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The activation domain of the maize transcription factor Opaque-2 resides in a single acidic region

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ABSTRACT

The maize (*Zea mays* L.) endosperm specific transcription factor, encoded by the *Opaque-2* (*O2*) locus, functions *in vivo* to activate transcription from its target promoters. *O2* regulates the expression of a major storage protein class, the 22 kDa zeins, and of a type I ribosome inactivating protein, b-32, during maturation phase endosperm development. The coding sequence of *O2*, which indicates it to be a member of the basic region-leucine zipper (bZIP) class of DNA-binding proteins, contains a number of regions rich in either proline or acidic residues which are candidates for activation domains. In functional assays using tobacco mesophyll protoplasts, the level of transactivation conferred by a series of O2-deletion constructs was tested using as a reporter a fusion of the b-32 target promoter to β-glucuronidase (GUS). The results indicate that O2 has a single acidic activation domain, located near the N-terminus of the protein (amino acids 41–91). The ability of a shorter part of this domain (amino acids 39–82) to confer transactivation was also demonstrated in domain swapping experiments, using fusions of the O2 polypeptide sequence to the DNA-binding domain of the parsley (*Petroselinum crispum*) transcription factor CPRF1.

INTRODUCTION

An essential component of gene regulation is transcriptional activation. The process is dependent on the formation on regulatory DNA elements of multiprotein complexes, which include transcription factors responsible for cell-type specific and temporal gene regulation. They direct the transcriptional machinery, composed of RNA-polymerase II and of additional factors, to the start site of gene transcription (1). The identification of distinct domains within the polypeptides of transcription factors has allowed the investigation of interactions with other proteins present in initiation complexes. Domains reported to be responsible for transcriptional activation contain acidic, glutamine-rich or proline-rich stretches of amino acids (2). These activation domains interact with other factors including TFIID or TFIIB, and enable or facilitate the binding of the RNA polymerase II at the start site for mRNA synthesis.

The *Zea mays* (L.) protein Opaque-2 (O2) is a member of the bZIP transcription factor family. It is involved in the regulation of seed storage protein synthesis, modulating the transcription of the 22 kDa α-zeins (3,4) and of the b-32 albumin (5). The 22 kDa α-zeins are the most abundant endosperm storage proteins in maize. The function of the b-32 albumin in endosperm development remains to be established, but the protein shares homology to type I ribosome-inactivating proteins (6). *O2*, which is located on chromosome 7, has been cloned by transposon tagging (7,8). A number of distinct functional domains have been identified on the O2 protein. In addition to the basic region followed by a leucine zipper, which is responsible for DNA-binding and dimerization (9,10), two nuclear localization signals have been identified on the polypeptide (11).

Our goal was to identify the transcriptional activation domain of O2 by deletion experiments in a transient expression system. To confirm that the region identified was sufficient to confer transcriptional activation in the absence of other parts of the sequence, domain swapping experiments were carried out. We demonstrate that O2 has an acidic activation domain located near the N-terminus of the peptide and show that the ability of this domain to activate transcription is retained in a hybrid protein composed of the O2 acidic activation domain and the bZIP domain of the parsley (*Petroselinum crispum* Bernh. ex Rchb.) common plant regulatory factor 1 (CPRF1) (12).

MATERIALS AND METHODS

Plasmid constructions

Effector constructs. Derivatives of O2 were expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter using pRT100 (13). The O2 sequences were fused at the 3′-end to the polyadenylation site of CaMV. C-terminal deletions were prepared from the vector pCaMVO2 (5), a plasmid containing the full length O2 cDNA (9), by using the natural restriction enzyme sites within the coding sequence of O2. $pO2_{1–412}$ was constructed by cutting with *Bam*HI and religation (Fig. 1), whereas $pO2_{1–284}$ and $pO2_{1–265}$ were prepared by cutting with *Sal*I/*Xba*I and *Eco*RI respectively, followed by religation. The leucine zipper of these two constructs is truncated: $pO2_{1–265}$ contains two leucine

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repeats, $pO2_{1–284}$ four repeats instead of seven. During the construction of N-terminal deletions of O2, the translation initiation start codon was lost and replaced by the ATG codon of the *Nco*I site of the pRT100 vector. For the construction of $pO2_{192–460}$, pCaMVO2 was digested with *Xba*I and *Stu*I, and the resulting 1 kb fragment was inserted in the filled-in *Bam*HI site of pRT100. $pO2_{125–460}$ was derived from inserting the 510 bp O2 fragment of *Bst*EII/*Sal*I digested pCaMVO2 in the *Sal*I/*Sma*I restriction sites of $pO2_{192–460}$. In order to delete acidic region 3, a *Kpn*I site between this domain and the bZIP region was inserted by directed *in vitro* mutagenesis at aa 226. To introduce the site, the 300 bp *Pst*I/*Sal*I fragment of O2 was cloned in M13mp19 (38) and the mutagenesis was carried out using the oligonucleotide 5′-TCTTTCCTCGGTACCCATCTTGAACCCC-3′ (the mutant nucleotides are underlined). The mutagenized fragment was integrated in pRT100 and the construct was designated $pO2_{\Delta 226–283}$. From this plasmid $pO2_{\Delta 226–460}$ was made by inserting the *Sal*I/*Xba*I O2 fragment of pCaMVO2 between the corresponding restriction sites.

The internal deletions of O2 were made as follows. $pO2_{\Delta 92–192}$ and $pO2_{\Delta 142–192}$ were prepared by cutting pCaMVO2 with *Kpn*I/*Stu*I thereby removing the N-terminal part of O2 and religating with the 200 bp O2-fragment of *Kpn*I/*Nar*I digested pCaMVO2 or the 400 bp O2 fragment of *Kpn*I/*Xho*I-digested pCaMVO2. $pO2_{\Delta 40–192}$ was made by religation of *Bst*EII/*Stu*I digested pCaMVO2. By digesting $pO2_{\Delta 40–192}$ with *Bst*EII and *Kpn*I and religating the deletion construct $pO2_{\Delta 1–40\ \Delta 92–192}$ was generated. All deletion constructs were checked by sequencing to verify fusion junctions.

The various O2 domains used to construct the O2–CPRF1 hybrids were cloned by PCR from pCaMVO2. The oligonucleotides for the amplification reaction (Table 1) carried an additional restriction site, an *Asp*718 site for 5′ primers and a *Bsp*EI site for 3′ primers with the exception of #4352, which carried a *Bst*EII restriction site. The PCR products were gel-purified and cloned via the appropriate restriction sites into the effector plasmid $pF1_{bZIP}$ (kindly provided by M. Sprenger MPI f. Züchtungsforschung, Cologne, Germany). This vector is designed for the expression in plants of polypeptide fusions of the CPRF1 DNA-binding domain [amino acids (aa) 258–337; 15]. The gene fusion is flanked by the CaMV 35S promoter and the *nos* terminator (Fig. 3). The O2 peptide sequence is cloned downstream of the nuclear localization signal (NLS) of the simian virus 40 large-T antigen (SV40 NLS) and upstream of the DNA-binding domain of the parsley transcription factor CPRF-1. The plasmids constructed were designated $pO2_{12–228}F1_{bZIP}$ (oligonucleotides #1832 and #2402), $pO2_{12–197}F1_{bZIP}$ (#1832 and #1829), $pO2_{12–123}F1_{bZIP}$ (#1832 and #1693), $pO2_{39–228}F1_{bZIP}$ (#1398 and #2402), $pO2_{39–197}F1_{bZIP}$ (#1398 and #1829), $pO2_{39–147}F1_{bZIP}$ (#1398 and #3009), $pO2_{39–123}F1_{bZIP}$ (#1398 and #1693), $pO2_{39–82}F1_{bZIP}$ (#1398 and #4355), $pO2_{83–228}F1_{bZIP}$ (#4356 and #2402), $pO2_{124–228}F1_{bZIP}$ (#2703 and #2402) and $pO2_{203–228}F1_{bZIP}$ (#2401 and #2402). The effector $pO2_{39–82/124–228}F1_{bZIP}$ was made by inserting the amplification product of #1398 and #4352 in the *Asp*718 and *Bst*EII restriction sites of $pO_{39–228}F1_{bZIP}$. The numbers in subscript are the amino acid coordinates of the O2-ORF (9).

Reporter constructs. The reporter plasmids for transient expression analysis contained the GUS gene under the control of either the b-32 (pb32-GUS) or a part of the chalcone synthase (pBTACEchsII-4GUS) promoters. The plasmid pBTACE-$_{chsII}$-4GUS (kindly provided by M. Sprenger, Cologne, Germany) (19) contains a tetramer repeat of a DNA-binding sequence for CPRF1 (Fig. 3). The CPRF1 G-box motif II, 5′-TCCACGTGGC-3′, derived from the chalcone synthase promoter (20) was fused to the TATA-box containing minimal promoter (–46 to +8) of CaMV 35S and the open reading frame of the GUS gene. pb32-GUS (5) contains the promoter of the b-32 gene (31) from base –396 to +4 fused translationally to GUS in the plant vector pBI201.1 (39) (Fig. 2). All five O2 binding sites as identified by Lohmer *et al.* (5) are present on this promoter fragment.

For expression of the SV40 NLS–O2 region–CPRF1 bZIP triple fusion hybrid in *E.coli*, the plasmid $pO2_{39–197}F1_{bZIP}$ was used. The portion of O2 was selected to omit the proline-rich segment at the N-terminus of O2 as the instability of fusion polypeptides containing this region suggested it was acting as a PEST site (40,41). The coding sequences were amplified by PCR for cloning in the *E.coli* expression plasmid pTrxFus (Invitrogen, Netherlands) using the primers #3099 (5′-GTGGTCGACCCACCGGTTGCA-TTTTTC-3′; containing a *Sal*I site) and #3100 (5′-CGTGGATC-CTATGACGCACAATCCCAC-3′; containing a *Bam*HI site). The 5′ primer starts at the SV40 NLS, the 3′ primer at the end of the bZIP domain of CPRF1 in pF1. The PCR product was gel-purified and after digestion with *Bam*HI and *Sal*I, integrated in the *Bam*HI/*Sal*I sites of pTrxFus and designated $pTrxFusO2_{39–197}$-$F1_{bZIP}$ (Fig. 3). The DNA of all constructs was sequenced to verify fusion junctions.

Table 1. Oligonucleotides used to prepare O2/CPRF1 hybrids

number	sequence	O2 AA start
#1398	5′- CC**GGTA**CCGGCATCGTCGTC -3′	5′-AA 39
#1693	5′- CC**TCCGGA**TACTACGGCACT -3′	3′-AA 123
#1829	5′- CC**TCCGGA**GCCTGCAGTTTG -3′	3′-AA 197
#1832	5′- GC**GGTAC**CGGGCCCTTCTGG -3′	5′-AA 12
#2401	5′- CC**GGTAC**CGCTACTAGCTCT -3′	5′-AA 203
#2402	5′- CC**TCCGGA**CTTGAACCCCAG -3′	3′-AA 228
#2618	5′- CC**TCCGGA**TTCCTCGGTAGG -3′	3′-AA 229
#2703	5′- CC**GGTACC**GGTGACCCCATG -3′	5′-AA 124
#4352	5′- GT**GGGTCACC**GCTTGTCGTCAGAGCCT -3′	3′-AA 82
#4355	5′- CTC**TCCGGA**GCTTGTCGTCAGAGCCT -3′	3′-AA 82
#4356	5′- CTC**GGTACC**CTAAATGCTGACCGGCC -3′	5′-AA 83

Bold, restriction sites introduced for cloning; underlined, O2 sequence.

Transient expression analysis

The transient expression of the plasmids in tobacco protoplasts cv. Petit Havana SR1 (41) was performed as described by Negrutiu *et al.* (42). Ten µg each of the effector and reporter plasmid DNA was used for 3×10^5 protoplasts. The transformed protoplasts were harvested and extracts were prepared for measurement of GUS activity as described by Jefferson (39). GUS activity in the extracts was measured using the fluorogenic substrate 4-methyl-umbelliferylglucuronide (4-MUG; Sigma) and measured in a luminescence spectrometer LS30 (Perkin

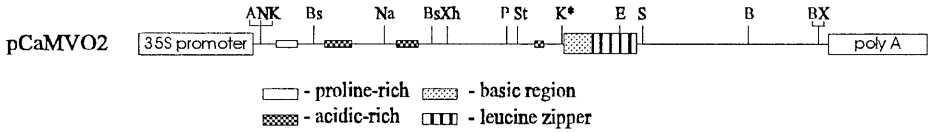

Figure 1. The restriction enzyme sites used for preparing the deletion constructs from the starting plasmid pCaMVO2. The deletions abut the pRT100 polylinker at the 5′-end (designated by sites 'ANK'), and are expressed from the flanking CaMV 35S promoter. The ATG codon at the *Nco*I site is the start codon for all deletions. The *Kpn*I site introduced by *in vitro* mutagenesis in the constructs $pO2_{226–283}$ and $pO2_{226–460}$ is marked with an asterisk and is only present in these constructs. The putative activation domains rich in proline or acidic aa's as well as the bZIP domain are boxed. The restriction enzymes are abbreviated as follows: *Apa*I (A), *Bam*HI (B), *Bst*EII (Bs), *Eco*RI (E), *Kpn*I (K), *Nar*I (Na), *Nco*I (N), *Pst*I (P), *Sal*I (S), *Stu*I (St), *Xba*I (X) and *Xho*I (Xh).

Elmer). Each assay was carried out with extracts containing 5 µg protein, estimated according to Bradford (44). The relative GUS activity were calculated for equimolar concentrations of the constructs.

Expression of fusion proteins in *E.coli*

The ThioFusion™ expression system (Invitrogen, Netherlands) was used for expressing an O2–CPRF1 hybrid as a C-terminal fusion to mammalian thioredoxin. The *E.coli* expression plasmid with the integrated O2–CPRF1 domains, pTrxFusO2 $_{39–197}F1_{bZIP}$, was transformed in *E.coli* GI724 by electroporation [25 µF, 1600 V, 200 Ω using the GenePulser™ (BioRad)]. The thioredoxin fusion protein located to the periplasmic space, allowing purification by an osmotic shock procedure (45).

SDS–PAGE and immunoblotting analysis

SDS–PAGE was carried out as described (46). For immunoblot analysis the proteins were transferred electrophoretically onto nitrocellulose overnight at 4°C using an immersed sandwich setup and the filter was blocked for 1 h in T-TBS [0.5% (v/v) Tween 20, 20 mM Tris–HCl pH 7.6, 137 mM NaCl] at room temperature containing 5% (w/v) non-fat dried milk. Incubation with rabbit anti-O2 serum (1:5000) in T-TBS for 1 h at room temperature was followed after three washing steps by incubation with horseradish peroxidase coupled anti-rabbit antibodies (1:5000). After washing for three times in T-TBS, bound antibody was detected using enhanced chemiluminescence (ECL Western Blotting Detection System, Amersham) and the resulting image recorded on Kodak Xomat-AR X-ray films. Rabbit polyclonal anti-O2 antibodies were raised to the *E.coli*-expressed O2 protein and kindly provided by Dr J. Muth (MPI f. Züchtungsforschung, Cologne, Germany).

In vitro DNA binding assays

Binding of $pTrxFusO2_{39–197}F1_{bZIP}$ proteins to a b-32 promoter fragment was carried out using 1 ng end-labelled DNA (5×10^4 c.p.m.) and the fusion protein (amounts as stated in legend of Fig. 4C) for 15 min at room temperature in 20 µl binding buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 50 mM KCl, 10% glycerol, 2 µg sonicated salmon sperm carrier DNA, 0.1 µg BSA). The binding assay was loaded on a native 3.5% PAA gel in 0.25× TAE and the electrophoresis was carried out at 10 V/cm. The gel was dried on Whatman paper and exposed on autoradiographic films (Kodak Xomat-AR). The b-32 fragment from –265 to –16 upstream of the TATA-box was isolated as previously described (5).

RESULTS

To characterise the transcriptional activation domain of O2 a transient expression system in tobacco mesophyll protoplasts was used. The b-32 promoter is highly responsive to expression *in trans* of Opaque-2 protein in this assay, thus permitting a fine structure analysis which would not have been practicable in the homologous system (5). Different regions of O2, expressed from the CaMV 35S promoter, were tested for their ability to transactivate a reporter plasmid, consisting of the b-32 promoter fused to β-glucuronidase (GUS), upon cotransfection. Every O2 construct (Fig. 2) retained the bZIP domain to ensure specific DNA-binding and nuclear localization properties were always present in the protein (9,11).

Transactivation of the b-32 promoter by O2 deletion derivatives

A series of deletions of O2 were generated by using internal restriction enzyme sites within the O2 sequence and cloned under the control of the CaMV 35S promoter into pRT100 (13) to create pCaMVO2 (Fig. 1) and derivatives of it (Fig. 2). The reporter gene used for assaying the O2 derivatives consisted of a translational fusion of the b-32 promoter, from –396 to +4, to GUS (5). Co-transformation of tobacco protoplasts with various O2 constructs and the pb32-GUS reporter plasmid led to an up to 80-fold increase in GUS activity over cells transfected with the reporter plasmid alone (Fig. 2). The transactivation obtained with the O2 wild-type (WT) construct was used as reference and was set as a relative activity of 100%. A deletion of O2 extending from the start of the leucine-zipper domain to the C-terminus of O2 (designated $pO2_{1–265}$) reduced the enzyme activity to 10% of the WT value. However, a deletion of part ($pO2_{1–412}$) or all ($pO2_{1–284}$) of the molecule C-terminal to the leucine zipper did not reduce activation; surprisingly, $pO2_{1–284}$ showed a 200% increase in activation. In contrast, an N-terminal deletion of the first 225 aa, $pO2_{226–460}$, decreased the transactivation to 11%. The addition of further aa, N-terminal to the bZIP region, in $pO2_{192–460}$ and $pO2_{125–460}$, also gave transactivation values of only 10% of wild type. These results narrow down the activation domain to the region N-terminal of the bZIP domain in O2.

Previously identified transcriptional activation domains have been classified into acidic, glutamine-rich and proline-rich types (Mitchell and Tjian, 1989). Based on a computer homology search with these activation domain types, four putative activation domains were identified within aa 1–192, in the N-terminal half of O2 (Fig. 2), one proline-rich domain, P1, and the acidic domains S1–3. Neither the proline-rich domain nor the acidic domain 3 is sufficient for transactivation, as they are both

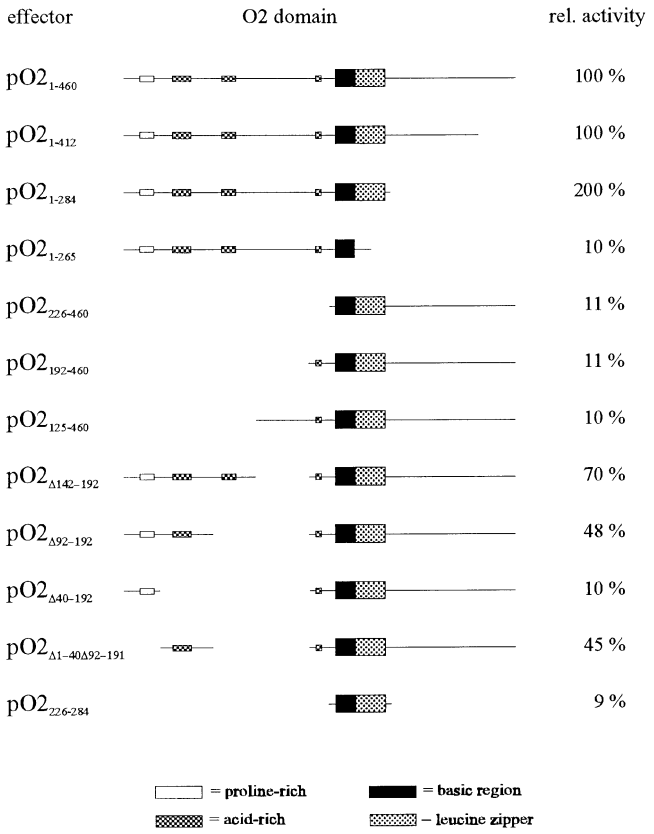

Figure 2. Transactivation of b32-GUS by pCAMVO2 and its deletion derivatives. The relative enzyme activity from co-transfections of O2-deletion constructs with the b32–GUS reporter plasmid. The relative transactivation potential is calculated from five independent transient assays, with a standard error of ±10% or less. The activation by $pO2_{1–460}$ of pb32-GUS was 45-fold over the value with the reporter construct pb32-GUS alone.

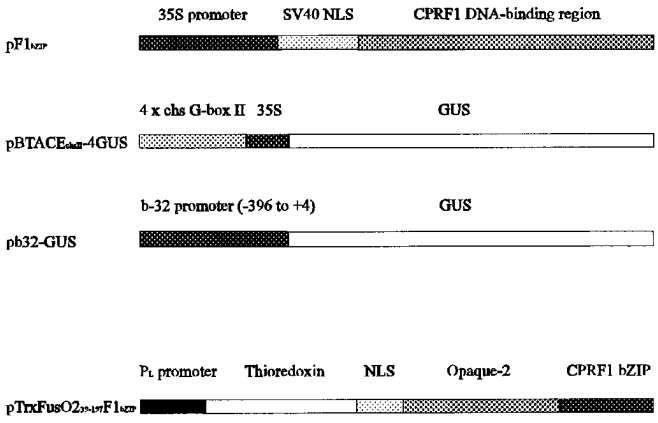

Figure 3. Schematic representation of the plasmids used for transient expression assays and *E.coli* expression. The effector plasmid $pF1_{bZIP}$, which was developed for domain swapping experiments, contains the NLS of the simian virus 40 T-antigen, and the DNA-binding region of the parsley transcription factor CPRF1, under the control of the CaMV 35S promoter. The CPRF1 DNA-binding region used is aa 260–340, starting 11 aa before the basic region. Domains tested were inserted between the SV40 NLS and the CPRF1 bZIP domain. The reporter plasmid $pBTACE^{chsII}$-4GUS has four tandem copies of the CPRF1 G-box binding motif under the control of the CaMV 35S minimal promoter (–48 to +8), in-frame with GUS open reading frame. In the reporter plasmid pb32-GUS, the b-32 promoter region from –396 to +4 is located in frame with the GUS gene. The *E.coli* expression plasmid $pTrxFusO2_{39–197}F1_{bZIP}$ contains the SV40–O2–CPRF-1 hybrid sequence derived from plasmid $pO2_{39–197}F1_{bZIP}$, fused C-terminal to thioredoxin, expressed from PL.

retained in the inactive deletion construct $pO2_{\Delta 40–192}$. This O2 mutant activated to only 10% of the wild-type activity, thus indicating that an O2 domain located between the proline-rich domain and acidic domain 3 must be required for transactivation. This basal activity could be increased to 48% by introducing the first acidic domain ($pO2_{\Delta 92–192}$). The same activity was exhibited by a construct possessing acidic domain 1 but lacking the Proline-rich domain ($pO2_{\Delta 1–40\ \Delta 92–191}$). The combination of the first and second acidic domains gave up to 70% of the WT activity ($pO2_{\Delta 142–192}$). Together, these results suggest that O2 has an activation domain largely comprised of acidic domain 1, with a further contribution from acidic domain 2.

Transient expression of O2 domains as hybrid proteins with the DNA-binding domain of CPRF1

The activation domains of a number of different transcription factors (C1, GAL4, GCN4, Vp1 and VP16) confer promoter activation when fused to heterologous DNA-binding domains in ‘domain swapping’ experiments (14–18). To confirm that the regions of O2 mapped as conferring activation in deletion experiments indeed constitute autonomous activation domains, fusions were made to the bZIP domain of the parsley transcription factor CPRF1, which appears to lack an intrinsic activation domain (19). The resulting hybrid factors were assayed by transient expression in tobacco protoplasts. The DNA-binding domain of CPRF1 used in these experiments binds to a G-box motif of the CHS promoter, the box II motif, which consists of the sequence 5′-TCACGTGGCC-3′ (20). In addition to the bZIP domain of CPRF1, the hybrid constructs derived from $pF1_{bZIP}$ contain at their N-termini the SV40 nuclear localization signal (NLS), to confer efficient nuclear localization of the hybrid polypeptides independently of their molecular weight (21).

O2 domains to be tested were amplified by PCR on pCaMVO2 template DNA (5), and fused into $pF1_{bZIP}$ (Fig. 3) between the SV40 NLS and the CPRF1 bZIP domain. Tobacco protoplasts were co-transformed with these effector clones and a reporter plasmid containing the open reading frame of the GUS gene under the control of one of two target promoters (Fig. 3). In the first series of experiments the reporter plasmid $pBTACE^{chsII}$-4GUS, containing a promoter fragment with a tetramer repeat of the CHS promoter box II motif was used. This motif is recognized by CPRF1 *in vivo* and *in vitro* (19).

Transient expression of the reporter plasmid $pBTACE^{chsII}$-4GUS resulted in a high background enzyme activity in the presence of the basal effector construct $pF1_{bZIP}$, which lacks any O2 segment (Table 2). This high basal activity, presumably due to recognition of the $pBTACE^{chsII}$-promoter by endogenous factors, could only be doubled in co-transfections with an O2 polypeptide running from aa 39 to 228 ($pO2_{39–228}F1_{bZIP}$), or even with a construct having the strong VP16 activation domain ($pF1_{bZIP}VP16_{AD}$). As a consequence, another DNA target promoter with lower background activity in tobacco protoplasts was employed. The deletion experiments had established that the maize b-32 promoter was inactive when introduced into tobacco

Table 2. Transactivation of the pBTACE^{chsII}-4GUS reporter by the O2/CPRF1 effector deletion series

effector	relative activity
$pO2_{39-228}F1_{bZIP}$	1.0
$pF1_{bZIP}$	0.5 +/- 0.1
$pF1_{bZIP}VP16_{act.dom.}$	1.0 +/- 0.1
none	0.03 +/- 0.01

The relative transactivation, measured by GUS activity, is calculated from five independent transient assays.

leaf protoplasts in the presence of the O2 bZIP domain alone ($pO2_{226–284}$, Fig. 2). Therefore, if it could be demonstrated that CPRF1 was capable of binding to this promoter *in vitro*, it could serve to assay the fusion constructs.

Mapping O2 activation domains in O2/CPRF1 hybrids using a b-32 promoter/β-glucuronidase reporter plasmid

To test for CPRF1-binding to the b-32 promoter *in vitro*, the O2–CPRF1 hybrid sequence was expressed in *E.coli* as a fusion to thioredoxin (Fig. 3), and the recombinant protein tested for binding specificity in electrophoretic mobility shift assays (EMSAs). The fusion protein derived from $pO2_{39–197}F1_{bZIP}$ had a predicted molecular weight of 40 kDa, but an apparent molecular weight of 50 kDa based on its mobility in SDS–PAGE (Fig. 4A). This hybrid protein, which lacks the O2 DNA-binding domain, was recognised by polyclonal anti-O2 antibodies on immunoblots (Fig. 4B). Using the protein in EMSA, binding to a b-32 promoter fragment was observed (Fig. 4C), which was not exhibited by purified thioredoxin or a crude extract from the pTrxFus-transformed cells. The O2–CPRF1 hybrid protein ($pO2_{39–228}F1_{bZIP}$) was shown to activate a b-32 promoter/GUS-fusion 30-fold over a control without transactivator in a transient expression system (Fig. 5). In addition, $pO2_{39–228}F1_{bZIP}$ was unable to activate b32*GUS, in which the O2 binding sites have been mutated (36), suggesting activation is mediated by binding at one or more of these sites. Once established, the system was used to test the transactivation conferred by different O2 domains, all lacking the O2 DNA-binding domain. To rule out differential stability of the fusion proteins leading to variation in transactivation, extracts from [^{35}S]methionine-labelled protoplasts transformed with different constructs were immunoprecipitated with O2 antibodies. The amount of precipitated fusion protein was similar between constructs, indicating them to be of comparable stability (data not shown).

The GUS activity produced by co-transfection with the hybrid construct containing the longest O2 fragment tested, $pO2_{12–228}F1_{bZIP}$, was set as 100% relative activity. A deletion derivative of this construct, which lacks the proline-rich region, $pO2_{39–228}F1_{bZIP}$, was as active as wild-type (Fig. 5). Similarly the transactivation seen was unchanged with constructs lacking the third acidic domain ($pO2_{12–197}F1_{bZIP}$) alone, or lacking the proline-rich domain and third acidic domain together

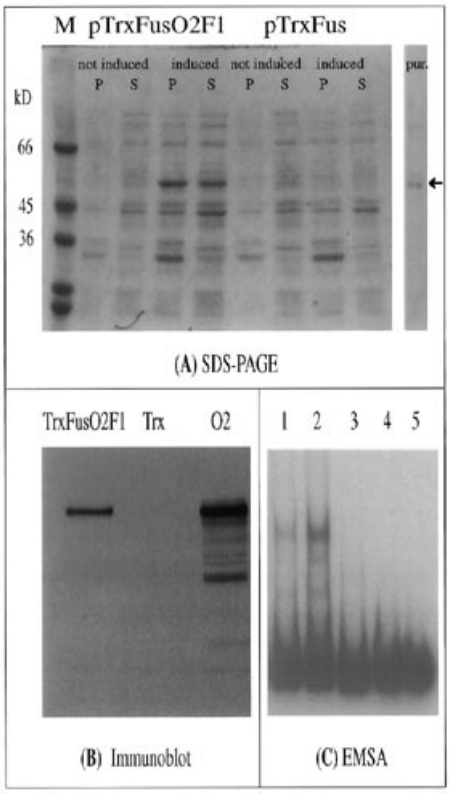

Figure 4. Expression of the TrxFus$O2_{39–197}F1_{bZIP}$ fusion protein in *E.coli*, and its binding to the b-32 promoter demonstrated by EMSA. (**A**) SDS–PAGE of cell extracts of pTrxFus$O2_{39–197}F1_{bZIP}$ or pTrxFus-transformed *E.coli* cells. M, molecular weight marker; P, pellet (insoluble fraction) of cell extracts from induced or not induced transformed cells; S, supernatant (soluble fraction); pur, osmotic shock-purified extract of pTrxFus$O2_{39–197}F1_{bZIP}$-transformed cells. (**B**) Immunoblot of purified fusion protein (TrxFus$O2_{39–197}F1_{bZIP}$), purified thioredoxin, and O2 (150 ng each). Detection with anti-O2 serum and ECL. (**C**) EMSA of radiolabelled b-32 promoter fragment with different cell extracts. Lane 1, 100 ng purified fusion protein (TrxFus$O2_{39–197}F1_{bZIP}$); lane 2, 500 ng purified fusion protein; lane 3, 1000 ng protein extract from pTrxFus-transformed cells; lane 4, 500 ng purified thioredoxin, lane 5, no protein added.

($pO2_{39–197}F1_{bZIP}$): both activated to the same extent as $pO2_{12–228}F1_{bZIP}$. Thus, the O2–CPRF1 hybrids, possessing the proline-rich domain, transactivated to similar extents as those lacking this domain irrespective of the other components of the fusions, as also shown for $pO2_{12–228}F1_{bZIP}$ and $pO2_{39–228}F1_{bZIP}$, $pO2_{12–197}F1_{bZIP}$ and $pO2_{39–197}F1_{bZIP}$, $pO2_{12–123}F1_{bZIP}$ and $pO2_{39–123}F1_{bZIP}$. The level of GUS activity was also not affected by acidic domain 3: the transactivation of the b-32 promoter using $pO2_{39–197}F1_{bZIP}$, which lacks this domain, was as high as that obtained using $pO2_{39–228}F1_{bZIP}$, which contained the third acidic domain. Also the third acidic domain alone ($pO2_{203–228}F1_{bZIP}$) did not increase the enzyme activity over background. Similarly, the segments of O2 located between acidic domain 1 and the O2 bZIP domain ($pO2_{83–228}F1_{bZIP}$ and $pO2_{124–228}F1_{bZIP}$) alone were unable to activate the reporter plasmid over the value obtained with the control. Transcriptional activation was seen in constructs containing the region covering the first acidic domain ($pO2_{39–82}F1_{bZIP}$). This 44 aa region conferred 50% of the transactivation of the longest O2 domain tested so far. The addition of further amino acids C-terminal to the first acidic region ($pO2_{39–123}F1_{bZIP}$ and $pO2_{39–147}F1_{bZIP}$) slightly

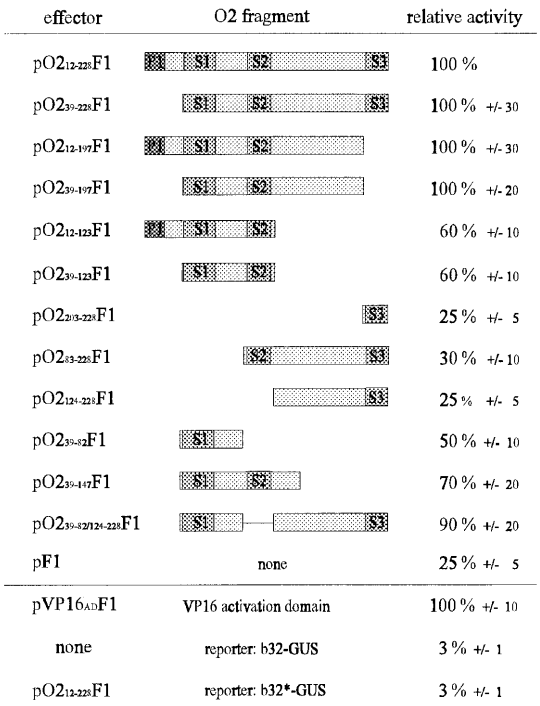

Figure 5. Transactivation of the pb-32GUS reporter by the O2/CPRF1 effector deletion series. The relative transactivation, measured by GUS activity, is the mean of at least 10 independent transient assays, standard errors as given. The activation by pO2$_{12–228}$ of pb32-GUS was 5-fold over the value with the reporter construct pb32-GUS alone.

increased the GUS activity to 60 and 70% respectively. Strong activation at 90% of that obtained with the longest fragment was obtained with the construct pO2$_{39–82/124–228}$F1$_{bZIP}$, which lacks the second acidic domain, suggesting this region does not contribute to the transactivation potential of O2. This was confirmed by the effector pO2$_{83–228}$F1$_{bZIP}$ which did not confer transactivation significantly over background.

The transactivation ability of O2 in the O2–CPRF1 hybrids is thus attributable to a region between the aa 39 and 82, containing the acidic domain S1, a result consistent with that seen in the O2 deletion experiments.

DISCUSSION

Assays of O2 activity in a number of heterologous systems in yeast and tobacco, and transient expression in the homologous system, maize, show that O2 is able to confer transcriptional activation in a variety of environments (10,22–24). O2 has also been shown to partially substitute GCN4 function in yeast cells (25). These observations suggest that an activation domain is an integral function of the O2 protein.

Mapping O2 transactivation domains by analysis of O2 deletions

It is known from comparisons of different bZIP proteins (9) and from the analysis of o2 mutants (10) that the DNA-binding region of O2 is located in the basic region between aa 228 and 247. One of the two nuclear localization signals (NLS) of O2 lies in the same region (aa 223–254), while the second NLS is located between aa 101 and 135 (11). In this study, the region responsible

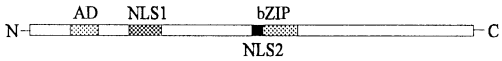

Figure 6. Modular structure of the Opaque-2 polypeptide. The 460 aa protein Opaque-2 has an N-terminal acidic activation domain (AD) located at position 39–82 and a DNA-binding region located in the basic leucine zipper domain (bZIP) between 228 and 247. Two nuclear localization signals (NLS) are located at aa 101–135, and in the bZIP region, at aa 223–254.

for transcriptional activation has been located using deletion constructs of O2 retaining the bZIP region, and with the aid of domain swapping analysis. C-terminal deletions which did not affect the leucine-zipper (pO2$_{1–284}$ and pO2$_{1–412}$) were able to transactivate as well as O2 WT (pO2$_{1–460}$). The deletion derivative pO2$_{1–284}$, which lacks the entire region C-terminal to the bZIP domain, transactivated 2-fold higher than the WT protein. The truncated O2 polypeptide may be sterically improved for efficient transactivation, or it may lack a domain capable of repressing transcription. A significant reduction in transactivation was seen only with either the removal of the leucine repeat or with deletions of the N-terminal part of O2. In the N-terminal region, one proline-rich and three acidic-rich putative activation domains were identified on the basis of sequence comparisons (2). The proline-rich sequence (8/19 consecutive aa are proline), has similarities to the proline-rich activation domain of CTF/NF1(26), and is located at the N-terminus, between aa 13 and 31 (Fig. 2). Acidic domain S1, covering aa 42–71, has a net negative charge of –8. Acidic domain S2, between aa 102 and 122, has net charge of –4. The last acidic domain, S3, is just upstream of the bZIP domain at aa 204–227 and has a net charge of –8). S3 does not function as an activation domain when present as the only acidic domain in the O2 polypeptide (constructs pO2$_{Δ125–460}$ and pO2$_{Δ192–460}$ in Fig. 2). Similarly, the proline-rich domain alone did not confer on the O2 fusion the ability to transactivate (construct pO2$_{Δ40–192}$ in Fig. 2). Addition to the basal construct (pO2$_{Δ193–460}$) of a fragment containing both acidic domains 1 and 2 (pO2$_{Δ142–192}$), however, increased transactivation to 70% of the wild-type activity. Further deletions demonstrated that addition of acidic domain 1 alone to the basal construct raises the transactivation seen to 48% of the WT activity.

Interestingly, acidic domain 1 has sequence homology to a region of the maize transcription factor C1 which has also been shown to function as an activation domain (14). The homology of these acidic regions between O2 and C1 proteins, which lack further homology elsewhere in the sequences, suggests structural conservation, although acidic activation domains in general share no significant sequence homology. The addition of acidic domain 2 to the acidic domain 1-containing effector construct resulted in an increase of the activation from 48 to 70% of WT level. The increase could result from improved spacing between acidic region 1 and the DNA-binding domain which might allow a more productive interaction with proteins of the transcription machinery. The results obtained are summarized in the modular structure of O2 shown in Figure 6.

Assay for O2 transactivation in hybrids with CPRF1, using the b-32 promoter reporter

The promoter of the maize b-32 gene is inactive in tobacco leaf protoplasts in the absence of exogenous transactivator (5). EMSA

assays were performed to test whether the parsley CPRF1 DNA-binding domain could bind to the b-32 promoter, since if this were the case, fusions containing this DNA-binding domain could also be assayed with this system. The experiments showed an *in vitro* binding of the O2–CPRF1 hybrid to a 300 bp b-32 promoter fragment which contains an ACGT core element (5′-TGACGTGA-3′) (9). This ACGT-element is not perfectly palindromic, but binding of bZIP proteins to non-palindromic motifs has been demonstrated, for example for the Arabidopsis transcription factor GBF1 (27), or the wheat bZIP protein EmBP-1 (28). The ACGT-core element could therefore function as a target for CPRF-1 which has been shown to bind to different ACGT-elements (19,20). The DNA-binding domain of CPRF1 may also be capable of binding to non-ACGT elements, as has been demonstrated for O2 (5,29) and Em-BP1 (28). The assumption that the DNA-binding domain of CPRF1 binds to the O2 target sequence(s) within the b-32 promoter was supported by its inability to transactivate the reporter pb32*-GUS, in which the O2 target sites have been mutated (Fig. 5).

Mapping O2 activation domains in hybrids with CPRF1

The N-terminal segment of O2 can stimulate transcription independent of its own DNA-binding domain (Fig. 5), a feature common to most activation domains described so far. The same transactivation as that obtained for O2 is seen for the truncated version pO212-228F1bZIP. The proline-rich domain of O2 is not required for transcriptional activation in the CPRF1 fusion (construct pO2 $_{39–228}$), confirming the result of the O2-deletion series. Together, the results obtained rule out a major role in transcriptional activation of either the acidic domain 3 (construct pO2 $_{203–228}$) or the proline-rich domain.

In contrast, constructs containing acidic domain 1 were able to activate target promoters to give 50% of the activity elicited by the complete O2 region when fused N-terminal to the bZIP domain of CPRF1. A C-terminal extension of this O2-fragment stimulated the transactivation further: the O2-region with the acidic domains 1 and 2 (pO2 $_{39–123}$F1$_{bZIP}$) conferred 60% and pO2 $_{39–147}$F1$_{bZIP}$ 70% of the activity of the control construct. Although the fragment from acidic domain 2 through acidic domain 3 increased transactivation when fused with acidic domain 1, this fragment alone could not stimulate the reporter significantly over background activity, and therefore does not represent a further autonomous activation domain. The deletion of the acidic domain 2 alone reduced transactivation only slightly to 90% of that observed with the control construct. The disparity of 50% activity seen for pO2 $_{39–82}$F1$_{bZIP}$, which contains only the S1 domain, compared to 100% for the control construct could be due either to the absence of additional domains which function only in the presence of the 39–82 region, or to suboptimal spacing or presentation of the activation domain to the transcriptional machinery in the O2$_{\Delta 39–82}$ protein. However, as no effector constructs containing regions outside of S1 were able to activate measurably, the existence of additional independent activation domains is unlikely. We have not seen large additive effects for segments beside S1, making it unlikely that O2 has an extended activation domain analogous to that reported for GCN-4 (30), which consists of the N-terminal one-third of the protein. Bobb *et al.* (31) have also reported a 243 aa acidic activation domain for a plant transcription factor, PvAlf; however, a more detailed dissection of this region may yield a smaller fragment sufficient for function. In contrast, Goff *et al.* (14) show that a 10 aa peptide is sufficient to activate transcription in plants when fused to the GAL4 DNA-binding domain. The 80 aa acidic domain from VP16 and a proline-rich segment from the plant transcription factor GBF1 have both also been shown to activate transcription in tobacco protoplasts (27,32).

The spacing between the DNA-binding domain and the activation domain seems to be critical for the level of activation conferred by a transcription factor (33,34). Support for a spacing effect for S1 is supplied by construct pO2$_{\Delta 39–82/124–228}$F1$_{bZIP}$, which does not contain the acidic region 2. It increased the relative activity of this construct by 40% over that of pO2$_{\Delta 39–82}$F1$_{bZIP}$, though the spacing fragment from 124 to 228 alone was not able to transactivate over background (pO2$_{\Delta 124–228}$F1$_{bZIP}$).

The domain swapping experiments (Fig. 5) confirm the findings of the O2 deletion series that O2 activates transcription by an acidic domain located at the N-terminus, and that this activation does not depend on the presence of a particular O2 *cis*-acting sequence. The significance of this lies in the ability of O2 to form heterodimers with other transcription factors (35), which may give rise to transcriptional activation from promoters not recognized by O2 alone. The ability of O2 to transactivate when located at varying distances to the TATA box has been demonstrated by altering the position of the binding site in the reporter construct (36). O2 polypeptide can be phosphorylated *in vitro* by nuclear extracts of maize endosperm or recombinant casein kinase II of Arabidopsis (37, our unpublished results). The major activation domain identified in O2 contains a potential casein kinase II phosphorylation site at Ser45, which may play a role in modulating activation from this protein.

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