Wild-Type Opaque2 and Defective opaque2 Polypeptides Form Complexes in Maize Endosperm Cells and Bind the Opaque2-Zein Target Site1[OA]

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The Opaque2 (O2) basic leucine (Leu)-zipper transcriptional activator controls the expression of several genes in maize (Zea mays). We investigated the phosphorylation extent of wild-type O2 and mutant-defective or mutant-truncated o2 polypeptides in endosperm cells, their subcellular localization, participation in complex formation, and involvement in functional activity. Besides wild type, four mutant alleles (o2T, o2-52, o2It, and o2-676) producing o2 polypeptides and a null transcript allele (o2R) were considered. Observing the effects of these mutations, multiphosphorylation events in O2 or o2 proteins were confirmed and further investigated, and the involvement of both the nuclear localization signal (NLS)-B and Leu-zipper domains in proper targeting to the nucleus was ascertained. The absence of these domains in the o2T and o2It-S mutant-truncated forms holds them within the cytoplasm, where they are partially phosphorylated, whereas the presence of NLS-B and a partial Leu-zipper domain in o2-52 distributes this mutant-truncated form in both cytoplasm and nucleus. Although mutated in the NLS-B domain, the o2It-L and o2-676 mutant-defective forms are, respectively, partially or completely distributed into the nucleus. Only wild-type O2 and mutant-defective o2 polypeptides bearing the Leu-zipper are able to form complexes whose components were proven to bind the O2-zein target site by in vitro analyses. The transcription of a subset of H-zein genes as well as H-zein polypeptide accumulation in several o2-mutant-defective genotypes indicate the in vivo involvement of o2-mutant-defective proteins in O2-zein target site recognition. The gathered information broadens our knowledge on O2 functional activity and our view on possible quality protein maize trait manipulation or plant transformation via the utilization of cisgenic elements.

The O2 transcriptional activator regulates the transcription of several structural genes coding for a subset of the zein multigene family, for the ribosome-inactivating protein b-32, and for a number of enzyme activities involved in different metabolic pathways (Rossi et al., 2001). Both b-32 and zein polypeptides are recovered in mature seeds, where they display their functional role against pathogen attack and as a source of storage products, respectively. Zein genes can be divided in sequence subfamilies: SF1, SF2, and SF3, coding mainly for heavy-class polypeptides, and SF4, coding mainly for heavy-class polypeptides (Viotti et al., 1985; Rubenstein and Geraghty, 1986). Sequences of the different subfamilies do not cross-hybridize and are regulated by different loci (Soave and Salamin, 1984). In particular, O2 controls the expression of the SF4 genes coding for various size zein polypeptides of the 22-kD type belonging to the heavy zH1 and zH2 classes (Viotti et al., 1985; Ciceri et al., 2000). In vitro experiments showed that O2 homodimers are able to bind the already described O2 target site (5′-TCCAC-GTGA-3′; Schmidt et al., 1990). This sequence is an imperfect A-box-type palindrome located in the P2 promoter regions of most of the heavy-zein genes at about −300 bp from the start codon (Sturaro and Viotti, 2001). However, a further, more distal, promoter, P1, is also able to drive zein gene transcription. P1 and P2 share their consensus sequences, are able to give rise to long and short transcripts, respectively, and both promote reporter gene expression in homologous or
heterologous cell-tissue systems (Langridge and Feix, 1983; Quattrocchio et al., 1990; Giovinazzo et al., 1992).

Several O2 wild-type alleles are known to code for polypeptides of different length, with two hypervariable regions occurring in the first 60 amino acids of the amino-terminal region. In addition, several o2 mutant alleles were characterized. These contain specific nucleotide insertions or deletions, causing frame shifts that result in the translation of mutant-truncated or mutant-defective polypeptides (Lazzari et al., 2002). More in detail, as schematically reported in Figure 1, the o2T allele produces a truncated polypeptide (o2T) missing the second half of the protein and, as a consequence, the NLS-B and the Leu-zipper motifs; the o2-52 allele codes for a differently truncated polypeptide that lacks the carboxy-terminal region downstream of the second Leu of the Leu-zipper motif; the o2lt allele produces two polypeptides: the former, referred to as long (o2lt-L or mutant-defective), migrating in SDS-PAGE as the wild-type protein (67–68 kD), and the latter, referred to as short (o2lt-S or mutant-truncated), with an SDS-PAGE relative mobility of about 47 kD; the o2-676 allele produces a polypeptide that contains an amino acidic substitution (R249K) in the basic region of the bZIP domain; and, finally, the o2R allele is defective in transcription and was defined as a null transcript allele (Schmidt et al., 1987).

Most of the o2 mutant alleles were shown to severely reduce the expression of the heavy-class zeins and only to slightly affect the expression of the light class. The o2-676 mutation, however, surprisingly reduces the expression of a group of zeins belonging to the light class, only slightly affecting some components of the heavy class (Aukerman et al., 1991). In homozygous lines for any of the o2 mutants, the observed seed phenotype is opaque and, even crossing different o2 alleles (Ciceri et al., 2000), complementation was never observed, the translucent and vitreous phenotype typical of the wild-type alleles never being restored (Schmidt, 1993). Mutant o2 alleles were recovered in different genetic backgrounds and found in different maize collections (Schmidt et al., 1987; Bernard et al., 1994). Our previous results clearly show that the genetic background can differently influence the transcription of the zH genes in the absence of the O2 protein—as in o2R genotypes—or even in the presence of defective o2 polypeptides, as in those genotypes carrying the o2It or o2-676 alleles (Dolfini et al., 1992; Ciceri et al., 2000). We suggested that these differences could be attributed to the presence of putative O2 vicarious protein factors able to partially complement o2 function or to other helper factors interacting with the o2-defective proteins (Ciceri et al., 2000; Locatelli et al., 2001).

The regulation of the expression of the O2 gene occurs at different levels. O2 transcription is tissue specific and developmental stage specific: O2 transcription mainly occurs in the subaleurone layers of the endosperm (Dolfini et al., 1992), starting from about 10 d after pollination (DAP; Gallusci et al., 1994). Moreover, the steady-state level of the O2 transcript is also subject to diurnal changes and appears to be regulated by a circadian clock. The highest quantity of O2 transcript is observed at midday and the lowest at midnight (Ciceri et al., 1999). A translational control of O2 expression is exerted by three short upstream open reading frames located in the leader sequence of the mRNA (Lohmer et al., 1993). At the posttranslational level, phosphorylation of the O2 protein modulates its DNA-binding affinity (Ciceri et al., 1997). The O2 polypeptide, in fact, exists in the endosperm cells as a pool of differentially phosphorylated forms that oscillate in their relative abundance, as well as in the extent of phosphorylation. The nonphosphorylated and hypophosphorylated forms bind the target DNA sequence with high affinity, whereas the hyperphosphorylated forms bind the target with low affinity or acquire the capacity to bind it only after in vitro enzymatic dephosphorylation (Ciceri et al., 1997). In addition, the O2 phosphorylation pattern changes diurnally: nonphosphorylated and hypophosphorylated forms accumulate during the day, whereas hyperphosphorylated forms are present mainly at night. This suggests that O2 activity is down-regulated at night both by a reduction in the transcript level and by hyperphosphorylation of the O2 protein (Ciceri et al., 1997). It was shown that O2-binding activity is also influenced by the methylation state of the O2-binding region located in the promoter of the 22-kD zein genes. This region is less methylated in the endosperm than in sporophytic tissues and methylation severely reduces the O2 affinity for its binding site (Sturaro and Viotti, 2001). Furthermore, it was

Figure 1. Schematic representation of an O2 wild-type (O2wt) polypeptide and location of each mutation. For each mutation, an arrow points to the position of the truncated polypeptides. White arrowhead indicates the amino acidic substitution (R249K) of the o2-676 polypeptide. Black bar indicates the first 24 amino acids recognized by the O2-NT antiserum, white bar the last 18 amino acids recognized by the O2-CT, and gray bar the last 18 amino acids recognized by the O2-CT2. Drawing not to scale.
suggested that the activity of the O2 protein depends upon regulatory mechanisms involving protein-protein interaction. In fact, in vitro experiments demonstrated that O2 interacts with another O2-like bZIP protein (OHPI) that is able to bind the O2-binding site, both as homodimer and heterodimer with O2 (Pysh et al., 1993). In addition, O2 interacts with another trans-acting factor, prolamin box-binding factor (PBF), which binds to the P-box motif located in the promoter region of the 22-kD zeins about 30 bp upstream of the O2-binding site (Vicente-Carbajosa et al., 1997; Wang et al., 1998; Wang and Messing, 1998). However, the in vivo occurrence of these or other heterodimers was never demonstrated. In support of the protein-protein interaction hypothesis, cooperative binding of O2 to multiple target sites in the promoters of the regulated genes was demonstrated (Yunes et al., 1998). Moreover, it is known that O2 binding to DNA is, in some way, promiscuous because O2 binds in vitro to different target sequences (Lohmer et al., 1991; Schmidt et al., 1992; Izawa et al., 1993; Yunes et al., 1994). Finally, O2 is able to complement in vivo the bZIP function of the GENERAL-CONTROL-NONDEPRESSIBLE4 (GCN4) gene product in gcn4 yeast (Saccharomyces cerevisiae) strains (Mauri et al., 1993), as well as to activate in the endosperm different genes bearing variable O2 core-binding sequences in their promoter region (Maddaloni et al., 1996; Kemper et al., 1999).

In this article, we analyzed and compared the characteristics of the O2 wild-type and mutant polypeptides with respect to their cellular localization, differences in binding activity, and phosphorylation state, and to their influence on zein gene expression. We also recovered protein complexes containing the O2 wild type at different seed developmental stages that were proven to be able to bind the O2 target site. Similarly, we showed that defective o2 polypeptides containing the Leu-zipper domain form complexes and their isoforms bind the O2 target site upon dephosphorylation. The overall recruited information allowed us to extend the knowledge of the O2 protein function in the maize endosperm.

RESULTS

Analysis of O2 Wild-Type and o2 Mutant Proteins in Different Maize Lines

Nucleotide sequence analysis of O2 genomic fragments and cDNA clones reveals a high degree of heterogeneity among wild-type and mutant alleles (Schmidt et al., 1990; Henry et al., 2005). We characterized three wild-type sequences, having slightly different coding capacities, due to insertion or deletion of a few amino acids in the amino-terminal region. They were classified as O2w1, O2w2, and O2w3 and identified to occur, respectively, in the W64A, W22, and A69Y lines (Ciceri et al., 2000; Lazzari et al., 2002). A number of mutated o2 alleles were also collected and characterized, bearing either few changes or severe alterations in the deriving polypeptides (Bernard et al., 1994; Lazzari et al., 2002).

These O2 and o2 polypeptides were analyzed by western blot with two different polyclonal antibodies: O2-NT, specific for the first 24 amino acids of the N-terminal part of the O2 protein, and O2-CT, specific for the last 170 amino acids of the carboxy-terminal part of the protein. The immunoblot analysis, carried out on O2- and o2-enriched protein extracts from immature endosperms from various genetic backgrounds (GBs), showed a specific polypeptide pattern for each O2 or o2 allele (Fig. 2A). The o2R allele, an o2 null transcript mutant introgressed in the A69Y GB, produced no immune-reacting band with both O2 antisera, with the exception of a faint unspecific band with a relative mobility of about 53 kD that was revealed in the various extracts with variable intensities. The O2wt allele produced a band migrating with a relative molecular mass of about 68 kD that was detected by both O2 antisera. Actually, this band was composed of a closely spaced doublet that became evident under particular electrophoretic conditions (Bernard et al., 1994; Ciceri et al., 1997). In A69Y and Bianchi o2 GBs, the o2It allele produced two bands, both detectable with O2-NT antiserum, the former migrating slightly faster than the wild type and the latter with a relative molecular mass of about 47 kD; this second band was not revealed by the O2-CT antiserum. The product of the o2T allele, in the A69Y and W64A GBs, was detected only by the O2-NT antiserum, showing a closely spaced doublet with relative molecular mass of about 43 kD. When A69Y o2It was crossed with W64A o2T, a pattern consisting of all three expected bands (two specific for the o2It allele plus the lowest migrating one, specific for the o2T allele) was detected with the O2-NT antiserum; in this cross, only the uppermost band, with relative molecular mass of about 67 kD, was detected by the O2-CT antiserum. Also, the 52-kD band produced by the A69Y o2-52 line was detected only by the O2-NT antiserum. Even in this case, in the cross between A69Y o2It and A69Y o2-52, the expected three-band pattern was recovered with the O2-NT antiserum. As usual, only the uppermost band of the o2It allele was detected by the O2-CT antiserum. Finally, the A69Y o2-676 allele produced a polypeptide that was detected by both O2 antisera and migrated slightly slower than the wild type, according to its coding capacity (Aukerman et al., 1991; Lazzari et al., 2002).

The above-mentioned closely spaced doublet is a result of the coexistence in the endosperm cells of differentially phosphorylated O2 or o2 forms (Ciceri et al., 1997, 1999). To better investigate the phosphorylation extent in the O2 and o2 lines, we analyzed the charge heterogeneity of the O2 wild-type and o2 mutant proteins by isoelectric focusing (IEF) in a narrow immobilized pH gradient (IPG; pH 4.0–5.0; Righetti, 1990; Bjellqvist et al., 1993; Ciceri et al., 1997). Figure 2B shows the isoelectric pattern of the O2 and
o2 alleles, whose migration in SDS-PAGE is displayed in the top images (Fig. 2A). After IPG-IEF electrophoresis, proteins were blotted onto nitrocellulose membrane and O2 polypeptides were revealed by immunoreaction with the O2-NT and O2-CT antisera. The o2R null transcript allele produced no immunoreacting band, with the exception of a few faint unspecific bands in the most basic or acidic regions of the gradient. The O2wt, o2It, and o2-676 alleles, which produce polypeptides in the 67- to 69-kD range, showed isoelectric patterns composed of seven to eight isoforms distributed along the 4.2 to 4.7 pH range and detectable by both O2 antisera. The o2T and o2-52 alleles, which produce truncated proteins, showed a lower number of bands, revealed only with O2-NT antiserum and occurring, respectively, in the most acidic or basic region of the gradient. The o2It and the o2T alleles showed the same behavior in two different genetic backgrounds: A69Y or Bianchi o2 and A69Y or W64A, respectively. The crosses between A69Y o2It and W64A o2T or between A69Y o2It and A69Y o2-52 showed additive patterns with bands of higher intensity in the most acidic part of the gradient in the first cross with respect to the second. To support this evidence, a blotted filter from a 3.5 to 5.0 pH gradient containing the wild-type O2 protein and the o2It and o2T mutants was at first immunodecorated with O2-CT, then stripped and reprobed with O2-NT (Fig. 2C). Clearly, in the most acidic region of the gradient, more clean-cut bands were detected for the o2T and o2It with the O2-NT as compared to the O2-CT. This indicates that the truncated o2T and o2It-S polypeptides undergo two or three phosphorylation events.

Variations in the Primary Structure of the O2 Protein Affect Its Cellular Localization

O2 localizes within the cell nucleus and contains two NLSs, NLS-A and bipartite NLS-B, located, respectively, in the first and second halves of the protein (Varagona et al., 1991, 1992). In this article, we analyzed and compared the localization patterns of the O2 and o2 polypeptides presented in Figure 1 in maize endosperm cells. O2 polypeptides were immunodetected by O2-NT and O2-CT antisera on sections from O2 wild-type and o2 mutant maize seeds at 17 to 20 DAP (Fig. 3). The same result was obtained in the o2-676 line, whose o2 polypeptide preserves the two NLSs in its primary sequence
null transcript o2R allele, as expected, did not show any immunofluorescent signal and was considered a negative control. The two o2 polypeptides from the o2-52 and the o2T lines were not revealed by the O2-CT antiserum because they lack the carboxy-terminal region, whereas they were detected by the O2-NT antiserum. In particular, the o2-52 polypeptide was detected both within the nuclei and in the cytoplasm, whereas the o2T that lacks the bipartite NLS-B and the Leu-zipper was detected only in the cytoplasm. The o2It allele produces two polypeptides, the o2It-L, which can be revealed with both the O2 antisera, and the o2It-S, which is revealed only with the O2-NT antiserum (Fig. 2A; Lazzari et al., 2002). Polypeptides immunoreacting with both O2 antisera were detected both in the cytoplasm and in the nucleus, with comparable quantitative distribution. Because o2It-S is not revealed by the O2-CT antiserum (Fig. 2A), which also does not reveal the most acidic bands in o2It extracts (Fig. 2C), the fluorescent signal recovered in the cytoplasm and in the nucleus with the O2-CT should be a result of the presence of the o2It-L polypeptide in both cellular compartments.

**O2 Protein Expression Occurs at Early Developmental Stages during Seed Development and Remains until Maturity**

O2 gene expression starts at about 10 DAP (Galluscì et al., 1994). O2 regulation occurs at the translational level, but phosphorylation of O2 proteins also contributes to regulate O2 activity at the posttranslational level. In this work, the presence and phosphorylation state of the O2 protein in the time course of seed development were investigated.

Protein extracts from wild-type seeds or endosperms at various developmental stages were fractionated according to their molecular masses and charge heterogeneity and revealed by O2-NT antiserum (Fig. 4). As shown in Figure 4A, the O2 protein is present in seeds from about 8 to 9 DAP to the later stages of development and can be detected also in fully dry seeds (data not shown). The relative abundance of all the O2 isoforms (whose presence is evident at 14 DAP) remains fairly constant during seed development (Fig. 4B). Actually, with longer exposures, faint bands of the O2 isoforms are visible from 8 to 10 DAP onward (data not shown).

**O2 Is Recovered as a Complex in Cross-Linked Endosperm Extracts at Early and Late Stages of Development**

The activity of the O2 protein was suggested to depend upon regulatory mechanisms involving protein-protein interaction. In fact, in vitro experiments demonstrated that O2 can interact with another O2-like bZIP protein (Pysh et al., 1993) and with a trans-acting factor, PBF, which binds to the P-box motif that lies 30 bp upstream of the O2 target site within the P2
promoter region of the 22-kD zeins (Vicente-Carbajosa et al., 1997; Wang et al., 1998; Wang and Messing, 1998). However, the occurrence in vivo of these or other heterodimers was never demonstrated.

Here, we report evidence of the existence in vivo of one or more protein complexes containing the O2 transcriptional activator. Protein extracts from O2 wild-type and o2R endosperms were treated with two different cross-linking reagents, dithiobis[succinimidy]propionate (DSP) and 1,4 bismaleimidyl-2,3-dihydroxybutane (BMDB), fractionated by SDS-PAGE, and blotted onto a nitrocellulose membrane. Immuno-reaction with the O2-NT antiserum revealed, only in the wild-type genotype, one or more diffused bands with relative mobility around 180 and 280 kD and an additional faint band at about 68 kD, corresponding to the O2 monomer (Fig. 5A).

To confirm the presence of the O2 protein in the immunodetected complex, the gel lane carrying the fractionated DSP protein complex was sliced from the gel and then treated with the reducing reagent dithiothreitol (DTT). A second fractionation was performed by SDS-PAGE on the treated gel slice. After immunoblotting of the resulting filter with the O2-NT antiserum, it was evident that the intensity of the O2-containing complex had decreased in favor of the monomeric form (Fig. 5C).

Results shown in Figure 5D indicate that, from early to late seed developmental stages, most of the O2 protein is embodied in complexes that always release the O2 monomeric form by treatment with the reducing agent.

Because the DSP molecule contains a disulfide bond and is cleaved by thiol-reducing agents, the O2 cross-linking extracts were analyzed by gel electrophoresis either before or after treatment with β-mercaptoethanol. In these analyses (Fig. 5B), we observed that the faint band corresponding to the O2 monomer in the non-reduced extract became more intense after reduction treatment, whereas the intensity of the O2 complexes severely decreased.
o2 Mutant Polypeptides Containing the Leu-Zipper Domain Form Complexes

The o2 mutant alleles contain base changes or specific insertions/deletions that cause either amino acid substitution or introduction of in-frame stop codons and consequently the formation of defective/truncated polypeptides (Lazzari et al., 2002). The o2It-S and the o2T polypeptides lack both the bipartite NLS-B and the Leu-zipper domain, whereas the o2-52 polypeptide is interrupted downstream of the second Leu of the Leu-zipper motif (Lazzari et al., 2002). The o2-676 polypeptide has an amino acid substitution in the NLS-B/DNA-binding domain that severely reduces its affinity for the O2 box, but does not affect its nuclear localization (Aukerman et al., 1991; Fig. 3). The o2It allele produces two polypeptides: a short one (o2It-S), a consequence of a frame shift due to a 10-bp deletion within the first half of the NLS-B coding sequence that introduced a stop codon; and a long one (o2It-L), with an almost identical relative mobility as the wild type. Experimental data (immunoreaction with the O2-CT antiserum and partial localization in the nucleus) suggest that o2It-L contains part of the NLS-B and the downstream canonical amino acid carboxy-terminal sequence. This can be explained by assuming a read-through of the frame shift in the basic region that restores the original translation frame. This interpretation was supported by the positive immunodetection of the o2It-L and negative immunodetection of o2It-S bands obtained with another antiserum, O2-CT2, which recognizes the last 18 amino acids (Fig. 1) of the O2 protein (data not shown).

To investigate whether the o2 mutant polypeptides were able to form complexes, protein extracts from O2 wild-type and o2 mutant endosperms were treated with the DSP cross-linking reagent, fractionated by SDS-PAGE, and blotted onto a nitrocellulose membrane. The O2 and o2 polypeptides were revealed by the O2-NT antiserum (Fig. 6A). Immunodetection showed the occurrence of complexes only for O2 or o2 polypeptides containing or supposed to contain a Leu-zipper domain. In fact, we observed the presence of the most prominent diffuse band of about 180 kD in the O2wt, o2-676, and o2It genotypes, and its absence in the other o2 extracts. The faint complex band recovered in the o2It genotype was probably due to the dimerization of the o2It-L polypeptide that should contain the Leu-zipper domain. From our data, however, it is impossible to know whether o2It-L contains a complete Leu-zipper or only a partial structure. Treatment of these extracts with reducing agents (Fig. 6B) eliminated the 180-kD diffuse band and released more prominent monomeric forms (as in Fig. 5, B and D). When we analyzed the diffuse band of the cross-linking extracts after reducing agent treatment and carried out a second fractionation, in both the o2-676 and o2It lines the monomeric form was revealed, whereas the complexes almost completely disappeared (Fig. 6, C and D).

O2 Complex Binds the O2 Target Site In Vitro

In vitro experiments showed that O2 binds the motif 5'-TCCACGTAGA-3', located in the promoter of the heavy-zein genes at about −300 bp from the ATG start codon (Schmidt et al., 1990; Sturaro and Viotti, 2001).

Considering this evidence, we investigated whether the O2 complex was also able to bind in vitro to the O2 target site. Cross-linking extracts from two O2 wild types (O2wt and O2w2 in Lazzari et al., 2002) and o2R were fractionated by SDS-PAGE before and after treatment with β-mercaptoethanol. Extracts from an Escherichia coli strain expressing the O2w2 protein (O2be) were also loaded as control (Ciceri et al., 1997). The filter was checked at the end of the Southwestern experiments for proper protein transfer by immunoreaction with O2-NT antiserum (Fig. 7C). The Southwestern experiments were performed using a double-stranded oligonucleotide containing two O2-binding sites (see “Materials and Methods”; Ciceri et al., 1997). In Figure 7A, we observed an intense hybridization signal for the O2be extract corresponding to the O2 monomer. In treated or untreated endosperm extracts, no signal was observed in the o2R sample, whereas faint hybridization signals were revealed in correspondence of the O2 complexes and monomers, with some hybridizing band in the lower region of the filter.

It is known that phosphorylation of the O2 protein modulates its DNA-binding affinity and that the hyperphosphorylated forms acquire a higher binding activity after potato acid phosphatase (PAP) treatment (Ciceri et al., 1997). Therefore, the probe was stripped from the filter, which was then treated with PAP and rehybridized with the same probe (Fig. 7B). The O2 wild types from endosperm extracts showed an increase of hybridization after treatment, both at the complex and the monomer expected relative mobility, whereas the signal of the O2be remained almost constant. From these data, we can infer that the O2 ability to bind the O2 target site is far more influenced by its phosphorylation state than by its monomeric/complex state and is increased by in vitro enzymatic dephosphorylation. Similar results on binding activity were obtained by analysis of the O2 complex at later developmental stages, indicating that O2 functional activity is mainly exerted as a complex partner (data not shown).

Heavy-Type Zein Gene Transcription in O2 Wild-Type and o2 Mutant Maize Lines

Alcoholic extracts from endosperms of maize seeds contain two major protein fractions: the sulfur-rich glutelins, known as β-, γ-, and δ-zeins, and the zein polypeptides sensu stricto, known as α-zein (Shewry and Tatham, 1990; Shewry et al., 1995). α-Zeins can be resolved by SDS-PAGE and IEF into several polypeptides with line-specific patterns (Viotti et al., 1985; Lund et al., 1995). To verify a potential transcriptional
activity of o2-defective proteins that, however, form complexes, we analyzed the SDS-PAGE pattern of zein extracts from O2 wild-type and o2 mutant lines in the A69Y genetic background (Fig. 8A). In the wild-type line, α-zeins were resolved into five discrete bands with different relative abundance: two of heavy type (H1 and H2) and three of light type (L1, L2, and L3). The pattern of each o2 mutant shows a characteristic spectrum of combinations reflecting the presence/absence and the abundance of the five bands. In particular, the L1 band was more intense in the o2T than in the O2 wild type, and the H1 and H2 bands were not detectable in the o2T, o2-52, and o2R mutants. In the o2It mutant, H1 and H2 bands were reduced with respect to the O2 wild type. In mutant lines that do not show H1 and H2 bands (o2T, o2-52, and o2R), the γ-zein band showed higher intensity than in the O2 wild type. Finally, in the o2-676, we observed the presence of both H1 and H2 bands with a noteworthy increase in their relative abundance with respect to those of the wild-type line, as already reported for the o2-676 allele in different GBs (Aukerman et al., 1991). This feature was evident also in the cross between o2-676 and o2It lines.

To extend a possible functional role of defective o2 proteins able to form complexes, we analyzed the heavy-type zein gene expression in different GBs carrying the o2It allele. The original o2It allele, recovered in the Bianchi o2It line, was introgressed in five GBs: W64A, A69Y, NYR, W22, and 3316 (Bernard et al., 1994; Ciceri et al., 2000). In any case, H-zeins were recovered in mature seeds, mainly H1 (Fig. 8B), indicating that in each of the GBs a subset of heavy-type zein genes is transcribed, as previously evidenced in RNA gel-blot analysis (see Dolfini et al., 1992; Ciceri et al., 2000).

Figure 7. DNA-binding analysis of O2 complex. A, Approximately 30 μg of protein cross-linking extracts from O2w1, O2w2, and o2R lines were loaded before (−) and after (+) treatment with β-mercaptoethanol, fractionated by SDS-PAGE, and blotted onto a nitrocellulose membrane. On the same gel, about 5 and 10 μg of the bacterial extract from E. coli expressing the O2w2 protein (O2be) were loaded. The protein blot was probed with the oligonucleotide containing the O2 target sequence, as described in “Materials and Methods.” B, The filter was washed in high-salt buffer to remove the probe, treated with PAP, and then reprobed as in A. Immunodetection of O2 protein. After removal of the probe, the filter was incubated with O2-NT antibody.
The Mutant Complex-Forming o2 Polypeptides Bind the O2 Target Site in in Vitro Functional Analysis

Previous experimental data indicated that the two o2-defective polypeptides, o2-676 and o2It-L, were unable to bind the O2 motif 5'-TCCACGTAGA-3' (Aukerman et al., 1991; Ciceri et al., 1997). The observed presence of H-zeins in the different GBs carrying the o2-676 (Fig. 8A; Aukerman et al., 1991) and o2It (Fig. 8B) alleles strongly suggests on the contrary that o2-676 and o2It-L—which are localized internal to the nucleus and were proven to be able to form complexes—might bind, by themselves or in concert with other protein factors, to target sequences within the promoters of some of the H-zein genes. To validate this interpretation, we performed a Southwestern experiment. A protein gel blot of O2 wild type, o2-676, and o2It extracts, resolved by IPG-IEF, was probed with the double-stranded oligonucleotide containing the O2 target site (Fig. 9A). The membrane was then treated with PAP and reprobed with the same oligonucleotide (Fig. 9, B and C). In Figure 9D, the same membrane was tested for protein loads by immunodetection with O2-NT antiserum. As previously shown (Ciceri et al., 1997), dephosphorylation increases the radioactive signals of the O2 wild type’s most basic isoforms and lights up those of the most acidic ones. Also, the o2-676 and o2It-L isoforms acquire the ability to bind to the O2 target sequence after PAP treatment, even if with reduced efficiency as compared to the wild type. This can explain the presence of H-type zeins in the GBs carrying the o2-676 and o2It alleles.

DISCUSSION

Involvement of O2 Modifications and Domains in Its Cellular Compartmentalization

The genotypes analyzed in this study derive from maize lines in which the introgression of the various o2 alleles was obtained with at least six backcrosses, as reported in Ciceri et al. (2000). Most of the results were obtained from the A69Y GB and are highly consistent in all the experiments.

Immunofluorescence data suggest that the localization of O2 and o2 polypeptides is correlated with the primary protein structure. It is known that the O2 protein contains two NLSs (Varagona et al., 1992). In histochemical localization of O2/GUS fusion protein in transformed tobacco (Nicotiana tabacum) and onion (Allium cepa) tissues, it was observed that bipartite NLS-B is more efficient than NLS-A in targeting O2 to the nucleus (Varagona et al., 1992). Our data show that in maize endosperm the o2T mutant polypeptide, which contains only NLS-A, is retained in the cytoplasm, whereas the O2 wild-type localizes for more than 90% into the nucleus. On the other hand, even though the o2-52 polypeptide contains NLS-B, its fluorescent signal was recovered both in the nuclei and in the cytoplasm, showing that the lack of the polypeptide sequence downstream of the second Leu of the Leu-zipper domain influences to a certain extent protein localization. The 10-bp deletion in the coding part of the basic domain of o2It also affects the localization of its gene products. In fact, the o2It-L polypeptide is detected both in the nuclei and in the cytoplasm, with comparable quantitative distribution, whereas o2It-S remains almost completely confined to the cytoplasm. Furthermore, we observed that the amino acid substitution in the NLS-B/DNA-binding domain of the o2-676 polypeptide, which severely reduces its affinity for the O2-box (Aukerman et al., 1991), does not affect its nuclear localization, being the o2-676 almost entirely revealed in the nucleus. These data suggest that, in the maize endosperm, the two functions of the bipartite basic domain (nuclear localization and DNA binding) are probably separated, as already verified in onion tissues with GUS fusion proteins (Varagona and Raikhel, 1994), and that the R249K substitution partially affects the DNA-binding affinity in in vitro experiments. In summary, the presence of all three NLS-A, NLS-B, and Leu-zipper subdomains is relevant for proper nuclear localization and any alteration in their sequence or location and any alteration in their sequence or
relative position (Varagona et al., 1992) may hamper O2 localization.

IEF data (Fig. 2B) showed that all the o2 polypeptides are present in the endosperm cells in several phosphorylated forms. Because o2T shows at least three phosphorylated forms and is unable to enter the nucleus (Figs. 2C and 3), we deduced that these phosphorylations are due to kinase activities that are present in the cytoplasm. This evidence does not exclude the presence of additional kinase activities able to phosphorylate the O2 protein in the nuclei. In fact, most of the O2 polypeptides are nuclear and the various isoforms are in almost equal relative amounts. A good candidate for O2 phosphorylation seems to be the CKII Ser-Thr kinase that is localized both in the cytoplasm and in the nuclei of maize endosperm cells (Grasser et al., 1989; Zhang et al., 1993). By comparison with the Prosite database (Bairoch, 1991), putative target sites for protein kinases were found in the deduced amino acid sequences of the O2 proteins (Lazzari et al., 2002). Eight locations for CKII were found, four of which occur close to or within NLS-B (Unger et al., 1993; Varagona et al., 1991, 1992). It is not clear whether phosphorylation has an effect on the translocation of the O2 protein to the nucleus, but its involvement in the modulation of O2 activity is demonstrated (Ciceri et al., 1997). We observed that all the phosphorylated isoforms of the O2 protein are present from early to late stages of kernel development (Fig. 4B). Consequently, modulation of the O2 activity by phosphorylation is exerted all along the endosperm development.

O2 and o2-Defective Proteins Form a Complex with Functional Activity

In the endosperm cell, the O2 polypeptide mainly localizes into the nucleus (Fig. 3), where it plays its essential role as transcriptional activator. Indeed, we were able to recover the O2 protein in a nuclear complex. In preliminary experiments on nuclei-enriched fractions treated with DSP, we found the O2 complex tightly bound to chromatin, forming a supercomplex that was unfractionable by SDS-PAGE even after treatment with DNase I (data not shown). However, this chromatinic complex releases the O2 polypeptide as a monomeric form after reducing agent treatment (data not shown). Furthermore, the complex was present from early to late stages of maize kernel development, indicating that O2 carries out its function in constant partnership with other factors. We do not know which are the O2 partners in the complex, but we can suppose that other Leu-zipper proteins are involved in the dimerization, as suggested from in vitro and in vivo evidence (Pysh et al., 1993; Vicente-Carbajosa et al., 1997; Wang et al., 1998; Wang and Messing, 1998). In fact, only the o2 mutant polypeptides containing the entire Leu-zipper motif are able to form the complex (Fig. 6), providing evidence that this motif is really involved in vivo in the protein-protein interaction. In our experiments, two different cross-linking agents were used with analogous results, further demonstrating the complex specificity. Moreover, no complex is recovered in the o2R line or in those lines producing o2 polypeptides defective in the Leu-zipper, supporting the assumption that the presence of a functional Leu-zipper motif is crucial for complex formation.

It is known that the O2 transcriptional activator is able to interact with other bZIP proteins; for instance, O2 can form a heterodimer with Mybleu, a polypeptide encoded by the unspliced myb7 mRNA and composed by a Myb domain followed by the Leu-zipper motif. This interaction was demonstrated in transiently transformed tobacco protoplasts, where Mybleu enhances the transcription of GUS fused to the promoter of the b-32 gene, an endosperm O2-regulated gene (Locatelli et al.,
2000). Furthermore, O2 is able to recruit the maize coactivators GCN5 and ADA2 to modulate transactivation of the GUS gene fused to the b-32 promoter in transiently transformed tobacco protoplasts (Bhat et al., 2004), showing that O2 is able to interact with proteins other than bZIP type in heterologous systems. Our data provide evidence of the presence of a complex involving the O2 protein in the tissue where O2 is typically expressed, the maize endosperm.

Southwestern results supply other important information: the O2 complex is able to bind the zein target sequence and this binding activity significantly increases after in vitro enzymatic dephosphorylation (Fig. 7), indicating that the complex-bound O2 isoforms are to a great extent hyperphosphorylated. This suggests that phosphorylation controls O2 activity also as a complex component.

Evidence of functional activity of o2-676- and o2Ilt-L-defective proteins derives from the analysis of the expression of a group of genes, among the several O2 targeted genes: the H-zeins. This evidence is further supported by the recovery of complexes in o2-676 and o2Ilt lines with comparable mobility to that of the O2 line, and by the fact that this o2-defective polypeptide binds to a certain extent the O2 target site. Heavy-type zein genes were clearly proven to be O2 regulated by the analysis of o2T and o2R genotypes (Viotti et al., 1982; Schmidt et al., 1987). In general, the upstream sequences of zein genes contain two promoters, the distal P1 and the proximal P2, separated by about 1,000 nucleotides (Langridge and Feix, 1983; Quattrocchio et al., 1990; Giovinazzo et al., 1992). Experimental evidence indicated that, in both promoter regions, functional binding sites for O2, O2 coactivators such as PBF, or other transcriptional activators are present (Maier et al., 1987; Schmidt et al., 1990; Vicente-Carbajosa et al., 1997). P1 and P2 are responsible for the production of long- or short-zein transcripts, P2 being the strongest one (Langridge and Feix, 1983). On the other hand, O2 binds in vitro any type of ACGT box in perfect or slightly imperfect surrounding palindromes showing higher affinity for the G type (Izawa et al., 1993). However, an A type with an imperfect palindrome is present in the zein gene promoter. Analyses of genomic clusters of heavy-type zein sequences reported the occurrence of genes coding for mature heavy-class (245 amino acids) and mature light-class (216 amino acids) zein polypeptides, which are transcribed (Song and Messing, 2003) and contain the canonical PBF and O2 boxes in the P2 region or even an additional PBF box in the P1 region. The overall picture suggests that zein genes contain a variety of similar, but not identical, regulatory motifs, and that O2 has promiscuous behavior toward target sites and also toward eventual partners. Partner-specific O2 and even o2-defective complexes could also exhibit different specificity toward the various target sites in vivo.

A flow chart of O2 transcription, posttranslational modification, nuclear import, and nucleus activity, as also deduced from o2 mutant behavior, is reported in Figure 10. This overview on O2 behavior broadens our knowledge about O2 activity and specificity toward its target genes and might be helpful in arranging its utilization for the manipulation of the quality protein maize trait (Gibbon and Larkins, 2005) and the achievement of cisgenic plants with proper regulators, modified regulatory elements, and even H-zein genes modified in their coding capacity for essential amino acids.

MATERIALS AND METHODS

Genetic Material and Protein Extract Preparation

Maize (Zea mays sp. mays) plants were grown in the field during the summer or in the greenhouse during February to May of 2000 and 2001. Genotypes carrying either the wild-type O2 alleles or the various o2 mutant alleles were described by Bernard et al. (1994), Ciceri et al. (2000), and Lazzari et al. (2002). Plants of various genotypes were self-pollinated and seeds were harvested at noon at different days after pollination, immediately frozen in liquid nitrogen, and stored at ~80°C until use. Total protein extracts, zein extracts, and O2-enriched protein extracts either from whole seed or dissected...
endosperms were prepared as previously described by Viotti et al. (1985) and Ciceri et al. (1997).

Gel Electrophoresis and Immunoblotting

O2 protein extracts were fractionated by SDS-PAGE or by IPG-IEF and immunoblotting reactions with O2 antisera (O2-NT, O2-CT) were performed as described previously (Ciceri et al., 1997; Lazzari et al., 2002). O2 polyclonal antibodies specific for the amino-terminal part (O2-NT) were obtained by immunizing rabbits with a synthetic oligopeptide containing the first 24 amino acids of the N-terminal region deduced sequence of the O2 wild-type allele reported by Schmidt et al. (1990), and named O2u2 by Lazzari et al. (2002). The carboxy-terminal O2 antisera (O2-CT) was from the R.J. Schmidt laboratory (University of California, San Diego). In some experiments, an affinity-purified carboxy-terminal antibody, O2-CT2, was utilized. The serum was obtained by immunizing rabbits with a synthetic oligopeptide of 19 amino acids containing the last 18 amino acids, underlined (C-PODYELL-GPNGAHMDMY), of the O2 protein, and the antibody was purified by chromatography on cyanogen bromide-Sepharose coupled with the 19-amino acid oligopeptide.

After electrophoresis, proteins were transferred to nitrocellulose membranes. O2 polyclonal antibodies were revealed by immunoreaction as described previously (Ciceri et al., 1997; Lazzari et al., 2002). Zein extracts were analyzed by fractionation on SDS-PAGE as reported by Viotti et al. (1985) and Lund et al. (1995). Gels were stained with Coomassie Brilliant Blue R250.

Immunofluorescence

O2 wild-type and o2 mutant maize immature seeds were harvested at 17 to 20 DAP and fixed in 10 mM sodium phosphate buffer, 4% (w/v) paraformaldehyde, pH 7 (fix solution). Vacuum infiltration was performed for 6 h on ice; the solution was then removed and the fixation reaction was continued overnight at 4°C. The fix solution was replaced with cold 0.85% (w/v) NaCl and, after 30 min at 4°C, with 70% (v/v) ethanol. The seeds were stored in 70% (v/v) ethanol at 4°C until use. For inclusion, seeds were dehydrated through an ethanol (alcohol/ter-butylc alcohol) series, included in paraffin blocks, and stored at 4°C until use. For the preparation of endosperm sections, seeds included in paraffin blocks were sliced with a microtome to approximately 8 μm in thickness. Sections were placed on glass slides and incubated overnight at 37°C to adhere. Each slide contained two sections of O2 and two of each o2 genotype. To remove paraffin, glass slides with sections were dipped into 100% xylol for 8 min at room temperature. Sections were rehydrated through an ethanol decreasing series, followed by treatments in 0.085% (w/v) NaCl, then in Tris-buffered saline (20 mM Tris-HCl, 0.9% [w/v] NaCl, pH 7.2) for 2 min each, and finally in Tris-buffered saline with 20 mM Glycerol for 30 min. Samples were permeabilized three times in TSB buffer (10 mM Tris-HCl, 0.9% [w/v] NaCl, 0.25% [w/v] gelatin, 0.02% [w/v] SDS, 0.2% [v/v] Trition X-100, pH 7.4) for 10 min each, then preincubated in TSB buffer in the presence of 4% (v/v) goat serum (G9023, Sigma) for 40 min at room temperature. The appropriate primary antibodies (O2-NT or O2-CT) were diluted in TSB buffer with 0.05% (v/v) instead of 0.2% (v/v) Trition X-100, in the presence of 1% (v/v) goat serum, applied on sections, and the incubation was protracted at room temperature for at least 1 h, into a moist box. After primary antibody incubation, the sections were washed three times in TSB buffer for 10 min each at room temperature. Sections were then treated in the dark at room temperature for 90 min with the secondary antibody goat anti-rabbit Alexa Fluor 488, diluted in TSB buffer with 0.05% (v/v) instead of 0.1% (v/v) Trition X-100, followed by the addition of 1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI). Incubation was extended for 10 min, then over-glass was applied to the sections. Sections were analyzed using a Leica confocal microscope and the images were acquired with a Leica confocal software.

Probing Protein Gel Blots with DNA, Southwestern Analysis

The double-strand DNA probe was prepared as already described (Ciceri et al., 1997) by annealing the oligonucleotides 5′-GCGGCTCCAGCTGGAT-AAGCTCTACCTGACGTTGCC-3′ and 5′-CTACAGTCTACCTGCTGAA-GCTTATACCTCCTGAGC-3′ containing two O2-binding sites (underlined).

Filling in labeling with [32P]-dCTP was performed by Klenow. According to the procedure outlined by Ciceri et al. (1997), IEF filters or O2 complex filters were hybridized with a radionulealed O2 probe, washed as described, and exposed to X-ray films. After appropriate exposure time, filters were treated with PAP and then rehybridized with the O2-probe (Ciceri et al., 1997). Finally, O2 and o2 were revealed by immunodetection with O2-NT antisera.

O2 Complex Formation and Analysis

Dissected endosperms were homogenized in the presence of 0.1 M sodium phosphate buffer, pH 8, with or without 3 mM thiol- cleavable DSP cross-linker (D(3669; Sigma), or in the presence of 10 mM HEPES-NaOH, 100 mM NaCl, 5 mM EDTA, pH 6.5, with or without 2.6 mM BMD cross-linker (22332; Pierce). The cross-linking treatments were performed at room temperature for 30 min. The active group of the DSP was blocked with Tris-HCl, pH 7.5, to a final concentration of 35 mM, according to manufacturer procedure. O2 complexes were analyzed by SDS-PAGE on 10% polyacrylamide with or without treatment with thiol-reducing agents. For two-dimensional analysis of DSP extracts, gel strips were incubated in 120 mM Tris-HCl, 3% (w/v) SDS, 20% (v/v) Suc, 0.02% (v/v) bromphenol blue, pH 6.8, at 37°C for 40 min. Gel strips were applied to an SDS-containing gel 10% in polyacrylamide and electrophoresed at room temperature. In both cases, proteins were blotted on nitrocellulose filters and O2 or o2 was revealed by immunodetection with O2-NT antisera.

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