Chromatin and DNA Modifications in the Opaque2-Mediated Regulation of Gene Transcription during Maize Endosperm Development

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The maize (Zea mays) Opaque2 (O2) gene encodes an endosperm-specific bZIP-type transcription activator. In this study, we analyzed O2 targets for chromatin and DNA modifications and transcription factors binding during endosperm development and in leaves. In leaves, O2 targets exhibit high cytosine methylation levels and transcriptionally silent chromatin, enriched with histones H3 dimethylated at Lys-9 (H3K9me2) and Lys-27 (H3K27me2). Transcriptional activation in the endosperm occurs through a two-step process, with an early potentiated state and a later activated state. The potentiated state has cytosine demethylation at symmetric sites, substitution of H3K9me2 and H3K27me2 with histones H3 acetylated at Lys-14 (H3K14ac) and dimethylated at Lys-4 (H3K4me2), and increased DNaseI sensitivity. During the activated state, the mRNA of O2 targets accumulates in correspondence to RNAPII, O2, and Ada2/Gcn5 coactivator binding. The active state also exhibits further increases of H3K14ac/H3K4me2 and DNaseI accessibility levels and deposition of histone H3 acetylated at Lys-9 and trimethylated at Lys-4. Analysis of o2 mutants revealed that O2 targets differ in their dependence on O2 activity for coactivator recruitment and for formation of specific chromatin modification profiles. These results indicate gene-specific involvement of mechanisms that modify chromatin states in the O2-mediated regulation of transcription.

INTRODUCTION

The genomic DNA of eukaryotes is compacted into chromatin that forms a barrier for the accessibility of transcription factors toward the binding of their DNA target elements. In the past decade, it has been clearly demonstrated that epigenetic regulatory pathways influence gene transcription by modulating chromatin structure without altering primary DNA sequences (Li et al., 2002, 2007). The mechanisms that regulate chromatin structure include ATP-dependent chromatin remodeling, replacement of major histones with variants, cytosine methylation, the small RNA pathway, and covalent posttranslational modifications of histones (Goldberg et al., 2007). These mechanisms can directly alter chromatin. However, posttranslationally modified histones can also constitute an intricate histone language that, depending upon the type and combination of different histone marks within a specific chromosomal region, serve as binding platforms to recruit additional effectors, which, in turn, determine changes in the chromatin structure (Strahl and Allis, 2000; Berger, 2007).

In plants, the studies of the regulation of gene transcription through modification of chromatin structure in response to environmental and developmental cues are still limited in comparison to yeast and mammals. One of the best-characterized examples is represented by the Arabidopsis thaliana floral repressor FLOWERING LOCUS C (FLC), where signals from vernalization, daylength, and autonomous pathways induce specific changes in the histone modification profile, thus determining a mitotically stable silent chromatin structure that enables flowering (Boss et al., 2004; Schmitz and Amasino, 2007). An additional example is the phaseolin (phas) gene, encoding a major seed storage protein in French bean (Phaseolus vulgaris) seeds (Li et al., 2001). Ng et al. (2006) demonstrated that phas transcription occurs in two phases, one for poising and the other for activation, characterized by distinct arrays of histone modifications in the phas promoter and possibly associated with ordered recruitment of different histone modifiers. In maize (Zea mays), the first correlation between tissue-specific gene transcription, cytosine demethylation, and changes in DNaseI hypersensitivity sites was reported by analyzing the standard and epimutated alleles of the P locus (Lund et al., 1995b). More recently, it was shown that the light-induced transcription of the maize C₄-specific phosphoenolpyruvate (C₄-Pepc) gene is associated with specific histone modifications at the proximal promoter, which differ from the histone modification profile observed in promoter distal elements in response to metabolic activation (Danker et al., 2008; Offermann et al., 2008).

In this study, we analyzed the role of chromatin and DNA modifications in the Opaque2 (O2)–mediated regulation of gene transcription during maize endosperm development. By choosing the O2 system, we can take advantage of good mechanistic knowledge of various aspects of the O2 activation of transcription (Próna et al., 2005). The O2 gene encodes a DNA binding
protein, belonging to the bZIP class of transcription activators, and its expression is restricted to endosperm from 10 to 45 d after pollination (DAP; Schmidt et al., 1987, 1990; Hartings et al., 1989). O2 can form a homodimer that activates transcription by binding to a conserved DNA motif, containing the ACGT core sequence (hereafter named the O2-box), within the promoters of its target genes (Lohmer et al., 1991; Schmidt et al., 1992; Maddaloni et al., 1996; Muth et al., 1996). Transcriptome analysis revealed that O2 regulates the transcription of several targets involved in various pathways (Hunter et al., 2002). Among these targets, the best characterized are the loci encoding for the zein family of proteins, which are the major maize endosperm storage proteins (Pirona et al., 2005). O2 activates mainly genes encoding the α-zein 22-kD subfamily (Kodrzycki et al., 1989). In the maize B73 inbred line, this zein subfamily is formed by a cluster of 15 intronless loci, spanning a region of ~112 kb, and by one single locus, located at two different sites at the short arm of chromosome 4 (azs22 loci; Song and Messing, 2003). The cluster contains both intact genes and pseudogenes with premature stop codons or large rearrangements, as well as various retroelements, which are interspersed between the azs22 loci. Some of the azs22 loci have a conserved O2-box, located ~300 bp upstream of their ATG translational start codon, and a prolamine box (P-box), positioned 20 bp upstream of the O2-box and bound by the prolamine binding factor, which contains a DOF domain and can cooperate with O2 in regulating transcription (Vicente-Carbajosa et al., 1997; Wang et al., 1998). Additional O2 targets, containing the O2-box and characterized by O2-mediated activation are the b-32 gene, encoding a type I ribosome inactivating protein of 32 kD that is involved in pathogen defense (Lohmer et al., 1991); the cyPPDK1 gene, encoding the endosperm-specific cytosolic isoform of the pyruvate orthophosphatase dikinase and required for carbon partitioning (Sheen, 1991; Maddaloni et al., 1996); and the LKR/SDH gene, encoding the bifunctional enzyme Lys ketoglutarate reductase/saccharopine dehydrogenase involved in Lys catabolism (Kemper et al., 1999). Preliminary observations suggest that mechanisms modifying chromatin states and cytosine methylation are involved in O2-mediated regulation of transcription. The first observation of developmental and tissue-specific changes in demethylation of zein genes was reported by Spena et al. (1983). Subsequently, it was shown that, in specific genetic backgrounds, the demethylation of selected maternally inherited azs22 loci correlates with allele-specific expression (Lund et al., 1995a). Furthermore, the in vitro binding of O2 at the O2-box sequence is impaired by high cytosine methylation (°C) levels that characterize sporophytic tissues, where O2 and its targets are not expressed (Rossi et al., 1997; Sturaro and Viotti, 2001). Finally, it was reported that O2 protein interacts with the maize homologs of the yeast transcriptional coactivator ADA2 (Ada2) and of the histone acetyltransferase GCN5 (Gcn5) and that the three proteins cooperate in driving the transient expression of O2 targets in a heterologous system, thus suggesting that O2 is implicated in modulating histone modifications (Bhat et al., 2003, 2004).

In this study, we provide evidence that O2-mediated transcriptional activation occurs in two phases, first a potentiated and second a transcriptional activated phase, both characterized by a specific profile of chromatin modifications. The dependency on O2 activity in the establishment of these chromatin states was different for distinct subsets of O2 targets, indicating a gene-specific interaction of O2 with chromatin modifying mechanisms in driving transcription.

RESULTS

Selection of O2 Target Genes and Analysis of Their Transcript Levels during Maize Endosperm Development

To analyze the role of chromatin modifying mechanisms in the O2-mediated regulation of transcription, we selected a number of maize sequences, representing different O2 target genes well characterized at molecular level for O2-mediated transcriptional activation. Since we aimed at detecting changes in the chromatin states across different genomic regions, a first criterion for O2 target selection was the availability, at time when this work started, of their complete genomic sequence in the B73 inbred line (see Supplemental Table 1 online). The B73 line was chosen because it is the only line for which was available previously sequenced BAC clones spanning the chromosomal region that contains all the azs22 loci (Song and Messing, 2003) and because this line was used in the maize genome sequencing initiatives (http://www.maizesequence.org), thus also providing information regarding the genomic sequence of most of the other O2 targets. Among azs22 loci, we selected three actively transcribed sequences with an intact coding region and a canonical O2-box within their promoter regions (Song and Messing, 2003). These sequences differ from each other in their genomic localization: azs22.4 and azs22.9 are both in the azs22 cluster, but the latter has a TNP2-like transposon located ~250 bp upstream of the O2-box; azs22.16 is a single-copy gene located outside of the cluster and is allelic to the floury2 allele. In addition, three azs22 pseudogenes that did not exhibit transcription in the study performed by Song and Messing (2003) were selected. These loci differ with respect to the O2-box sequence and the presence of premature stop codons in the coding sequence (CDS): azs22.5 has a canonical ACGT core in the O2-box, but premature stop codons; azs22.12 carries a C-to-A transversion in the O2-box core but has an intact CDS; azs22.11 has both a C-to-A transversion in the O2-box and premature stop codons in the CDS. We also selected single copy O2 targets, including cyPPDK1 (Sheen, 1991) and LKR/SDH (Kemper et al., 1999). In previous studies, cyPPDK1 showed an expression that was restricted to endosperm (Gallucci et al., 1996), while LKR/SDH was transcribed preferentially in the endosperm under O2 control but also exhibited a minor level of O2-independent expression in other tissues (Kemper et al., 1999). Finally, two genes with an O2-independent transcription were selected: the azs19.8-1-4 locus of the zI8 subfamily of 19 kD α-zeins (corresponding to the Z49M16-4 clone in Song and Messing, 2002) as an example of an endosperm-specific gene, and MAc1 (Shah et al., 1983), encoding actin 1, as an example of a constitutively expressed gene.

Quantitative RT-PCR (qRT-PCR) experiments, with locus-specific primer combinations (see Methods and Supplemental Figures 2A and 2B for details), were performed to analyze the
transcript levels of the selected O2 targets in wild-type endosperms harvested at various developmental stages, in o2 null mutant endosperms, and in nucellar and young leaves (V2/V3 stage) as examples of sporophytic tissues. The results were in good agreement with the reports from the previous studies described above (Figure 1A). The transcription of azs22.4, azs22.9, azs22.16, and cyPDK1 was restricted to endosperm, starting from 12 DAP and depended on O2 activity because their mRNAs were not detected in o2 mutant endosperms. O2 showed a similar pattern, which is in agreement with a previous report (Gallusci et al., 1994), although mRNA traces were detectable also in 8-DAP endosperms. However, at this stage, the O2 protein was not detected in immunoblots with anti-O2 antibody (see Supplemental Figure 1A online), indicating that 8-DAP endosperms used in this study represent an early endosperm developmental stage where the O2-mediated transcriptional activation did not occur. As expected, the LKR/SDH gene exhibited a certain degree of O2-independent transcription in wild-type 8 DAP and in o2 mutant endosperms, as well as in sporophytic tissues. The transcription of azs19.B-1-4 and MAC1 was endosperm specific and constitutive, respectively, and for both genes it was independent of O2 activity. In agreement with previous observations (Liu and Rubenstein, 1993; Song and Messing, 2003), we detected transcripts corresponding to azs22.5, azs22.12, and azs22.11 pseudogenes in wild-type endosperms, while their accumulation was abolished in the o2 mutant. Nevertheless, the comparison of transcript levels of O2 targets after the standardization of qRT-PCR results for primer amplification efficiency indicated that the mRNA amount of azs22 pseudogenes was very low (at least 40 times less compared with the azs22 genes; Figure 1B). This observation suggests that azs22 pseudogenes are transcribed only at a basal level or that posttranscriptional regulation accounts for the low amount of their transcripts.

**RNPII and O2 Binding to O2 Target Genes**

Chromatin immunoprecipitation (ChiP) assays, followed by quantification of the immunoprecipitates by quantitative PCR (qPCR), were employed to analyze the timing of the in vivo RNPII and O2 binding to O2 targets. This and subsequent experiments were performed using chromatin extracted from (1) wild-type 8-DAP endosperms, as an example of early endosperm development where O2 activation does not occur; (2) wild-type 15- and 23-DAP endosperms, representing endosperm developmental stages with O2 expression; and (3) o2 mutant 15-DAP endosperms and young leaves, where O2 is not expressed. The results showed that RNPII binding to the promoter (containing the O2-box) and to the CDS of azs22 genes (azs22.4, azs22.9, and azs22.16), azs22.5 pseudogene, cyPDK1, and LKR/SDH occurred only in 15- and 23-DAP wild-type endosperms, concomitantly with the O2 binding to the promoter of the same genes (Figure 2). The comparison of these results with the mRNA levels of O2 targets during endosperm development indicated that the binding of RNPII and O2 correlated with the accumulation of transcripts (cf. Figures 1 and 2). The binding was abolished in 8-DAP wild-type and in 15-DAP o2 mutant endosperms, as well as in leaves, where O2 protein is not present and transcription activation of O2 targets does not take place. The LKR/SDH gene represents an exception because weak RNPII binding was observed also in tissues lacking O2 expression, thus confirming previous observations of a certain degree of O2-independent transcription. Our results furthermore showed that O2 binding was specific for the promoter region containing the O2-box because, when we checked for possible O2 enrichment in a promoter region located ~350 bp upstream of the O2-box of azs22 loci, no signal above the background level was detected (see Supplemental Figure 3 online). Only a weak, if any, binding of both RNPII and O2 was observed for the other two azs22 pseudogenes: the azs22.12 and azs22.11 loci, which have a mutated O2-box. As expected for genes expressed independently of O2 activity, O2 binding was not observed for azs19.B-1-4 and MAC1 promoters. Collectively, our findings provide support for specific binding of O2 protein at the O2-box of its targets, with concomitant RNPII recruitment and accumulation of transcripts.

**Cytosine Methylation in O2 Target Genes**

The quantitative analysis of the mC level was performed by restriction of genomic DNA with the McrBC enzyme, followed by PCR quantification of the McrBC-treated and untreated DNA. McrBC digests DNA that is methylated at two or more cytosines, thus reducing PCR amplification proportionally to the mC level in the sequence encompassed by the selected primers (Rossi et al., 2007). The results indicated that mC in the promoter of O2 targets and of the azs19.B-1-4 locus was higher in leaves compared with endosperms (Figure 3). The mC of the promoter of these genes was always low in endosperms, independent of the developmental stage and of o2 mutation. This finding provides evidence that a low mC level in the promoter of O2 targets does not necessarily correlate with transcription.

Information about which specific cytosine in the sequences of O2 targets undergoes changes in its methylation level was obtained by sequencing of bisulphite-treated genomic DNA. Results from a selected number of sequences representing different O2 targets are summarized in Supplemental Table 2, while their graphical representation is reported in Supplemental Figures 4A and 4B. Indications from bisulphite genomic sequencing corroborated the findings from McrBC treatments regarding changes of the total mC level. Interestingly, we observed that a significant level of mC was present in the CDS of some sequences (particularly in azs22.9 and azs22.11) in both endosperms and leaves. In addition, bisulphite analysis revealed that the higher mC level of promoters observed in leaves compared with endosperms was due almost exclusively to methylation of cytosine in CpG and CpWpG (W is A or T) sequences, including the CpG dinucleotide within the ACGT core of the O2-box. Similarly, CpG and CpWpG methylation was the predominant methylation type also in CDS.

**Analysis of Nucleosome Density at O2 Target Genes**

Nucleosome density in O2 target genes was measured with ChiP assays using an antibody against the histone H3 C-terminal region (H3cter), which recognizes both modified and unmodified
Figure 1. Transcript Profile of O2 Target Genes.
histone H3. This experiment provided evidence for possible changes in nucleosome occupancy during transcription activation and was also relevant to appropriately correct ChIP data obtained using antibodies against modified histones. Indeed, nucleosome density may not be homogeneous across the genome in different transcriptional states or in mutants affecting histone modifier activity (Ng et al., 2006; Rossi et al., 2007). This implies that nucleosome-rich and nucleosome-poor regions may appear as more and as less modified than they really are, respectively. The results indicated that nucleosome occupancy

Figure 2. O2 and RNAPII Binding at O2 Target Promoters.

Bar diagrams represent real-time PCR quantification of ChIP DNA, reported as percentage of the chromatin input, from assays performed using the indicated antibodies (RNAPII and O2) to assess binding in promoter and CDS region (see legend in the top left corner) for the indicated genes and for the tissues reported in the graphs. Horizontal black line shows the background signal, measured by omitting antibody during ChIP procedure. Data are average values from two independent ChIP assays and from three PCR repetitions for each ChIP assay; standard errors are reported.

Figure 1. (continued).

(A) Real-time RT-PCR was performed using locus-specific primer combinations to analyze the mRNA level of O2, O2 target, and control (azs19.B-1-4 and MAct) genes in maize endosperm harvested at various DAP from wild-type and o2 mutant (15 DAPo2 and 23 DAPo2) plants and in sporophytic tissues (L, leaf; N, nucellar). Bar diagrams represent the mean value (expressed as arbitrary units, calculated by a standard curve obtained employing as PCR templates plasmids containing cDNA fragments corresponding to the amplicon; see Methods for details) of mRNA level obtained by two different cDNA preparations and three PCR replicates for each preparation. Standard errors are reported.

(B) Relative levels of assayed genes, normalized as follows: data from qRT-PCR, performed using cDNA prepared from 15-DAP endosperms, were standardized to qRT-PCR data, performed using as template plasmids containing cDNA sequences corresponding to the amplified fragments for each of the analyzed genes. This standardization allows comparison of the qRT-PCR data from different genes, after correction for differences in the primer amplification efficiency. Ordinates are fold differences compared with the sample with lowest transcript level (azs22.11), which was set equal to 1. Standard errors are reported. Fold differences are indicated on the top of each bar diagram.
in both promoter and CDS regions of $O_2$ targets did not show relevant changes in endosperms at various developmental stages or in leaves (see Supplemental Figure 5 online). A statistical analysis of the data corroborated this conclusion because statistically significant changes of H3cter enrichment were randomly observed and occurred only for some sequences and in some tissues/developmental stages, independently of the transcriptional state of the genes. The observation that, with all primer combinations, no particular tissue or developmental stage showed a peculiar H3cter enrichment also suggests that chromatin extracted from different tissues has similar precipitation efficiencies in ChIP assays. Nevertheless, to consider also small differences in nucleosome density, data from ChIP with H3cter were employed to correct ChIP-derived data for the analysis of the modified histone levels (see Methods for further details).

**Histone H3 Acetylation in O2 Target Genes**

To analyze the correlation between transcriptional activity of $O_2$ targets and changes of posttranslational histone modifications, we focused on different types of modifications occurring in the N-terminal residues of the histone H3. First, we performed ChIP assays to investigate changes in histone H3 acetylation, which
occurs primarily at Lys-9 and -14 (H3K9ac and H3K14ac) and that usually correlate with transcriptional activity (Kouzarides, 2007; Zhang, 2008). To this end, we employed antibodies specific for H3K9ac, H3K14ac, and histone H3 diacetylated on K9 and/or K14 (H3ac). The results revealed that, in 8-DAP endosperms, the promoter of azs22 genes (azs22.4, azs22.9, and azs22.16) and of the azs22.5 pseudogene showed a level of H3K14 and H3ac significantly above the background signal (Figure 4). The amount of these histone marks increased in later endosperm developmental stages (15 and 23 DAP) in an O2-dependent manner because it was strongly reduced in 15-DAP o2 mutant endosperm. O2-dependent accumulation in 15- and 23-DAP endosperms was observed also for H3K9ac, although this histone mark was not detected in 8-DAP endosperms. Histone acetylation was not or only slightly detected in leaves. Similar changes in the histone acetylation profile were observed for the promoter of cyPPDK1 and LKR/SDH. However, in these sequences hyperacetylation in 15- and 23-DAP endosperms was not related to O2 activity because, similar to the O2-independent azs19.B-1-4 promoter, it was maintained also in 15-DAP o2 mutant endosperms. Finally, azs22.12 and azs22.11 pseudogene promoters exhibited very low histone acetylation levels in all tissues analyzed, while histone acetylation in the CDS of MAc1 sequence occurred both in endosperms and leaves as expected for a housekeeping gene.

Taken together, these results show that H3K9ac and H3K14ac accumulate during different stages of endosperm development and that this accumulation is related to O2 activity only in a subset of O2 targets. Histone acetylation was also detected in the CDS regions of the sequences mentioned above (see Supplemental Figure 6 online), and results showed that promoter and CDS regions exhibited similar histone acetylation profiles during endosperm development.

**Histone H3 Methylation in O2 Target Genes**

The levels of histone H3 di- and trimethylated at Lys-4 (H3K4me2 and H3K4me3), which are modifications usually related to

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**Figure 4.** Histone H3 Acetylation Levels in the Promoters of O2 Target Genes.

Bar diagrams represent qPCR results from ChIP assays performed using the indicated antibodies (H3ac, H3K9ac, and H3K14ac) and reported as percentage of chromatin input after correction for nucleosome density, measured using H3cter antibody in separate ChIP assays (see text and Methods for details). Data are average values from two independent ChIP assays and three PCR replicates for each assay; the standard error is reported on the top of each diagram. Horizontal black line shows the background signal, measured by omitting antibody during ChIP procedure.
transcriptional activation, and the levels of histone H3 dimethylated at Lys-9 (H3K9me2) and Lys-27 (H3K27me2), which are considered to be heterochromatic silencing-related chromatin marks (Kouzarides, 2007; Zhang, 2008), were analyzed by ChIP assays using specific antibodies. In the promoters of all O2 targets, with the exception of the azs22.12 and azs22.11 pseudogenes, H3K4me2 was detected in 8-DAP endosperms, and its level increased in 15- and 23-DAP endosperms (Figure 5). Similarly to H3K14ac, a subset of O2 target promoters (azs22.4, azs22.9, and azs22.16 genes and the azs22.5 pseudogene) showed a strong decrease of the H3K4me2 level in 15-DAP o2 mutant endosperms, while this reduction was not observed for a second group of promoters (cyPPDK1 and LKR/SDH). Conversely, all O2 targets, with exception of azs22.12 and azs22.11 pseudogenes, exhibited an accumulation of H3K4me3 only in 15- and 23-DAP endosperms from wild-type plants, and its amount was severely reduced in the presence of the o2 mutation. Only traces of H3K4me2 and H3K4me3 were observed in the

![Figure 5. Histone H3 Methylation Levels in the Promoters of O2 Target Genes.](image)

Bar diagrams represent qPCR results from ChIP assays performed using the indicated antibodies (H3K4me2, H3K4me3, H3K9me2, and H3K27me2) and reported as percentage of chromatin input after H3cteri correction. Data are average values from two independent ChIP assays and three PCR replicates for each assay; standard error is reported on the top of each diagram. Horizontal black line shows the background signal.
endosperms for the promoter of azs22.12 and azs22.11 pseudogenes. In the promoters of all O2 targets and of azs19.B-1-4, H3K4me2 and H3K4me3 were not detectable in leaves, whereas in the CDS of the constitutively expressed MAC1 gene, they accumulated both in leaves and in endosperms at all developmental stages. An opposite profile was observed for H3K9me2 and H3K27me2, whose accumulation in the promoters of all O2 targets and of azs19.B-1-4 was detected only in leaves. Finally, the analysis of the histone H3 methylation levels in the CDS of O2 targets showed that the changes in the histone methylation profile during endosperm development reported for the promoters are also observed in CDS regions (see Supplemental Figure 7 online).

To summarize, our findings indicate that (1) the accumulation of H3K4me2 occurs in early stages of endosperm development and is dependent on O2 only in a subset of O2 targets; (2) the accumulation of H3K4me3 is always related to O2 activity and occurs concomitantly with gene transcriptional activation (cf. Figures 1 and 5); and (3) the accumulation of H3K9me2 and H3K27me2 occurs only in leaves, in parallel with an increase of the \(^{14}C\) level (cf. Figures 3 and 5).

**DNasel Sensitivity in O2 Target Promoters**

To analyze the degree of chromatin decondensation in the O2 target promoters during endosperm development and in leaves and in correspondence to deposition of different chromatin and DNA marks, we performed DNasel sensitivity assays. These experiments were done using the method of McArthur et al. (2001), that employs real-time PCR to quantify the sensitivity of chromatin to digestion by DNasel. This method permitted the use of locus-specific primers to detect changes in chromatin condensation between genes, such as those at the azs22 loci, which have a very high nucleotide sequence homology. Moreover, it improves the quantification accuracy with respect to the more conventional use of the DNA gel blot hybridization assay. The results are illustrated graphically by plotting the percentage of remaining DNA after DNasel treatment against an increasing number of DNasel units (Figure 6). In these curves, the level of sensitivity of the site to digestion is proportional to the rate of loss of copies for a given sequence (McArthur et al., 2001). Our findings indicate that the highest rate of loss of copies for promoters of azs22.4, azs22.9, and azs22.16 genes and of azs22.5 pseudogene was observed in 15-DAP wild-type endosperms (Figure 6). The degree of DNasel accessibility was very limited in leaves, while it exhibited an intermediate level in 8-DAP wild-type and 15-DAP o2 mutant endosperms. A similar profile was observed also for cyPPDK1 and LKR/SDH promoters. However, in comparison to azs22 loci, these sequences are characterized by a less pronounced difference in the degradation rate observed in 15-DAP wild-type compared with o2 mutant endosperms. This difference was completely absent for the azs19.B-1-4 promoter, which drives transcription independently of O2 activity. The rate of copies loss for the azs22.12 and azs22.11 pseudogenes was very low at all endosperm developmental stages and in o2 mutant endosperms and was similar to that observed in leaves.

Taken together, these results indicate that the chromatin state at O2 target promoters is poorly accessible to DNasel in leaf tissues, while, with exception of azs22.12 and azs22.11 pseudogenes, it becomes more decondensed in endosperm. The degree of DNasel sensitivity increases in later stages of endosperm development and correlates with O2 activity for a subset of genes, whereas other sequences achieve a major degree of chromatin decondensation also in the absence of O2.

**Binding of Maize Ada2 and Gcn5 at the O2 Target Promoters**

To assess whether changes in histone H3 acetylation and methylation levels correspond to the binding of specific histone modifiers at the O2 target promoters, we performed ChIP assays using maize-specific antibodies (see Supplemental Figure 1B online). In particular, we analyzed the binding of the coactivator Ada2 and of the histone acetyltransferase Gcn5. Both factors have previously been shown to physically interact with each other. Ada2 in addition was found to bind to O2 protein (Bhat et al., 2003, 2004). The results showed that, in 8-DAP endosperms, low levels of Ada2 and Gcn5 enrichment, only slightly over the background signal, were detected for most of the sequences analyzed (Figure 7). The level of Ada2 and Gcn5 binding at these sequences, with exception of the azs22.12 and azs22.11 pseudogenes, increased and was evident during later stages of endosperm development, while it was not detectable in leaves. In 15-DAP o2 mutant endosperms, the binding at the promoter of azs22 genes and of the azs22.5 pseudogene, but not that of the other O2 targets, was abolished. Therefore, these results indicate that Ada2 and Gcn5 are recruited to O2 target promoters during endosperm development and that O2 protein is required for this binding only for a subset of targets.

**DISCUSSION**

In this study, we analyzed changes of the mRNA, \(^{14}C\), and histone H3 acetylation and methylation levels, as well as the binding of transcription factors and DNasel sensitivity, at different O2 target genes during endosperm development. Our results are indicative of an ordered sequence of changes in these chromatin and DNA marks that occur corresponding to different states in transcriptional activity. These findings provide, therefore, evidence for the role of chromatin modifying mechanisms in the O2-mediated regulation of gene transcription.

**The O2 Dependency for Deposition of Chromatin Marks Defines Distinct Groups of O2 Target Genes**

The comparison of the chromatin modification profiles formed during endosperm development in different O2 targets allowed their subdivision into distinct groups (see Supplemental Figure 8 online). Group 1 contains the three azs22 genes (azs22.4, azs22.9, and azs22.16) and the azs22.5 pseudogene, while cyPPDK1 and LKR/SDH genes belong to group 2. The other sequences analyzed (azs22.12 and azs22.11 pseudogenes, and azs19.B-1-4 and MAC1 genes) displayed differences in their chromatin modification profiles, but they can be grouped together because all shared the feature of a transcriptional
Figure 6. DNaseI Accessibility at the O2 Target Promoters.

Graphs represent the plot of percentage of remaining DNA after DNaseI digestion and qPCR amplification, corrected for DNA content, against the number of DNaseI units used for the treatments of nuclei prepared from 8- and 15-DAP wild-type endosperms, 15-DAP o2 mutation endosperms, and leaves. Data are average values from two different DNaseI treatments and three PCR replicates for each treatment; standard errors are reported. Correction for DNA content was obtained by normalizing the qPCR data for each sample and each DNaseI treatment to the corresponding data obtained using the identical sample and primers specific for the sequence of the Opie2 Copia-like retroelement, which was inaccessible to DNaseI also at the highest number of DNaseI units.
activation not, or only very weakly, mediated by O2. Indeed, O2 binding to the promoters of these sequences was not detected by ChIP assays. The mRNA level and chromatin marks associated with transcription activation of azs22.12 and azs22.11 were very low in all tissues analyzed, suggesting that they are not or only basally transcribed, whereas azs19.B-1-4 and MAc1 genes are O2 independent actively transcribed genes. By contrast, transcription of members of groups 1 and 2 showed dependency on O2 activity as evidenced by O2 binding to their promoters and by the finding that RNPII recruitment and mRNA accumulation were impaired in o2 mutant endosperms. These genes also exhibited similar changes of the levels of mC, histone H3 modifications, DNasel sensitivity, and Ada2 and Gcn5 binding during endosperm development and in endosperms compared with leaves. However, they differ regarding the O2 dependency in the later stages of endosperm development (15 and 23 DAP). For group 1 sequences, the o2 mutation strongly reduced accumulation of modified histone H3 associated with transcription (H3K9ac, H3K14ac, H3K4me2, and H3K4me3), the degree of DNasel accessibility, and the recruitment of Ada2 and Gcn5. Conversely, with the exception of H3K4me3 deposition, the o2 mutation had no or only very weak effects in genes of group 2. This observation suggests that O2 can activate transcription of its targets through different mechanisms, which are specific for subsets of genes on the basis of different modalities of interaction with chromatin regulators. A similar scenario was also reported for other transcription activators, including the bZIP class yeast GCN4, which shows several structural and functional homologies with O2 (Mauri et al., 1993; Pirona et al., 2005). It was shown that GCN4 protein is able to recruit multiple coactivators, including yeast ADA2 and GCN5, at its target promoters, but the requirements for these coactivators varies greatly among individual promoters and in many cases is not sufficient to drive transcription (Swanson et al., 2003; Yu et al., 2006).

**Different Regulatory Mechanisms for azs22 Pseudogenes with Distinct Chromatin Modification Profiles**

Although all three azs22 pseudogenes exhibited very low mRNA levels in endosperm, they showed relevant differences in their chromatin modification profiles and in binding of transcription factors during endosperm development (see Supplemental Figure 8 online). As previously mentioned, the azs22.12 and azs22.11 loci exhibit only a basal transcriptional activity. This activity seems to be partially related to O2 because the o2 mutation fully abolished the accumulation of their transcripts in

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Figure 7. Ada2 and Gcn5 Binding at O2 Target Promoters.

Bar diagrams represent qPCR results from ChIP assays, performed using anti-Ada2 and anti-Gcn5 antibodies and reported as percentage of chromatin input. Data are average values from two independent ChIP assays and three PCR replicates for each assay; standard error is reported on the top of each diagram. Horizontal black line shows the background signal.
endosperm. The O2 binding to the O2-box of these promoters was affected by a C-to-A transversion within the ACGT core sequence (Rossi et al., 1997). However, Muth et al. (1996) reported that in vitro binding of O2 can also occur with decreased efficiency in sequences located outside the O2-box. The in vivo O2 binding to these elements may be weak or unstable and thus not detectable in ChIP assays and insufficient to effectively recruit the machinery required to drive transcription over a basal level. Conversely, the azs22.5 pseudogene has a conserved O2-box and displayed changes in its chromatin state and of transcription factors binding during endosperm development similar to those of actively expressed azs22 genes (see Supplemental Figure 8 online). This observation is indicative of an active transcription of this locus, followed by a subsequent reduction of its mRNA level due to posttranscriptional regulation. We were unable to prove this conclusion definitively because we could not distinguish between different azs22 loci with very high nucleotide sequence homology using the conventional nuclear run-on technique to measure the rate of RNA transcription (Kodrzycki et al., 1989). However, previous observations suggested that posttranscriptional mechanisms play an important role in the regulation of azs22 expression (Kodrzycki et al., 1989; Plotnikov and Bakaldina, 1996). In particular, according to a previous suggestion (Song et al., 2001), the premature stop codons within azs22.5 CDS may constitute the signal for the RNA surveillance system named nonsense-mediated RNA decay, which is conserved in eukaryotes, including plants and identifies and eliminates these types of aberrant RNAs (Kertesz et al., 2006).

Chromatin and DNA Modification Profiles of O2 Targets Promoters and Coding Regions

In this study, similar changes of the histone H3 modification profile were detected in promoter and CDS of O2 targets. This is in agreement with recent findings from a genome-wide ChIP analysis in rice (Oryza sativa; Li et al., 2008), indicating that, although H3K4me2 and H3K4me3 accumulate preferentially at the 5′-end of genes, relevant amounts were also detected in the coding region. It was proposed that the distribution across the entire genic region may be related to a requirement of these marks for both transcriptional initiation and elongation (Li et al., 2007). A different situation was reported for 14C. Consistent with indications from genome-wide analyses in Arabidopsis and rice (Zilberman and Henikoff, 2007; Cokus et al., 2008; Li et al., 2008), our data from bisulphite genomic sequencing showed that, in endosperm tissues, the CDS but not the promoter of some O2 targets had high 14C levels, mainly due to methylation at CpG and CpWpG sites. The azs22.9 and azs22.11 loci are the sequences with a pronounced 14C level within their CDS. It was evident that this methylation does not affect transcription because azs22.9 is actively transcribed in endosperms. The accumulation of 14C in these loci may be related to their peculiar genomic location. For example, azs22.9 lies very close to a transposon (Song and Messing, 2003), which contains highly methylated sequences that can spread chromatin and DNA marks to adjacent genes (Weil and Martienssen, 2008). On the other hand, more than one-third of the Arabidopsis transcribed genes showed significant levels of 14C in coding regions, independent of their location (Zilberman and Henikoff, 2007), and the exact role of this methylation in regulating transcription remains unknown (Zhang, 2008).

Chromatin Modifying Mechanisms Integrate O2-Mediated Activation of Gene Transcription through a Two-Phase Process

The results from this study allow us to formulate a molecular model that integrates the role of chromatin modifying mechanisms into the O2-mediated regulation of transcription (Figure 8). In sporophytic tissues, such as leaves, O2 targets exhibited high 14C level and enrichment of the heterochromatin-related H3K9me2 and H3K27me2 marks, thus being embedded into a

![Figure 8. Molecular Model for the Two-Phase O2-Mediated Transcriptional Activation.](image)
tightly packaged and transcriptionally silent chromatin structure, which was poorly accessible to DNase1.

In early developmental stages of endosperm development (8 DAP) the silencing-related histone marks were substituted with H3K4me2 and H3K14ac, and chromatin was more accessible to DNase1. Furthermore, demethylation at CpG and CpWpG sites, which when occurring in the O2-box ACGR core favors the in vitro binding of O2 (Rossi et al., 1997; Sturaro and Viotti, 2001), took place. Since at this stage the accumulation of O2 target transcripts was not detected, this state corresponds to a potentiating phase that is poised for O2 binding at its DNA target elements. According to indications from different authors (Liu et al., 2005; Ng et al., 2006; Li et al., 2007), this implies that the histone modification pattern of the potentiated state (the deposition of H3K14ac and H3K4me2 for O2 targets) is permissive but not instructive for transcription. The potentiated state might be formed during either gametogenesis or in very early stages of endosperm development. The former possibility is compatible with the finding that parent-specific FIE1 methylation and expression pattern was already established in maize gametes, before endosperm formation (Gutiérrez-Marcos et al., 2006). The factor or factors involved in the establishment of the potentiated state at O2 targets are currently unknown (the X factor in Figure 8). Specific regulators may selectively recognize O2 target sequences to promote local chromatin changes. Alternatively, the potentiated state may originate by a global chromatin rearrangement of the endosperm genome, as suggested by observations of an extensive cytosine demethylation in maize endosperm and of a less condensed chromatin in Arabidopsis endosperm compared with other tissues (Lauria et al., 2004; Baroux et al., 2007).

In any case, our results indicate that in the later stages of endosperm development a switch from a potentiating to a transcriptionally activating phase occurs. This corresponds to a further accumulation of H3K14ac and H3K4me2 and novel deposition of H3K9ac and H3K4me3, with a concomitant increase of DNase1 sensitivity and recruitment of O2, RNPII, Ada2, and Gcn5 to the O2 target promoters. In accordance with reports from various transcription activation models (Li et al., 2007), the activation very likely occurs via a cascade of events leading first to the binding of O2 at the O2-box and then to the O2-mediated recruitment of RNPII for transcription initiation. Since previous reports indicated that Ada2 interacts with Gcn5 and O2 proteins (Bhat et al., 2003, 2004), O2 may directly recruit histone modifiers, such as the Ada2/Gcn5 complex, at group 1 O2 targets to promote accumulation of histone marks, thus forming a chromatin environment that facilitates RNPII targeting and the establishment of the activated state. Accordingly, the o2 mutation affects the formation of a transcriptionally activated chromatin environment and the Ada2/Gcn5 targeting in group 1 sequences (see Supplemental Figure 8 online). By contrast, in group 2 O2 targets, factors distinct from O2 seem to be involved in the formation of a more decondensed chromatin structure and in Ada2/Gcn5 recruitment (the Y factor in Figure 8) because the o2 mutation does not impair these events. The factor responsible of local chromatin changes may bind a promoter site separate from O2-box (as depicted in Figure 8) or may be another bZIP protein, such as OHP1 (Pysh et al., 1993), which is expressed in endosperm and could bind the O2-box. However, also in group 2 O2 targets, if O2 is lacking, RNPII cannot be recruited and the formation of the transcriptionally activated state is impeded. In the model shown in Figure 8 we speculate that, for group 2 sequences, a further degree of chromatin decompaction with respect to the potentiated state is required to allow O2 access to its target elements. Transcription is then activated through direct O2 interaction with RNPII or recruitment of additional coactivators to form the transcription initiation complex. However, alternative models and the participation of additional factors (e.g., prolamine binding factor, histone methyltransferases [HMTs], and ATP-dependent remodeling complexes) can be envisaged. Biochemical characterization of multiprotein complexes at different O2 targets is needed to reveal the precise mechanisms of O2-mediated transcriptional activation in distinct sequences.

Nevertheless, observations regarding the deposition of H3K4me3 may provide additional information. The accumulation of this mark in the activated state is the only mark to be impaired by o2 mutation in both group 1 and group 2 O2 target genes. This indicates that HMTs, such as the Arabidopsis SET-domain Trithorax 1 trimethylase (Saleh et al., 2007; Pien et al., 2008), may be common components of complexes required for transcription initiation at different subsets of O2 targets. Alternatively and in accordance with previous reports from mammal and plant systems, H3K4me3 accumulation may also occur after O2-induced transcription initiation, due to recruitment of HMTs by the Pol II-associated factor 1 that deposits H3K4me3 during RNA elongation (Krogan et al., 2003; He et al., 2004). It was shown that accumulation of H3K4me3 principally results from trimethylation at Lys-4 of the variant histone H3.3, which replaces the canonical H3 form during transcription (Ahmad and Henikoff, 2002; Loyola and Almouzni, 2007). To test whether specific deposition of the H3.3 variant occurs at O2 targets during endosperm development, we performed ChIP assays with a plant H3.3-specific antibody (Sano and Tanaka, 2007). Unfortunately, this antibody did not work in ChIP assays performed using various technical conditions (data not shown), possibly because it was raised against the unique H3.3 peptide that discriminates against canonical H3 but that corresponds to an epitope located in the globular domain and is hardly available for antibody binding after cross-linking.

Specificities of Histone Modification Profiles for Different Regulatory Systems

Comparison of the temporal order of changes of the chromatin modification profiles between different plant transcription regulatory systems reveals the existence of system-specific peculiarities. For example, the sequence of changes at the bean phas promoter resembles that reported for the human IFN-β promoter after infection of Sendai virus (Agalioti et al., 2002). Ng et al. (2006) reported that the AB13-like factor is responsible for establishment of the phas potentiated state, characterized by an enrichment of H3K9ac, while an abscisic acid-related signal transduction pathway induces the activated state with an accumulation of H3K14ac and H3K4me3. By contrast, our results indicate that H3K14ac is related to the potentiated state in the O2 system and the activated state is associated with H3K9ac and H3K4me3. Similar indications were reported for the maize
C2-Pepc gene, where the deposition of H3K14ac is not related to transcripational activation and the accumulation of H3K9ac and H3K4me3 in the proximal promoter only occurs upon light induction and in C2-Pepc-specifically expressing mesophyll cells, respectively (Danker et al., 2008; Offermann et al., 2008). Genome-wide analyses in yeast also provided evidence that H3K14ac only weakly correlates with the transcriptional state (Liu et al., 2005). Although still numerically limited, these studies suggest that in plants, as in yeast and mammals, specific histone modifications can have different functional readouts. Indeed, according to the histone language hypothesis, the functional outcome of a specific histone modification is not fully definable and predictable but depends on the combination with adjacent chromatin marks and on the genomic context (Berger, 2007). In addition, different regulatory systems can drive transcription through distinct sequences of events. For example, in different regulatory pathways, modification of histones can occur before or after ATP-dependent chromatin remodeling (Fry and Peterson, 2001; Li et al., 2007). This implies that each regulatory system needs to be characterized for its specific interaction with chromatin modifying mechanisms to fully understand whether and how chromatin-related regulation plays a role in that system.

METHODS

Plant Materials and Primer Design

For this study we used plants from the maize B73 inbred line. The o2 mutant corresponds to the o2mt/m allele originated by a stable insertion of the nonautonomous element of the Bg transposon system (the Bg receptor named rbg) in the untranslated leader sequence of the O2 gene (242 bp upstream of the ATG), which completely abolishes O2 transcription (Hartings et al., 1995). The o2mt/m mutant allele was previously introgressed into the B73 line through several subsequent backcrosses. The endosperms were obtained from manually dissected frozen kernels, harvested at various DAP from plants pollinated by means of sibbing crosses performed during field trials. Leaf tissues were obtained from greenhouse grown seedlings harvested at the V2/V3 stage (approximately corresponding to 2-week-old plants).

The primers used for qPCR were designed to span different genomic regions of the genes analyzed (see Supplemental Table 1 online). The specificity of each primer combination was verified by cloning and sequencing the amplified fragments. For primer combinations used to amplify azs22 loci and the azs19B-1-4 locus, at least 10 clones were sequenced and the specificity was verified by checking for the presence in all 10 clones of single nucleotide polymorphisms and small insertions/deletions characteristic of a given locus (Song and Messing, 2002, 2003; see Supplemental Figures 2A and 2B online for alignment of promoter and CDS region of azs22 loci and indications of single nucleotide polymorphisms and insertions/deletions). PCR conditions are reported in Supplemental Table 1 online.

RT-PCR

Total RNA extraction and first-strand cDNA synthesis and quantification were performed as described by Varotto et al. (2003). qRT-PCRs were performed using locus-specific primers, SYBR Green I (Sigma-Aldrich), and a real-time iCyclerIQ (Bio-Rad) as previously described (Rossi et al., 2007), and threshold cycles (TCs) were obtained for each sample. For each tissue/developmental stage, two cDNA preparations, from two distinct biological samples, were made and three replicates of qRT-PCR were performed for each cDNA preparation (a total of six replicates). For each primer combination, the amplification efficiency was measured using plasmids containing cDNA fragments corresponding to the amplification; a series of five different 10-time dilutions of the template, starting from 500 pg, was used to achieve the standard curve. For all the primer combinations, the amplification efficiency was 0.85 to 1.1. The standard curve was also used to calculate the relative amount of the mRNA of O2 targets in each sample, which was then reported as arbitrary units in bar diagrams (Figure 1). To compare the amount of mRNAs for different O2 targets, we used data (TC mean values) from real-time PCR performed using cDNA prepared from 15-DAP endosperm. Then, fold difference between TCs of the two primer combinations to be compared was determined by raising 2 to the ΔTC power (2ΔTC), followed by normalization for differences in primer amplification efficiency (2ΔCt value obtained using 5 pg of plasmids containing the two amplicons to be compared as templates in real-time PCR; see Supplemental Figure 2 online).

Protein Extraction and Immunoblotting

Preparation of crude protein extracts and immunoblotting were performed as previously reported (Varotto et al., 2003). The antibodies used for immunoblots were prepared and tested for specificity as described by Muth et al. (1996) for anti-O2 and Bhat et al. (2003) for anti-Ada2 and anti-Gcn5 and as reported in Supplemental Figure 1 online.

ChiP Assays

ChiP assays were performed as previously reported (Rossi et al., 2007) with minor modifications. In this study, cross-linking was performed using tissues immediately frozen in liquid N2 after harvesting because this allowed us to collect large amounts of materials from summer field trials, available for ChiP assays during all our working period. We performed previous tests that indicated that ChiP data obtained with frozen tissues and fresh materials were comparable (for example, materials, technical conditions, and primer combinations described in Rossi et al. [2007] gave similar results with fresh and frozen tissues). Frozen leaves from 2-week-old seedlings (cut in pieces of ~1 cm2) and endosperms harvested at various DAP (15- and 23-DAP endosperms were dissected into three pieces, while the entire 8-DAP endosperm was used) were fixed using 1% formaldehyde in TS buffer (200 mM NaCl and 20 mM Tris-HCl, pH 7.6) immediately after thawing. Fixation was conducted at 4°C, using two cycles each consisting of 2 min of vacuum infiltration plus 8 min of rest in TS buffer, for a total of 20 min. Fixation was blocked with 0.1 M glycine, and the materials were washed three times with water, dried with towels, frozen, and conserved at ~80°C. Chromatin was extracted by grounding fixed tissues to powder in liquid N2, followed by homogenization in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 mM Na butyrate, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin). The homogenate was heated 5 min at 60°C, centrifuged 2 min at 500g, filtered through two layers of miracloth, washed four times with lysis buffer without inhibitors (each wash corresponding to 20 min of centrifugation at 5000g and 4°C) to remove cytoplasmic contaminations, and the pellet suspended in lysis buffer. The extracted DNA was sheared by sonication to ~350- to 800-bp fragments and centrifuged; supernatants were carefully quantified using a spectrophotometer. Usually, 0.3 g of 2-week-old leaves and 0.2 g of endosperms are required to obtain ~12 μg of chromatin, corresponding to the amount required for one ChiP assay. A fraction of the sonicated supernatants was saved and after cross-linking reversion (see below), used as input control in qPCR evaluation. For any ChiP assay, 12 μg of chromatin were precleared with 60 μL salmon sperm DNA/Protein A agarose (Upstate) for 3 h at 4°C and centrifuged, and the protein-DNA complexes were...
immunoprecipitated overnight at 4°C adding the appropriate antibody. Typically, the following amount of the different antibodies was used for immunoprecipitation: 3 μg of α-H3cter (Abcam; ab1791), 5 μg of α-H3ac (Upstate; 06-599), 8 μg of α-H3K9ac (Upstate; 07-352), 3 μg of α-H3K14ac (Upstate; 07-353), 8 μg of α-H3K4me2 (Upstate; 07-030), 3 μg of α-H3K36me3 (Abcam; ab8580), 8 μg of α-H3K9me2 (Upstate; 07-441), 12 μg of α-H3K27me2 (Upstate; 07-452), 10 μg of α-RNPII (Santa Cruz Biotechnology; C-21, sc-900), 10 μL of α-02 (Muth et al., 1996), 15 μL of α-ZmAda2 (Bhat et al., 2003), 15 μL of α-ZmGcn5s (Bhat et al., 2003). A no-antibody negative control was performed by omitting antibody addition during incubation. Sixty microliters of salmon sperm DNA/Protein A agarose was then added and incubation continued for 2 h. The agarose beads were then subsequently washed once with low salt buffer (20 mM Tris-HCl, pH 8, 50 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 0.1% SDS), two times with high salt buffer (20 mM Tris-HCl, pH 8, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 0.1% SDS), once with LND buffer (20 mM Tris-HCl, pH 8, 250 mM LiCl, 1% Nonidet P-40, and 1% Na deoxycholate), and twice with TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA); the chromatin was eluted in a two-step incubation with a 0.1 M NaHCO₃ and 1% SDS solution to obtain a final volume of 500 μL. Cross-linking was reversed 4 h at 65°C in the presence of 0.2 M NaCl; samples were treated with 2 μg of proteinase K for 1 h, extracted twice with phenol-chloroform and once with chloroform, and DNA was precipitated and suspended in 75 μL of water. One microliter of ChIP DNAs and one-tenth dilution of inputs were used for qPCR analysis. Two independent ChIP experiments were performed for each antibody and each plant material, and three repetitions of qPCR analysis were performed for each ChIP assay and for each sequence analyzed (a total of six qPCR replicates).

Real-time PCR was employed for qPCR as described by Rossi et al. (2007), and the TC mean value and standard error were obtained for the six qPCR replicates. The data were expressed as a percentage of immunoprecipitated compared with input, obtained by calculating the ∆TC between the average TCs of immunoprecipitate and of the corresponding input and by raising 2 to the ∆TC power (2^∆TC). For H3cter correction, the 2^∆TC by ChIP assay with a given antibody and amplification with a given primer combination was divided by the corresponding 2^∆TC from ChIP assay with H3cter. To maintain the same numerical magnitude for H3cter-corrected and uncorrected data, the values achieved from H3 correction were multiplied by a constant representing the mean value of H3cter 2^TC calculated for all samples for a given primer combination (Rossi et al., 2007). Data from ChIP assays with H3cter were subjected to analysis of variance. Specifically, the TC values from the three replicates for each of the two independent ChIP assays were used for calculation of F test in analysis of variance, which was performing assuming biological sample replications as random effects and treatment effects (data from various tissues and/or developmental stages) considered as fixed effects. Since dissimilar precipitation efficiencies were reported for ChIP assays performed using chromatin extracted from various tissues (Offermann et al., 2008), we verify that this does not occur with the tissues/developmental stages and with technical conditions employed in this work. Differences in precipitation may be tested using transcriptionally active control genes, such as MAc1; however, these genes exhibited also differences in mRNA accumulation across the technical conditions employed in this work. Differences in precipitation were treated with 2 μg of proteinase K for 1 h, extracted twice with phenol-chloroform and once with chloroform, and DNA was precipitated and suspended in 75 μL of water. One microliter of ChIP DNAs and one-tenth dilution of inputs were used for qPCR analysis. Two independent ChIP experiments were performed for each antibody and each plant material, and three repetitions of qPCR analysis were performed for each ChIP assay and for each sequence analyzed (a total of six qPCR replicates).

Real-time PCR was employed for qPCR as described by Rossi et al. (2007), and the TC mean value and standard error were obtained for the six qPCR replicates. The data were expressed as a percentage of immunoprecipitated compared with input, obtained by calculating the ∆TC between the average TCs of immunoprecipitate and of the corresponding input and by raising 2 to the ∆TC power (2^∆TC). For H3cter correction, the 2^∆TC by ChIP assay with a given antibody and amplification with a given primer combination was divided by the corresponding 2^∆TC from ChIP assay with H3cter. To maintain the same numerical magnitude for H3cter-corrected and uncorrected data, the values achieved from H3 correction were multiplied by a constant representing the mean value of H3cter 2^TC calculated for all samples for a given primer combination (Rossi et al., 2007). Data from ChIP assays with H3cter were subjected to analysis of variance. Specifically, the TC values from the three replicates for each of the two independent ChIP assays were used for calculation of F test in analysis of variance, which was performing assuming biological sample replications as random effects and treatment effects (data from various tissues and/or developmental stages) considered as fixed effects. Since dissimilar precipitation efficiencies were reported for ChIP assays performed using chromatin extracted from various tissues (Offermann et al., 2008), we verify that this does not occur with the tissues/developmental stages and with technical conditions employed in this work. Differences in precipitation may be tested using transcriptionally active control genes, such as MAc1; however, these genes exhibited also differences in mRNA accumulation across the technical conditions employed in this work. Differences in precipitation were treated with 2 μg of proteinase K for 1 h, extracted twice with phenol-chloroform and once with chloroform, and DNA was precipitated and suspended in 75 μL of water. One microliter of ChIP DNAs and one-tenth dilution of inputs were used for qPCR analysis. Two independent ChIP experiments were performed for each antibody and each plant material, and three repetitions of qPCR analysis were performed for each ChIP assay and for each sequence analyzed (a total of six qPCR replicates).

McrBC Assays and Bisulphite Genomic Sequencing

The genomic DNA was prepared as previously reported (Hartings et al., 1995). The analysis of cytosine methylation by restriction with McrBC enzyme followed by real-time quantification of McrBC-treated and untreated DNA was performed as previously described (Rossi et al., 2007).

The treatment of genomic DNA with bisulphite converts unmethylated cytosines to uracil, while methylated cytosines remain unmodified. This treatment was performed as described by Jacobsen et al. (2000), with exception that genomic DNA was sheared by sonication to ~300- to 1500-bp fragments prior to bisulphite treatment to improve the denaturation efficiency. One microliter of bisulphite-treated DNA was used for each PCR reaction. Primers used for PCR amplification are reported in Supplemental Table 1 online. Specificity and amplification efficiency of primer combinations, particularly for the azs22 loci that exhibit high nucleotide sequence homology, were improved using nested or semi-nested PCR amplification (see primer combinations listed in Supplemental Table 1 online), and specificity was verified by checking for characteristic nucleotide polymorphisms in the amplicons after sequencing. PCR conditions are reported in Supplemental Table 1 online. After purification of the PCR products on PCR quick spin columns (Qiagen), the fragments were cloned in pGEM-T plasmid (Promega), and eight independent clones were sequenced for each primer combination. Statistical analysis to identify significant differences between tissues/developmental stages for each genomic region was performed by applying Student’s t tests.

DNasel Sensitivity Assays

Nuclei preparation for DNasel assays was performed using a modification of the protocol from Luthe and Quatrano (1980). Twelve milligrams of frozen 2-week-old leaves or endosperms were homogenized at 4°C with 6 mL of Honda buffer (0.44 M sucrose, 2.5% Ficoll, 5% Dextran 40, 25 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5% Triton X-100, 0.1 mM PMSF, and 2 mM spermine). The homogenate was diluted with 12 mL of Honda buffer and filtered first with two layers of miracloth and then with a 100-μm mesh nylon membrane. The filtrate was centrifuged at 6000g for 5 min at 4°C, and the pellet was suspended in 20 mL of Honda buffer without spermine and filtered through a 100-μm mesh nylon membrane. The nuclear suspension was further purified by centrifugation in a discontinuous gradient of Percoll. For each tube, an aliquot of 5 mL of the nuclear suspension was stratified on the top of the Percoll gradient, which contained 5-μL layers of 40, 60, and 80% Percoll (with Percoll dissolved in a buffer containing 0.44 M sucrose, 25 mM Tris-HCl, pH 7.6, and 10 mM MgCl₂) on a 5-μL layer of 2 M sucrose cushion. The gradients were centrifuged at 6000g for 20 min at 4°C and the nuclei-enriched fraction recovered from a band located in the 80% Percoll layer, just above the 2 M sucrose cushion. The nuclei fraction was washed two times with equal volume Honda buffer without spermine to remove Percoll, followed by two washes with NR buffer (50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 10 mM β-mercaptoethanol, and 20% glycerol) and suspension of the pellet arising from the total of the 12 g of starting material in 3.5 mL of NR buffer. Nuclear preparations were evaluated by staining samples with 0.1% toluidine blue (Sigma-Aldrich) and examination with a light microscope. To correct for initial differences in nuclei contents among samples, genomic DNA was isolated from 80 μL of nuclear suspension by adding 400 μL of digestion buffer (10 mM Tris-HCl, pH 8, 100 μM NaCl, 25 mM EDTA, 0.5% SDS, and 100 μg of proteinase K), followed by incubation at 50°C for 6 h. The DNA was then extracted with phenol-chloroform, precipitated, suspended in 100 μL of water, and its amount evaluated at the spectrophotometer. Usually, a DNA concentration of 50 to 150 ng/μL was obtained from 3.5 mL of a nuclear suspension prepared from 12 g of starting material. Before DNasel assays, treatment with micrococcal nuclease was performed on a fraction of nuclear preparations; this
treatment generated a typical nucleosome ladder when the DNA was extracted and fractionated on the agarose gel, thus ensuring the integrity of chromatin from nuclear preparations. The micrococcal nuclease digestion was performed using 300 μL of the nuclear suspension, which was centrifuged 2 min at 2000g and 4°C, suspended in micrococcal nuclease buffer (0.3 M sucrose, 50 mM Tris-HCl, pH 8, 5 mM MgCl₂, 1.5 mM NaCl, 0.1 mM CaCl₂, and 5 μM β-mercaptoethanol), and precipitated twice with phenol-chloroform, resuspended in 30 μL of water. The results of the digestion were visualized by separation of treated DNA in agarose gel, followed by ethidium bromide staining.

The treatment with DNasel was adapted from published methods (Wurtzel et al., 1987; McArthur et al., 2001). Equal amounts of nuclear suspension, corresponding to 240 μg of DNA, were used for each material. The nuclear suspension was centrifuged for 1 min at 3000g and 4°C, and the pellet was suspended in 1.8 mL of DNasel buffer (0.3 M sucrose, 50 mM Tris-HCl, pH 8, 5 mM MgCl₂, 4 mM CaCl₂, 15 mM NaCl, and 0.05 mM β-mercaptoethanol), and subdivided into six fractions of 300 μL corresponding to treatments with six different DNasel unit numbers. Meanwhile, a series of DNasel (Invitrogen) dilutions in DNasel buffer was prepared to add 10 μL of the diluted enzymes for each of the six fractions of the nuclear suspension. The DNasel treatment was performed using 0, 0.125, 0.250, 0.500, 0.750, and 1.500 units of DNasel. The six fractions of the nuclear suspension were precipitated twice at 37°C and incubation continued for 5 min after addition of DNasel; the reaction was then stopped by adding 300 μL of 0.1 M EDTA, and the DNA was extracted twice with phenol-chloroform, precipitated, and suspended in 30 μL of water. The six qPCR replicates. For each DNaseI unit, three repetitions of qPCR analysis of the nuclear suspension. The DNaseI treatment was performed using 0, 100, 200, 300, 400, and 500 units of Micrococcal nuclease (GE Healthcare). The digestion was then stopped by adding 100 μL of 0.1 M EDTA, and the DNA was extracted twice with phenol-chloroform, precipitated, and suspended in 30 μL of water. The results of the digestion were visualized by separation of treated DNA in agarose gel, followed by ethidium bromide staining.

The treatment with DNasel was adapted from published methods (Wurtzel et al., 1987; McArthur et al., 2001). Equal amounts of nuclear suspension, corresponding to 240 μg of DNA, were used for each material. The nuclear suspension was centrifuged for 1 min at 3000g and 4°C, and the pellet was suspended in 1.8 mL of DNasel buffer (0.3 M sucrose, 50 mM Tris-HCl, pH 8, 5 mM MgCl₂, 4 mM CaCl₂, 15 mM NaCl, and 0.05 mM β-mercaptoethanol), and subdivided into six fractions of 300 μL corresponding to treatments with six different DNasel unit numbers. Meanwhile, a series of DNasel (Invitrogen) dilutions in DNasel buffer was prepared to add 10 μL of the diluted enzymes for each of the six fractions of the nuclear suspension. The DNasel treatment was performed using 0, 0.125, 0.250, 0.500, 0.750, and 1.500 units of DNasel. The six fractions of the nuclear suspension were precipitated twice at 37°C and incubation continued for 5 min after addition of DNasel; the reaction was then stopped by adding 300 μL of phenol-chloroform and putting the sample at 4°C. The DNasel-treated samples were extracted twice with phenol-chloroform, precipitated, and suspended in 30 μL of water, and its concentration was measured with the spectrophotometer. Two microliters of a 5 ng/μL DNasel-treated DNA dilution were employed for qPCR. Two independent nuclei preparations and DNasel treatments were performed for each sample, and three repetitions of qPCR analysis were performed for each DNasel treatment (a total of six qPCR replicates). Real-time PCR was employed for qPCR as described by Rossi et al. (2003), and the TC mean value and standard error were obtained for the six qPCR replicates. For each DNasel unit, ΔTC was calculated with respect to untreated sample and the 2ΔTC obtained by raising 2 to the ΔTC power. Correction for possible difference in DNA content was further considered and calculated by dividing the 2ΔTC for each treated sample and each primer combination to 2ΔTC obtained using the identical sample but primers specific for the sequence of the Opaque2 Copia-like retroelement, which was inaccessible to DNasel also at the highest number of DNasel units (Figure 6).

Accession Numbers
Accession numbers for sequences described in this article can be found in Supplemental Table 1 online.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Immunoblots with Anti-O2, Anti-Ada2, and Anti-Gcn5 Antibodies.

Supplemental Figure 2. Alignment of Promoter and CDS Region of azs22 Loci.

Supplemental Figure 3. Specificity of O2 Binding for the O2-Box Region within O2 Target Promoters.

Supplemental Figure 4. Bisulphite Sequencing of O2 Target Promoters and CDSs.

Supplemental Figure 5. Histone Density in O2 Target Genes.

Supplemental Figure 6. Histone H3 Acetylation in the CDS of O2 Target Genes.

Supplemental Figure 7. Histone H3 Methylation in the CDS of O2 Target Genes.

Supplemental Figure 8. Different Profiles of Chromatin and DNA Marks and Transcription Factor Binding Characterize Distinct Groups of O2 Target Genes.

Supplemental Table 1. List of Sequences Analyzed and Primers.

Supplemental Table 2. Analysis of the Cytosine Methylation Profile by Bisulphite Sequencing.

Supplemental Data Set 1. Text File Corresponding to the Sequence Alignment in Supplemental Figure 2A.

Supplemental Data Set 2. Text File Corresponding to the Sequence Alignment in Supplemental Figure 2B.

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