The Wheat Transcriptional Activator SPA: A Seed-Specific bZIP Protein That Recognizes the GCN4-like Motif in the Bifactorial Endosperm Box of Prolamin Genes

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The conserved bifactorial endosperm box found in the promoter of wheat storage protein genes comprises two different cis elements that are thought to be involved in regulating endosperm-specific gene expression. Endosperm nuclear extracts contain binding activities. One is called ESBF-I, which binds to the endosperm motif (EM), and the other is called ESBF-II, which binds to the GCN4-like motif (GLM). Here, we present a functional analysis of the endosperm box of a low-molecular-weight glutenin gene found on the 1D1 chromosome of hexaploid wheat (LMWG-1D1) in transgenic tobacco plants. Our analysis demonstrates the necessity of the EM and GLM for endosperm-specific gene expression and suggests the presence in tobacco of functional counterparts of wheat ESBF-I and ESBF-II. Furthermore, we describe the isolation and characterization of cDNA clones encoding SPA, a seed-specific basic leucine zipper protein from wheat that can activate transcription from the GLM of the -326-bp LMWG-1D1 promoter in both maize and tobacco leaf protoplasts. This activation is also partially dependent on the presence of functional EMS, suggesting interactions between SPA with ESBF-I-like activities.

INTRODUCTION

Developing seeds of flowering plants accumulate and store carbohydrates, lipids, and proteins that subsequently are used by the germinating seedlings as a source of energy, carbon, and nitrogen. Seed storage proteins are synthesized in the endosperm of monocotyledonous seeds and in the embryonic axis and cotyledons of dicotyledonous seeds. These seed storage proteins are not synthesized in other tissues. The major seed storage proteins in wheat (the most important and widely grown crop plant) are the prolamins, which account for >90% of the total protein content of the grain. Wheat prolamins consist of two major groups of proteins, the gliadins and the glutenins, which differ in their ability to form polymers. The gliadins are a complex mixture of monomeric proteins subdivided into α-, β-, γ-, and ω-gliadins on the basis of their electrophoretic mobilities. The glutenins are composed of high-molecular-weight and low-molecular-weight (LMW) subunits that assemble into high-mass polymers stabilized by intermolecular disulfide bonds (Colot, 1990). Comparisons of the promoter sequences of various cereal prolamin genes have identified a conserved region located ~300 bp upstream of the transcriptional start; this region has been named the endosperm box (Kreis et al., 1985). In members of the grass tribe Poideae, which includes wheat, barley, and rye, the conserved region contains two different conserved motifs, called the endosperm motif (EM) and the GCN4-like motif (GLM), which are very similar to the binding sites of the yeast GCN4 and mammalian AP1 factors (Müller and Knudsen, 1993). The sequence of these elements is shown in Figure 1A.

The development of efficient transformation protocols for cereals has recently permitted the first analysis of a rice seed storage protein glutenin promoter in a stable homologous transgenic system (Zheng et al., 1993), but detailed promoter analyses of cereal storage protein genes have only been performed in model dicot species such as tobacco (Colot et al., 1987; Marris et al., 1988; Schernthaner et al., 1988; Robert et al., 1989; Thomas and Flavell, 1990; Takaiwa et al., 1991). Expression in the heterologous dicot host was restricted to the endosperm, suggesting a conservation of the cis elements responsible for cell-type specificity in monocots and dicots. Furthermore, deletion analysis of a wheat LMW glutenin (LMWG-1D1) promoter demonstrated that a fragment located from positions -326 to -160 and containing the bifactorial endosperm box was essential for expression in tobacco endosperm (Colot et al., 1987). In vivo dimethyl sulfate footprinting of the same LMW glutenin
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Figure 1. Functional Analysis of the −326-bp LMWG-7D7 Promoter in Transgenic Tobacco Plants.

(A) Structure of the promoter fused to the GUS marker gene and sequences of the putative cis elements in the wild-type and mutated promoter constructs. The G residues previously footprinted in vivo (Hammond-Kosack et al., 1993) are shown in boldface italics, whereas the mutated residues are outlined. WT, wild type; GLM−, mutated GLM; EM−, mutated EM; CCAAT−, mutated CCAAT motif.

(B) Histochemical analysis of GUS activity in a cross-section of a tobacco seed transformed with the wild-type −326-bp LMWG-7D7 promoter construct. e, endosperm.

(C) GUS activity in extracts from seeds and leaves of multiple independent tobacco transformants containing various promoter constructs. GUS activities in untransformed tobacco plants are shown for reference. The analysis was performed on seeds collected 15 DAA. 4MU, 4-methylumbelliferone.

promoter in developing wheat endosperm detected the sequential binding of putative trans-acting factors to the EM and GLM (Hammond-Kosack et al., 1993). This work identified an additional footprinted copy of each motif in an adjacent upstream region of the promoter that, together with the downstream elements, forms a so-called long endosperm box (LEB). Electrophoretic mobility shift assays (EMSAs) using wheat endosperm nuclear protein extracts showed the specific binding of two different factors, ESBF-I and ESBF-II, to the EM and GLM, respectively (Hammond-Kosack et al., 1993).

The endosperm box of the prolamin genes of members of the grass tribe Andropogonoideae, which includes maize, sorghum, and Coix, contains a conserved EM. In vitro footprinting experiments have shown that the EM of a zein promoter is bound by a nuclear protein from maize endosperm nuclear extracts (Maier et al., 1987). This binding activity may correspond to the maize counterpart of wheat ESBF-I. Maize opaque2 (o2) mutants show an overall reduction of α-zein content, with a particularly strong effect on the expression of the 22-kDa α-zeins (Kodrzycki et al., 1989). O2 is an endosperm-specific member of the basic leucine zipper (bZIP) family of transcription factors that has been shown to bind specifically the 22-kDa zein promoter and activate transcription in vitro (Schmidt et al., 1992). O2 has been shown to activate endosperm-specific transcription of the b-32 gene, a type I ribosome-inactivating protein, via a distinct cis sequence (Lohmer et al., 1991). Several lines of evidence suggest that O2 may be functionally related to the
wheat ESBF-II factor. First, O2 can bind to the GLM of the LMW glutenin endosperm box and activate transcription in yeast and plants through this element (Holdsworth et al., 1995). Second, O2 expressed in yeast complements a gcn4 mutant by binding and activating the HIS3 gene at its GCN4 binding site (Mauri et al., 1993), indicating that the GCN4 motif, the GLM sequence, and the O2 binding sites in maize genes are functionally related. Finally, the recombinant O2 protein has been shown to bind in vitro to a GLM sequence of a pea lectin gene that is necessary and sufficient for seed-specific expression in transgenic tobacco plants (de Pater et al., 1993, 1994).

In this study, we describe a functional analysis of the bifactorial endosperm box of the wheat LMWG-1D1 promoter in transgenic tobacco plants that defines the importance of both the EM and GLM for endosperm-specific expression and the LEB as a functional unit. In addition, we describe the isolation and characterization of cDNA clones encoding SPA (for storage protein activator), a novel seed-specific transcriptional activator from wheat that shows partial sequence similarity to the maize O2 transcription factor. SPA can bind to both GLMs of the LEB and is able to transactivate a reporter gene linked to a minimal promoter containing the LEB sequence. SPA requires an intact EM motif for maximal transcriptional activation. In addition, SPA can activate transcription of a maize 22-kD zein gene, and in the transient assay systems employed, SPA and O2 activated transcription to similar levels.

RESULTS

Functional Analysis of the LMWG-1D1 Endosperm Box in Transgenic Tobacco Plants

A deletion analysis of the LMWG-1D1 promoter in transgenic tobacco previously indicated that sequences located constructs in transgenic plants were verified from a few randomly selected transgenic plants by polymerase chain reaction (PCR) amplification and direct sequencing.

Quantitative and histochemical analyses of GUS activity in seeds of transgenic plants containing the wild-type promoter construct were performed. As shown in Figure 1B, the LMWG-1D1-GUS fusion was specifically expressed in the endosperm of transgenic seeds. GUS activities were determined in extracts from leaves and seeds of approximately nine to 15 independent transformants for each construct at 15 days after anthesis (DAA) and are summarized in Figure 1C. Similar relatively high levels of GUS activity were observed in the seeds of plants transformed with the wild type and CCAAT box mutated constructs, whereas plants transformed with constructs containing mutations in the EMs or the GLMs showed only background levels of expression.

It appears that the mutations introduced at the CCAAT box did not significantly affect the activity of the -326-bp LMWG-1D1 promoter in transgenic tobacco seeds. Very low levels of GUS expression were detected in the leaves of some of the plants transformed with the wild type and the CCAAT promoter constructs. Therefore, it is possible that the activity of the -326-bp LMWG-1D1 promoter in transgenic tobacco is not as strictly seed specific as are longer promoter constructs (Colot et al., 1987). It is clear that the two conserved motifs contained in the bifactorial endosperm box are essential for expression in tobacco endosperm and are probably recognized by tobacco homologs of ESBF-I and ESBF-II, because the introduced promoter mutations, based on in vivo footprinting data, specifically abolished in vitro binding of the ESBF-I and ESBF-II activities (Hammond-Kosack et al., 1993).

Isolation of a cDNA Encoding a bZIP Protein from Wheat Endosperm

Because of the potential functional similarity between maize
were used in 3' RACE (for rapid amplification of cDNA ends) reactions performed with wheat seed poly(A)+ mRNA. As a positive control, similar reactions were performed using maize seed poly(A)+ mRNA. The resulting PCR products were resolved electrophoretically and hybridized with an O2 probe. The wheat 3' RACE sample obtained with one of the degenerate primers showed a faint but clear hybridization of a band ~750 bp, which demonstrated the presence of an O2-like bZIP in wheat seeds (data not shown). Hybrid selection of the PCR products by using an immobilized O2 probe, followed by PCR amplification, enriched the O2-specific protein.

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Ten individual clones were sequenced, and all corresponded to nearly identical RACE products encoding the C terminus of a bZIP protein (data not shown). This partial protein showed only limited sequence similarity to O2 (46% identity) but had a much more striking homology to the maize OHP1 and OHP2 proteins (84% identity). OHP1 and OHP2 are not specifically expressed in endosperm, but they show considerable sequence similarity to O2 in their bZIP region and have been shown to heterodimerize with O2 (Pysh et al., 1993). The partial-length cDNA clone was tentatively called wOHP because of its close sequence similarity to the maize OHP clones.

To isolate full-length wOHP clones, we screened a wheat endosperm cDNA library in λgt10 with the partial wOHP cDNA insert. The screening was performed at a slightly reduced stringency to avoid discriminating against possible homologous classes of bZIP proteins. Two longer cDNA clones of different size that, according to partial sequencing data, had an identical overlapping nucleotide sequence, were isolated. Surprisingly, they did not correspond to longer wOHP cDNA clones but encoded a potential bZIP

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The SPA protein shows limited but significant sequence similarity to O2, OHP1, and OHP2 from maize and to the 02 homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994). SPA homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994). SPA homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994). SPA homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994). SPA homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994). SPA homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994). SPA homologs Sbopa2 and Cljop2 from sorghum and Coix, plants closely related to maize (Pirovano et al., 1994).
Wheat Transcription Factor SPA

In addition, limited sequence similarity (38% overall amino acid identity and 63% amino acid identity in their bZIP regions) was found between SPA and common plant regulatory factor 2 (CPRF-2), a parsley bZIP protein that binds to the G-box of the chalcone synthase promoter (Weisshaar et al., 1991). SPA has lower sequence similarity to other wheat bZIP proteins such as HBP-1 (23%; Tabata et al., 1989) and EmBP-1a (22%; Nui and Guiltinan, 1994) than to O2 and O2-like proteins. In addition, Figure 3 shows that SPA and the other cereal bZIP proteins share some small conserved domains outside their bZIP regions, which extend predominantly toward the N terminus and which are not conserved in the parsley protein. These small conserved regions are relatively rich in negatively charged amino acids, a feature associated with acidic activation domains of transcription factors.

SPA Transcripts Are Specifically Expressed in Wheat Seeds

The temporal and spatial pattern of expression of a tran-
The binding of His–SPA protein to the GLMs of the *LMWG-1D1* endosperm box was determined by EMSAs. The probe used in EMSAs corresponded to the LEB region extending from positions −302 to −231 in the *LMWG-1D1* promoter (wild type) containing two intact GLMs and a probe (GLM−) containing mutations in both GLMs corresponding to those shown in Figure 1A. These probes had been used previously in gel shift assays with endosperm nuclear extracts to demonstrate binding of the ESBF-II activity to the GLMs (Hammond-Kosack et al., 1993). Figure 5B shows that the His–SPA fusion protein binds to the wild-type probe but not to the GLM− sequence. The two protein–DNA complexes formed with the wild-type probe could possibly be derived from the binding of SPA homodimers at only one of the two GLMs in the probe, whereas the other complex could represent binding at both motifs. Previously, Hammond-Kosack et al. (1993) proposed the binding of the same activity, ESBF-II, to both GLMs, but no direct evidence for this was available. To determine whether SPA could bind to both motifs, we used oligonucleotide probes corresponding to the individual GLMs in their wild type or mutated forms in EMSAs. Figure 5C shows that both wild-type probes GLM1 and GLM2 give rise to a similar retarded complex that was not observed with the mutant probes. The GLM1 probe showed an additional, faster migrating complex that was specific to the GLM1 wild-type probe, but the significance of this retarded complex is unclear. The similar mobilities of the His–SPA complexes with the wild-type LEB probe (Figure 5B) and with the individual GLM1 and GLM2 probes indicate that the two bound complexes formed on the LEB probe probably correspond to His–SPA binding to both GLMs in the LEB probe.

**Figure 4.** Developmental and Spatial Pattern of Expression of SPA mRNA.

RNA gel blot analysis was performed with poly(A)⁺ RNA (500 ng) isolated from developing wheat seeds staged according to the Zadoks scale (Zadoks et al., 1974) and on poly(A)⁺ RNA from leaves (L), roots (R), and coleoptiles (C). After hybridization with the SPA probe, the same blot was subsequently hybridized with an LMW glutenin (LMWG) probe and a ubiquitin probe from Antirrhinum (UBIQ). The positions of the 2.4- and 1.4-kb RNA markers are shown at left.

**SPA Protein Can Bind in Vitro to the GCN4-like Motifs of the *LMWG-1D1* Endosperm Box**

To verify the potential involvement of SPA in the regulation of glutenin genes, we assessed the binding of SPA to potential target sites in the *LMWG-1D1* promoter. A recombinant SPA protein containing an N-terminal fusion of six histidine residues was expressed in *Escherichia coli* by using a single NcoI restriction site at the putative translational start to clone the SPA coding region in the expression vector pSET-A (Invitrogen, San Diego, CA). As shown in Figure 5A, isopropyl β-D-thiogalactoside treatment induced expression of a protein of ∼55 to 60 kD that was not present in uninduced cell extracts. The apparent size of the recombinant protein determined by SDS-PAGE was slightly larger than the expected 49-kD molecular mass of the His-tagged SPA fusion protein. A similar apparent increase in size on SDS gels is not uncommon for recombinant proteins containing His tags because it has been previously reported for another bacterially expressed recombinant bZIP protein (Niu and Guiltinan, 1994).

The recombinant His–SPA protein was purified to near homogeneity by nickel ion chelate chromatography and used directly for DNA binding assays. Previous studies using another His-tagged bZIP protein (Niu and Guiltinan, 1994) indicated that the His tag at the N terminus of SPA might not affect its DNA binding specificity.

**Transient Expression of the SPA Protein Transactivates from the GCN4-like Motifs of the *LMWG-1D1* Promoter**

Having determined the in vitro binding of SPA homodimers to the GLMs of the *LMWG-1D1* LEB, protoplast transient expression assays were performed to determine whether the SPA protein could activate expression from these target sites in vivo. The expression assays were conducted with protoplasts from maize black Mexican sweet (BMS) cell suspension cultures and from tobacco mesophyll cells. Figure 6A shows the reporter constructs used in this analysis. The wild type and versions of the *LMWG-1D1* LEB containing mutations of the EM sequences (EM−) and mutations of the GLM1- and GLM2-containing sequences separately or together were fused in the native orientation 5' of a −67-bp cauliflower mosaic virus (CaMV) 35S promoter–GUS reporter gene. In transactivation experiments, the effector plasmid consisted of the entire SPA coding region placed under the control of the double CaMV 35S promoter in the expression plasmid pJIT60. All experiments were performed in replicates of five, using the same batch of protoplasts, and each experiment
Figure 5. Binding of Recombinant SPA to the GLMs in the LEB of the LMGW-1D1 Promoter.

(A) Expression and purification of recombinant HIS-SPA protein. Crude extracts from uninduced and induced bacteria harboring the pHIS-SPA expression vector were resolved on an SDS–polyacrylamide gel along with an aliquot (1 μg) of the eluted protein. The molecular mass markers are indicated at left in kilodaltons.

(B) EMSA showing the binding of the His–SPA protein to the wild-type (WT) LEB probe. Binding was abolished by the mutations introduced into the two GLMs (GLM–), as described in Figure 1A. The arrows show the positions of the two LEB–SPA complexes. (−) indicates absence and (+) indicates presence of the LEB–SPA protein.

(C) Binding of the His–SPA protein to the individual wild-type (WT) GLMs as shown by EMSA. Yeast or rabbit reticulocyte lysate is recognized by the recombinant protein. The single GLM2–SPA complex and the two GLM1–SPA complexes are indicated by arrows. The sequences of the individual probes are given in Methods.

was repeated between three and six times with consistent results. Differences in GUS enzyme levels were significant at P < 0.01.

Figure 6B shows that SPA was able to transactivate the wild-type LEB–CaMV 35S construct (4.5-fold; section i) in tobacco mesophyll cells. In BMS cells, SPA transactivated the wild-type LEB–CaMV 35S construct 11-fold (Figure 6C, section i). Transactivation in tobacco cells and in BMS protoplasts depended on the presence of both copies of the GLMs in the LEB, as shown in Figures 6B and 6C, sections ii, iv, and v. In addition, mutation of the EMSs reduced transactivation by SPA of the LEB–CaMV–GUS fusion (from 4.5- to twofold; Figure 6B, section iii) in tobacco mesophyll cells and also reduced SPA-mediated activation of the LEB–CaMV 35S construct in BMS cells (from 11-fold to sixfold; Figure 6C, section iii).

As shown in Figures 5B and 5C, SPA can bind to the individual GLM probes lacking the EMS sequences but cannot bind to the LEB sequence in which both GLMs are mutated. As determined in EMSA analyses conducted with wheat endosperm nuclear extracts, the ESBF-II binding activity specifically recognizes the GLMs and is not affected by the mutations in the EMSs (Hammond-Kosack et al., 1993). Similarly, the binding of SPA to the GLMs was not expected to be influenced by the mutations in the EMSs. To verify this, additional EMSAs were performed using the same LEB probes as described before and using a LEB probe mutated in the two EMSs. Figure 7 shows protein–DNA complex formation with both the wild-type probe and the probe mutated in the EMSs (EMS–), demonstrating that SPA binding at the GLMs is independent of the presence of functional EMSs.

This finding suggests that factors present in tobacco and maize leaves that recognize the EMSs are necessary for full activation by SPA from the GLMs. Nevertheless, the transactivation of the chimeric LEB–CaMV 35S promoter in both maize and tobacco protoplasts clearly demonstrates that SPA is a transcriptional activator that can activate via the conserved GLMs contained in the bifactorial endosperm box of several prolamin genes.

Comparison of SPA- and O2-Activated Transcription Levels

The similarities between SPA and O2 led us to compare the relative levels of expression directed by these two transcription
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Activation of Transcription from the GLM Sequences by SPA.

(A) Structure of the LEB reporter constructs used in this study. Section i, wild-type LEB-CaMV-GUS fusion; section ii, both GLMs mutated in the LEB fusion; section iii, both EMS mutated in the LEB fusion; section iv, GLM1 motif mutated in the LEB; section v, GLM2 motif mutated in the LEB. The sequences of wild-type and mutant LEBs are shown in Figure 1A.

(B) Transient expression of constructs in tobacco mesophyll protoplasts without or with (+SPA) coexpression of a CaMV 35S:SPA plasmid is shown in sections i to v.

Figure 6. Activation of Transcription from the GLM Sequences by SPA.

The information obtained about the function of GLM and EM cis sequences in the LMWG-1D1 promoter and the isolation of a seed-specific transcription factor that specifically activates transcription from the GLM has led us to the following conclusions. First, the GLM sequences in the context of the −326-bp LMWG-1D1 promoter are necessary for seed-

DISCUSSION

The information obtained about the function of GLM and EM cis sequences in the LMWG-1D1 promoter and the isolation of a seed-specific transcription factor that specifically activates transcription from the GLM has led us to the following conclusions. First, the GLM sequences in the context of the −326-bp LMWG-1D1 promoter are necessary for seed-

(C) Transient expression of constructs in maize BMS protoplasts without or with (+SPA) coexpression of a CaMV 35S:SPA plasmid is shown in sections i to v. GUS activities were determined in duplicates of each sample, which was replicated five times in each experiment. Each experiment was repeated at least three times with similar levels of expression (P < 0.01).
Figure 7. Binding of Recombinant SPA to the GLMs of the LEB Is Not Affected by Mutations in the EMS.

The radiolabeled wild-type (WT) LEB probe, a LEB probe with mutated endosperm motifs (EM-), and a LEB probe with mutated GCN4-like motifs (GLM-) were used for EMSA experiments. (−) or (+) indicates whether the HIS-SPA fusion protein (300 ng) was added. The sequences of the probes are shown in Figure 1A. The arrows indicate the LEB-SPA complexes.

specific expression. Second, the seed-specific SPA transcription factor binds to and activates transcription from the GLM sequences. Third, this activation is enhanced by the adjacent EM sequences, which are not required for SPA binding.

Isolation of a cDNA Encoding SPA

The adoption of a PCR approach for the amplification of bZIP-encoding genes from wheat endosperm, coupled to a round of hybrid selection, resulted in the isolation of two different wheat bZIP cDNA clones encoding proteins showing partial amino acid sequence similarity to the maize transcriptional activator O2. wOHP, the partial cDNA clone initially obtained by PCR, is most similar to the maize bZIP proteins OHP1 and OHP2, and the similar expression patterns observed for both wheat and maize genes indicate that wOHP may be a possible ortholog of OHP1 and OHP2. In maize, OHP1 is expressed at a much lower level than is O2 in developing endosperm, and it is not able to transactivate the O2 target site in transient expression assays. It is thought that OHP1 and OHP2 act as negative modulators of O2 transactivation (Aukerman and Schmidt, 1994). The other wheat bZIP clone, SPA, subsequently was isolated by screening an endosperm cDNA library with the wOHP probe. Our inability to amplify the SPA clone in initial PCR reactions could have resulted from the change of the highly conserved amino acid sequence NRE(S/A)A, from which the sequence of the degenerate primers was derived, to NRDSA in the SPA protein. In addition, SPA is expressed at a level much higher than that of wOHP in developing wheat seeds (data not shown); this fact explains the relative ease of finding SPA in the endosperm cDNA library.

The SPA protein shows a higher overall sequence similarity to OHP1/OHP2 than to O2, but unlike OHP1 and OHP2, SPA is a strong transcriptional activator. The isolation of O2-like clones from other cereals has been reported recently in the case of Coix and sorghum, which belong to the same grass tribe as maize. The close phylogenetic relationship of

Figure 8. Transactivation by SPA and O2.

(A) Comparison of the activation potential of SPA (+SPA) and O2 (+O2) from the wheat LEB-60CaMV-GUS construct and a maize 22-kD zein maize22Z460CaMV-GUS construct by transient expression in maize EMS cells. EM*GLM+ is the wild-type LEB, EM-GLM- has the two GLM sequences mutated, EM-GLM+ has the two endosperm motifs mutated, and maize22Z460CaMV-GUS is the maize zein reporter construct. Each experiment was repeated three times with five replicates of each GUS assay. Differences in GUS enzyme levels were significant at P < 0.01. (B) Sequence comparison of the maize maize22Z460CaMV-GUS and wheat LMWG-1D1 promoters in their endosperm box regions. The wheat GLM and EM sequences are shown in boldface, and the corresponding elements in the maize promoter as well as the canonical O2 binding site are underlined.
these three plant species is reflected in the extensive sequence conservation, in both coding and regulatory regions, that exists among the 22-kD α-zein genes of maize, an α-kafirin gene from sorghum, and an α-coilin gene from Coix (Ottoboni et al., 1993).

In view of the conservation of putative cis elements in the promoters of these 22-kD-like α-prolamins genes, the presence of O2-related proteins in Coix and sorghum is not surprising. These proteins show 70% (Cljop2) and 63% (Sbopa2) amino acid identity to O2, but their pattern of expression has not been reported (Pirovano et al., 1994; Yunes et al., 1994). Nevertheless, their high sequence similarity to O2 and the ability of the sorghum clone to transactivate the maize b-32 promoter to a similar extent as O2 in a transient expression assay strongly suggest that they are likely to be orthologs of O2. A potential functional relationship between O2 and SPA may be reflected by the level of sequence conservation within the DNA binding domain of SPA and O2. However, this degree of sequence similarity is also shared by the parsley CPRF-2 protein (Weisshaar et al., 1991), the rice seed protein OsbZIPp, and the rice transcriptional activator RITA-1 (Izawa et al., 1994). Little information is available about the tissue specificity and transcription targets of OsbZIPp. RITA-1 is predominantly but not specifically expressed in rice aleurome and endosperm, whereas CPRF-2 is expressed in cell cultures. Therefore, it is unlikely that SPA is an ortholog of O2, based on sequence comparisons and expression patterns. This conclusion is supported by other functional information, such as the differences in the requirement of SPA and O2 for an intact EM for transcriptional activation. It is possible that SPA may have an ortholog in maize that may activate transcription from the GLM motifs present in several classes of maize storage protein genes, in contrast to the specific role of O2 in activating the 22-kD class of zein genes.

The spatial and temporal pattern of SPA expression is consistent with its involvement in the regulation of seed-specific gene expression, and its ability to bind and activate transcription from the GLMs of the LMWG-1D1 promoter indicates that SPA may correspond to the DNA binding factor ESBF-II previously detected in wheat endosperm nuclear extracts (Hammond-Kosack et al., 1993). This hypothesis needs to be proven by independent tests using antibodies in "supershift EMSA" experiments, for example. SPA may be involved in the activation of many wheat prolamin genes, because most of them contain conserved GLMs in the proximal regions of their promoters that are possible binding sites with the ESBF-I factor bound at both GLM sites with the ESBF-I factor bound at them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bombardment assay also suggested an interaction between the activities binding to them. Analysis of a barley c-hordein promoter in an endosperm transient bom
homologous and stable transgenic system (Zheng et al., 1993) demonstrated that different cis elements direct the endosperm-specific expression of a rice glutelin promoter in rice and tobacco. Several synergistic regulatory elements important for expression in rice were identified, which some showed sequence similarity to the GLMs. It is possible, therefore, that the regulatory mechanisms directing seed-specific expression in monocots and dicots are partially overlapping and that the lack of effect of mutating the footprinted CAAT box in transgenic tobacco may reflect these differences. Transformation of these promoter deletions into wheat plants would address this issue. The demonstration of a GLM in a pea lectin promoter fragment that is able to confer seed-specific expression in transgenic tobacco plants (de Pater et al., 1993) suggests that the GLMs are a major determinant of seed-specific expression in tobacco plants and probably exert a similar function in monocot plants.

Interestingly, mutations in the EMs of the LEB considerably lowered the transactivating potential of SPA in the transient expression assays. This finding is consistent with the important role of the EM and its cognate binding activity, ESBF-I, in tobacco seed expression. EMSA analyses demonstrated that the binding of SPA to the GLMs in the context of the LEB is not affected by mutation of the EMs and that SPA can specifically bind to the individual GM1 and GM2 sequences. These results reflect the binding activities of the ESBF-II factor in wheat nuclear extracts, and mutations of the EMs are known to abolish the binding of the wheat endosperm nuclear factor ESBF-I (Hammond-Kosack et al., 1993). Therefore, the effect of these mutations in the transactivation assays could result from the abolition of binding of a similar factor present in tobacco and maize leaves. The requirement for both GLMs for SPA-mediated activation, together with the requirement for the EM sequences, indicates that complex interactions between SPA binding at both GLMs and with the activity bound at the EMs (ESBF-I) may be required for gene activation. Testing this hypothesis requires the isolation and characterization of the ESBF-I binding activity.

The observed similarities between SPA and O2 do not extend to their interactions with the EM. SPA-mediated activation from the LEB–CaMV 35S construct was enhanced twofold by an intact EM, whereas O2-mediated activation was not affected by the EM. Because both factors activated transcription from the same GLMs on the LEB–CaMV 35S construct (Holdsworth et al., 1995; this study), there appears to be a fundamental difference in their mode of action upon the LEB, and this may also extend to their activities on other promoters. This aspect of SPA and O2 function will be better understood when the proteins corresponding to the EM and the LEB box are cloned and expressed in transgenic plants. These observations and the data presented here agree with a model in which ESBF-I binds to the EM and promotes subsequent binding of SPA to the GLM to achieve high-level expression.

METHODS

Plasmid Constructions and Transformation

Mutations in the –326-bp LMWG-1D1 promoter fragment were introduced by polymerase chain reaction (PCR) (modified from Ho et al., 1989). The resulting PCR fragments were cloned into the BamHI site located upstream of the β-glucuronidase (GUS) gene in the plasmid pKGT (Jefferson, 1987) and sequenced. For Agrobacterium tumefaciens-mediated plant transformation, the GUS constructs were excised as HindIII–EcoRI fragments and introduced into the binary vector pBIN19, and Nicotiana tabacum cv Samsun was transformed, as previously reported (Bie van, 1984). Histochemical analysis of GUS activity in developing seeds and replicated fluorometric analyses of GUS activity in seeds and leaves of at least 10 independent transplants was performed as described by Jefferson (1987). The chimeric long endosperm box (LEB)-cauliflower mosaic virus (CaMV) 35S promoter constructs were obtained by cloning the 37-bp LEB fragments into the HindIII–BamHI sites upstream of the –67 35S CaMV–GUS gene fusion in the plasmid pBI221.9. The 22Z4 zein reporter construct was obtained by cloning the double-stranded oligonucleotide 5′-agcttAGGTTGTCATGATGAGATGTTGAAGAGATCATGTCACTCCAGTACGTTGAAATTCTG-3′ in the same vector. The nucleotides in lowercase are cloning sites. For the construction of the effecter plasmid, the same Sall–EcoRI fragment used for the construction of the bacterial expression vector was cloned into the corresponding restriction sites in the polylinker of the CaMV 35S expression vector pJT60. Transformation of tobacco mesophyll and maize black Mexican sweet (BMS) protoplasts was performed using polyethylene glycol–CaCl₂, as described by Negrutiu et al. (1987). Fluorometric detection of GUS activity was determined in replicate samples, according to Jefferson (1987).

RNA Isolation and RNA Gel Blot Analysis

Seeds of wheat plants (cv Chinese Spring) were collected at five stages of development, according to the Zadoks scale (Zadoks et al., 1974), ranging from 10 to 28 days after anthesis (DAA), and leaves were collected at 10 DAA. The earliest stage from which seed components could be recognized on the ear was 10 DAA. Roots and coleoptiles were obtained from 10-day-old germinated seeds. Total RNA was isolated by the hot-phenol method (Shirzadegan et al., 1991), Poly(A)⁺ RNA was isolated using Dynabeads oligo(dT)₂₅ (Dynal, Oslo, Norway). Poly(A)⁺ mRNA samples were resolved in formaldehyde gels and transferred to Hybond-N membranes (Amersham, Bucks, UK). RNA hybridization, autoradiography, and quantitation were performed as described by the manufacturer.
PCR Isolation of a Partial wOHP cDNA

To amplify wheat basic leucine zipper (bZIP) cDNAs expressed in endosperm, two degenerate primers were devised based on the highly conserved amino acid sequence NRE(A/S) found in the basic domain of most plant bZIP proteins. The first degenerate primer (DA1) contains the sequence 5'-AAGGGATCCAAYMGIGARKCIGC-3' (where I is inosine, Y is A/G, M is A/C, K is G/T, R is C/T, and italicized letters represent the bases of the cloning site), whereas the second primer (DA2) contains the sequence 5'-AAGGGATCCAAYMGIGARAGYGC-3'. These two primers were used in 3' rapid amplification of cDNA ends (RACE) reactions conducted using poly(A)+ RNA from stage 3 wheat seed (18 DAA). As a positive control, similar reactions were performed using poly(A)+ RNA from maize seed to verify the amplification of opaque2 (02) cDNA 3' ends. Reverse transcriptions were performed for 1 hr at 42°C in a volume of 20 µL, using 500 ng of poly(A)+ RNA, 100 ng of the poly-(dT), adapter (Frohman et al., 1988), and 200 units of Superscript reverse transcriptase (Gibco BRL, Paisley, UK). PCR reactions were performed in a volume of 50 µL, using 5 µL of each reverse-transcribed sample diluted 10-fold with H2O, 5 µL of 10 × PCR buffer (Perkin-Elmer, Warrington, UK), 5 µL of 25 mM MgCl2, 5 µL of 2 mM deoxyribonucleotide triphosphates (dNTPs), 75 ng of adapter primer (Frohman et al., 1988), and 500 ng of degenerate primer. After incubation at 94°C for 5 min, 2.5 units of AmpliTaq (Perkin-Elmer, Warrington, UK) was added, and PCR was performed with three cycles of 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C.

An aliquot of the PCR products was resolved on an agarose gel, blotted, and hybridized at low stringency (50°C, with 5 × SSPE [20 × SSPE is 3 M NaCl, 0.2 M NaH2PO4, pH 7.4, 0.02 M EDTA]) with a cDNA encoding O2 (Lohmer et al., 1991). Weak hybridization of an ~750-bp band was detected. Hybrid selection was then performed to isolate the 750-bp products hybridizing with the O2 clone. Approximately 2 µg of pJ768 plasmid containing O2 cDNA was digested to 1 µg (µL) in 10 × SSC (30 × SSC is 0.3 M NaCl, 0.3 M trisodium phosphate, pH 7.0) and spotted on membrane. After denaturation for 0.5 min and neutralization for 1 min, the membrane was prehybridized for 30 min at 50°C in 1.5 mL of 2 x SSC and 0.1 % SDS and twice for 1 hr at 50°C in 1.5 mL of 2 x SSC and 0.1 % SDS. After a brief wash twice at 4°C for 5 min and neutralization for 1 min, the membrane was washed twice at 4°C for 0.5 hr in 2 x SSC and 0.1 % SDS. Two weakly hybridizing plaques were purified. The plasmids containing the SPA cDNAs were excised in vivo according to the manufacturer's protocol, and sequencing was performed on both strands of the longest cDNA clone. The sequence has an EMBL accession number of Y09013.

Expression and Purification of Recombinant SPA

A subclone of the longest SPA cDNA was used for expression in Escherichia coli. The 5' untranslated region containing an additional ATG codon was removed by ligating the blunt-ended NcoI site located at the putative start codon into the blunt-ended Clal site of the upstream polylinker. The entire SPA coding region was then isolated as a Sall-EcoRI fragment and cloned into the XhoI and EcoRI sites in the polylinker of the His-tagged fusion vector pRSET-A (Invitrogen, San Diego, CA). The resulting plasmid (pHIS–SPA) was introduced into E. coli BL21(DE3)pLyS3 for protein expression. The recombinant product consists of the N-terminal peptide with the sequence 5'-MRGSHHHHHGMSMTGGQMGRLYD0D0KRWGSELD-3' fused to the entire SPA protein. For expression of the HIS–SPA protein, cells were grown in 50 mL of Luria-Bertani medium with ampicillin (100 µg/mL) to an OD600 of ~0.6 and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 1 to 3 hr before harvesting. Pelleted cells were resuspended in 800 µL of lysis buffer (50 mM NaPO4, pH 8.0, and 300 mM NaCl) containing 4 mM imidazole and 0.5 mM 4-(2-aminoethyl)benzenesulfonfluoride (AEBSF) and then frozen in liquid nitrogen. The thawed suspension was sonicated three times for 5 to 10 sec and then centrifuged for 20 min at 12,000g at 4°C. The resulting supernatant (600 µL) was incubated for 90 min with constant movement at 4°C with 200 µL of a 1:1 slurry of NINTA (Qiagen, Dorking, UK) resin preequilibrated in lysis buffer. The resin was then pelleted, washed two to three times with binding buffer (500 mM NaCl, 50 mM NaPO4, pH 8.0, and 300 mM NaCl), and treated with 250 mM imidazole. The resins were washed again and then eluted with 500 mM imidazole, and the recombinant product was concentrated by ultrafiltration.
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