Synergistic Activation of Seed Storage Protein Gene Expression in Arabidopsis by ABI3 and Two bZIPs Related to OPAQUE2

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The expression of many seed storage protein genes in cereals relies on transcription factors of the bZIP class, belonging to the maize OPAQUE2 family. Here, we describe a survey of such factors in the genome of Arabidopsis thaliana, and the characterization of two of them, AtbZIP10 and AtbZIP25. Expression analysis by in situ hybridization shows that the occurrence of their mRNAs in the seed starts from early stages of development, peaks at maturation, and declines later in seed development, matching temporally and spatially those of the seed storage protein genes encoding 2S albumins and cruciferins. Gel mobility shift assays showed that AtbZIP10 and AtbZIP25 bind the ACGT boxes present in At2S and CRU3 promoters. Moreover, using the yeast two-hybrid system we show that AtbZIP10 and AtbZIP25 can interact in vivo with ABI3, an important regulator of gene expression in the seed of Arabidopsis. Transient expression analyses of a reporter gene under the control of the At2S1 promoter in transgenic plants overexpressing ectopically AtbZIP10, AtbZIP25, and ABI3 reveal that none of these factors could activate significantly the reporter gene when expressed individually. However, co-expression of AtbZIP10/25 with ABI3 resulted in a remarkable increase in the activation capacity over the At2S1 promoter, suggesting that they are part of a regulatory complex involved in seed-specific expression. This study shows a common mechanism of ABI3 in regulating different seed-specific genes through combinatorial interactions with particular bZIP proteins and a conserved role of O2-like bZIPs in monocot and dicot species.

During seed development, storage reserves accumulate mostly in the form of carbohydrates and proteins, whose degradation upon germination will provide nutrients to the growing seedling before the photosynthetic capacity is fully acquired. Seed storage proteins (SSP) are specifically synthesized in developing seeds, both in the endosperm and in the embryo. In the seed of monocotyledonous species, the former is the predominant reserve tissue, whereas in dicotyledonous plants the endosperm is commonly re-absorbed as maturation proceeds, and storage proteins are preferentially accumulated in the embryo. Because SSP-encoding genes are specifically induced and tightly regulated during seed development, they represent an interesting model system for studying the mechanisms of temporal and tissue-specific gene regulation.

In the developing seed, different programs of gene expression have been defined that comprise distinct classes of genes that are coordinately regulated (1, 2). The MAT (maturation) class includes major SSP genes (like 2S albumins and 12S globulins) expressed at early and mid-maturation phases, whereas the LEA (late embryogenesis abundant) class includes primarily genes involved in the acquisition of desiccation tolerance expressed at later stages of maturation (3, 4). Unraveling the molecular basis of seed-specific gene expression has been mainly focused on the identification of cis-acting promoter elements and associated transcription factors (TFs). Both aspects have been considerably studied in monocots due to the importance of cereal seeds as crops, but in dicot species the molecular aspects of this regulation are still less understood. In cereals, the bipartite endosperm box (5, 6) and the ACAA motif (7, 8) are important regulatory elements, repeatedly found in the promoters of SSP genes. These cis-elements are bound by TFs of the bZIP, DNA binding with one finger (DOF), and MYB classes (9, 10). One of the first-described transcription factors in plants and probably one of the best studied, involved in this regulation is the bZIP protein from maize OPAQUE2 (O2). Mutations in either the O2 gene or in its binding sites within the endosperm box of zein promoters, result in a dramatic reduction in the level of zein transcription (12). The central role of O2-like factors in seed-specific expression has been well documented in other cereal species, such as wheat (SFA), barley (BLZ1 and BLZ2), rice (RISBZ1 and RITA1), Sorghum (SBO2), and Coix (CJIQ2) (5, 13–18).

In dicot plants, the study of SSP gene promoters by deletion analysis, site-specific mutagenesis or gain-of-function approaches has revealed the importance of bZIP target sites to provide full activity and in conferring tissue specificity (19–22). It has been also demonstrated that these sites are bound in vitro by maize O2 or O2-like factors from cereals, resulting in efficient activation in planta when they are co-transformed into the dicot plant tissues (19, 23). Moreover, the fact that target sites of O2-like bZIPs in the promoters of monocot SSP genes are necessary for seed expression in transgenic dicot plants (24, 25) suggests that similar, yet uncharacterized, factors are involved in electrophoretic mobility shift assay; aa, amino acids; MAT, maturation gene class; AD, activation domain; BD, binding domain; GUS, β-glucuronidase.

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important in the regulation of SSP genes in the dicot embryos. In Arabidopsis and in closely related species from the genus Brassica, regulatory cis-acting elements required for specific expression of SSP genes have been determined (22, 26, 27). Both in 25S albinum and napin gene promoters these include the RY motif and the ACCT box, representing putative targets for B3 and bZIP transcription factors, respectively. Alternative strategies, like the analysis of genetic mutants with altered patterns of gene expression in the seed, have also led to the discovery of genes corresponding to TFs responsible for these variations. Remarkably, factors with a B3 domain such as ABI3, FUS3, or LEC2, potentiell implicated in the regulation of SSP genes through the RY element, were found among them (28–30).

Concerning the interaction with the ACCT boxes in these promoters, several bZIP proteins of the ABI5 group expressed in the seed have been identified as putative candidates to participate in this regulation (31, 32). However, mutations in the ABI5 and EEL genes were reported not to affect the expression of early and mid-maturation SSPs (e.g. 25S albinum gene) but instead of those genes expressed at late embryogenesis (e.g. LEA protein genes), suggesting that different bZIP factors should be involved in their regulation (32).

As part of a project devoted to the study of seed-specific transcriptional control, we focused on the identification of Arabidopsis bZIP transcription factors related to OPAQUE2, as putative candidates for the regulation through ACCT boxes of early and mid-maturation SSP genes. Here, we present evidence that two bZIP factors of the OPAQUE2 family in Arabidopsis are involved in MAT SSP gene activation in a concerted manner with ABI3.

EXPERIMENTAL PROCEDURES

Plant Material—Arabidopsis thaliana seeds from Columbia (Col-0) and C24 were obtained from the Arabidopsis Biological Resource Center (Ohio). Seeds from A. thaliana C24 expressing ectopically ABI3 (2) were obtained from the authors. Plants were grown under standard conditions in growth chambers at 22 °C with a 16-h photoperiod.

Isolation and Analyses of cDNAs and Genomic Clones of O2-like bZIPs—cDNAs encoding AtbZIP9, -10, -25, and -63 were obtained from public Expressed Sequence Tag (EST) collections or amplified by reverse transcriptase-PCR. The corresponding genomic clones were isolated from an A. thaliana library prepared in gEM11 (Promega), kindly donated by J. Paz-Ares. Screening of the Arabidopsis genomic library was performed following general procedures (33) with gene-specific probes corresponding to cDNA fragments encoding the following protein regions: AtbZIP9, aa 1–100; AtbZIP10, aa 1–125; AtbZIP25, aa 300–403; AtbZIP63, aa 220–314 and previously shown not to cross-hybridize. DNA sequences were obtained using the ABI PRISM 377 dye terminator sequencing system and the ABI PRISM 377 DNA sequence analyzer (PerkinElmer- Applied Biosystems). Analyses of DNA and deduced protein sequences were done with computer services under the European Bioinformatics Institute (www.ebi.ac.uk/). Sequence alignments and phylogenetic analyses were performed using ClustalW and Phylipp packages, respectively, at the server of the DNA Data Bank of Japan (www.ddbj.nig.ac.jp/). Detection of conserved domains in protein sequences was done with MAST/MEME tools (meme.sdsc.edu/ meme/website/meme.html).

Northern Blots and in Situ Hybridization Analyses—Northern blot analysis was performed using total RNA from different Arabidopsis tissues as described in Ref. 2. Gene-specific probes (see above) were labeled by random priming following standard procedures (35). In situ hybridization was performed according to Ref. 34 with digoxigenin-labeled antisense RNA probes generated according to the manufacturer’s specifications (Roche Diagnostics). Gene-specific probes, described above, were cloned into pGEM-T easy (Promega), and labeled RNA was synthesized on linearized templates using T7 or SP6 polymerase depending on insert orientation. An ABI3-specific probe was obtained by PCR amplification of a DNA fragment corresponding to the last 103 aa of the ABI3 protein. This fragment was cloned into pGEM-T easy and used to synthesize RNA probes following the same procedure.

Electrophoretic Mobility Shift Assays (EMSAs)—The cDNAs encoding AtbZIP10, AtbZIP25, and ABI3 proteins were cloned into the expression vector pRSET-B (Invitrogen), and O2 cDNA into pGEX-2T vector (Amersham Biosciences) as a translational fusion to glutathione S-transferase (GST). After transformation into the appropriate strains, BL21(DE3)/pLyS8 and XL1-Blue, recombiant proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h, and cellular pellets were used to prepare crude protein extracts. Alternatively, proteins were synthesized using the TNT system (Promega).

Experimental conditions for electrophoretic mobility shift assays were essentially as previously described (30, 14). Probes were generated by annealing overlapping oligonucleotides, corresponding to specific regions of the AtSKI1 and CRU3 promoters. The following oligonucleotides were used: ATSIWTAS, 5′-CTCTTTACCGTGAGA-3′; ATSIWTAS, 5′-GATTGCCAGTGAAAGA-3′; ATSIWTAS, 5′-TCTTTACAGGAAG-3′; ATSIWTAS, 5′-GAGTCCCTGCTATAAA-3′; and CRU3WTAS, 5′-TAATCTTCTAAGGG-3′. Probes were end-labeled with [32P]dATP by the fill-in reaction (Klenow exo-free DNA polymerase; United States Biochemical) and purified from an 8% polyacrylamide gel electrophoresis (39:1 cross-linking). Binding reactions contained 0.5 ng of [32P]labeled probes.

Yeast Strains and Methods—Strains HF7c and SFY526 of Saccharomyces cerevisiae carrying LacZ (β-galactosidase) and HIS3 (imidazole glycerol phosphate dehydratase) reporter genes under the control of a truncated Gal1 promoter that contains Gal4-responsive elements (Gal1UAS) were obtained from Clontech. The effector plasmids pGBT9 and pGAD424 (Clontech), which contain the alcohol dehydrogenase 1 (Adh1) promoter fused to the Gal4 DNA binding domain (Gal4DBD; pGBT9 vector) or to the Gal4 DNA activation domain (Gal4AD; pGAD424 vector), respectively, were used to generate translational fusions with AtbZIP10, AtbZIP25, or ABI3 or with particular fragments derived from them (ABI3 N: aa 1–525, ABI3 C: aa 401–720; AtbZIP10 C: aa 184–410; and AtbZIP25 C: aa 206–403). All constructs were generated by PCR amplification with selected primers and verified for in-frame fusions and sequence fidelity. Yeast transformation, activity assays for β-galactosidase in liquid cultures and colony growth in His-depleted medium, were done at 30 or 23 °C, according to the manufacturer’s instructions with minor modifications (13, 14).

Particle Bombardment in Arabidopsis Leaves—Particle coating and bombardment were carried out basically according to Ref. 13 with a biolistic Helium gun device (DuPont PDS-1000, BioRad), using rupture disks of 1,100 psi and a 6-cm distance between macrocarrier and target samples. Leaves from Arabidopsis plants grown under sterile conditions were isolated from a rosette stage, placed onto a 5-cm dish agar MS/2 medium, and shot in sets of six. After bombardment leaves were incubated in the same plate for 24 h, and GUS activity was quantified by chemiluminescence (GUS light kit, Tropix) and expressed as relative luminescence units (RLU) per µg of protein. AtS1:GUS reporter vector is a pUC19-derived plasmid containing the β-glucuronidase reporter gene under the control of the AtS1 promoter (31) fused to the 3′-terminator of the nopaline synthase gene (3′nos). Effector plasmids pGBT9 for the expression of AtbZIP10, AtbZIP25, and ABI3 were generated by cloning the corresponding cDNAs under the control of the CaMV35S promoter and a 3′nos terminator in pBSII (Stratagene). A. thaliana transgenic plants overexpressing AtbZIP10 and AtbZIP25, were generated by Agrobacterium transformation following procedures referred to in Ref. 34 and using the vector pHOKII, in which the expression of both cDNAs is driven by the CaMV35S promoter. Plants overexpressing ABI3, previously described (2), were a kind gift from the authors.

RESULTS

The Arabidopsis Genome Contains Four Genes Encoding OPAQUE2-related bZIPs—The completion of the A. thaliana sequencing project (35) facilitates the identification of genes encoding defined protein families within the genome. O2-related bZIPs have several features that make them distinguishable from other members of the bZIP group of TFs (Fig. 1A). The bZIP region has an extended zipper comprising up to nine leucine heptad repeats with a high degree of sequence conservation; outside this domain, other sequence signatures are also shared specifically within the O2 family. Among 75 bZIP-encoding genes annotated in the Arabidopsis genome (36, 37), comprehensive sequence searches (see “Experimental Procedures”) using both the conserved bZIP region and the different signatures (1–6) schematically represented in Fig. 1A, allowed
the identification of four genes, AtbZIP10, AtbZIP25, AtbZIP63, and AtbZIP9 as the only members of the O2 family in Arabidopsis. Naming of these genes followed the nomenclature proposed by the EU-Regulatory Gene Initiative in the Arabidopsis project (37). Amino acid sequence alignments and comparisons with other O2 members in different plant species, led to the recognition of similar structural signatures common to these TFs. The results of such analyses are summarized in Fig. 1A, where the occurrence of domains 1–6, alongside the conserved bZIP region, is indicated for each individual member of the family. Among the four Arabidopsis O2-like bZIP proteins, AtbZIP10 shares the maximum number of conserved domains with members of the family in other species, and in particular with maize O2 and O2-like cereal proteins. The phylogenetic tree in Fig. 1B, based on sequence similarities of the bZIP region, shows that AtbZIP25 is very closely related to AtbZIP10, suggesting that both genes might represent redundant functions. AtbZIP63 forms a clade together with parsley CPRF2 and soybean G/HBF, two dicot bZIPs putatively involved in responses to pathogen and environmental stress (38, 39). Finally, AtbZIP9 is related to RITA1 and other rice bZIPs of yet unknown functions (15).

AtbZIP10 and AtbZIP25 Are Expressed in the Developing Seed—For a transcription factor to participate in the regulation of seed storage protein genes, a necessary requisite is to be expressed in the developing seed. Consequently, the expression patterns of the four O2-like bZIPs from Arabidopsis were investigated by Northern blot analysis. Total RNA samples isolated from different tissues, including siliques at two developmental stages, were hybridized after electrophoretic separation to gene-specific probes corresponding to AtbZIP10, AtbZIP25, AtbZIP63, and AtbZIP9 genes. As shown in Fig. 2 the four genes are expressed in roots (R), shoots (S), rosette leaves (L), and flowers (F), but only hybridization to the AtbZIP10 and AtbZIP25 probes presented intense signals in lanes S1 and S2, containing RNAs from siliques.

If AtbZIP10 and AtbZIP25 were regulators of SSP gene expression, an overlapping pattern of expression of these genes should be anticipated. To explore this possible relationship, the same blot was subsequently hybridized with a probe specific for a 2S albumin gene. The peak for the TF genes was at the S1 stage whereas that for the 2S gene was at the later S2 stage of siliques development. The patterns of AtbZIP10 and AtbZIP25 mRNAs accumulation are therefore consistent with the possibility of the former being regulators of this SSP gene.

To clarify whether the signals detected in Northern blots derived specifically from the seed or from other siliques tissues, a more precise expression analysis, namely in situ mRNA hybridization, was done for these genes (Fig. 3). AtbZIP63 and AtbZIP9 mRNAs could not be detected in developing seeds, AtbZIP9 expression being restricted to the valve vascular veins in the siliques (data not shown). In contrast, expression in the seed could be clearly detected for AtbZIP10 and AtbZIP25, whose patterns of expression were found to be identical throughout seed development. AtbZIP10/25 transcripts were present from early stages of development both in the developing embryo and, at lower levels, in the endosperm. Expression of AtbZIP10/25 decreased as development proceeded. At later stages, they were expressed at very low levels in the mature embryo and the remaining endosperm, but were strongly up-regulated in the inner layer of the seed coat. AtbZIP10/25 RNA could also be detected in the lignified inner subepidermal layer of the valves. To test for possible overlapping patterns of expression of AtbZIP10/25 and ABI3, an important regulator of gene expression in the Arabidopsis seed (28), in situ hybridiza-
finding that the Arabidopsis AtbZIP10 and AtbZIP25 spatial and temporal patterns of expression in developing embryos overlap with those of At2S and At12S genes, prompted us to investigate whether these factors could recognize in vitro the conserved ACGT boxes present in these SSP gene promoters. To this end, AtbZIP10 and AtbZIP25 proteins expressed in Escherichia coli or produced in vitro, were tested for their ability to bind different oligonucleotide sequences containing ACGT boxes derived from At2S1 and CRU3 promoters. These boxes present adjacent RY regulatory elements (CATGCRY) in these promoters (schematically represented in Fig. 4A). In Fig. 4B the results of EMSA demonstrate that the AtbZIP10 and AtbZIP25 binding to these sequences occurs specifically through the ACGT boxes, since mutations (AAAG) introduced at this core abolished the interaction in the context of both At2S and CRU3 promoters.

AtbZIP10 has been previously demonstrated to be an important regulator of 2S albumin and cruciferin genes, since abi3 mutants display a significant, although not complete, reduction in the expression levels of SSP genes (2). The AtbZIP10 protein has a modular structure with high sequence similarity to the maize Viviparous1 (Vp1) gene product (40). Similar to Vp1, AtbZIP10 has a B3 domain, but its ability to bind DNA through this domain has not been described. In addition, both Vp1 and ABI3 contain a B2 domain, reported in VP1 to increase the DNA binding affinity of different proteins, including O2 (41). We have detected that AtbZIP10 and AtbZIP25, as well as ABI3 are co-expressed in the same tissues during seed development, and that AtbZIP10 and AtbZIP25 bind to ACGT core sequences in the promoters of SSP genes expressed herein too. It was pertinent, therefore, to check whether possible changes in their binding affinities were produced in the presence of ABI3. In vitro, EMSA experiments shown in Fig. 4C, display the binding behavior of different amounts of AtbZIP10 or O2 proteins incubated with target DNA sequences. A clear increase in the binding of both proteins to their target sites was obtained in the presence of ABI3 (see lanes under +ABI3 in Fig. 4C) as compared with controls lacking the ABI3 protein. In analogous experiments performed with AtbZIP25, we were unable to see an increase of the binding affinity in the presence of ABI3 (data not shown).

**In Vivo Interactions between AtbZIP10/25 and ABI3 in the Yeast Two-hybrid System**—AtbZIP10 and AtbZIP25 display high sequence similarity between them and are closely related to other bZIPs of the O2 family over particular protein regions. Two of the shared signatures (1 and 2 in Fig. 1A) span the activation domain AD-1. To test their potential as transcriptional activators, as well as their putative interactions with ABI3, the yeast one- and two-hybrid systems were used (Fig. 5). Yeast plasmid vectors were generated to express proteins containing the GAL4 DNA-binding domain as N-terminal fusions to the complete AtbZIP10 and AtbZIP25 factors or their C-terminal regions. When these vectors were transformed into appropriate yeast strains, activation of the reporter activity was detected in both cases (see Fig. 5D), AtbZIP10 being a much stronger activator than AtbZIP25 in this system as shown by the higher concentration of 3AT required to counteract its growth in His-depleted medium. As in other O2-like factors, we could map the activation capacity in the N-terminal region of both proteins, since GAL4BD-fusions to the C-terminal fragments fail to induce reporter activities compared with the complete proteins (e.g. see Fig. 5C for AtbZIP25).

Yeast two-hybrid experiments were performed at two different temperatures, 30 °C (standard conditions) and 23 °C (commonly used to disclose weaker interactions). The results of a systematic analysis to check for possible interactions between
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FIG. 4. DNA binding of ATBZIP10 and ATBZIP25 to SSP gene promoters. A, promoter structure of SSP genes corresponding to albumin At2S1 and cruciferin CRU3. The location of G-boxes (solid circles) and RY elements (open circles) relative to the TATA box is shown. Nucleotide sequences of the oligonucleotides used in EMSAs are displayed, indicating the G-boxes (bold) in native promoters (WT) and the point mutations introduced to alter the ACGT core sequences (mt). B, mobility shift assays of the oligonucleotides described above, derived from the 2S and CRU3 promoters, incubated with recombinant proteins AtbZIP10 and AtbZIP25. The presence of a particular protein in the in vitro reactions with labeled oligonucleotides is indicated with + signs. (C), DNA binding of AtbZIP10 to the G-box of the At2S1 promoter in the presence of ABI3. Upper panel, retardation bands obtained in EMSAs for decreasing quantities (1 to 1:9) of AtbZIP10 protein assayed in the absence (−) or presence (+) of ABI3 in the binding reaction. An increase in the intensity of the bands is observed when ABI3 is added to the reaction. The lower panel shows a similar effect exerted by ABI3 on the maize protein OPAQUE2.
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In Arabidopsis, major seed storage protein genes are expressed in the embryo at early and midstages of maturation under a tight temporal and tissue-specific regulation. Here, we present several lines of evidence on support that the TFs, AtbZIP10 and AtbZIP25, characterized in this study, participate in this regulation. First, they are structurally very closely related to OPAQUE2-like factors involved in the regulation of seed storage protein genes in cereal species. Likewise, they are transcriptional activators, expressed in the seed at the maturation phase and bind to regulatory sequences in transcribed activators, expressed in the seed at the maturation phase and bind to regulatory sequences in transcriptional activators, expressed in the seed at the maturation phase and bind to regulatory sequences in transcriptional activators, expressed in the seed at the maturation phase and bind to regulatory sequences in transcriptional activators, expressed in the seed at the maturation phase and bind to regulatory sequences in transcriptional activators, expressed in the seed at the maturation phase and bind to regulatory sequences in...
We have determined that AtbZIP10 and AtbZIP25 are highly expressed in the developing seed from very early stages of development to mid-maturation, then decaying as desiccation proceeds. This pattern of expression is consistent with a putative participation in the regulation of SSP genes, such as 2S albumins and cruciferins. Since the expression of AtbZIP10 and AtbZIP25 is not restricted to the seed, an important matter of further study, outside the scope of this work, is the function of these factors in organs unique to the seed.

**Activation of SSP Genes Results from the Combinatorial Effects of Different Regulatory Factors**—Systematic analyses of different SSP gene promoters, both from monocot and dicot species, have led to the identification of multiple protein binding sites responsible for their regulation. Among them, ACGT core elements are critical in conferring seed-specific expression, as determined in functional assays of mutated promoters or in gain-of-function experiments (6, 20, 22). Remarkably, in monocot species, transcription factors responsible for SSP gene activation through ACGT elements have been clearly identified as members of the O2-like bZIP class; maize O2 being originally isolated from seed mutants defective in SSPs (9). In *Arabidopsis* and other dicot species, a number of mutants are known also with altered expression programs at early and midstages of maturation, affecting MAT genes like 2S albumins. Interestingly, none of the mutants characterized so far (*e.g.* abi3, fus3, or lec1 and 2) correspond to a TF interacting with ACGT boxes, despite unequivocal participation of this element in the regulation of these genes. It was shown, however, that coupling of additional elements to these boxes in the context of native promoters was essential for promoter hormonal modulation and resulted in synergistic activation effects (22), indicating that expression changes could arise from mutations in any of the participating components.

According to a previously proposed model of 2S regulation (27), full activation of 2S promoters results from the interaction of diverse factors acting on defined elements organized into two major regulatory domains. In particular, the RY-G domain consists of a G-box (the target of a yet uncharacterized protein), flanked by RY elements (putatively bound by B3 domain containing factors, like ABI3). The analysis of abi3 mutants revealed the participation of the ABI3 protein in the regulation of 2S promoters (2), which was presumed to occur through its direct interaction with the RY elements (27). In support of that, transient expression experiments with 2S promoters demonstrated that ABI3 activation capacity was lost when mutations in the RY elements were introduced, although direct DNA binding was never observed for the ABI3 protein. Interestingly, despite the integrity of RY elements, ABI3 could not activate the RY-G sequences, indicating that other factors interacting with this element are crucial in the ABI3-mediated activation of 2S promoters, perhaps by tethering and stabilizing ABI3 into a protein complex acting on the RY-G sequences.

On this basis, we searched and identified O2-like factors from *Arabidopsis* and tested their putative contribution to the activation of 2S albumin promoters, particularly in relation to ABI3. Our *in situ* hybridization results show that AtbZIP10 and AtbZIP25 are highly expressed in developing seeds at a time when 2S albumin expression takes place and overlapping with that of the ABI3 gene. Gel retardation assays confirmed that AtbZIP10 and AtbZIP25 bind in a sequence-specific manner to the G-box within the RY-G regulatory domain, and most significantly, using the yeast two-hybrid system we demonstrated that AtbZIP10/25 and ABI3 are able to physically interact. The use of experimental conditions with lower incubation temperatures (23 °C), allowed us to detect consistently a
weaker interaction between ABI3 and AtbZIP25 and to map the interacting domain of the later to the C-terminal region. All these findings are consistent with the established idea of a regulatory complex acting on 2S promoters and support that the O2-like factors identified herein represent the ACGT-interacting factors implicated in 2S activation. If this was so, full activation of 2S promoters should be achieved in the presence of both ABI3 and AtbZIP10/25. Transient expression analyses with the 2S promoter driving GUS reporter activity corroborate these expectations. Activity levels obtained with this construct in leaves of transgenic plants overexpressing AtbZIP10, AtbZIP25, or ABI3 alone were higher than the basal ones obtained in wild-type backgrounds, although of limited range, as previously reported by Northern blot analysis in CmV35S::ABI3 lines (2). However when AtbZIP10/25 were included as co-effectors in CaMV35S::AtbZIP10/25 plants, a considerable synergistic effect on the activity of the 2S promoter was observed. These results demonstrate that neither AtbZIP10/25 nor ABI3 overexpression is sufficient to attain the full induction of 2SS genes, and that a combined effect of these factors is required for complete activation. It is also worth mentioning that these observations reflect a parallelism in the mode of action of ABI3 in controlling both MAT and LEA genes through specific interactions with different bZIP proteins (42) and are in support of the importance of combinatorial control in the regulation of transcription in plant promoters (43).

Although the positive effect of ABI3 on 2S activation has been consistently demonstrated in the past, the mechanisms underlying this process were obscured by the fact that no DNA binding capacity could be demonstrated for ABI3. This capacity, presumed on the basis of structural similarities of this factor to maize VP1, is a matter of controversy even for the kind, are the cereal VP1-like factors and ABI3 in development in monocot and dicot species. Examples of such a kind, are the cereal VP1-like factors and ABI3 in Arabidopsis, and their interacting bZIP proteins TRAB1 and ABI5 (of rice leek, since only a truncated version of VP1 spanning the B3 factor to maize VP1, is a matter of controversy even for the kinds, are the cereal VP1-like factors and ABI3 in Arabidopsis, and their interacting bZIP proteins TRAB1 and ABI5 (of rice 1279–1287).

In conclusion, this study allowed the identification in Arabidopsis of four bZIPs belonging to the O2 family. A detailed characterization of two of them, AtbZIP10 and AtbZIP25, showed that they are both structurally and functionally the most closely related to O2-like cereal TFs, and might represent their Arabidopsis counterparts involved in SSP gene regulation. In this respect, it is worth mentioning the conservation discovered among TFs controlling equivalent processes of seed development in monocot and dicot species. Examples of such a kind, are the cereal VP1-like factors and ABI3 in Arabidopsis, and their interacting bZIP proteins TRAB1 and ABI5 (of rice 1279–1287).

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Synergistic Activation of Seed Storage Protein Gene Expression in *Arabidopsis* by ABI3 and Two bZIPs Related to OPAQUE2

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