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The maize EmBP-1 orthologue differentially regulates Opaque2-dependent gene expression in yeast and cultured maize endosperm cells

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Abstract

In addition to the bZIP protein Opaque2 (O2), there are other maize endosperm nuclear proteins that recognize the O2 box in 22 kDa zein gene promoters. In an effort to understand the effect of these factors on 22 kDa zein expression, we have cloned one of these and identified it as the putative maize (*Zea mays* L.) orthologue of the wheat bZIP protein EmBP-1 (mEmBP-1). The mEmBP-1 protein exhibits 52% sequence identity and 68% similarity with the wheat protein and recognizes a similar spectrum of DNA sequences, albeit with slightly altered specificity. The *mEmBP-1* gene exists as duplicate loci in maize on chromosomes 7S (*mEmBP-1a*) and 2L (*mEmBP-1b*). The *mEmBP-1* genes are expressed in endosperm, embryo, immature ears, tassel, roots, and seedling shoots at low levels. Although mEmBP-1 binds to the O2 box from the 22 kDa zein gene promoter as a homodimer, it is unable to heterodimerize with O2. The mEmBP-1 protein can activate transcription from a truncated promoter containing a pentamer of the O2 site in yeast cells; however, it inhibited regulated transcription of a 22 kDa zein promoter in a transient expression assay using cultured maize endosperm cells.

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; bZIP, basic leucine zipper; cDNA, complementary DNA; DAP, days after pollination; DAPI, 4,6-diamidino-2-phenylindole; DTT, dithiothreitol; EmBP-1, embryo maturation binding protein 1; GST, glutathione *S*-transferase; GUS, β-glucuronidase; NOS, nopaline synthase; NLS, nuclear localization signal; O2, Opaque2; OHP1, O2 heterodimerizing protein 1; RACE, rapid amplification of cDNA ends; RPA, ribonuclease protection assay; SDS, sodium dodecyl sulfate; TUSC, Trait Utility System for Corn.

Introduction

Wheat EmBP-1 is a basic leucine zipper (bZIP) protein which has been implicated in the mechanisms of abscisic acid (ABA)-mediated expression through its ability to bind specifically to the ABA response element (ABRE) from the wheat *Em* gene (Guiltinan *et al.*, 1990). As a major class of eukaryotic transcription factors, bZIP proteins are defined by a region rich in basic amino acids involved in base-specific DNA contacts adjacent to a series of heptameric leucine repeats which are required for protein dimerization (Landschulz *et al.*, 1988; reviewed in Hurst, 1994).

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number Y15165.

In plants, bZIP proteins have been isolated with overlapping DNA-binding specificities (Izawa *et al.*, 1993; Foster *et al.*, 1994), and many of these proteins recognize sequences with an ACGT core. Plant bZIP proteins have been classified into three groups according to binding preferences for base sequences flanking the core (Williams *et al.*, 1992); wheat EmBP-1 binds the G-box sequence (5′-CACGTG-3′; Niu and Guiltinan, 1994) and is classified as a group 1 protein. G-box binding sequences are found in the promoter regions of a large number of plant genes regulated by diverse environmental stimuli such as light and temperature, and osmotic and pathogenesis-related effects such as drought, wounding, and salinity (Menkens *et al.*, 1995). The ability of such a broad range of bZIP proteins to form heterodimers as well as homodimers may serve to diversify control of gene transcription in response to a variety of stimuli by altering DNA-binding sequence specificities and protein-protein interactions.

Although the *in vivo* functions of EmBP-1 and other plant bZIP proteins have yet to be fully elucidated, the role of the maize bZIP protein Opaque2 (O2) as a *trans*-acting regulator of 22 kDa zein gene expression has been defined using molecular genetic approaches. Mutations at the *o2* locus cause a severe reduction in the 22 kDa zein mRNA, while having less significant effects on the closely related 19 kDa and more distantly related classes of zeins (Pedersen *et al.*, 1980; Burr *et al.*, 1982; Langridge *et al.*, 1982). This 50–80% reduction in total zein protein accumulation converts the normally translucent kernel to an opaque kernel (Mertz *et al.*, 1964), an effect which is at least in part due to a lower rate of 22 kDa zein gene transcription (Kodrzycki *et al.*, 1989). The O2 bZIP domain footprints over a sequence from the 22 kDa α-zein promoter containing an imperfect palindrome (5′-TCCACGTAGA-3′) referred to as the O2 target site or the O2 box (Schmidt *et al.*, 1990). The O2 box is not present in the promoter of the related 19 kDa class of zein genes nor in the genes for the γ- or α-zeins (Aukerman and Schmidt, 1994). However, a closely related sequence (5′-TCCACGTCAT-3′) is present in the β-zein gene promoter, and O2 is capable of binding to this site as well (Cord-Neto *et al.*, 1995). Interestingly, a similar site (5′-GCCACGTCAT-3′, probe 9 herein) with only one base difference is preferentially bound by the wheat EmBP-1 protein (Niu and Guiltinan, 1994).

By independent methods, our two laboratories have simultaneously isolated maize cDNAs (*mEmBP-1*) with high sequence similarity to wheat *EmBP-1*. Although we show that this gene exists as duplicate loci in the maize genome, we only observe a single transcript size of 1.6 kb under hybridization conditions which simultaneously detect both genes. The mEmBP-1 protein product binds DNA sequences similar to those bound by wheat EmBP-1 (Niu and Guiltinan, 1994) as well as the O2 box from the 22 kDa zein gene promoter. Our data indicate that mEmBP-1 can act as an O2 box-dependent positive activator of transcription in a heterologous system; however, it negatively affects gene expression in the context of the native 22 kDa zein gene promoter.

Materials and methods

Hybridization screen for EmBP-1 *homologues*

Maize (*Zea mays* L. W64A × 182E inbred) cDNA λgt10 libraries from endosperm 14, 22 and 29 days after pollination (DAP) were provided by the laboratory of Dr L. Curtis Hannah at the University of Florida. The GC19 fragment (Guiltinan *et al.*, 1990) encoding a portion of wheat *EmBP-1a* was labeled with α-^{32}P-dCTP using the random primer labeling kit from Boehringer Mannheim (Indianapolis, IN) according to the manufacturer's instructions. Plaques were transferred onto nylon membranes according to the protocol of Sambrook *et al.* (1989) and hybridized with the probe in a solution of 0.5 M sodium phosphate pH 7.2, and 7% sodium dodecyl sulfate (SDS) (Church and Gilbert, 1984) at 55 °C overnight. Hybridized filters were washed twice at 55 °C with 5% SEN (5% SDS, 1 mM EDTA, 0.04 M Na_2HPO_4 pH 7.2) for 30 min with gentle agitation. *Eco*RI fragments of positive plaques were subcloned into pUC19 (Yanisch-Perron *et al.*, 1985), and plasmid DNA was isolated for sequencing (Lee and Rasheed, 1990) by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 with ^{35}S-dATP. The sequences of both strands of the 1.6 kb clone were determined along the entire cDNA.

O2-box binding expression library screen

An endosperm expression library constructed from an R802 inbred line homozygous for the *o*2-676 allele (Aukerman *et al.*, 1991) was screened with α-^{32}P-labeled double-stranded oligonucleotide containing the O2 box. The probe was generated by

annealing the two oligonucleotides Nos. 52 (5′-GTTCATGCATGTCATTCCACGTAGATGAAAA-G-3′) and 53 (5′-TATTCTTTTCATCTACGTGGAAT-GACATGCATG-3′ together and filling in the overhanging ends with Klenow enzyme in the presence of α-^{32}P-dATP.

The library was screened according to the protocol of Singh (1991) with minor changes. The blocking buffer was composed of 25 mM HEPES pH 7.5, 40 mM KCl, 1 mM EDTA, 5% nonfat dry milk, and 1 mM dithiothreitol (DTT). Protein-DNA interaction was allowed to proceed overnight in 10 ml of this solution containing 5 ng/ml of the labeled double-strand O2 box probe and salmon sperm DNA at 5 μg/ml. The membranes were washed 4 times at room temperature in blocking buffer for 5 min each wash. Positive plaques were identified and subsequently reacted with O2 heterodimerizing protein 1 (OHP1) antibody to eliminate those clones expressing the previously characterized OHP1 protein. To verify that none of the positives represented revertants of the *o2-676* point mutation, the blot was hybridized with O2 cDNA. Of the three remaining identical isolates, the final lambda clone 41 containing a 1.1 kb *Eco*RI insert was subcloned into the *Eco*RI site of pBluescript II (Stratagene, La Jolla, CA) to produce lab clone 541 (LC541) and sequenced as above.

Southern blot analysis and mapping

Conditions for Southern blot analyses were as previously described (Pysh *et al.*, 1993) with the exception that the hybridizations were performed at 42 °C with 50% formamide (moderate stringency) or at 50 °C or 57.5 °C with 50% formamide (high stringency). The genomic map locations of LC541 were obtained by Southern analysis with DNA isolated from the maize recombinant inbred family CM37 and T232 digested with *Bam*HI (Burr and Burr, 1991) and the 1.1 kb *Eco*RI fragment or the 540 bp 3′ *Kpn*I fragment from LC541 as probes. Computational analysis of the results was performed by Dr Benjamin Burr, Brookhaven National Laboratory, Uptown, NY.

Northern blot analyses

Total RNA from 16 DAP endosperm, 14 DAP embryo, tassel, 4 cm ear, 5-day old seedling shoot, root, and leaves (inbred Oh43) was isolated as described by Cone *et al.* (1986). Northern blot analyses were performed as described previously (Pysh *et al.*, 1993) with the exception that hybridizations were incubated at 57.5°C using the random primed probe generated from the 1.1 kb insert of LC541. To ensure that similar amounts of RNA were transferred to the membrane, the blot was stripped and reprobed with tubulin. Poly(A)$^+$ RNA was isolated from 18 DAP endosperm, and northern blot analysis was completed using either the 1.1 kb *mEmBP-1* cDNA or O2 probes. The O2 probe consisted of a 1.1 kb *Bgl*II/*Bam*HI fragment from LC218 (Schmidt *et al.*, 1992) containing the intact 5′ UTR but lacking ca. 500 bp from the 3′ end. For quantitation after autoradiography, the corresponding bands were excised from the blot and counted in a scintillation counter.

Gel shift analysis

An *mEmBP-1-GST* fusion clone was constructed by ligating the 1.1 kb *Eco*RI fragment from LC541 into the *Eco*RI site of pGEX1 (Pharmacia, Piscataway, NJ), and the fusion protein was prepared as detailed in the manufacturer's instructions. The O2 and OHP1 proteins were produced *in vitro* as previously described (Pysh *et al.*, 1993), while the gel shift conditions were as specified by Schmidt *et al.* (1992).

Binding specificity analysis

The full-length 1.6 kb *mEmBP-1* cDNA clone was inserted in frame into the histidine fusion expression vector pXN11, which originally contained the wheat version of *EmBP-1*, using *Eco*RI restriction enzyme sites (Niu and Guiltinan, 1994). The resulting histidine fusion was extracted and purified on a nickel ion column as instructed by the manufacturer (Novagen, Madison, WI). Dialysis, elution, and DNA binding analysis were completed as described previously (Niu and Guiltinan, 1994).

Nuclear localization analysis

The pUC118 β-glucuronidase (GUS) cloning vector containing an in-frame *Xho*I site inserted immediately in front of the GUS stop codon was kindly provided by Mark Shieh (Shieh *et al.*, 1993). The full-length 1.6 kb *mEmBP-1* cDNA *Eco*RI fragment was made blunt by filling in with Klenow polymerase (Promega, Madison, WI), partially digested with *Pst*I to produce a 1.4 kb fragment with a 0.2 kb C-terminal deletion, and finally treated with T4 polymerase (Promega) to remove the *Pst*I 3′ overhangs. The resulting fragment was cloned into the pUC118 GUS vector which was

linearized with *Xho*I, made blunt with mung bean nuclease (Boehringer Mannheim, Indianapolis, IN), and treated with calf intestinal phosphatase (Boehringer Mannheim). The in-frame fusion of the C-terminus of GUS with mEmBP-1 was confirmed by DNA sequencing and transferred into the plant expression vector pMF6 (Shieh *et al.*, 1993) using a *Bam*HI-*Eco*RI double digest. Biolistic particle bombardment of onion epidermal cells was completed as described previously (Shieh *et al.*, 1993); however, GUS activity was detected with the fluorometric substrate ImaGene Green (Molecular Probes, Netherlands) under the manufacturer's instructions. Localization was observed using a Nikon DIAPHOT-TMD epi-fluorescence inverted microscope with Nomarski optics and a B-1E filter set to detect ImaGene Green fluorescence. Nuclear staining with DAPI (4,6-diamidino-2-phenylindole) was visualized with a UV-2A filter set.

Yeast transactivation assay

The full-length *mEmBP-1* cDNA was cloned downstream of the yeast phosphoglycerate kinase promoter to generate the plasmid pYP′*mEmBP-1* (Schmidt *et al.*, 1992). This plasmid also relied on a pentamer of O2 boxes upstream of a minimal yeast promoter to control expression of a β-galactosidase reporter gene (sequence and manner of construction are described in Schmidt *et al.*, 1992). The yeast strain RH151-7B was transfected with either the empty plasmid pYP′, pYP′ *mEmBP-1* (expressing the mEmBP-1 protein), or pYP′O2Δ1 (expressing the maize O2 protein) to test their ability to activate transcription. β-galactosidase activity was measured as described previously (Schmidt *et al.*, 1992).

Maize endosperm transient expression assay

The 22 kDa zein::luciferase reporter plasmid, pRLP73, is functionally identical to LC175 (Unger *et al.*, 1993), and the two plasmids behave identically in transient expression assays (data not shown). Excess restriction sites in the polylinker sequence between the coding sequence for firefly luciferase and the nopaline synthase (NOS) 3′ termination and polyadenylation sequences have been removed. The Wx::GUS control plasmid pPHI4866 has been previously defined (Unger *et al.*, 1993). The O2 effector plasmid LC181 contains the *Bgl*II/*O2*Δ*1* cassette described by Schmidt *et al.* (1992) inserted into the *Bam*HI site of pMF6 (Goff *et al.*, 1990), placing the O2 coding sequence under the regulation of a derivative of the CaMV 35S promoter. The mEmBP-1 effector plasmid contains a *Bgl*II/*mEmBP-1* cassette inserted into the same *Bam*HI site in pMF6.

The biolistic particle bombardment protocol and maize endosperm suspension cell cultures used in this study and their maintenance were as described by Unger *et al.* (1993) with minor changes mentioned herein. A total of 10 μg of a plasmid DNA mixture (2 μg luciferase reporter, 2 μg GUS control, and 6 μg pMF6 expression plasmids) was precipitated onto 1 μm tungsten particles using a two-fold scale-up of the procedure described by Sanford *et al.* (1993). A DNA aliquot of 8 μl of a total of 60 μl was used for each shooting. After a 13 day subculture, 75 mg of a 150 mg/ml cell preparation was bombarded and incubated for 20–24 h at 29 °C. Cells were disrupted on ice in 300 μl of buffer containing 0.1 M potassium phosphate pH 7.8, 1 mM DTT, and 0.5% (v/v) Triton X-100 using a Sonifier Cell Disrupter (Branson, model W140D). Luciferase assays were performed with 25 μl of extract to 200 μl of Luciferase Assay Buffer (20 mM Tricine pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 500 μM ATP, and 270 μM Coenzyme A). Following the addition of 100 μl of 1 mM K+ luciferin, light output was measured using a 20 s integration in a luminometer (model 2010, Analytical Luminescence, Ann Arbor, MI). GUS assays were performed according to the manufacturer's instruction with the GUS-light kit (Tropix, Bedford, MA). The luciferase activity was corrected for changes in bombardment based on the GUS activity control (Schledzewski and Mendel, 1994), and the results were normalized to the 22Z-4 promoter activity in the absence of effector plasmids.

Results

The maize homologue of wheat EmBP-1 is capable of binding to the O2 box

Maize endosperm libraries 14, 22, and 29 DAP were screened using a fragment of the wheat *EmBP-1* cDNA as a hybridization probe to isolate its maize homologue. Of the seven strongly hybridizing clones isolated from a total of 270 000 plaques, sequence data from a representative clone revealed a full-length insert of 1610 bp (EMBL accession number Y15165). The full-length cDNA contains an 1161 bp open reading frame predicting a 386 amino acid polypeptide beginning at 73 bp and ending with TGA at 1233 bp.

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                10        20        30        40
maize   MASSSDEQSKPPESPAAAAVVTAAAPPQTHAEWVASLQAY
        MASSS   S     PAA       A  Q HAEW AS  AY
wheat   MASSSAT-SGDDRPPAAGGGGGTPA--QAHAEWAASMHAY

                    50              60        70
maize   YAAA----GHPY-AW----PAQ-HLMAAAAAGAHFGTPVP
        YAAA    GHPY AW     AQ H   AA  GA  G PVP
wheat   YAAAASAAGHPYAAWPLPPQAQQHGLVAAGPGAAYGAPVP

                80          90        100
maize   FPVYHPGAAAAY-YAHASMAAGVPYPTCEAVPAVALPTVP
        FP YH  AAA Y  AHAS AAGVPY   E
wheat   FPMYHHPAAAYYAHAHASNAAGVPYMAGE--------SA

        110       120       130       140
maize   EGKGKGKGGGASPEKGSSGAPSGEDASRSDDSGSDESSET
           GKGK  G       SG        S   D GS  SSE
wheat   SAAGKGKRAGKTR-RVPSGEINSSSGS--GDAGSQGSSEK

        150       160       170       180
maize   RDDDTDHKD-SSAPKKRKSGNTSAEGEPSQATVVRYAAVE
              K  SS  K RKSG    EGEPSQAT V  A  E
wheat   GNAGANQKGSSSSAKRRKSGAANTEGEPSQATTVQNAVTE

         190       200       210       220
maize   SPYPAKGRSASKLPVSAPGRAALPSATPNLNIGMDIWNAS
         P   K RSASKL V APGRAAL SA PNLNIGMD   AS
wheat   PPLEDKERSASKLLVLAPGRAALTSAAPNLNIGMDPLSAS

         230       240       250       260
maize   PALAVPAVQGEVSPGLALARRDGVTQLDEREIKRERRKQS
        P      VQGEV              Q DERE KRERRKQS
wheat   P---SSLVQGEVNAAASSQSNASLSQMDERELKRERRKQS
O2                                 MPTEERVR-KRKES
OHP1                               NPVQQRQQ-RRKQS

         270       280       290       300
maize   NRESARRSRLRKQQECEELARKVADLTTENSALRAELDNL
        NRESARRSRLRKQQECEELA KV  LT  N  LR ELD L
wheat   NRESARRSRLRKQQECEELAQKVSELTAANGTLRSELDQL
O2      NRESARRSRYRKAAHLKE
OHP1    NRESARRSRSRKAAHLNE

         310       320               330       340
maize   KKACQDMEAENSRLLGGV-------ADAQVPSVTTTLGMS
        KK C  ME EN  L G ___           PSV TTL
wheat   KKDCKTMETENKQLMGKILSHDDKMQQSEGPSVVTTLSIQ

                350       360       370       380
maize   IEPPKLQLQLQQHHDEEGQLHKKSSNNSNGNCAGGSHKPE
         E P
wheat   VEAPDPHQGGDGKAS

            386
maize   ANTTR
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Figure 1. Amino acid comparisons of maize EmBP-1a, wheat EmBP-1a, and the basic regions of Opaque2 and OHP1. Identical amino acids are identified by repetition between the compared EmBP-1 protein sequences. The basic region and the heptameric hydrophobic repeats are indicated by underlining. Alignment was performed using BLAST at the National Center for Biotechnology Information (NCBI) and the GCG Wisconsin package. Dashes indicate spacings necessary for optimal alignment.

BLAST analysis indicates that this cDNA most likely represents the maize homologue of the wheat DNA-binding protein EmBP-1 (mEmBP-1; Figure 1). The 386 amino acid deduced polypeptide contains a basic motif (from D256 to E286) and a heptameric repeat of hydrophobic residues (L287, L294, L301, L308, M315, and L322) consistent with the peptide being a member of the bZIP family of proteins. It has 52% identity and 68% similarity with wheat EmBP-1; however, similarity to O2 and OHP1 is restricted to the basic motif of the bZIP domain (Figure 1). The similarity of the two proteins within the leucine zipper motif is very limited (28%), unlike the previously described OHP1 (Pysh *et al.*, 1993) which has 75.5% identity to the O2 protein in the zipper motif. The middle of the mEmBP-1 protein is interspersed with conserved regions of unknown function, most notably the NLNIGMD sequence beginning at position 217 (Figure 1).

Electrophoretic mobility shift assays with labeled O2 box oligonucleotides and null *o2* kernel extracts revealed the presence of nuclear factors that recognize the O2 box in the absence of O2 (Parsons and Schmidt, unpublished; Schmidt *et al.*, 1992; Pysh *et al.*, 1993); thus, other proteins in addition to O2 may regulate 22 kDa zein gene expression. To identify clones encoding these proteins, an endosperm expression library from a null *o2* background was screened with a radiolabeled oligonucleotide containing the O2-binding site. Of the eight positive isolates detected from a total of 200 000 plaques, five were identified as encoding the known O2 heterodimerizing protein 1 (OHP1; Pysh *et al.*, 1993). The remaining three isolates encoded a novel protein with O2 box binding activities which possessed the same restriction enzyme patterns and probably represented multiple isolations of the same clone. A 1.132 kb representative clone (LC541) was identified as a partial cDNA of maize EmBP-1 homologue, as it precisely matched the full-length cDNA from position 478 to 1610. Additionally, the context of the first ATG codon of LC541 was not favorable for initiating translation and subsequent northern analysis (see below) demonstrated a message length of 1.6 kb.

Two mEmBP-1 *genes are present in the maize genome*

Southern blot analysis of maize genomic DNA hybridized with the 1.1 kb insert of LC541 at moderate stringency revealed two strongly hybridizing bands

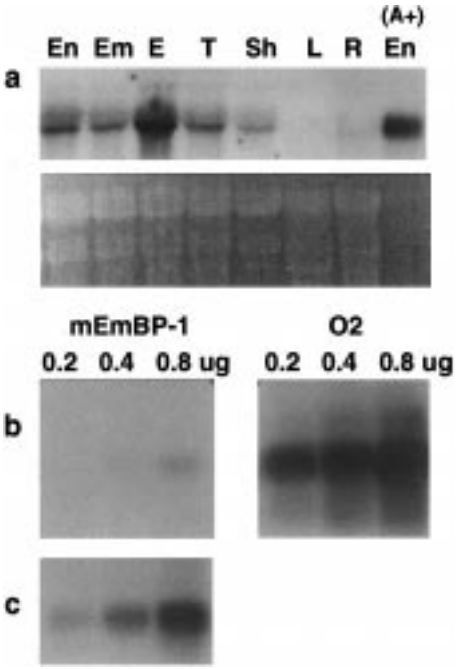


Figure 2. mEmBP-1 is expressed in several tissues of the maize plant. a. Total RNA (12 μg) from wild-type (inbred Oh43) maize 16 DAP endosperm (En), 14 DAP embryo (Em), 4 cm ear (E), mature tassel (T), 5-day old seedling shoot (Sh), leaf (L), and root (R), and 12 μg poly(A)$^+$ RNA from 18 DAP wild-type endosperm were examined by northern blot analysis using the 3′ 540 bp *Kpn*I fragment of *mEmBP-1* cDNA as a probe. The bottom panel depicts the ethidium bromide-stained gel. b. Dilutions of poly(A) RNA (0.2, 0.4, and 0.8 μg) from 18 DAP wild-type endosperm were hybridized with a 1.1 kb *mEmBP-1* cDNA probe or a 1.1 kb truncated O2 cDNA probe to compare levels of expression by scintillation counting. Adjustments for minor differences in the probe specific activity were taken into account when estimating message levels derived from the scintillation counter. c. An 8-fold longer exposure of the radioactive filter used to generate the autoradiograph in panel b.

suggesting that *mEmBP-1* is a duplicate locus. Following the segregation of *Bam*HI-generated restriction fragment length polymorphism (RFLP) within a recombinant inbred family allowed us to map *mEmBP-1a* to position 20 on the short arm of chromosome 7 and a second locus (*mEmBP-1b*) to approximately position 90 on the long arm of chromosome 2 (data not shown). Increasing the incubation temperature to 57.5 °C resulted in hybridization only to the restriction fragment mapping to chromosome 7 (data not shown). This permitted us to unequivocally determine that the *mEmBP-1* cDNA we isolated is the product from the gene on chromosome 7.

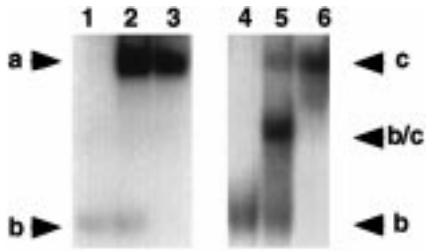


Figure 3. mEmBP-1 can bind the O2 box but does not form heterodimers with O2. Gel mobility shift assays with *in vitro* translated O2s (truncated O2), mEmBP-1, and OHP1 were completed with the O2 box probe as follows: O2s (lanes 1 and 4), O2s and mEmBP-1 (lane 2), mEmBP-1 (lane 3), O2s and OHP1 (lane 5), and OHP1 (lane 6). Lanes 1 and 4 show the presence of homodimeric O2s (b), while lanes 3 and 6 indicate the presence of homodimers of mEmBP-1 (a) and OHP1 (c), respectively. In lane 2, intermediate complexes were not observed when O2s and mEmBP-1 were mixed and incubated together in the presence of the probe. However, the intermediate complex formed by the heterodimer of O2s and OHP1 can be seen in lane 5 (b/c). Free probe is not depicted in this figure.

mEmBP-1 *is expressed in endosperm and several maize organs*

Northern blot analysis of total RNA probed at high stringency with the 1.1 kb *Eco*RI fragment corresponding to *mEmBP-1a* cDNA demonstrated that a 1.6 kb transcript is present in developing endosperm. Whereas *O2* expression is seen only in the endosperm, *mEmBP-1* transcript was observed in immature ear, tassel, embryo tissues, root, and young shoot; it was not detected in leaf (Figure 2a). Quantitation of gene transcripts indicated that the relative amount of *mEmBP-1* is ca. 10-fold lower than the expression level of *O2* (Figure 2b and 2c).

mEmBP-1 *protein is unable to heterodimerize with O2 protein*

We have previously postulated that O2 may bind to the O2 box in the 22 kDa zein gene promoter as a heterodimeric complex with another bZIP factor (Schmidt *et al.*, 1990; Aukerman *et al.*, 1991). Since both *O2* and *mEmBP-1* are expressed in the same tissue and bind the O2 box individually, we examined whether mEmBP-1 could heterodimerize with O2. Since the relative migration of the shifted complexes of the GST-mEmBP-1 fusion protein and the *in vitro* translated O2 protein were similar (data not shown), a truncated O2 protein (O2s; Schmidt *et al.*, 1990) was utilized in mobility shift assays with an O2 box probe to distinguish heterodimeric complexes from those generated by homodimeric proteins. The O2s

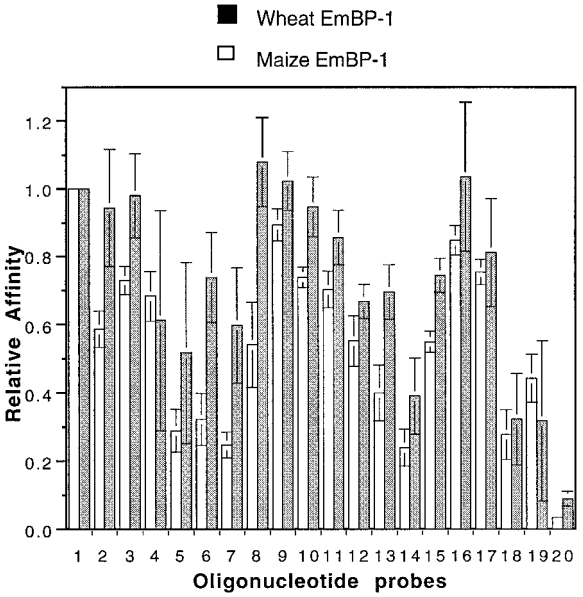


Figure 4. DNA binding specificity of mEmBP-1 is similar to that of wheat EmBP-1. Using mobility gel shift analyses, the relative binding affinities of maize EmBP-1 protein (open bars) were determined in triplicate and normalized to oligonucleotide 1 with the 19 oligonucleotides and a pUC18 polylinker control previously examined with wheat EmBP-1 protein (solid bars; data and sequences originally published in Niu and Guiltinan, 1994). The relative levels of bound and unbound probe were determined using a Phosphorimager 445 SI with ImageQuant software (Molecular Dynamics). All of the oligonucleotides originally selected by wheat EmBP-1 are also bound by the maize version of EmBP-1, although with slightly different affinities. The depicted results are the means of three separate experiments with error bars representing their standard deviations. The oligonucleotide numbers correspond to the following sequences with the lower-case letters indicating changes from the palindromic G-box of oligonucleotide 1: 1, GCCACGTGGC; 2, GCCACGTGtC; 3, GCCACGTGGa; 4, GCCACGTGta; 5, GaCACTGtC; 6, GaCACGTGGa; 7, tCCACGTGGa; 8, GCCACGTcaC; 9, GCCACGTcat; 10, GCCACGTcag; 11, GCCACGTaag; 12, GCCACGTGtg; 13, GCCACGTGtt; 14, GCCACGTtGC; 15, GACACGTGtg; 16, tCCACGTcag; 17, taCACGTcag; 18, GCCAgcTGGa; 19, GCCACGcTGGC; 20, pUC18 polylinker.

protein consists of a 0.3 kb fragment (*Pst*I/*Sal*I) from the O2 cDNA (nucleotides 566–855) that includes the bZIP domain cloned into a pBluescript II plasmid for *in vitro* transcription and translation. As shown in Figure 3, O2s (lanes 1 and 4 labeled b), mEmBP-1 (lane 3 labeled a), and OHP1 (lane 6 labeled c) bind to the O2 box as homodimers. However, under the conditions promoting heterodimer formation of O2s-OHP1 (lane 5, intermediate complex b/c), heterodimerization between mEmBP-1 and O2s cannot be detected (lane 2). Furthermore, interactions between EmBP-1 and OHP1 were absent under similar conditions (data not shown).

mEmBP-1 protein binds to the same oligonucleotides selected by wheat EmBP-1

Since the DNA-binding domains of wheat and maize EmBP-1 are similar, we speculated that their binding specificities also would be comparable. To relate these binding specificities, we tested the binding of purified histidine-tagged mEmBP-1 protein to a series of oligonucleotides that were selectively bound by wheat EmBP-1 (Niu and Guiltinan, 1994). Although the same group of probes (1, 2, 3, 4, 8, 9, 10, 11, 12, 15, 16, 17) was bound to mEmBP-1 with relative affinities of at least 50%, wheat EmBP-1 had the greatest affinity for probe 1 (Figure 4 and Niu and Guiltinan, 1994). In contrast, maize EmBP-1 demonstrated greater binding to probes 8, 16, and 9 which contained alterations in the $+2$, $+3$, $+4$, and -4 positions of the G-box consensus sequence when compared to probe 1.

mEmBP-1 activates gene expression in yeast from an O2 box and possesses a potential nuclear localization signal (NLS)

To test the ability of mEmBP-1 protein to activate gene expression *in vivo*, a composite reporter/expression plasmid was introduced into the yeast strain RH151-7B (Schmidt *et al.*, 1992). The reporter consisted of a pentamer of O2 boxes upstream of a minimal yeast promoter fused to the β-galactosidase (β-gal) gene, while *mEmBP-1* and *O2* were expressed from the phosphoglycerate kinase promoter (pYP′mEmBP-1 or pYP′O2Δ1) contained within the same plasmid. As depicted in Figure 5a, β-gal activity was stimulated ca. 10-fold by mEmBP-1 and nearly 40-fold by O2. Supporting the ability of mEmBP-1 to regulate gene expression, we show that the GUS::mEmBP-1 fusion protein is localized to the nucleus of onion epidermal cells in a transient assay system using biolistic particle bombardment. Figure 5b depicts the cytoplasmic partitioning of the GUS marker gene alone, while nuclear localization of the GUS::mEmBP-1 fusion construct containing the entire open reading frame is shown in Figure 5c.

mEmBP-1 does not activate a 22 kDa zein promoter in a transient assay system

To further examine the activation potential of mEmBP-1, we tested its capacity to activate transcription in the context of an intact, endosperm-specific promoter using biolistic particle bombardment (Unger *et al.*, 1993). Cultured maize endosperm

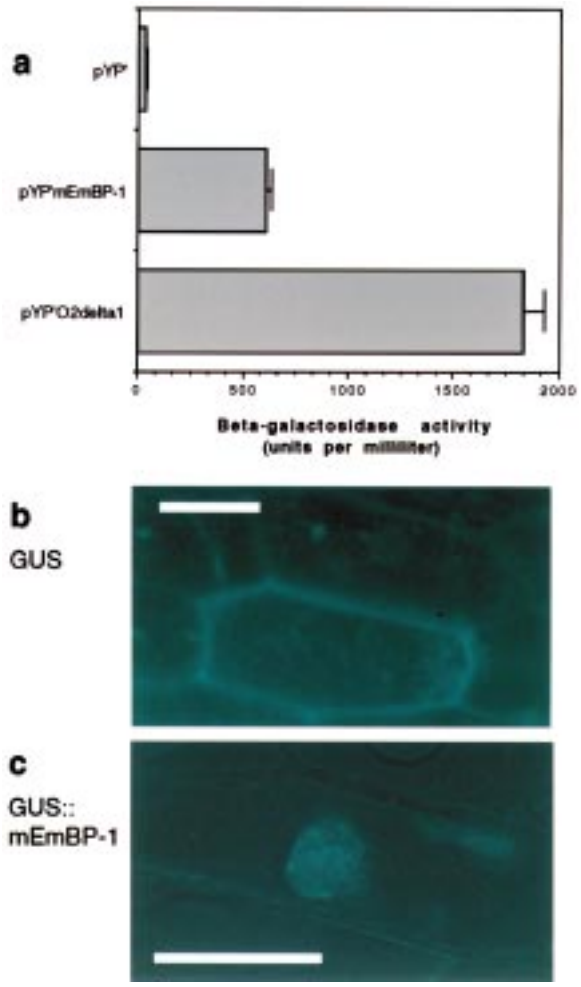
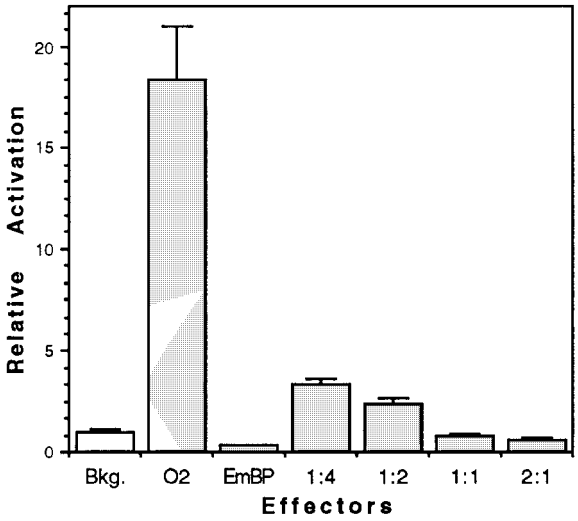


Figure 5. mEmBP-1 regulates gene expression in yeast and is nuclear-localized in transient assay. a. The yeast strain RH151-7B was transformed with a composite reporter/expression construct (pYP′) composed of a pentamer of O2 box sequences upstream of a minimal yeast promoter fused to the β-galactosidase gene (β-gal) and the yeast phosphoglycerate kinase promoter for effector expression. The amount of β-gal activity produced by the pYP′ expression plasmid by itself or containing *mEmBP-1* or a truncated *O2Δ1* (labeled O2delta1) was measured to determine O2 box-dependent transcriptional activation. The data represent the means of three to five experimental repetitions with at least three independent transformants, and the error bars depict their standard deviations. Both mEmBP-1 and O2 activate transcription from the constructed O2 box promoter. b. Biolistic particle bombardment of onion epidermal cells with a control β-glucuronidase (GUS) marker gene demonstrates cytoplasmic staining as detected by the fluorometric GUS substrate ImaGene Green (Molecular Probes, Netherlands). The white magnification bar indicates a length of 100 μm. c. Transient transformation of onion epidermal cells using a GUS::mEmBP-1 fusion construct containing the entire open reading frame produces nuclear localization of the fusion construct. The white magnification bar indicates a length of 100 μm.



	DNA per shooting in micrograms						
pMF6	.8	.53	.53	.47	.40	.27	0
O2	0	.27	0	.27	.27	.27	.27
EmBP	0	0	.27	.07	.13	.27	.53

Figure 6. mEmBP- 1 does not activate 22 kDa zein gene expression in endosperm cells. Cultured maize endosperm cells were transiently transfected by biolistic particle bombardment in quadruplicate with a luciferase reporter gene under control of a 22 kDa zein gene promoter (22Z-4) containing an intact O2 box, a GUS control, and effectors expressing *O2* and *mEmBP-1* (labeled EmBP). The table indicates the amounts of effector DNA in μg, while the LUC and GUS plasmids were present in all shootings in the amount of 0.27 μg. The activation of luciferase relative to the co-bombarded GUS control is shown either in the absence (Bkg.) or presence of the *O2* and *mEmBP-1* effectors. mEmBP-1 acted as a dominant inhibitor of O2 activation. The ratios indicate concentrations of *mEmBP-1* DNA relative to a constant level of *O2* DNA such that increasing the *mEmBP-1* effector reduces the level of expression of luciferase. Error bars represent the standard error of the mean.

cells were transiently transformed with a mixture of up to five plasmids: a luciferase reporter under control of a 22 kDa zein gene promoter (22Z-4), an O2-independent GUS control, an effector expressing either EmBP-1 or O2 protein, and the empty construct (pMF6) to control for the level of CaMV 35S promoter. Unlike O2, mEmBP-1 did not activate transcription from 22Z-4 (Figure 6). When the *mEmBP-1* DNA was co-bombarded with *O2*, mEmBP-1 acted as a dominant inhibitor of O2 function, resulting in a dosage-dependent reduction in the ability of O2 to activate transcription (Figure 6). This experiment demonstrates that mEmBP-1 is being expressed in these cell types and that it binds the O2 box *in vivo*.

Discussion

From sequence analysis and amino acid predictions, the similarity of O2 and mEmBP-1 appears to lie strictly within the basic region (88%; Figure 1). However, we expected their basic motifs to be comparable since both are capable of binding the O2 box sequence (Schmidt *et al.*, 1992, and this work), and this region of the bZIP proteins confers DNA binding specificity. Based on comparisons to known nuclear localization signals (Dingwall and Laskey, 1992; Varagona *et al.*, 1992), we postulate the main NLS of mEmBP-1 protein resides in the conserved basic region which contains the bipartite NLS structure of KRERRK-X_{10}-RLRK from 261 to 280 (Figure 1). Analysis of more extensive C-terminal *mEmBP-1* deletions removing the basic motif indicated that the alternate SV40-like NLS (Boulikas, 1994; PKKRK from 163 to 166; Figure 1) was insufficient to direct nuclear partitioning (data not shown).

Previous mapping studies with numerous genes and RFLP probes have established that there are regions of the maize genome that appear to be the result of duplication and *EmBP-1b* coincides with these known regions of chromosomal duplications in the maize genome (Helentjaris *et al.*, 1988; Dowty and Helentjaris, 1992). The hybridization conditions used in the RNA expression analyses were sufficiently stringent to detect only the 7S gene transcript (*mEmBP-1a*); however, conditions of moderate stringency adequate enough to detect both genes in genomic Southern analysis revealed only a single transcript length of 1.6 kb. These results suggest that the transcripts of the two genes cannot be resolved with northern analysis, especially since preliminary RNase protection assays (RPAs) demonstrate that both genes are expressed in most tissues (data not shown). A partial cDNA *mEmBP-1b* clone generated by using 3′ RACE exhibits ca. 85% homology to *EmBP-1a*, although the 3′ ends are increasingly disparate (data not shown). In the future, we plan to isolate genomic sequences of *mEmBP-1a* and *mEmBP-1b* genes to study their promoters and intron/exon compositions to elucidate duplicate gene expression patterns. Since the molecular characterization of O2 as a transcriptional activator has relied on an association with a mutant phenotype, a similar approach may be necessary with EmBP-1. With the newly developed Pioneer Trait Utility System for Corn (TUSC), we hope to identify Robertson's *Mutator* element insertions in both *mEmBP-1a* and *mEmBP-1b* via PCR technology and to associate gene inactivation with plant phenotypes.

Whereas mEmBP-1 can activate O2-dependent gene expression in a yeast expression system (Figure 5), it demonstrates a dominant inhibitory effect on a 22 kDa zein gene promoter in transient assays when using cultured endosperm cells (Figure 6). These disparate effects may simply be attributed to the effects of promoter context. Previous literature readily supports the interplay of several DNA-binding elements and their cognate binding factors in defining promoter specificity; these complex associations may not be represented in both promoter constructs. Generally, a coupling element appears to enhance the specificity of gene regulation via G-boxes, since the DNA-binding affinities of bZIP proteins seem too relaxed to provide adequate specificity (Lamb and McKnight, 1991; de Vetten and Ferl, 1994). Although wheat and maize EmBP-1 proteins demonstrate high sequence specificity for their target sequences, some relaxed DNA specificity can be observed, since they bind to a variety of oligonucleotides including those with alterations flanking the ACGT core of the palindromic G-box (Figure 4; Niu and Guiltinan, 1994). The barley *HVA22* promoter clearly demonstrates the requirement for a *cis*-coupling element and a G-box element to confer ABA-inducible gene expression (Shen and Ho, 1995). Direct interactions between the *Arabidopsis* DNA-binding factors OBF and OBP1 of different protein classes (bZIP versus zinc finger) are required to stimulate activity of the *GST6* promoter (Zhang *et al.*, 1995; Chen *et al.*, 1996). Futhermore, O2-dependent zein activation in transient assays using suspension cultures relies on the presence of the neighboring prolamin box and protein contacts with the prolamin box binding factor (Vicente-Carbajosa *et al.*, 1997) found only in endosperm cells (R. Parsons and R. Schmidt, unpublished).

Although the complete story regarding the action of EmBP-1 on O2-box sequences remains unclear, our ability to demonstrate activation or inhibition of O2-regulated gene expression depending on promoter context raises many interesting questions. On artificial promoters such as that used in the yeast expression system, the requirements for interactions with other DNA-binding factors may be bypassed by virtue of the multiple complexes formed. O2 may activate the zein promoter because it can interface with other protein factors that bind in the proximity of the promoter, whereas bZIP proteins like OHP1 and mEmBP-1 may be unable to form these important contacts. The bind-

ing of mEmBP-1 to the O2 box and its ability to affect transcription in an O2-box-dependent manner suggests the possibility that mEmBP-1 has a role in regulating 22 kDa zein gene expression. However, mEmBP-1 is expressed at 10-fold lower levels and in a wider variety of tissues than the endosperm-specific O2 protein, suggesting additional functions beyond zein regulation. It is likely that mEmBP-1 (like OHP1) is one of the proteins observed in the *o2* null mutant extracts which bind to the O2-box probe (Schmidt *et al.*, 1992), yet confirmation of this awaits the generation of antibodies to mEmBP-1. Future analyses based on protein-protein interactions such as those completed in rice by Nantel and Quatrano (1996) could potentially identify a protein that heterodimerizes with mEmBP-1, increasing our understanding of the complete spectrum of genes regulated by this bZIP protein. Further molecular and genetic studies are needed to elucidate the actual role of EmBP-1 in the endosperm and other tissues, especially regarding its involvement in ABA signal transduction.

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