2 protein recognizes neither box O2a nor O2b in the α-coixin promoter but rather a third sequence, which we designated the O2c box. The overlapped region protected by the O2 protein on both strands of the α-coixin promoter comprises 23 nucleotides and has an inner palindromic sequence 5′-GACATGTC-3′, which presumably represents the most important requirement for O2 binding effectiveness. Although we did not perform a mutational analysis of the O2c box, further support for its importance could be indirectly addressed because (1) it is highly homologous to already published O2 binding sites in the *b-32* gene; (2) in an in vivo dimethylsulfate footprinting of a glutenin gene in wheat endosperm, it was demonstrated that a region including the motif GACATGTA is protected at the time when the gene is highly transcribed (Hammond-Kosack et al., 1993); and (3) the naturally occurring mutations in the GACATGTC-like sequence of the 22-kD α-zein promoter were sufficient to prevent the O2 protein binding.

As shown in Figure 9, the 19-kD α-zein clone *ZE19* (Spena et al., 1982) contains three highly homologous GACATGTC sequences. Such sequences are also found in other 19-kD α-zein clones (Brown et al., 1986a). One of these, the CACATGTG sequence located at the prolamin-box (Figure 9), is also present in several 22-kD α-zein genes (see Figure 1).

However, in spite of the exhibited homology, no apparent O2 binding occurs in such GACATGTC-like sequences of the 19-kD or in that of the 22-kD α-zein clones (Schmidt et al., 1992). Because the CATG core is conserved in all sequences that are not recognized by O2, we concluded that the nucleotides at positions −3, −2, +2, and +3 are also important in determining O2 binding specificity. Strikingly, even a variation that preserves the palindromic trait, as in the case of the CACATGTG sequence mentioned above, can be sufficient to abolish O2 binding. Paradoxically, a mutation that disrupts the palindrome, as occurred with the O2c box of the *α-3A* coixin gene (Figure 9), does not prevent O2 binding (Figure 2A). Therefore, it may be assumed that not only the palindromic trait but also some nucleotides at certain positions play an important role in the DNA–O2 protein interaction.

Interestingly, the protected sequences flanking the GACATGTC palindrome in the *α-3B* O2c box comprise two other smaller motifs (Figure 9). The first is TGACT, which resembles the GCN4 binding site (Hill et al., 1986), and the second is CATCTCTA, a sequence common to the 5′-flanking region of prolamin genes from several cereals, called CATC box (Kreis et al., 1986). The relevance of such motifs on the O2 protein binding is under investigation.

Surprisingly, the GACATGTC sequence showed high homology with the O2 binding sites B1 and B4 identified in the promoter of the *b-32* gene (Lohmer et al., 1991). Such a finding helps to resolve the conflicting results concerning the O2 target site reported by two different laboratories (Lohmer et al., 1991; Schmidt et al., 1992). By using the same experimental procedures and conditions reported for the identification of the O2 box in the α-zein promoter (Schmidt et al., 1992), we found a *b-32*–like target site in the α-coixin promoter. This clearly denotes that the apparently conflicting results cannot be attributed to the different experimental conditions (Schmidt et al., 1992; Izawa et al., 1993), and it is more likely that all the binding sites identified thus far can be recognized by the O2 protein even though they have different binding affinities.

The fact that in the 22-kD prolamin genes of maize and *Coix*, two different sequences can be recognized by the O2 protein raises a very interesting evolutionary issue. Because the 22-kD class of prolamin-coding genes are present in multicopy in both plants, one would suppose that the duplication events giving rise to these genes would have occurred before the events that separated the two plant species. Therefore, the regulatory system between the O2 and its prolamin-dependent genes would be the same in the two plant species. Our data support the fact that they are not. In addition, the *pGK1* 22-kD α-prolamin gene of sorghum exhibits an α-coixin–like O2 box. The issue could be solved if in the 22-kD α-prolamin multigene family both kinds of O2 target sites were present in the ancestor of maize, sorghum, and *Coix*. Increasing the number of sequences of 22-kD–like α-prolamin promoters could provide more information to confirm our hypothesis. Interestingly, the *ψgZ22.8* α-zein clone seems to be an intermediate between the *α-3B* α-coixin and the *22Z-4* α-zein genes because it contains an *α-3B*–like O2a box and a *22Z-4*–like O2c box (see Figure 1). In contrast, the contiguous *gZ22.8* α-zein clone (Thompson et al., 1992) presents the O2a and the O2c target sites similar to those of the *22Z-4* α-zein clone (Figure 1).

```
                          |  Footprinted region   |
α-3B   -188 CAAAATTGACTAGGA GACATGTC ATCTCTAGCTTA
α-3A   -190 CAAAATCGACTAGGA GCCATGTC ATCTCTAGCTTA
ZE19   -185 CAAAACCAACTAGAT ACCATGTC ATCTCTACCTTA
       -309 GTCACAACATTGTCA CCCATGTA TTTGGACAATAC
       -344 ATGTGGCTATCGTTA CACATGTG TAAAGGTATTGC
22Z-4  -340 TCATGTTAAGGTTGT CACATGTG TAAAGGTGAAGA
```

Figure 9. Comparison of the α-Coixin O2c Site with Homologous Sequences of 22- and 19-kD Zein Promoters from *22Z-4* and *ZE19*.

Positions are relative to the translation start. The O2 target sequence footprinted in the α-coixin promoter is indicated. The GCN4-like motif, the O2c palindromic core, and the CATC box are indicated by underline, boldface italic letters, and a double underline, respectively. The sources are *α-3A*, *α-3B* (Ottoboni et al., 1993), *ZE19* (Spena et al., 1982), and *22Z-4* (Schmidt et al., 1992).

The O2 Protein Binds Target Sites without ACGT Core

Without losing binding affinity, the O2 protein supports a relatively high degree of degeneracy at the nucleotides flanking the ACGT core (Izawa et al., 1993). However, the replacement of the ACGT core by ACAT was sufficient to abolish binding in vitro (Schmidt et al., 1992) and transactivation in vivo (Ueda et al., 1992) of the α-zein promoter by the O2 protein. Nevertheless, this conflicts with the fact that the B1, B2, B3, and B4 sites to which O2 is reported to bind in the promoter of the *b-32* gene (Lohmer et al., 1991) and the O2c target site of the α-coixin do not contain an ACGT core. Thus, although most of the characterized plant bZIP proteins recognize the ACGT core, it does not imply that they necessarily require this core. For example, the TGACGT/C binding proteins also interact with the TGACGC motif of the cauliflower mosaic virus (CaMV) 35S promoter (Katagiri et al., 1989). Moreover, in most cases, the importance of the ACGT core was analyzed by mutations that create asymmetric cores (Oeda et al., 1991; Schindler et al., 1992; Izawa et al., 1993), whereas in the O2c α-coixin box, the CATG core remains perfectly symmetric. This does not mean, however, that the binding will remain unaffected by any alteration to the ACGT core that preserves symmetry. For example, the substitution of the ACGT core by GTAC in boxes II and III of the chalcone synthase parsley promoter prevents the recognition by the common plant regulatory factor bZIP proteins (Armstrong et al., 1992).

Can Low-Affinity Binding Sites in Vitro Mediate Transcriptional Activation in Vivo?

Using immature maize endosperm and tobacco protoplast, we demonstrated that the transiently coexpressed maize O2 protein enhanced the activity of the heterologous 22-kD–like α-coixin promoter at the same level as it does for the maize 22-kD α-zein promoter. Furthermore, even when the region upstream from the EcoRI site was deleted, thus preserving only the O2c box, the O2-mediated enhancement of expression was maintained on both promoters. Nevertheless, the resulting expression level was lower than that obtained with the nondeleted promoters. In this case, however, the truncated α-coixin promoter was transactivated to a higher level than the truncated

α-zein. From this data, we proposed that both the O2a box in the α-zein promoter and the O2c in the α-coixin promoter do not represent the unique *cis* elements (albeit being the most important) responsible for the resulting O2 transactivation.

In the binding assay in vitro, however, only the O2a box of the α-zein and O2c box of the α-coixin strongly interacted with the O2 protein. Because the transactivation experiments performed in maize endosperm and tobacco protoplasts in vivo gave similar results, an artifact seemed unlikely. This discrepancy could be explained if the conditions of the binding assay in vitro were too restrictive to detect weak but selective interaction of the protein to the target fragment. In fact, when we performed a footprint analysis of the α-coixin promoter without the DNA competitor poly(dI-dC), the sequence CATGCATG, located just downstream of the prolamin-box (Figure 1), was weakly protected by the β-Gal::O2 protein (data not shown).

It is also possible that the protein–DNA complex was stabilized in vivo by other proteins not present in the assay in vitro. In this instance, binding affinity may not be a good indication of transcriptional rates, the biologically relevant parameter. For example, in the ethyl methanesulfonate–induced *o2* mutant *o2-676* (Aukerman et al., 1991), members of the 22-kD α-zein class are expressed, while the rescued O2-676 protein is unable to bind the 22-kD α-zein promoter in vitro.

The results presented here showed that the O2 protein recognizes and binds to alternative sequences, even if they lack the ACGT core. This corroborates the hypothesis that one factor may have different affinities for multiple sites and points to the necessity of combining binding experiments in vitro with expression assays in vivo to dissect the biologically relevant parameters involved in the protein–DNA interaction. Moreover, we stress the importance of finding among related genes the occurrence of naturally preserved variants of functional *cis* elements to obtain insights into the understanding of the evolution of gene regulation mechanisms.

METHODS

Construction of Plasmids

All sequence position numbers refer to the ATG initiation codon. The pACT1-F plasmid (McElroy et al., 1990), which was used as a positive control in the endosperm transient expression assay, contained the β-glucuronidase (*GUS*) gene under the control of the strong constitutive rice *actin1* promoter.

P285α-CGUS is a 0.3-kb fragment that was amplified from an EcoRI-StuI *α-3B* promoter subclone (Ottoboni et al., 1993) using the universal sequencing primer and the primer Alfacoix–ATG 5′-TGCTGGATCCATGGCGACAAGATGCTGCT-3′ (New England BioLabs, Inc., Beverly, MA), which is complementary to the −18 to +8 fragment and creates NcoI and BamHI restriction sites over the ATG. The amplified fragment was digested with EcoRI and BamHI and inserted into pBluescript KS+ (Stratagene). The resulting clone *P285α-C* was verified by sequencing of both strands. The cloned fragment was then recovered by digestion with HincII and NcoI and inserted into the *pRT103* plasmid (Töpfer et al., 1987), which was digested with the same enzymes, replacing the cauliflower mosaic virus (CaMV) 35S promoter. The *GUS* coding region was excised from pJII140 (Gallie et al., 1987) by digestion with NcoI and BamHI and fused in the corresponding sites among the 285-bp *α-3B* promoter and the CaMV 35S poly(A) signal.

P285ΔO2b is the 285-bp promoter fragment of P285α-C that was amplified using the universal sequencing primer and the primer Alfacoix-272 5′-CTGCGAATTCAAGCGACACTTTTTTCTT-3′ (New England BioLabs, Inc.), which is complementary to the −285 to −262 and creates a deletion of the CAATAGA sequence in the O2b box. The amplified fragment was digested with EcoRI and BamHI and inserted into pBluescript KS+. The resulting clone was verified by sequencing both strands.

Pα-CxGUS is the *α-3B* promoter fragment that spans residues from −1084 to −285 and was excised from the *α-3C* coixin clone (Ottoboni et al., 1993) by digestion with HindIII and EcoRI. The 0.8-kb fragment was ligated into the corresponding sites of P285α-C, giving rise to the whole *α-3B* promoter clone Pα-Cx. Afterward, this promoter was recovered by HincII and NcoI digestion and inserted at the same sites of pRT103 replacing the CaMV 35S promoter, which resulted in the *Pα-Cx103* clone. The *GUS* gene was inserted as given above.

Pα-Cs(−)GUS is an EcoRI-Sau3AI fragment spanning nucleotides −285 to −50 of the *α-3B* (Ottoboni et al., 1993) promoter. It was subcloned in the EcoRI-BamHI fragment of the pBluescript KS+ vector. Subsequently, the upstream promoter fragment from −1165 to −285, which was obtained by digesting the *α-3C* clone (Ottoboni et al., 1993) with HincII and EcoRI, was joined, resulting in the *α-3B* promoter clone Pα-Cs. The whole fragment was excised with HincII and SstI, blunt ended with T4 DNA polymerase, and ligated with pRT103, which was linearized with HincII and XhoI and blunt ended with T4 DNA polymerase. Recombinant plasmids were screened by replica plate hybridization, and those with the promoter in the reverse orientation with respect to the CaMV 35S poly(A) signal were identified by restriction mapping. The *GUS* gene was then inserted as mentioned previously.

Pα-PCxGUS is an XbaI clone containing the pseudogene *α-3A* (Ottoboni et al., 1993) from positions −962 to +1081. It was used as a template for polymerase chain reaction amplification of the promoter region. The primers used were the reverse sequencing primer and the Alfacoix-ATG oligonucleotide. The amplified product was cloned using the TA cloning system K2000-01 (Invitrogen, San Diego, CA) and sequenced on both strands. After digestion with XbaI and treatment with T4 DNA polymerase, the promoter fragment was excised with NcoI and used to replace the CaMV 35S promoter of pRT103 linearized with HincII and NcoI, resulting in the *Pα-PCx103* clone. Subsequently, the *GUS* gene was cloned into the NcoI-BamHI sites as described above.

P276α-Z4GUS is the fragment spanning nucleotides −276 to +1 of the *22Z-4* α-zein gene (Schmidt et al., 1992) that was excised as an EcoRI-NcoI fragment from the vector PZ4Luc (kindly provided by R.J. Schmidt, University of California at San Diego) and used to replace the α-coixin promoter of P285α-CGUS.

Pα-Z4GUS was derived from the PZ4Luc plasmid that was digested with AccI, treated with the Klenow fragment of DNA polymerase I, and then restricted with NcoI to obtain an 840-bp *22Z-4* promoter sequence spanning nucleotides −841 to +1. Subsequently, in a tripartite ligation, this fragment was fused to pRT103 digested with HincII-BamHI and the NcoI-BamHI *GUS* coding sequences. The *Pα-Z4103* clone used in the DNA binding assay was obtained by fusing the above mentioned 840-bp *22Z-4* fragment to pRT103 digested with HincII and NcoI.

pRT103GUS was obtained by ligating the NcoI-BamHI *GUS* fragment into the corresponding sites of pRT103.

pRT101-O2 is the complete maize *Opaque2* (*O2*) cDNA from clone *O2cDNA1-4* (Schmidt et al., 1990) obtained by partial digestion with EcoRI and subsequently inserted into pRT101 (Töpfer et al., 1987) linearized with EcoRI and treated with calf intestinal alkaline phosphatase (New England BioLabs, Inc.) according to the manufacturer's instructions. A clone in which the *O2* cDNA entered in the correct orientation was identified by restriction mapping.

Transient Expression Assays in Immature Maize Endosperm

The commercial maize hybrid F-352 from Sementes Agroceres S/A (São Paulo, Brazil) was grown at the experimental field of University of Campinas and self-pollinated. Ears were harvested at 15 days after pollination, surface sterilized for 15 min with 5% commercial bleach, and rinsed four times in distilled water. Seeds were dissected from the cob and sectioned longitudinally; 16 sections were flattened on 100-mm-diameter Petri dishes containing 20 mL of MS medium (Murashige and Skoog, 1962), with the sliced surface facing upward. Five micrograms of CsCl-purified DNA alone or coprecipitated with 10 μg pRT101-O2 was used to coat 3 mg of 1- to 3-μm-diameter gold particles according to the method of Ye et al. (1990). The endosperms were bombarded twice with 0.5 μg DNA (or 1.5 μg when cotransfected) using a homemade helium particle delivery device (Ye et al., 1990). After bombardment, the samples were incubated for 48 hr in the dark at 27 ± 1°C. The endosperms were then stained for GUS activity according to the method of Jefferson (1987). To minimize experimental errors, all constructs were evenly analyzed using seeds of the same ear.

Transient Expression Assays in Tobacco Mesophyll Protoplasts

Nicotiana tabacum cv SR1 plants were grown in axenic conditions on MS salts (Murashige and Skoog, 1962). Mesophyll protoplasts were prepared from young leaves as described by Saul et al. (1988). Samples of 0.5×10^6 protoplasts were used for PEG transformation (Saul et al., 1988; Negrutiu et al., 1990). Ten micrograms of Pα-CxGUS DNA, or equimolar amounts of the other constructs, plus sonicated salmon sperm DNA to a final amount of 43 μg were used in the transformation of each aliquot of protoplast. After incubation for 24 hr, the protoplasts were pelleted, lysed in GUS extraction buffer (Jefferson, 1987), and centrifuged; the supernatant was used for protein estimation (Bradford, 1976). One microgram of protein was used in the fluorometric GUS assay (Jefferson, 1987).

DNA Binding Assay

The DNA binding assay was performed with a β-galactosidase–O2 (β-Gal::O2) fusion protein according to the method of Schmidt et al. (1992). Approximately 40 ng of ^{32}P-labeled Sau3AI, EcoRI-Sau3AI, or EcoRI-XbaI digests of plasmids Pα-Cx103, Pα-Z4103, Pα-PCx103, P285α-C, P285ΔO2b, and pBluescript KS+ (used as a negative control) was incubated with 15 μL protein A–Sepharose beads containing the β-Gal::O2 immunocomplex for 90 min at room temperature with intermittent agitation. Washes, processing, and agarose gel electrophoresis of the retained fragment were performed as described by Schmidt et al. (1992).

DNase I Footprint

The 260-bp Sau3AI restriction fragment from *α-3B*, which contained all the putative O2 target sites, was cloned into the BamHI site of pBluescript KS+. Either a HindIII-NotI or an EcoRV-XbaI fragment containing the 260-bp sequence and flanking polylinker sites was isolated from an agarose gel and 3′ end labeled by partial fill-in with α-^{32}P-dATP, the other deoxynucleotide triphosphates (when required), and the Klenow fragment of DNA polymerase I, thereby labeling the bottom or the top strand, respectively. Note that in contrast to the T4 polynucleotide kinase labeling procedure in the Klenow method that was just described, the 3′ end of the strand is labeled rather than the 5′ end. The labeled fragments were then purified from a 7.5% polyacrylamide gel, and for each reaction, 35,000 cpm of DNA was incubated with 0, 10, 20, and 30 μL of the protein A–Sepharose beads containing the immunoprecipitated β-Gal::O2 fusion plus 30, 20, 10, and 0 μL of the protein A–Sepharose beads, respectively, in a total volume of 50 μL of binding buffer containing 12.5 mM Hepes, pH 7.5, 50 mM KCl, 10% glycerol, 0.05% Nonidet P-40, 0.5 mM DTT, and 0.7 μg poly(dI-dC). Reaction mixtures were incubated for 15 min at 4°C and then digested with 0.3 units of DNase I according to recommendations of the Sure Track Footprinting Kit (No. 27-9101-01; Pharmacia LKB Biotechnology). The resulting fragments were separated by electrophoresis on a 5 or 6% sequencing gel. The same end-labeled DNA was treated with the G+A and G chemical cleavage reaction, according to the Sure Track Footprinting Kit or the Maxam-Gilbert Sequencing Kit (No. SEQ-1; Sigma) manufacturer's recommendations, respectively, and run on lanes adjacent to the footprinting reactions.

ACKNOWLEDGMENTS

We thank Edson L. Kemper for technical help. We are very grateful to Dr. Robert J. Schmidt, University of California at San Diego, for supplying pZ4Luc and *O2cDNA1-4* and for instruction in the DNA binding assays; to Dr. Reinhard Töpfer, Max Planck Institut, Cologne, Germany, for supplying pRT101 and pRT103 vectors; to Diatech Ltd., London, and Dr. T. Michael A. Wilson, Scottish Crop Research Institute, Invergowrie, Dundee, Scotland, for supplying the pJII140 plasmid; and to Dr. Ray Wu, Cornell University, Ithaca, NY, for providing the pACT1-F vector. We also thank Dr. Francisco G. Nobrega, Universidade de São Paulo, São Paulo, Brazil, for instruction in the Maxam-Gilbert reactions. This work was financed by grants to P.A. from Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)/ Projeto Temático de Equipe (PTE)-90/3808-5. P.A., A.L., and L.M.M.O. received research fellowships from CNPq. J.A.Y. and G.C.N. received postgraduate fellowships from CNPq and Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Received October 4, 1993; accepted December 6, 1993.

REFERENCES

Armstrong, G.A., Weisshaar, B., and Hahlbrock, K. (1992). Homodimeric and heterodimeric leucine zipper proteins and nuclear factors

from parsley recognize diverse promoter elements with ACGT cores. *Plant Cell* **4,** 525–537.

Aukerman, M.J., Schmidt, R.J., Burr, B., and Burr, F.A. (1991). An arginine to lysine substitution in the bZip domain of an *opaque-2* mutant in maize abolishes specific DNA binding. Genes Dev. **5,** 310–320.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Brown, J.W.S., Wandelt, C., and Feix, G. (1986a). The upstream regions of zein genes: Sequence analysis and expression in the unicellular green alga *Acetabularia*. Eur. J. Cell Biol. **42,** 161–170.

Brown, J.W.S., Wandelt, C., Feix, G., Neuhaus, G., and Schweiger, H.G. (1986b). The upstream regions of zein genes: Sequence analysis and expression in the unicellular alga *Acetobacteria*. Eur. J. Cell. Biol. **42,** 161–170.

Burr, B., and Burr, F.A. (1982). Three mutations in *Zea mays* affecting zein accumulation: A comparison of zein polypeptides, *in vitro* synthesis and processing, mRNA levels and genomic organization. J. Cell Biol. **94,** 201–206.

Burr, B., Burr, F.A., John, T.P., Thomas, M., and Davis, R.D. (1982). Zeins: Storage protein family of maize. J. Mol. Biol. **154,** 33–49.

DeRose, R.T., Ma, D.P., Kwon, I.S., Hasnain, S.E., Klassy, R.C., and Hall, T.C. (1989). Characterization of the kafirin gene family from sorghum reveals extensive homology with zein from maize. Plant Mol. Biol. **12,** 245–256.

Di Fonzo, N., Mandocchi, L., Salamini, F., and Soave, C. (1986). Purification and properties of an endospermic protein of maize associated with the *opaque-2* and *opaque-6* genes. Planta **167,** 587–594.

Entwistle, J., Knudsen, S., Müller, M., and Cameron-Mills, V. (1991). Amber codon suppression: The *in vivo* and *in vitro* analysis of two C-hordein genes from barley. Plant Mol. Biol. **17,** 1217–1231.

Esen, A. (1986). Separation of alcohol-soluble proteins (zeins) from maize into three fractions by differential solubility. Plant Physiol. **80,** 623–627.

Gallie, D.R., Sleat, D.E., Watts, J.N., Turner, P.C., and Wilson, T.M.A. (1987). A comparison of eukaryotic viral 5′-leader sequences as enhancers of mRNA expression *in vivo*. Nucl. Acids Res. **15,** 8693–8711.

Hagen, G., and Rubenstein, I. (1981). Complex organization of zein genes in maize. Gene **13,** 239–249.

Halford, N.G., Forde, J., Shewry, P.R., and Kreis, M. (1989). Functional analysis of the upstream regions of a silent and an expressed member of a family of wheat seed protein genes in transgenic tobacco. Plant Sci. **62,** 207–216.

Hammond-Kosack, M.C.U., Holdsworth, M.J., and Bevan, M.W. (1993). *In vivo* footprinting of a low molecular weight glutenin gene (LMWG-1D1) in wheat endosperm. EMBO J. **12,** 545–554.

Hartings, H., Maddaloni, M., Lazzaroni, N., Di Fonzo, N., Motto, M., Salamini, F., and Thompson, R. (1989). The *O2* gene which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. EMBO J. **8,** 2795–2801.

Hill, D.E., Hope, I.A., Macke, J.P., and Struhl, K. (1986). Saturation mutagenesis of the yeast *his3* regulatory site: Requirements for transcriptional induction and for binding by GCN4 activator protein. Science **234,** 451–457.

Izawa, T., Foster, R., and Chua, N.-H. (1993). Plant bZIP protein DNA binding specificity. J. Mol. Biol. **230,** 1131–1144.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The *GUS* gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Katagiri, F., Lam, E., and Chua, N.-H. (1989). Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340,** 727–730.

Kodrzycki, R., Boston, R.S., and Larkins, B.A. (1989). The *opaque-2* mutation of maize differentially reduces zein gene transcription. Plant Cell **1,** 105–114.

Kreis, M., Williamson, M.S., Forde, J., Schmutz, D., Clark, J., Buxton, B., Pywell, J., Harris, N., Shewry, P.R., Forde, B.G., and Miflin, B.J. (1986). Differential gene expression in the developing barley endosperm. Phil. Trans. R. Soc. Lond. **B314,** 355–365.

Leite, A., Ottoboni, L.M.M., Targon, M.L.P.N, Silva, M.J., Turcinelli, S.R., and Arruda, P. (1990). Phylogenetic relationship of zeins and coixins as determined by immunological cross-reactivity and Southern blot analysis. *Plant Mol. Biol.* **14,** 743–751.

Liu, C.-N., and Rubenstein, I. (1992). Genomic organization of an α-zein gene cluster in maize. Mol. Gen. Genet. **231,** 304–312.

Lohmer, S., Maddaloni, M., Motto, M., Di Fonzo, N., Hartings, H., Salamini, F., and Thompson, R.D. (1991). The maize regulatory locus *opaque-2* encodes a DNA-binding protein which activates the transcription of the *b-32* gene. EMBO J. **10,** 617–624.

McElroy, D., Zhang, W., Cao, J., and Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. Plant Cell **2,** 163–171.

Motto, M., Maddaloni, M., Ponziani, G., Brembilla, M., Marotta, R., Di Fonzo, N., Soave, C., Thompson, R., and Salamini, F. (1988). Molecular cloning of the *o2-m5* allele of *Zea mays* using transposon marking. Mol. Gen. Genet. **212,** 488–494.

Motto, M., Di Fonzo, N., Hartings, H., Maddaloni, M., Salamini, F., Soave, C., and Thompson, R.D. (1989). Regulatory genes affecting maize storage protein synthesis. In Oxford Surveys of Plant Molecular and Cell Biology, Vol. 6, B.J. Miflin, ed (Oxford: Oxford University Press), pp. 87–114.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Negrutiu, I., Dewulf, J., Pietrzak, M., Botterman, J., Rietveld, E., Wurzer-Figurelli, E.M., De Ye, and Jacobs, M. (1990). Hybrid genes in the analysis of transformation conditions: II. Transient expression vs. stable transformation—Analysis of parameters influencing gene expression levels and transformation efficiency. Physiol. Plant. **79,** 197–205.

Oeda, K., Salinas, J., and Chua, N.-H. (1991). A tobacco bZip transcription activator (TAF-1) binds to a G-box-like motif conserved in plant genes. EMBO J. **10,** 1793–1802.

Oliphant, A.R., Brandl, C.J., and Struhl, K. (1989). Defining the sequence specificity of DNA-binding proteins by selecting binding sites from random-sequence oligonucleotides: Analysis of yeast GCN4 protein. Mol. Cell. Biol. **9,** 2944–2949.

Osborne, T.B., and Mendel, L.B. (1914). Nutritive properties of proteins of the maize kernel. J. Biol. Chem. **18,** 1–6.

Ottoboni, L.M.M., Leite, A., Targon, M.L.P.N., Silva, M.J., and Arruda, P. (1990). Heterogeneity of *Coix*, maize and teosinte prolamins detected by isoelectric focusing. Rev. Bras. Genet. **13,** 313–322.

Ottoboni, L.M.M., Leite, A., Yunes, J.A., Targon, M.L.P.N., Souza Filho, G.A., and Arruda, P. (1993). Sequence analysis of 22 KDa-like α-coixin genes and their comparison with homologous zein and

kafirin genes reveals highly conserved protein structure and regulatory elements. Plant Mol. Biol. **21,** 765–778.

Pysh, L.D., Aukerman, M.J., and Schmidt, R.J. (1993). OHP1: A maize basic domain/leucine zipper protein that interacts with Opaque2. Plant Cell **5,** 227–236.

Rafalski, J.A. (1986). Structure of wheat *gamma*-gliadin genes. Gene **43,** 221–229.

Roth, B.A., Goff, S.A., Klein, T.M., and Fromm, M.E. (1991). *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian *myb* and *myc* binding sites. Plant Cell **3,** 317–325.

Saul, M.W., Shillito, R.D., and Negrutiu, I. (1988). Direct DNA transfer to protoplast with and without electroporation. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht: Kluwer Academic Publishers), pp. A1.1–A1.16.

Schindler, U., Beckmann, H., Kadesch, T., and Cashmore, A.R. (1992). DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor GBF1. EMBO J. **11,** 1275–1289.

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

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

Shotwell, M.A., and Larkins, B.A. (1989). The biochemistry and molecular biology of seed storage proteins. In The Biochemistry of Plants: A Comprehensive Treatise, Vol. 15, A. Marcus, ed (New York: Academic Press), pp. 296–345.

Soave, C., Righetti, P.G., Lorenzoni, C., Gentinetta, E., and Salamini, F. (1976). Expressivity of *opaque-2* gene at the level of zein molecular components. Maydica **21,** 61–75.

Spena, A., Viotti, A., and Pirrota, V. (1982). A homologous repetitive block structure underlies the heterogeneity of heavy and light chain zein genes. EMBO J. **1,** 1589–1594.

Steel, R.G.D., and Torrie, J.H. (1980). Principles and Procedures of Statistics: A Biometrical Approach, 2nd ed (New York: McGraw-Hill).

Targon, M.L.N., Ottoboni, L.M.M., Leite, A., Ludevid, D., Puigdomènech, P., and Arruda, P. (1992). Synthesis and deposition of coixin in seeds of *Coix lacryma-jobi*. Plant Sci. **83,** 169–180.

Thompson, G.A., Siemieniak, D.R., Sieu, L.C., Slightom, J.L, and Larkins, B.A. (1992). Sequence analysis of linked maize 22 kDa α-zein genes. Plant Mol. Biol. **18,** 827–833.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H.H. (1987). A set of plant expression vectors for transcriptional fusions. Nucl. Acids Res. **15,** 5890.

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

Viotti, A., Caloro, G., Vitale, A., and Sala, E. (1985). Each zein gene class can produce polypeptides of different sizes. EMBO J. **4,** 1103–1110.

Wandelt, C., and Feix, G. (1989). Sequence of a 21-kD gene from maize containing an in-frame stop codon. Nucl. Acids Res. **17,** 2354.

Ye, G.-N., Daniell, H., and Sanford, J.C. (1990). Optimization of delivery of foreign DNA into higher-plant chloroplasts. Plant Mol. Biol. **15,** 809–819.