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Interactions between Distinct Types of DNA Binding Proteins Enhance Binding to *ocs* Element Promoter Sequences

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Octopine synthase (*ocs*) elements are a group of promoter elements that have been exploited by plant pathogens to express genes in plants. *ocs* elements are components of the promoters of certain plant glutathione *S*-transferase genes and may function as oxidative stress response elements. Genes for *ocs* element binding factors (OBFs), which belong to a specific class of highly conserved, plant basic domain–leucine zipper transcription factors, have been isolated and include the Arabidopsis *OBF4* and *OBF5* genes. To characterize proteins that modulate the activity of the OBF proteins, we screened an Arabidopsis cDNA library with the labeled OBF4 protein and isolated OBP1 (for OBF binding protein). OBP1 contains a 51–amino acid domain that is highly conserved with two plant DNA binding proteins, which we refer to as the MOA domain. OBP1 is also a DNA binding protein and binds to the cauliflower mosaic virus 35S promoter at a site distinct from the *ocs* element in the 35S promoter. OBP1 specifically increased the binding of the OBF proteins to *ocs* element sequences, raising the possibility that interactions between these proteins are important for the activity of the 35S promoter.

INTRODUCTION

A number of bacterial and viral pathogens have used a family of related, 20-bp DNA promoter sequences to express their own genes in plants (Bouchez et al., 1989). These sequences are called *ocs* elements because they were first discovered in the promoter of the Agrobacterium octopine synthase gene (Ellis et al., 1987). The *ocs* element in the cauliflower mosaic virus (CaMV) 35S promoter is also called *as-1* (Lam et al., 1989). Genes containing *ocs* element sequences are activated by auxin and salicylic acid (SA) in a number of plants (Kim et al., 1994; Liu and Lam, 1994; Qin et al., 1994; Ulmasov et al., 1994; Zhang and Singh, 1994). In Arabidopsis, the activity of *ocs* element sequences in response to auxin is highest in the root tip region (Zhang and Singh, 1994). The induction of *ocs* element activity by auxin and/or SA may also be part of a stress response, a possibility stemming from the discovery that *ocs* element sequences play a role in the transcription of certain plant glutathione *S*-transferase (*GST*) genes (Ellis et al., 1993; Ulmasov et al., 1994) and that nonactive auxin analogs also activate the *ocs* element (Ulmasov et al., 1994).

GSTs are multifunctional proteins that play important roles in the detoxification of cytotoxic compounds produced during xenobiotic metabolism and in the protection of tissues against oxidative damage (reviewed in Tsuchida and Sato, 1992; Daniel, 1993). GST expression in animals and plants is induced by a range of factors, including growth factors, hormones, and cellular stress-inducing agents. It has been proposed that these factors activate GST expression by inducing conditions of oxidative stress (Daniel, 1993). An electrophile-responsive element (EpRE), which is present in the promoters of some animal *GST* genes, has been proposed to function as an oxidative stress response element (reviewed in Daniel, 1993). The EpRE and *ocs* elements share a number of features, raising the possibility that the *ocs* element may function as an oxidative stress response element in plants. The EpRE consists of two adjacent AP-1–like binding sites (Friling et al., 1992; Bergelson et al., 1994), and these sites bear some resemblance to an *ocs* element half site. Individually, the two AP-1–like sequences have low to no activity but act synergistically to form the EpRE (Friling et al., 1992). Similarly, both halves of the *ocs* element are required for in vivo transcriptional activity (Bouchez et al., 1989; Singh et al., 1989; Ellis et al., 1993). Finally, both the EpRE and *ocs* elements are binding sites for basic domain–leucine zipper (bZIP) transcription factors (reviewed in Daniel, 1993; Singh et al., 1994).

The ability of SA to induce *ocs* elements and *GST* expression may be important for the plant defense response in which SA plays pivotal roles (Gaffney et al., 1993). One way SA appears to act is by binding to and inhibiting the action of catalase, thereby causing an increase in H_2O_2, which in turn leads to

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the expression of plant defense genes (Chen et al., 1993). Redox perturbation is also important early in the plant defense response, with an oxidative burst occurring at the membrane of infected cells within a few minutes of pathogen infection. In soybean, a *GST* gene containing a functional *ocs* element sequence is induced soon after the oxidative burst, presumably to limit tissue damage to infected and neighboring tissue (Levine et al., 1994). Thus, pathogens may use *ocs* elements to key into plant transcription systems that are inducible at various stages during the plant defense response and, consequently, are active in infected tissue.

The interaction of *ocs* element sequences with plant DNA binding proteins has been analyzed in detail (Singh et al., 1989; Tokuhisa et al., 1990), and genes for *ocs* element binding factors (OBFs), belonging to a specific class of highly conserved, plant bZIP transcription factors, have been isolated in a number of plants. In Arabidopsis, five family members have been isolated: designated histone binding protein aHBP1b (Kawata et al., 1992), TGA1 (Schindler et al., 1992a), OBF4/OBF5 (Zhang et al., 1993), and TGA3 (Miao et al., 1994). The Arabidopsis proteins range in homology from 44 to 86% amino acid identity over their entire sequences. A tobacco member of this family, TGA1a, can activate transcription through *ocs* element sequences, and its activity has been determined by using in vitro and in vivo assays (Katagiri et al., 1990; Neuhaus et al., 1994). Reverse genetic experiments have provided further evidence linking this family of bZIP proteins with *ocs* element activity (Rieping et al., 1994). Specifically, a dominant negative mutant for a member of the OBF/TGA family, which could no longer bind DNA but could still dimerize, was able to suppress transcription from a minimal promoter containing an *ocs* element site in transgenic tobacco.

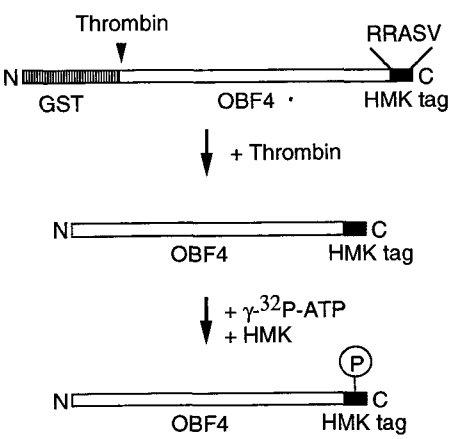

Figure 1. Schematic Drawing Outlining the Procedure Used to Generate the Labeled OBF4HMK Protein Used for Screening the Arabidopsis cDNA Library.

The diagram is not drawn to scale. The circled P represents the phosphorylation of the serine in the HMK tag. The N and C refer to the N and C termini of the proteins. RRASV indicates arginine, arginine, alanine, serine, and valine. (+), in the presence of.

The activity of specific transcription factors can be modulated significantly by interacting proteins. Such interactions are important for gene-specific regulation as well as for coupling the transcription factor to the transcription machinery. There are a number of reports demonstrating interaction of bZIP proteins with other proteins. For example, Jun/Fos family members have been found to interact with the NF-κB transcription factor to form NF–AT complexes (reviewed in Nolan, 1994). c-Jun also interacts with a novel protein kinase, JNK_1, that is stimulated by a number of factors, including UV light, and phosphorylates the c-Jun activation domain (Derijard et al., 1994). To analyze the *ocs* element further, we have attempted to characterize proteins that interact specifically with and modulate the activity of the Arabidopsis OBF proteins. In this report, we describe the isolation of a novel plant protein, OBP1 (for OBF binding protein), by screening an Arabidopsis cDNA library, using the labeled OBF4 protein as a probe. Interestingly, OBP1, which is also a DNA binding protein, was able to increase significantly the binding of OBF proteins to *ocs* element sequences.

RESULTS

Isolation of OBP1

The approach we have used to isolate OBPs involved screening Arabidopsis cDNA expression libraries with labeled OBF proteins. The procedure used to generate the labeled OBF4 protein is outlined schematically in Figure 1. The heart muscle kinase (HMK) phosphorylation site was placed as a kinase tag at the C terminus of OBF4, as described in Methods. The resulting product, OBF4HMK, was inserted into the pGEX-2T expression vector and expressed as a GST fusion protein in *Escherichia coli*. The recombinant GST–OBF4HMK protein was purified using standard procedures, and the OBF4HMK protein was separated from the GST peptide by thrombin digestion. The purified OBF4HMK protein was then labeled with HMK and used to screen an Arabidopsis callus cDNA expression library. *ocs* element sequences have been shown to be highly active in Arabidopsis callus tissue (Zhang and Singh, 1994). The screening conditions were similar to those described by Gu et al. (1994) and led to the isolation of a number of clones.

In this report, we focus on one of these clones, designated OBP1. As shown in Figure 2, DNA sequence analysis revealed that the *OBP1* cDNA insert is 923 bp long and contains a large open reading frame of 240 amino acids. An in-frame stop codon precedes the first methionine, suggesting that the complete protein coding sequence is present in the *OBP1* cDNA clone. The cDNA clone does not contain a poly(A) tail, and based on the size of the *OBP1* transcript obtained from RNA gel blot analysis, it is likely to be missing ~180 bp of the 5′ and/or 3′ untranslated sequences. The amino third of the OBP1 protein is hydrophilic. The OBP1 protein contains a number of cys-

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1   GCATCACTTC CCTTAAAGCT TCAACCTTCT CCGATATGCC GACGTCGACT CCGGTGAACC
61  ACGGCGAATA GCTATGAAAC CTAACGGCGT AACAGTTCCG ATTTCTGACC AGCAAGAACA
                M  K   P  N  G  V   T  V  P   I  S  D   Q  Q  E  Q   16
121 GCTTCCATGT CCTCGTTGTG ACTCATCCAA CACTAAGTTC TGTTACTACA ACAACTACAA
      L  P  C   P  R  C   D  S  S  N   T  K  F   C  Y  Y   N  N  Y  N   36
181 CTTCTCTCAG CCTCGTCACT TCTGCAAAGC TTGTCGTCGT TACTGGACTC ACGGTGGTAC
      F  S  Q   P  R  H   F  C  K  A   C  R  R   Y  W  T   H  G  G  T   56
241 TCTCCGTGAC GTTCCTGTCG GTGGTGGTAC TCGTAAAAGC GCGAAACGTT CCCGCACTTG
      L  R  D   V  P  V   G  G  G  T   R  K  S   A  K  R   S  R  T  C   76
301 CTCGAACTCT TCTTCCTCCT CTGTTTCTGG TGTCGTCTCT AACTCTAACG GTGTTCCGTT
      S  N  S   S  S  S   S  V  S  G   V  V  S   N  S  N   G  V  P  L   96
361 ACAAACGACG CCTGTTCTCT TCCCTCAGTC GTCAATCTCT AACGGCGTTA CTCACACAGT
      Q  T  T   P  V  L   F  P  Q  S   S  I  S   N  G  V   T  H  T  V  116
421 AACTGAAAGC GACGGAAAGG GAAGTGCTTT ATCTCTCTGT GGAAGTTTCA CCTCCACTCT
      T  E  S   D  G  K   G  S  A  L   S  L  C   G  S  F   T  S  T  L  136
481 GTTGAACCAT AACGCTCGTG CGACGGCTAC GCATGGATCC GGTTCGGTTA TTGGTATCGG
      L  N  H   N  A  R   A  T  A  T   H  G  S   G  S  V   I  G  I  G  156
541 AGGTTTTGGA ATCGGACTCG GGTCGGGTTT TGATGACGTC AGCTTTGGAC TCGGAAGAGC
      G  F  G   I  G  L   G  S  G  F   D  D  V   S  F  G   L  G  R  A  176
601 GATGTGGCCG TTTTCAACTG TTGGTACTGC GACAACGACG AATGTTGGGA GTAACGGTGG
      M  W  P   F  S  T   V  G  T  A   T  T  T   N  V  G   S  N  G  G  196
661 TCATCACGCT GTTCCAATGC CAGCCACGTG GCAGTTCGAG GGTTTAGAGA GCAACGCTGG
      H  H  A   V  P  M   P  A  T  W   Q  F  E   G  L  E   S  N  A  G  216
721 TGGTGGATTT GTCTCCGGTG AGTACTTTGC GTGGCCGGAT CTTTCCATCA CAACTCCGGG
      G  G  F   V  S  G   E  Y  F  A   W  P  D   L  S  I   T  T  P  G  236
781 AAACTCACTC AAATGAGAAA AGCTTCTGTC TTCATCAAAG CGCTTCAAAG TCTCTTCCTT
      N  S  L   K  *
841 TTTTTAGTCT CTATTGGTGA TGTTGATCTC TTAGTTTGTG TCTTGTCAAT TTAGAAACTT
901 GTCTTCGGAA ACTAGAAAAC AGA
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Figure 2. Sequence of the OBP1 cDNA Clone and the Predicted Amino Acid Sequence.

The predicted amino acid sequence for the longest open reading frame is shown directly below the nucleotide sequence. The in-frame stop codon preceding the first methionine is boxed. The cysteines in the potential zinc finger domain are circled, and the serine-rich region is underscored. The asterisk indicates the stop codon. The OBP1 GenBank, EMBL, and DDBJ data base accession number is X89192.

teines near the N terminus that may constitute a potential zinc binding motif (C-X_2-C-X_{21}-C-X_2-C, where X stands for any amino acid). There is also a serine-rich region, which is most pronounced in the stretch of amino acids from 77 to 91.

Interaction between OBF4 and OBP1 Can Be Detected Using an in Vitro Assay

The interaction between OBF4 and OBP1 was further analyzed in vitro. For these experiments, the OBP1 protein was expressed as a GST fusion in *E. coli.* The OBF4HMK protein was also initially expressed as a GST fusion in *E. coli*, and the GST peptide was removed after treatment with thrombin. The labeled OBF4HMK and GST–OBP1 proteins were incubated together for 30 min at room temperature, and then glutathione-agarose beads were added. After centrifugation and washing of the beads, the bound protein was recovered by elution with glutathione and analyzed by SDS-PAGE. The results shown in Figure 3A demonstrate that OBF4 interacts with OBP1 in vitro. OBF4 alone (Figure 3A, lane 1) or in the presence of the GST protein (Figure 3A, lane 2) or BSA (data not shown) has a low background level of binding to the glutathione-agarose beads. However, in the presence of the GST–OBP1 protein (Figure 3A, lane 3), the amount of OBF4HMK associated with the glutathione-agarose beads was increased significantly, presumably by binding to the GST–OBP1 protein, which in turn was bound to the beads. OBP1 was also found to interact with OBF4HMK as well as OBF5HMK purified from a baculovirus expression system (data not shown).

To determine whether the interaction of OBP1 with OBF4 and OBF5 was specific for these proteins, we tested whether

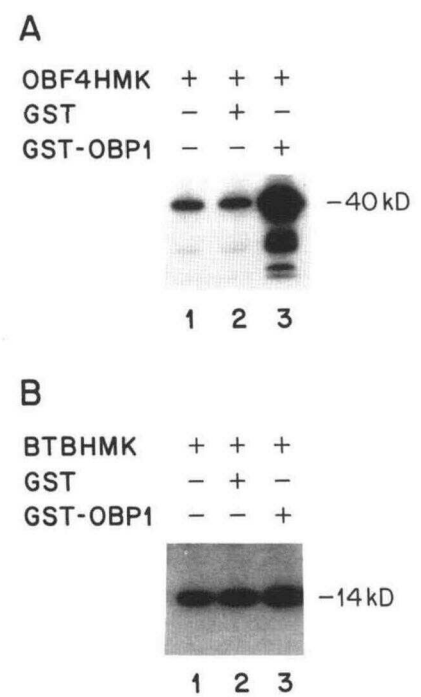

Figure 3. OBP1 and OBF4 Interact in an in Vitro Assay.

(A) OBP1 interacts specifically with OBF4HMK in vitro. Fifty nanograms of the OBF4HMK protein expressed in *E. coli* was labeled with γ-^{32}P-ATP and incubated with equal amounts of GST or GST–OBP1 for 30 min, followed by the addition of glutathione-agarose beads. The OBF4HMK protein that bound to the glutathione-agarose beads alone or with either GST (lane 2) or GST–OBP1 (lane 3) was purified and analyzed by SDS-PAGE, and the labeled OBF4HMK protein was then detected by autoradiography. Lanes 1 and 2 reflect nonspecific binding of OBF4HMK; lane 3 reflects the specific binding of OBF4HMK to GST–OBP1 bound to the glutathione-agarose beads. The size of the OBF4HMK protein is 40 kD.

(B) OBP1 does not interact with the BTBHMK domain. Fifty nanograms of the BTBHMK protein expressed in *E. coli* was labeled with γ-^{32}P-ATP and incubated with equal amounts of GST or GST–OBP1 as indicated. The interaction assay was performed as described for OBF4HMK in **(A)**. The amount of BTBHMK binding obtained in lane 3 is similar to the amount obtained with the nonspecific controls in lanes 1 and 2. The size of the BTBHMK domain is 14 kD.

(+) and (−) indicate the presence or absence of a particular protein, respectively.

OBP1 could interact with other proteins. The Drosophila bric-à-brac protein (Chen et al., 1995) has been shown to contain a 115–amino acid conserved motif, called the BTB domain, which is found in a variety of proteins, including a number of zinc finger proteins. The BTB domain, also called a POZ domain, has been shown to act as a protein–protein interaction domain (Bardwell and Treisman, 1994; Chen et al., 1995). Using a labeled BTBHMK protein and the same assay described for OBF4HMK in Figure 3A, we determined that OBP1 does not interact significantly with the BTB domain of the bric-à-brac protein (Figure 3B).

Analysis of the *OBP1* Gene Copy Number and RNA Expression Patterns

We analyzed the size of the Arabidopsis *OBP1* gene family by genomic DNA gel blot analysis. We used genomic DNA prepared from the Columbia and Landsberg *erecta* ecotypes. As shown in Figure 4A, there was only a single band that hybridized strongly with the *OBP1* cDNA clone. With EcoRI, a polymorphism was observed between the Columbia and Landsberg *erecta* ecotypes. A number of weakly hybridizing bands were observed, suggesting that there are genes related to *OBP1* in Arabidopsis. After longer exposure, additional weakly hybridizing bands were observed (data not shown). To determine the size of the *OBP1* transcript, the *OBP1* clone was used as a probe in RNA gel blot analysis with mRNA from callus tissue. *OBP1* detected a transcript of 1100 bases (Figure 4B). We used reverse transcriptase–polymerase chain reaction in a preliminary analysis of the expression patterns of *OBP1* in young seedlings (Figure 4C). *OBP1* was constitutively expressed in roots and the aerial portions of 8-day-old seedlings (Figure 4C, lane 1 versus 5), and *OBP1* RNA levels were not affected by treatment with SA or auxin (Figure 4C, lanes 2 to 4, and data not shown). Thus, where tested, the *OBP1* transcript appears to be synthesized constitutively.

OBP1 Shares Homology with Other Plant DNA Binding Proteins

Data base searches were performed with both the *OBP1* DNA and protein sequences to determine whether related sequences exist. OBP1 was found to have homology with the revised protein sequence for a maize DNA binding protein, MNB1a (Yanagisawa and Izui, 1993). MNB1a was isolated in a screen for DNA binding proteins to the CaMV 35S promoter. MNB1a recognizes a sequence in the CaMV 35S promoter (−276 to −252) that contains an AAGG motif critical for binding (Yanagisawa and Izui, 1993). The AAGG motif lies upstream of the *ocs* element in the CaMV 35S promoter (−82 to −63). As shown in Figure 5A, the MNB1a protein (238 amino acids) and the OBP1 protein (240 amino acids) are very similar in size and overall share 37% amino acid identity and 53% amino acid similarity.

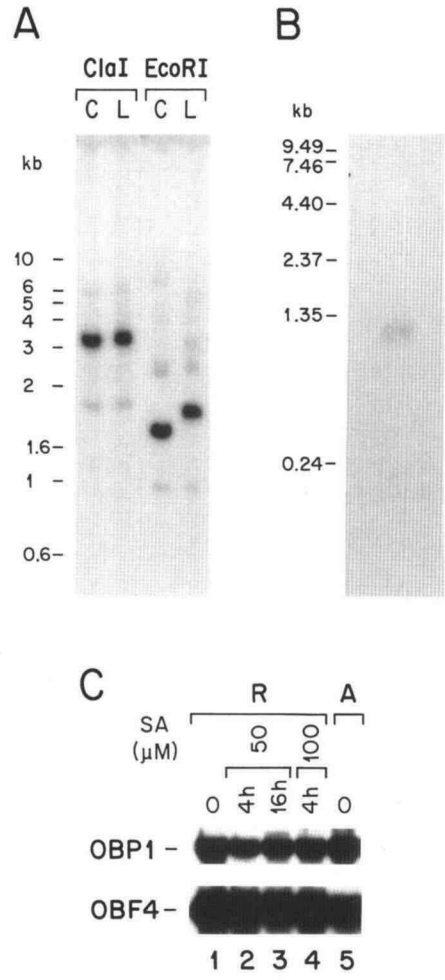

Figure 4. Analysis of the *OBP1* Gene Family and RNA Expression Patterns in Arabidopsis.

(A) DNA gel blot analysis of Arabidopsis genomic DNA, using the *OBP1* cDNA clone as a probe. Each lane contains 8 μg of genomic DNA from Columbia (C) and Landsberg (L) *erecta* ecotypes that was digested with ClaI or EcoRI. A 1-kb DNA ladder (Bethesda Research Laboratories) was used for the molecular length markers shown at left.

(B) RNA gel blot analysis using 2.5 μg of poly(A)$^+$ RNA from Arabidopsis callus tissue and the *OBP1* cDNA clone as a probe. A Bethesda Research Laboratories RNA ladder was used for the molecular length markers shown at left.

(C) Reverse transcriptase–polymerase chain reaction analysis of *OBP1* RNA levels in 8-day-old seedlings. Seedlings were treated with different concentrations of SA for 4 or 16 hr. RNA was isolated from root (R) or aerial (A) parts of the seedlings. The RNA was converted to single-stranded cDNA by reverse transcription with an oligo(dT) primer. Primers specific for *OBP1* and *OBF4* were used in the polymerase chain reaction with cDNA from each pool. The constitutive expression of *OBF4* serves as a control for the reverse transcriptase–polymerase chain reaction.

The OBP1 protein also shares homology with the unpublished sequence for a pumpkin DNA binding protein called AOBP (for ascorbate oxidase binding protein; GenBank, EMBL, and DDBJ accession number D45066). AOBP was isolated in a screen for DNA binding proteins that bind to the promoter of the ascorbate oxidase gene whose transcription is upregulated by auxin (Esaka et al., 1992). The protein sequence for AOBP is not complete, but AOBP is significantly larger than OBP1 and MNB1a. As shown in Figure 5B, there is a 51–amino acid stretch near the N terminus that is extensively conserved among the three proteins (86% amino acid identity and 92% amino acid similarity between OBP1 and MNB1a; 78% amino acid identity and 88% amino acid similarity between OBP1 and AOBP). We have called this highly conserved 51–amino acid region the MOA domain (for MNB1a, OBP1, and AOBP). Interestingly, the N-terminal half of this region contains a number of cysteines that are distributed in a manner similar to certain zinc finger domains.

A

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  1  ..............................MKPNGVTVPISDQQEQLPCP  20
  1  MQEASSAAAAGAEPGRRAAQHQFAGVDLRRPKGYAAPAPAVGEGDPCP  50

 21  RCDSSNTKFCYYNNYNFSQPRHFCKACRRYWTHGGTLRDVPVGGGTRKSA  70
 51  RCASRDTKFCYYNNYNTSQPRHFCKGCRRYWTKGGTLRNVPVGGGTRKKP  100

 71  KRSRTCSNSSSSSVSGVVSNSNGVPLQTTPVLFPQSSISNGVTHTVTESD  120
101  ........SSSSSSSSYVAAADADRQPKKKPASKKRRVVAPAPELATAAD  142

121  GKGSALSLCGSFTSTLLNHNARATATHGSGSVIGIGGFGIGLGSGFDDVS  170
143  PGKTATT.....TTTTSEITTETGALEDSDSLAHL....LLQPGTEDAEA  183

171  FGLGRAMWPPSTVGTATTTNVGSNGGHHAVPMPATWQFEGLESNAGGGFV  220
184  VALGLGLSDFPSAGKAVLDDEDS.....FVWPAASFDMGA..CWAGAGFA  226

221  SGE....YFAWPDLSITTPGNSLK  240
227  DPDPACIFLNLP.............  238
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B

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          1                                                  51
MNB1A     PCPRCASRDTKFCYYNNYNTSQPRHFCKGCRRYWTKGGTLRNVPVGGGTRK
OBP1      PCPRCDSSNTKFCYYNNYNFSQPRHFCKACRRYWTHGGTLRDVPVGGGTRK
AOBP      PCPRCNSMETKFCYYNNYNVNQPRHFCKACQRYWTEGGTIRNVPVGAGRRK
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Figure 5. Comparison of the Predicted Amino Acid Sequence of the Arabidopsis OBP1 Protein to Related Proteins in Other Plant Species.

(A) Comparison of OBP1 and the maize MNB1a protein (GenBank, EMBL, and DDBJ accession number X66076). The sequences were aligned using the Genetics Computer Group (Madison, WI) Gap program. Dots are inserted by the program to optimize alignment of the protein sequences.

(B) Sequence conservation in the MOA domain. The MOA domain of OBP1, the maize MNB1a, and the pumpkin AOBP (Genbank, EMBL, and DDBJ accession number D45066) were lined up using the Genetics Computer Group Gap program.

In both **(A)** and **(B)**, a vertical line indicates that the amino acid matched that in OBP1, and a double dot indicates a similar amino acid.

OBP1 Binds Specifically to the AAGG Motif in the CaMV 35S Promoter

We overexpressed the OBP1 protein in *E. coli* as a GST fusion protein. After removal of the GST peptide, we tested whether the OBP1 protein is able to bind specifically to the AAGG motif in the CaMV 35S promoter, which is recognized by the related MNB1a protein. As shown in Figure 6A, incubating the OBP1 protein with a 30-bp oligonucleotide probe containing the AAGG motifs resulted in a single retarded band. The band intensity could be correlated directly with the amount of OBP1 present (Figure 6A, lanes 2 and 3). In contrast, the binding of OBP1 to a mutant probe in which the AAGG motifs had been mutated to CATG was significantly reduced, although with high levels of OBP1, a low amount of binding was observed (Figure 6A, lanes 5 and 6). These results are similar to those obtained with the maize MNB1a protein (Yanagisawa and Izui, 1993). Because OBP1 is able to interact with OBF4 and OBF5, we tested whether these proteins when overexpressed in the baculovirus system had any effect on the binding of OBP1 to the AAGG motif. We tested a range of OBP1, OBF4, and OBF5 protein concentrations but were unable to observe any effect of OBF4 or OBF5 on the binding of OBP1 to the AAGG motif (Figure 6C and data not shown).

OBP1 Specifically Enhances the DNA Binding of OBF4 and OBF5 to ocs Element Sequences

We also tested whether OBP1 had any effect on the binding of the OBF4 and OBF5 proteins to *ocs* element sequences. In these experiments, OBP1 was overexpressed in *E. coli*, and OBF4 and OBF5 were overexpressed in the baculovirus system. As shown in Figure 7, we found that addition of OBP1 significantly increased OBF4 binding activity to the *ocs* element sequence from the promoter of the *ocs* gene (Figure 7, lanes 3 to 5). OBP1 had an even greater stimulating effect on the DNA binding of the OBF5 protein to this sequence (Figure 7, lanes 7 to 9). OBP1 was not able to bind to the *ocs* element probe (data not shown). The mobility of the retarded bands observed with OBF4 and OBF5 did not change with the addition of OBP1, at least at the resolution of the gel retardation conditions used. We obtained the same stimulation of OBF4 DNA binding by OBP1 with both the OBF4HMK protein and an OBF4 protein containing a Myc epitope tag at the C terminus (data not shown). The stimulation of DNA binding by OBP1 was most pronounced at low concentrations of the OBF proteins (data not shown). We tested whether a range of BSA or GST protein concentration had any effect on the DNA binding activity of the OBF proteins and were unable to observe any change in OBF DNA binding activity (data not shown).

The ability of OBP1 to increase the DNA binding of OBF5 was tested with different *ocs* element sequences. As shown in Figure 8, OBP1 was able to stimulate OBF5 binding to the *ocs* element sequences present in the CaMV 35S promoter (Figure 8A, lane 2 versus lanes 3 and 4) and to a lesser degree

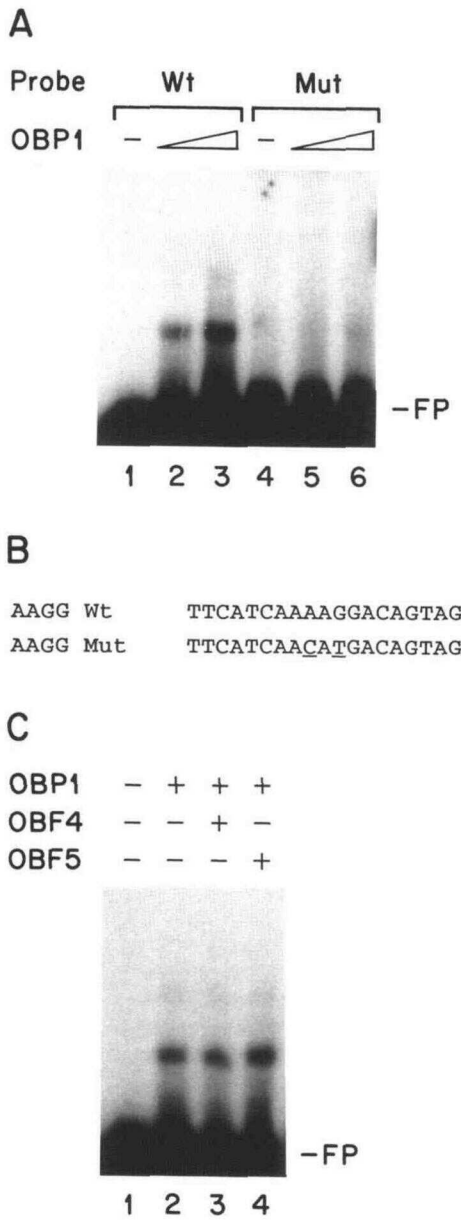

Figure 6. Analysis of the DNA Binding Properties of OBP1.

(A) Analysis of OBP1 binding to the AAGG wild-type (Wt) and AAGG mutant (Mut) sequences from the CaMV 35S promoter. Lanes 2 and 5 contain 5 ng of OBP1, and lanes 3 and 6 contain 25 ng of OBP1. Lanes 1 and 4 contain only the free probe.

(B) The oligonucleotide sequences for the AAGG wild-type and mutant (Mut) probes. The mutated residues in the AAGG mutant probe are underlined. The 5′ end of each probe contained an EcoRI site.

(C) The effect of OBF4 and OBF5 on OBP1 binding to the AAGG wild type probe. Lanes 2 to 4 contain equal amounts of OBP1 (5 ng), and lanes 3 and 4 contain 20 ng of OBF4 or OBF5 protein, respectively. (+) and (–) indicate the presence or absence of a particular protein, respectively. Lane 1 contains only the free probe.

FP, free probe.

to the *ocs* element sequence in the nopaline synthase (*nos*) promoter (Figure 8A, lane 6 versus lanes 7 and 8), which is a weaker binding site for OBF5. The ability of OBP1 to stimulate the binding of OBF5 to the *ocs* element sequence present in the 35S promoter is potentially significant because OBP1 also binds to an AAGG motif present in this promoter. OBP1 had no effect on the inability of OBF5 to bind to a mutant version of the *ocs* element in which both halves of the element had been mutated (Figure 8A, lanes 9 to 12). OBP1 was not able to bind to any of the probes used in Figure 8 (data not shown).

To determine whether the effect of OBP1 on OBF4 and OBF5 DNA binding was specific for these two DNA binding proteins, we tested the effect of OBP1 on the binding of other DNA binding proteins. The Arabidopsis CA-1 protein binds to the phytochrome-responsive chlorophyll *a/b cab140* promoter (Sun et al., 1993). A cDNA clone encoding a CA-1–like binding activity that is not a member of the bZIP family has been isolated in Arabidopsis (Dr. Elaine Tobin, personal communication). As shown in Figure 9A, OBP1 was unable to stimulate and instead caused a slight decrease in the binding of the CA-1–like protein over a range of protein concentrations tested. We also examined whether OBP1 could modulate the activity of another bZIP protein, Zebra. Zebra is an Epstein-Barr virus transactivator required during the viral lytic life cycle (Farrell et al., 1989; Lieberman and Berk, 1990). As shown in Figure

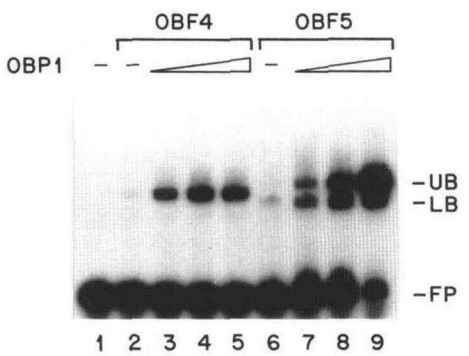

Figure 7. The Effect of OBP1 on OBF4 and OBF5 Binding to the *ocs* Wild-Type Sequence from the *ocs* Promoter.

The binding of equal amounts of OBF4 and OBF5 (5 ng) to the *ocs* wild-type sequence was analyzed in the presence of different concentrations of OBP1 using the gel retardation assay. Each triangle indicates increasing amounts of OBP1 (4, 10, and 25 ng of protein, respectively). No OBP1 protein was added in lanes 1, 2, and 6. The position of the free probe (FP), the upper band (UB), and the lower band (LB) for OBF5 are shown; the mobilities of these bands for OBF4 are slightly slower. The lower band is formed when the OBF protein binds to either half of the *ocs* element and the upper band when the OBF protein binds to both halves (reviewed in Singh et al., 1994). In this experiment, the binding of OBF4 and OBF5 by themselves resulted in only a small amount of lower band formation (lanes 2 and 6). Lane 1 contains only the free probe.

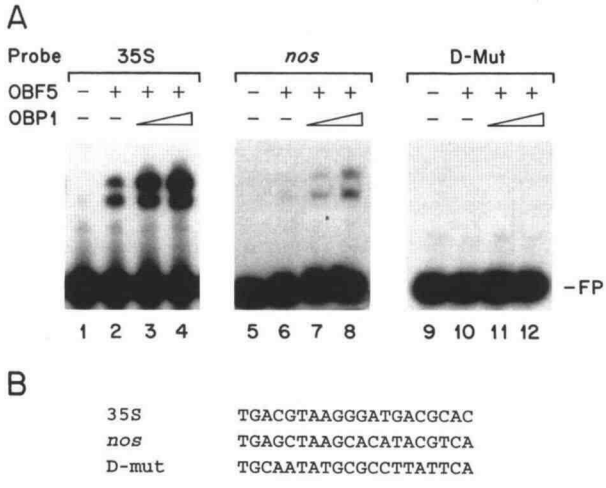

Figure 8. The Effect of OBP1 on OBF5 Binding to Additional *ocs* Element Sequences.

(A) OBF5 (1 ng) was added to the reactions used in lanes 2 to 4, 6 to 8, and 10 to 12. Each triangle indicates increasing amounts of OBP1 (10 and 25 ng of OBP1, respectively). (+) and (−) indicate the presence or absence of a particular protein, respectively. The positions of the free probes (FP) are shown. Lanes 1, 5, and 9 contain only the free probe.

(B) The sequences for the *ocs* element portions of the 35S, *nos*, and double mutant (D-mut) probes.

9B, OBP1 was able to cause only a small increase in Zebra binding to its recognition sequence.

DISCUSSION

Protein–protein interactions play important roles in many aspects of cellular activity. The isolation and characterization of proteins that interact with the Arabidopsis OBF proteins should lead to a better understanding of the role the OBF proteins play in plants. By using the labeled OBF4 protein as a probe and screening an Arabidopsis cDNA library, we isolated a protein, OBP1, that interacts specifically with the Arabidopsis OBF proteins. Similar screening approaches have been used successfully for the isolation of interacting proteins in other systems (for example, see Blackwood and Eisenman, 1991; Blanar and Rutter, 1992). There are a number of interesting features associated with the OBP1 protein. In addition to interacting with the Arabidopsis OBF proteins in vitro, OBP1 is a DNA binding protein that binds to a region of the CaMV 35S promoter containing an AAGG motif. OBP1 is also able to increase significantly the binding of OBF proteins to *ocs* element sequences, including the *ocs* element sequence in the 35S promoter. The function of OBP1 is not known, and additional studies are required to address whether the AAGG motif present in the 35S promoter is an optimal binding site for OBP1 and whether OBP1 acts as an activator or repressor of transcription. Studies are also needed to determine whether OBP1 interacts with the OBF proteins in vivo and to identify cellular genes regulated by OBP1. The interaction between OBP1 and the OBF4 proteins is not expected to have evolved for the viral CaMV promoter, but an interesting possibility is that it may have evolved to help recruit the OBF proteins to specific cellular promoters.

Genomic DNA gel blot analysis revealed that there are a number of *OBP1*-related genes in Arabidopsis. Using the *OBP1* cDNA as a probe, we have isolated a number of cross-hybridizing cDNAs (Kang and Singh, unpublished results). In addition, *OBP1* has homology at the DNA level with two Arabidopsis expressed sequence tag sequences in the data base (GenBank, EMBL, and DDBJ accession numbers T14116 and T88559); the homology is in the region coding for the MOA domain. The characterization of the *OBP1*-related clones may help in the identification of OBP1 domains responsible for DNA binding, the ability to interact with the OBF proteins, and the ability to stimulate OBF binding to *ocs* element sequences.

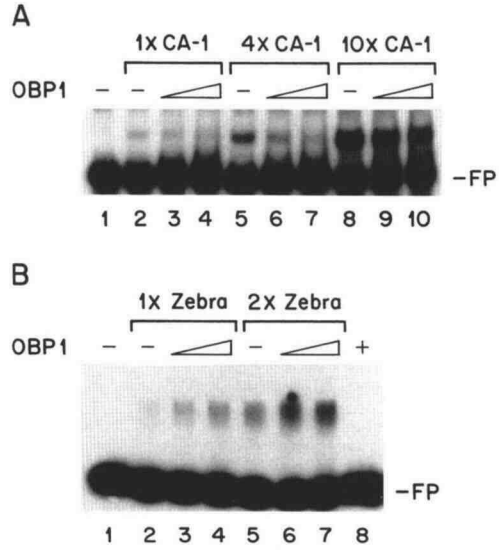

Figure 9. The Effect of OBP1 on the Binding of Other DNA Binding Proteins.

(A) Binding of the CA-1–like protein (CA-1) to the A2 DNA sequence. Different amounts of CA-1 were used (lanes 2 to 4, 1x indicates 1 ng; lanes 5 to 7, 4x indicates 4 ng; lanes 8 to 10, 10x indicates 10 ng). Lane 1 contains only the free probe (FP).

(B) Zebra binding to the Zm-1 DNA sequence. Different amounts of Zebra were used (lanes 2 to 4, 1x indicates 5 ng; lanes 5 to 7, 2x indicates 10 ng). Lane 8 contains 25 ng of the OBP1 protein and no Zebra protein. Lane 1 contains only the free probe.

In both **(A)** and **(B)**, the triangles indicate increasing amounts of OBP1 (10 and 25 ng of OBP1, respectively). The (−) indicates that no OBP1 was present. The positions of the free probes are shown.

The MOA Domain: A Putative Zinc Binding Domain Highly Conserved in Plants

The Arabidopsis OBP1, maize MNB1a, and pumpkin AOBP proteins share a highly conserved 51-amino acid region that we have called the MOA domain. All three proteins are DNA binding proteins, strongly suggesting that this region may constitute a DNA binding domain. The MOA domain contains the sequence $C\text{-}X_2\text{-}C\text{-}X_{21}\text{-}C\text{-}X_2\text{-}C$, which bears resemblance to a single zinc finger domain of the GATA1 type ($C\text{-}X_2\text{-}C\text{-}X_{17}\text{-}C\text{-}X_2\text{-}C$) (Omichinski et al., 1993; Klug and Schwabe, 1995), although the amino acid sequence and spacing between the two cysteine pairs is not conserved. Interestingly, the extensive conservation in the MOA domain extended for 21 amino acids after the last cysteine in the putative zinc finger. The GATA family of zinc finger proteins also contains a conserved region, required for DNA binding, on the C-terminal side of the zinc finger. Recently, the self-association of GATA-1 was found to be mediated by its zinc finger domains (Crossley et al., 1995), demonstrating that this region can also function in protein–protein interactions. A plant protein, NTL1, contains a putative, single zinc finger (Daniel-Vedele and Caboche, 1993) that matches the GATA family much more closely than the MOA domain. MOA domain proteins may therefore constitute a novel family of zinc finger–containing proteins in plants.

The MOA domain also has some resemblance to half a LIM domain. LIM domains are novel cysteine-rich, zinc binding domains found in a number of proteins, including transcriptional regulators (reviewed in Sanchez-Garcia and Rabbitts, 1994). Recently, the LIM domain has been shown to be a specific protein binding interface (Schmeichel and Beckerle, 1994), and a role in DNA binding has also been proposed (Feuerstein et al., 1994; Sanchez-Garcia and Rabbitts, 1994). The N-terminal half of the LIM domain consensus sequence, $C\text{-}X_2\text{-}C\text{-}X_{16\text{-}23}\text{-}H\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_{16\text{-}21}\text{-}C\text{-}X_2(C,H,D)$, bears some similarity with the MOA domain: $C\text{-}X_2\text{-}C\text{-}X_{19}\text{-}H\text{-}X\text{-}C\text{-}X_2\text{-}C$. Whether the MOA domain plays a role in protein–protein interactions and/or DNA binding remains to be determined.

OBP1 and MNB1a are essentially the same size and show significant homology, with 37% amino acid identity and 53% amino acid similarity, raising the possibility that OBP1 might be the Arabidopsis homolog for MNB1a. DNA binding studies revealed that OBP1, like MNB1a, binds to the AAGG motif, which lies upstream of the *ocs* element in the CaMV 35S promoter. These results raise the possibility that interactions between distinct types of DNA binding proteins, such as OBP1 and the Arabidopsis OBFs, play an important role in the activity of the 35S promoter. The 35S promoter is modular in structure and has been broken up into discreet domains (Benfey and Chua, 1990). The A domain (−90 to −46), which contains the *ocs* element sequence (−82 to −63), gives rise to root tip expression in transgenic tobacco. The B domain from −90 to −343 gives rise to expression in the aerial parts of the plant. When combined, the A and B domains are expressed throughout the plant, including the root. The B domain has been further broken up into five subdomains. The B4 subdomain (−301 to −208), which contains the AAGG binding site for MNB1a/OBP1, is active in developing vascular tissue in stems and leaves, primarily in vascular parenchyma and phloem element cells. Together, the A domain and B4 subdomain work synergistically, resulting in significantly stronger activity in vascular tissue in seedlings and mature plants both in the aerial tissues and the root. The role of the MNB1a/OBP1 binding site in B4 expression remains to be evaluated.

How Does OBP1 Stimulate the Binding of the OBF Proteins to *ocs* Element Sequences?

The mechanism by which OBP1 stimulates the binding of the OBF proteins to *ocs* element sequences remains to be elucidated. In our gel retardation experiments, the mobility of the retarded bands observed with OBF4 and OBF5 did not change with the addition of OBP1. This suggests that the complex of the OBF protein and OBP1 is not able to bind to the *ocs* element or is unstable and dissociates under the gel retardation conditions. The ability of proteins to stimulate the DNA binding activity of other proteins has been observed previously. The DNA binding activity of the serum response factor to the serum response element was stimulated by a cellular homeodomain protein, called Phox1 (Grueneberg et al., 1992). Interestingly, *E. coli*–produced Phox1 did not cause a change in the mobility of the serum response factor–serum response element complex. However, Phox1 expressed in yeast did cause a change in the mobility of the serum response factor/serum response element complex, suggesting that stable complex formation may require post-translational modifications. The region of Phox1 required to stimulate serum response factor binding was mapped to the homeodomain. Members of the AP-1 family (bZIP proteins) and steroid receptors (zinc finger proteins) have also been found to interact through their respective DNA binding domains, although in these cases the interactions led to the repression of DNA binding for both types of proteins (reviewed by Schule and Evans, 1991; Wagner and Green, 1994).

The hepatitis B virus X protein, which is not able to bind to DNA, has been shown to alter the DNA binding specificity of two bZIP proteins, CREB and ATF-2, by protein–protein interactions (Maguire et al., 1991). In this case, the hepatitis B virus X protein was found to be part of the protein–DNA complex detected in gel retardation experiments. Another viral protein, the Tax protein of human T cell leukemia virus type-1, has been found to stimulate the DNA binding activity of a number of bZIP proteins, including members of the CREB/ATF family required for transcriptional activation of the human T cell leukemia virus type-1 long terminal repeat (Zhao and Giam, 1992; Wagner and Green, 1993; Adya et al., 1994). Through such a stimulation, Tax, which is not a DNA binding protein, activates viral gene expression that leads to malignant leukemia.

There are a number of similarities in the way Tax and OBP1 stimulate the DNA binding activities of bZIP proteins. Tax and

OBP1 are unable to stimulate the DNA binding of proteins lacking a bZIP motif. Like OBP1, the stimulation of DNA binding by Tax was maximal at low concentrations of the bZIP protein. Wagner and Green (1993) found that Tax stimulates bZIP DNA binding activity by enhancing the dimerization of bZIP proteins in the absence of DNA. The Tax and OBP1 proteins also differ in a number of aspects. Tax has been reported to stimulate the DNA binding activity of all bZIP proteins tested (Wagner and Green, 1993), although others have reported a preference for CREB (Zhao and Giam, 1992; Adya et al., 1994). We observed only a slight stimulation of Zebra binding by OBP1, in clear contrast to the large stimulation observed with OBF5. It will be interesting to see if OBP1 stimulates the binding of other Arabidopsis bZIP proteins such as the G-box binding factors (Schindler et al., 1992b). A lack of specificity may be adequate for Tax, which functions as a viral transactivator, because a number of the genes involved in mammalian cell growth encode bZIP proteins. However, OBP1 is a cellular protein and may require a greater degree of specificity with regard to stimulating bZIP DNA binding. OBP1 is able to bind DNA directly, in contrast to Tax, and this may contribute further to specificity by targeting those bZIP proteins that bind to the same promoters as OBP1 does.

METHODS

Expression, Purification, and Labeling of Recombinant Proteins

The *ocs* element binding factors (OBFs) and OBF binding protein (OBP1) were overexpressed in *Escherichia coli* and/or the baculovirus system as glutathione S-transferase (GST) fusion proteins using the pGEX-2T and pAcG2T plasmid vectors, respectively. The coding regions of *OBF4* and *OBF5* were inserted into the BamHI-EcoRI site in pGEX-2T and pAcG2T, and the coding region of *OBP1* was inserted into the EcoRI site of pGEX-2T by using the polymerase chain reaction and the oligonucleotide sequences listed below. The 3′ oligonucleotides for *OBF4* and *OBF5* also contained the sequence for a heart muscle kinase (HMK) phosphorylation site resulting in a five-amino acid HMK tag at the C terminus of each of the OBF proteins.

The primer sequences for *OBF4* are 5′-GGGGGGATCCATGAATACAACCTCGACACATTTTGTTCCACCG-3′ and 5′-GGGGGAATTCAACAGATGCACGACGGGGCGTTGGTTCACGTTGCCTAGCCGC-3′. The primer sequences for *OBF5* are 5′-GGGGGGATCCATGGGAGATACTAGTCCAAGAAC-3′ and 5′-GGGGGAATTCAACAGATGCACGACGGGGCTCTCTTGGTCTGGCAAGCCATAG-3′. The primer sequences for *OBP1* are 5′-GGGGGAATTCCCATGAAACCTAACGGCGTAACAGTTCC-3′ and 5′-GGGGGAATTCGAGATCAACATCACCAATAGAGAC-3′.

The pGEX-2T constructs were transformed into *E. coli* XL-Blue competent cells for OBF4HMK and *E. coli* BL21(DE3) competent cells for OBP1. Five milliliters of an overnight culture was added to 500 mL of CircleGrow (Bio101, La Jolla, CA) and incubated on a shaking platform for 4 hr. The expression of the GST fusion proteins was then induced for 2 hr with the addition of 0.5 mM isopropyl β-D-thiogalactoside for OBF4HMK and 10 μM isopropyl β-D-thiogalactoside for OBP1. The OBF4HMK and OBF5HMK–GST fusion proteins were overexpressed using the Baculogold baculovirus DNA and Sf9 insect cells according to the manufacturer's instructions (PharMingen, San Diego, CA). The purification of the GST fusion proteins using glutathione beads and the digestion with thrombin (Sigma) were as described by Ausubel et al. (1994).

Two hundred nanograms of the OBF4HMK protein was incubated overnight with 3 units of HMK (Sigma) at 4°C in 20 mM Tris, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, 10 mM DTT, and 40 μCi of γ-^{32}P-ATP. The labeled proteins were separated from unincorporated γ-^{32}P using G-50 Sepharose columns and PBS.

Library Screening

Labeled OBF4HMK was used to screen 200,000 plaques from an Arabidopsis callus cDNA expression library (Zhang et al., 1993). The protein screening technique was based on the method described by Gu et al. (1994). Briefly, after 4 hr of growth at 42°C, the plaques were covered with dry filters previously soaked with 10 mM isopropyl β-D-thiogalactoside, and the plates were left overnight at 37°C. The filters were prehybridized for 2 hr at 4°C in 5% nonfat dried milk, 20 mM Hepes, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1% Nonidet P-40. The filters were then incubated in hybridization buffer (1% nonfat dried milk, 20 mM Hepes, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.1% Nonidet P-40, 10% glycerol, and 2 × 10^7 cpm of the probe) for 5 to 6 hr at 4°C. Filters were washed three times for 30 min each with hybridization buffer and then subjected to autoradiography.

DNA Sequence Analysis

Single-stranded DNA sequence reactions were performed using the Sequenase Version 2.0 DNA sequencing kit (Amersham) and the bacteriophage M13 vectors mp18 and mp19.

Protein–Protein Binding Studies

Fifty nanograms of labeled OBF4HMK or BTBHMK protein was incubated in 20 μL of PBS buffer for 30 min at room temperature either alone or in the presence of GST or GST–OBP1. Twenty microliters of 50% glutathione-agarose beads was added, and the solution was centrifuged at 500*g* followed by two washes with PBS. The bound protein was recovered by elution in 5 mM glutathione in 50 mM Tris, pH 7.5, and analyzed by SDS-PAGE followed by autoradiography.

Genomic DNA Gel Blot Analysis

Genomic DNA was extracted from *Arabidopsis thaliana* (ecotypes Columbia and Landsberg *erecta*) based on the method of Dellaporta et al. (1983). Genomic DNA (8 μg) was digested with ClaI and EcoRI. DNA gel blot analysis was performed as described by Foley et al. (1993), using a random-primed *OBP1* cDNA probe.

RNA Gel Blot and Reverse Transcriptase–Polymerase Chain Reaction Analyses

Poly(A)$^+$ RNA (2.5 μg) from Arabidopsis callus tissue was fractionated on a 1.4% agarose–formaldehyde gel with RNA molecular mass markers (Bethesda Research Laboratories). RNA gel blot analysis was as

described by Zhang et al. (1993), except that a random primed *OBP1* cDNA probe was used and hybridization was at 43°C. Reverse transcriptase–polymerase chain reaction was performed as previously described (Zhang and Singh, 1994). RNA samples were isolated from 8-day-old seedlings after auxin and salicylic acid (SA) treatment. cDNA was synthesized using reverse transcriptase and amplified by oligonucleotide primers specific for *OBF4* (for oligonucleotide sequences, see Zhang and Singh, 1994) and *OBP1* (the same oligonucleotide pair used to overexpress OBP1). After 20, 27, and 35 cycles, aliquots were run on an agarose gel, transferred to a nylon membrane, and probed with the corresponding labeled probe. Amplification products were visualized by autoradiography. To enable quantitation, a cycle number was chosen for each primer pair that was in the exponential phase of the polymerase chain reaction.

Gel Retardation Assays

For the gel retardation experiments, the OBF4HMK and OBF5HMK proteins were overexpressed in the baculovirus system and OBP1 was overexpressed in *E. coli*. The Zebra and CA-1–like proteins, which were overexpressed in *E. coli*, were gifts from Dr. E. Tobin and Dr. M. Carey (University of California–Los Angeles). For the *ocs* element DNA probes, the oligonucleotides shown below were synthesized with flanking BamHI and BglII sites and cloned into the BamHI site of the pUC118 polylinker as described previously (Singh et al., 1990). The *ocs* wild-type sequence is 5′-AAACGTAAGCGCTTACGTAC-3′. The cauliflower mosaic virus (CaMV) 35S sequence is 5′-TGACGTAAGGGATGACGCAC-3′. The nopaline synthase (*nos*) sequence is 5′-TGAGCTAAGCACATACGTCA-3′. The *ocs* double mutant sequence is 5′-TGCAATATGCGCCTTATTCA-3′.

EcoRI-HindIII fragments containing the oligonucleotides were gel purified, end labeled, and used in gel retardation experiments as previously described (Singh et al., 1989). The DNA sequence for the A2 and Zm probes are described by Sun et al. (1993) and Chi and Carey (1993), respectively.

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