

Characterization of Three Rice Basic/Leucine Zipper Factors, Including Two Inhibitors of EmBP-1 DNA Binding Activity*

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The promoter of the wheat *Em* gene contains elements with a CACGTG core sequence (G-boxes), which are recognized by EmBP-1, a wheat basic/leucine zipper (bZIP) protein. G-boxes are required for *Em* expression in response to the phytohormone abscisic acid and for transactivation by the *Viviparous-1* protein (VP1) using transient expression systems. In order to identify other factors that are part of the transcriptional complex that associates with G-boxes, we have screened a rice (*Oryza sativa*) cDNA library with biotinylated EmBP-1. We have isolated osZIP-1a, a homolog of EmBP-1 and other plant G-box-binding factors. We show that EmBP-1 and osZIP-1a will preferentially heterodimerize *in vitro*. Overexpression of osZIP-1a in rice protoplasts can enhance expression from the *Em* promoter only in the presence of abscisic acid. Two other clones have been identified by screening with EmBP-1: osZIP-2a and osZIP-2b. These osZIP-2 factors represent a novel class of bZIP proteins with an unusual DNA-binding domain that does not recognize G-boxes. The osZIP-2 factors can heterodimerize with EmBP-1 and prevent it from binding to the *Em* promoter. Interestingly, osZIP-1a does not heterodimerize with the osZIP-2 factors and its DNA binding activity is unaffected by their presence. Thus, osZIP-2 factors may be involved in sequestering a particular group of G-box-binding factors into inactive heterodimers.

Since they were first identified as a conserved sequence in several of the light-induced *rbcs* genes (1), G-boxes (CACGTG) and other *cis* response elements with an ACGT core sequence have been implicated in gene induction by a wide variety of response signals in plants (see Refs. 2 and 3). We are particularly interested in elucidating the role of these G-boxes in the regulation of the *Em* gene, which is expressed during the later stages of higher plant embryogenesis. Two major regulators of *Em* gene expression are the phytohormone abscisic acid (ABA)¹

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U04295, U04296, and U04297.

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¹ The abbreviations used are: ABA, abscisic acid; bZIP, basic/leucine zipper; GBF, G-box-binding factor; GUS, β -glucuronidase; ABRE, ABA response element; CaMV, cauliflower mosaic virus; MBP, maltose-bind-

(4) and the product of the maize *Viviparous-1* and *Arabidopsis* ABA-insensitive *Abi3* loci (5, 6). Using a transient expression assay in rice protoplasts, a 644-bp region of the wheat *Em* promoter (7), fused to a β -glucuronidase (GUS) reporter gene, can be induced ~25-fold by physiological levels of ABA (8). With this expression assay, a 66-bp ABA response element (ABRE) has been identified that, when fused to a minimal cauliflower mosaic virus (CaMV) 35S-promoter, conferred ABA inducibility to this normally non-responsive viral promoter (9). The ABRE contains three putative regulatory elements based on conserved sequence motifs: Em1a, Em2a, and Em1b. The two Em1 sequences are very similar to the G-boxes identified as regulatory elements in a wide variety of inducible plant promoters. Mutations in either the Em1a or Em1b elements greatly reduce but do not eliminate ABA inducibility (10, 11), and tetramers of Em1a or Em1b can confer ABA inducibility to a minimal CaMV 35S-promoter (11). Furthermore, transactivation of the *Em* promoter and a synergistic interaction with ABA by the VP1 protein in maize protoplasts also requires these same G-boxes (11).

The ABRE has been used to isolate *trans*-acting proteins that recognize the *Em* promoter. A cDNA encoding the bZIP factor EmBP-1, a class of transcription factors that are characterized by a DNA-binding domain rich in basic amino acids adjacent to a leucine zipper dimerization domain, has been characterized (10). Using methylation interference footprinting, it was demonstrated that EmBP-1 can specifically recognize the Em1a element in the ABRE. The resulting footprint is indistinguishable from the one obtained using wheat embryo nuclear extracts. A mutation in the ACGT core of the Em1a element, which abolished binding by EmBP-1, also caused a strong reduction in the ABA inducibility (10) and VP1 transactivation properties of the mutated promoter (11).

In addition to EmBP-1, more than 40 bZIP proteins from plants recognize elements with an ACGT core (for a review, see Ref. 2). Most of these bZIP factors can be classified in three major groups based on their overall structure and DNA-binding specificity (12). Group 1 factors, which include EmBP-1, are also called G-box-binding factors (GBF) since they will bind preferentially to ACGT elements with two G-box half sites, *i.e.* CACGTG. Using a variety of techniques, the *Arabidopsis* GBF1 (13) and the tobacco TAF1 (14) have been shown to act as transcriptional activators while overexpression of CPRF-1 in parsley protoplasts results in a 6-fold reduction in the light-induction of the chalcone synthase promoter (15). The second group of plant bZIP factors is not as well defined and mostly consists of homologs of the maize Opaque-2 (16) and rice RITA-1 transcriptional activators (17), as well as the parsley CPRF-2 protein (18). The third group comprises the TGA fac-

ing protein; GST, glutathione *S*-transferase; ORF, open reading frame; bp, base pair(s); kb, kilobase pair(s); NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

tors, which will recognize elements with a TGACG core sequence including several ACGT elements (19). The tobacco TGA1a is a transcriptional activator as determined both by *in vitro* transcription assays (20) and by transactivation experiments in plant (21) and yeast cells (22). Finally, some plant bZIP factors, such as the *Arabidopsis* GBF4 (23) and PosF21 (24), the maize OBF2 (25) and the rice lip19 (26), do not seem to belong to any of these groups.

The observation that many plant promoters have ACGT cores in elements responsive to different signals, and the fact that the bZIP proteins that recognize these similar elements are encoded by multigene families, suggest that combinatorial interactions between different bZIPs and other regulatory proteins are likely to be required for specificity of expression. Numerous examples exist where eukaryotic gene expression is regulated by the interaction of a transcription factor with either another factor from the same or different family, or with a specific regulatory protein such as a kinase, a DNA-binding inhibitor, a cytoplasmic anchor, etc. (see Ref. 27). This may also be the case in plants, since gel exclusion studies suggest that the size of the G-box-binding complex is approximately 160 kDa (28). Furthermore, it has been demonstrated that GF-14, a plant homolog of the 14-3-3 family of mammalian brain proteins, is part of that complex (29). All of these examples have prompted us to use a protein-protein interaction screen to isolate from an expression library, rice cDNAs whose products can interact with EmBP-1. As a result we have isolated the first rice gene encoding a Group 1 GBF, *osZIP-1a*, as well as two members of the *osZIP-2* gene family representing a novel group of plant bZIP proteins. In this report, we also present evidence that, unlike EmBP-1, overexpression of *osZIP-1a* can transactivate the ABA induction of the Em promoter in rice protoplasts. Also, the *osZIP-2* factors can prevent EmBP-1, but not *osZIP-1a*, from binding to the Em promoter in a gel shift assay. Thus, a possible role for the *osZIP-2* factors is that they can selectively inactivate some members of the Group 1 bZIP family.

MATERIALS AND METHODS

Production of Expression Plasmids—Subcloning of the EmBP-1 cDNA in the expression vector pPR997 (New England Biolabs) has been described previously (30). To obtain a plasmid expressing the MBP-*osZIP-1a* fusion, we flanked the *ZIP-1a* gene with *EcoRI* and *HindIII* sites by polymerase chain reaction amplification of the pE4D2 cDNA clone with the primers ANo-7 (GCGGAATTCATGGGTAGCAGTGG) and ANo-8 (GCGAAGCTTACAGTTGCGCCTGC). The amplified product was then digested with *EcoRI/HindIII* and ligated in the same sites of pPR997 yielding pAN72. Since yields of MBP-*osZIP-1a* were extremely low, we resorted to the purification of the ZIP-1aΔ107 partial protein with the pAN64 plasmid obtained by ligating the *NcoI/KpnI* insert of pE4-1 in the blunted *EcoRI* site of pPR997.

Purification and Biotinylation of the Fusion Proteins—Expression and purification of the MBP fusion proteins were performed as described (30). Purified fusion proteins were biotinylated with a 5-fold molar excess of NHS-LC-Biotin as recommended by the manufacturer (Pierce).

Far Western Slot Blot—Appropriate amounts of proteins were blotted to BA-S nitrocellulose with a Schleicher & Schuell slot blotter. The filter was then allowed to dry and probed with the appropriate biotinylated protein as described for the library screening (30), except that development in NBT/BCIP was allowed to proceed overnight.

DNA Sequencing—Plasmids from various subclones were isolated with the QIAprep-spin plasmid kit (Qiagen) and sequenced on both strands using a Sequenase kit (U. S. Biochemical Corp.) or at the UNC-CH Automated DNA Sequencing Facility on a model 373A DNA sequencer (Applied Biosystems) using the *Taq* DyeDeoxy™ terminator cycle sequencing kit (Applied Biosystems). Analysis of sequence data was performed with GCG (31) and DNA Strider software (32).

Northern and Southern Blots—Genomic DNA and total RNA were isolated from rice embryonic suspension cells as described (33, 34). Blots were probed using the manufacturer's recommended protocols for Hybond-N filters (Amersham) with DNA labeled using the Random

Primed DNA Labeling Kit (Boehringer Mannheim). For high stringency hybridization, conditions for the last wash were 10 min at 67 °C in 0.1 × SCC, 0.1% SDS.

In Vitro Transcription and Translation—*In vitro* transcription and translation in rabbit reticulocyte lysates (with or without [³⁵S]methionine) were performed according to the manufacturer's recommendations (Promega). For production of EmBP-1, we used the pAN10 plasmid which was produced by ligation of the *EcoRI/XbaI* insert of pAN7 (30) in the same sites of pBluescript KS⁻ (Stratagene). The pE4-1 and pE3-1 clones were used for production of *osZIP-1a*Δ107 and *osZIP-2a*, respectively. For production of *osZIP-2b*, a small *SacII* fragment, containing an out-of-frame ATG, was removed from the pE3A4 cDNA to give pAN75. Finally, pAN77 was constructed by removing a *BglII/XhoI* fragment from the pAN75 insert, resulting in the production of the *osZIP-2b*ΔLZ partial protein.

Electrophoretic Mobility Shift Assay—The strategy for production of the ABRE probe has been described previously (10). The following DNA oligonucleotides, when used as probes or in the competitive binding assay, were annealed and filled-in with Klenow and deoxynucleotides.

Em1a:	5'-GCGCTCGAGTGCCTGGACACGTGGC-3' 5'-CGCGTCGACGTGCGCCACGTGTGTC-3'
Em1b:	5'-GCGCTGAGCGTGCAACGGCC-3' 5'-CGCGTCGACGAGGCGGCACGTGTG-3'
Em1c:	5'-GCGCTCGAGACAAACGTACACGCGC-3' 5'-CGCGTCGACGTGCGCGGTGTACG-3'
Em1d:	5'-GCGCTCGAGCGCCATTACGTGTT-3' 5'-CGCGTCGACAAGACAACGTAAT-3'
Em2a:	5'-CGCGTCCGACGGACAACGAGCAGGC-3' 5'-CGCCTCGAGCGTCGGCCGATCCT-3'

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When the DNA was to be used as a probe, dCTP was replaced with a [³²P]dCTP and unincorporated nucleotides were removed with NucTrap push columns (Stratagene). For the actual gel shift assay, 20 ng of MBP fusion proteins, or 2–4 μl of reticulocyte lysate, were incubated for 20 min at room temperature with 1 ng of ³²P-labeled DNA and 1.0 μg of poly(dI-dC) in a final volume of 20 μl of Binding Buffer IV (12 mM Tris-HCl, pH 7.9, 45 mM KCl, 7.5 mM MgCl₂, 12% glycerol, and 1 mM dithiothreitol). When necessary, unlabeled competitors were added at the same time as the probe. In order to favor dimer exchange in the heterodimerization experiments, the proteins were preincubated for 20 min at room temperature prior to the addition of the other components. Gel electrophoresis was performed as described (35), and the dried gel was exposed to x-ray film for 1–3 days.

Resin Binding Assay—We followed a previously described protocol (36), except that we used MBP fusion proteins and an amylose resin (New England Biolabs) instead of GST fusions and glutathione-Sepharose.

Two-hybrid Assay—Two-hybrid analysis of the various bZIP factors was performed using protocols, plasmids, and yeast strains obtained from Dr. Roger Brent (37). *EcoRI* and *XhoI* restriction sites were inserted around the bZIP domains of EmBP-1 (amino acids 257–362) and *osZIP-1a* (amino acids 292–390) by polymerase chain reaction mutagenesis. The amplified products were then inserted in the same sites of the pEG202 and pJG4–5 vectors. Subcloning of the full-length *osZIP-2b* gene was accomplished by inserting the *EcoRI/XhoI* insert of the pE3A4 cDNA in pEG202 or pJG4–5. β-Galactosidase activity was measured by a permeabilized cell assay (38).

Transfection in Rice Protoplasts—The pCR349.11S plasmid, in which the *EmBP-1* gene is fused to the CaMV 35S-promoter, was constructed by inserting an *XmnI/HincII* fragment of pAN11 (30) into *SmaI* site of pDH51 (39). The *osZIP-1a* gene in pAN72 was isolated and reinserted in the *EcoRI/HindIII* sites of pBluescript KS⁻ to give pAN73. The *BamHI/SaI* insert of pAN73 could then be inserted next to the 35S-promoter in the same sites of the pDH51 expression vector yielding pAN74. The reporter plasmids pBM207 (9) and pACT-1D (40) have been described previously. Polyethylene glycol-mediated transfection of rice protoplasts and assays for GUS activity were performed as described (8), except that we produced our plasmids in the methylase-deficient *Escherichia coli* strain GM2163 (41). Statistical analysis of the results was performed with Microsoft Excel.

RESULTS

Isolation and Sequence Comparison of Rice bZIP Factors—EmBP-1 was expressed in *E. coli* as a fusion with the maltose-binding protein (MBP) and purified by affinity chromatography. The fusion protein (MBP-EmBP-1) was then biotinylated

A

CCTCGCTGCC CTFACGCTAG GCGTGCCTGA CCAGCCAGCA CAGGTGGTGA TCTCATCATC 60
 CCGCGCGCGC CGCGCGCGGC GTTCGGGCTC CCGCGCGCCC ACCACCCGAC ATCCGAGGCG 120
 CCGCGCTCCG CGCGCGCGGG ATCGCGCGCC CCAGCACAAG ATTTTGGCTA ATGCTACTTT 180
 ATGGTGTGTG GGATAATGGA GTAATCACTG CTCTGTGTAT CTGAGCTCAC ACCATGGGTA 240
 1 M G S
 CGAGTGGCGC AGACGCCACC ACTAAGACAA GCAAGGCATC TGACCTTCAG GAGCAACAGC 300
 S A P T K F S K A S A P P
 CACCTGCTAG TTCAAGCATC GCACACCGGG CTGTTTACC AGATTGGCCG AACTTTCAGG 360
 P A S S S T A T P A V Y P D W A N F Q G
 24 GATATCTCCG AATTCCACCA CATGGCTTCT TTCCATCAC CTGGCGGTCA AGCCACAGG 420
 Y P P I P P H G F F P S P V A S S P Q G
 44 GTCATCCTTA CATGTGGGGA GCTCAGCTA TGATACACC ATATGGAACC CCACACACC 480
 H P Y M W G A Q P M I P P Y G T P P P
 64 CATATGTGAT GTATCTFCCA GAGATATATG CTCACCTATC TATGCTCCCG GCGCAGACCC 540
 Y V W Y P P G V Y A H P S M P P G A H P
 CATTTACTCC ATATGCCATG GCCTCTCAA ATGGCAATCG TGATCTACT GGAACACAA 600
 F T P Y A M A S P N G N A D P T G T T T
 104 CTACTCTGCG TCGCTGCTGT CCGTGTGAGA CGGATGGCAA ATCTCTGAA GGAAAAGAAA 660
 T A A A A A G E T D G K S S E G K E K
 124 AAAGTCCCAT TAAAGAGTCT AAAGAGTCT TAGGTAGCTT GAATGATGAT ACAGGAAAGA 720
 S P I R S R K L M S L G S L N H I T G R N
 144 ACTCTACTGA ACTGTGTAAA ACTCTGTGGG CATCGACTAA TGAGCAACTT TCTCAAGTGT 780
 S T E H G K T S G A S A N G A I S Q S G
 164 GGGAAAGTGG AAGTGAAGT TCTAGTGAAG GAAGTGAAGC AAATCTCAGC AATGATTCAC 840
 E S G S E S S E G S E A N S Q N D S H
 184 ATCAACAAGA AAGTGGACAA GAGCAAGTGC GAGAGTGTTC AAGTCCCGAC AATGTGTGAT 900
 H K E S G Q E Q D G E V R S S Q N G V S
 204 CAGCTTACC ATCCAGGACA AAGTGTATC AAATCTATGC AATCATGCCA ATGACATGAA 960
 R F H I M H M L R L K Q A E I C F E D
 224 GTGTCCTCAG ACTGCTCTCA ACTCAACTT TGAACATGAG AATGACACT TGGGCCAACA 1020
 G P V P A P T T N L N I G M D Y W A N T
 244 CAGCATGCTC CACTCCAGCA ATACATGGA AAGCAACCCC AACTCGAGCT CCAGGGTCTA 1080
 A S S T P A I H G K A T P T A A P G S M
 264 TGGTCCAGG AGACAGTGG GTCCAGGATG AACCGGAACT CAAAAGGACG AGAAGAALAC 1140
 V P G E Q W V Q D E R E L K R Q R R K Q
 284 AAATCCACAG GGAGTCTCT CCAGATCTA GGTTCGATTA GCAGGCTGAA CTGACAGAT 1200
S N R E S A R F E R L R K Q A E I C F E D
 304 TGCTCAAGC TGCTATAGT TAAAGTGAAG AAATACTTC ACTTASAGAT GAAGTGAACC 1260
A O R A E V I K Q E N T S I R D E V N R
 324 GGATCAGAAA GAGATGATG GAGCTCTAT CAAGAATAG CTCATTAAG GAATAACTTG 1320
R K R E Y D E L S K N S S I K E K L E
 344 AGGCAAAACA ACAAAACAT GATGAGGAC GAGTGGACAA TAAAGTGCAC CATTCTGCTG 1380
D K Q H K K T D E A G V D N K L Q H S G D
 364 ATGACAGCCA AAAAAAGGA AATCAATGA GCTATGCTGC AATGCTCTCG AGCATACCC 1440
D S Q G
 384 TAACATGATT ATGGCAGCG GCAACTGTAG TATGTGATT AACTTCAAC TTATGCGAGT 1500
TAATTTGTC CAGGTTGTA TAGTGTAT GCCAACTTG TGGGCTGGT TCTGTPTCA 1560
GATCTCTCT GCTTATCTT TTAGAAGTCA AGATATGACT GCCAAATGAG GTGTGACCC 1620
AGTTCACCT ACTGTATGA AATAGTATT AAACTATGT TTACAAACT GTATTTGTA 1680
CTACTATGT TATGTACAC TGAAGATGA CCAGTATTT ATATATAAGA TGACGTACAG 1740
TTGAACAGT GGTTTAA 1757

B

AGATGGCGAG CACAGAGGCTG AGCCTCGAGC TGGGGAAAGT CGGAATCCAG TCGTCCCGCC 60
 1 M A S T R L S L E L G R V G I Q S S P P P
 CTRGCTCCAG CAGCTTAAGC CGCGCGCAC CCCTGATGCA CCGTCCCGCC CGCGCCACGG 120
 21 C S S S S A G H P A M Q P A A A A T A
 CGCGCGCGGG CTACGCCCCC AGGCCACGCC ACATCTCTAC CGAGCCCGAG AAGGAGCGCA 180
 41 A P G Y G P R P R H M L T E A E K E A K
 ACCGCTCCCG GCGATGCTC GCCACCGGAG AATCCCGCGG GAAAACCATC CTCGCGCGCC 240
 61 R L R L R V L A N R E S A R K K T I L R R Q
 AGGCGATCCG AGATGAATGG GAAAGAAAAG TTGCGATGTT GTCTGCGGAC AGACAGACCA 300
A T A A R K V A D S S S H E T I
 81 TGAARAGCA GAGGAGTGT TTAGAGTCA AGTATCTCTC ACTGAAAGCA ACAAACAA 360
K K E K D V I M Q E Y L S I K E T N K Q
 101 AGCTGAAGA ACAGTGTGG ATCAAGTGA TCAAGAAGCC GCGCCGTGGC GAGCCGATGG 420
K E Q V A I R T I K K A A V A E P M D
 121 ACACGGCGCC CGCGCGCGAG CACCAACCGG AGACGCGCGC GCGCCGTGGC CGCCCGACGA 480
T A P P A Q Q O A E T A A A V A P P T T
 141 CGGCGACCCC CGGCTCGAG GCGCCGCGAC CGAGTCTTCT GTACACCGCC GCGCCGCGGG 540
T P S T A P O P S F L Y T A P A G Q
 161 CGTGGCGCC GGTCCGCTAC GTGCGGCGCT GGTCCGCGC GTGCGCGCC ACGSGTACG 600
V A P V Y V W G S L P A C G P T G Y E
 181 AGCCGCGCC GCCGCTCTG CTCCCGGCT CGCGGTGTA CTACCCCGT GTCCCGCATG 660
P P P P L C L L P P C A W Y Y P V V A D P
 201 CGCGCTGTC TTCCGCGCGC ACGTGCGACT ACCCCGATCT GTACCAAGA CAGCCGACGA 720
R V S S P P T S T S Y P O S Y Q E O P T S
 221 GCAGCCCTGG CGGCGGAGG GCCAGCGAG ACACCGACGA CGACCCGTC TGCTCTGAC 780
P G G A F L D F D E H A T S L T L
 241 TGCGCATCGA CTGCAAGAC AGAGCGCGC CTGGCCCGCG CGGACGCGC CGAGCTGGA 840
A I D V D K R S A P G A G G S A P S W T
 261 CAGCAGCCT CCATAGAGG ACAGGAGAAA GCGCAGCGCG GCGCGCGAG CGAGGAAGCG 900
A R L H K A T G R R R R R R R R R R G S Q
 281 GAGGAAGGAG CTACCAAGC TGAAGAGAT GCAAGCGCGC GCGCGCGGCT CGTCCCGCGC 960
G R S
 301 CGGCGCGCGC AGACAGTGG AGCCTGTGAC ATCTCATAC CACAACAGC GAACAAACG 1020
ACAGACAGAC ACGATGTGG ACACAGGTGA GGAAGCAGAT GATGACTTC GATGTGTTT 1080
TGGAGGCAA ACACAGAAA CACCAACTAG ATATATAGT TCTCTGATT TSTTGCATCA 1140
CGTGTACT AGTAAAGT ACTCGACTAT CGATCTTTT AATATAGAT AGCTTAGGTT 1200
TGCATCTG GTCAAAAGT CTTAACTAGT CAAGGA 1236

C

CGCGGAGCAG CCAGATGCCA AGATCTCCCC CGCGGCTACT CCTCCCATGT CGGACAGCA 60
 1 M S D D H
 CAGCGCGCGC GGAGCGGAGC ACCGCAATG CCGCGGAGG TTCGCGTGG ACTGTGGGA 120
 6 S G G G G E Q R Q C R R G F A V D V V E
 GTCGCTGTCG GCGCTGGCTC TGCGCGACAT GCGCGCGCCC GCGCTCAAC CGCCATGCA 180
 26 L G A A L P L A D M A G A V K P A M Q
 CTRGACTG CAGCGGAGC CCCTGATGCA GAGAGCGAG GAGATGCGA CAGAGGCT 240
 L Q L O P T A E F D E H A T S L T L
 46 GAGCCTTCAG CTCGCGAAC ACCTGGGAGC CATCTCCAG TCTCTTCTT GCTCCAGAG 300
 S L Q L G N N V G S I I Q S S S C S S S
 66 CGCTTCCAGC GCGCGCTCC CCGCGCACCC CCGCGCGCGG CCGCGCGCGG CCACCGCTA 360
 G S S A G L P A P P A A A A P A T A Y
 86 CGGACCAAG CCCTCTHCA TCGTACCGA GGAGGAGAAG GAGCGAAGC GCTTCCGCGC 420
 S T R P S L M L T E E K E A K R L R
 106 AGTCTCCG ACCGCGAAT CGGACGCA ACACTACTC CGGCTCAGG CTATTGAGA 480
V L A N R E S A R Q T L R Q T L R Q T L R D
 126 TGAATGCGA AGAAGGTGG CGATCTGTC AACACAGAT GAGAGCATCA AGAGGAGAG 540
E A R K V A D S T Q N E S M K K E R
 146 GGAGCAGTG ATGCAAGAT ACCTCTACT GAGGAGACA AACAAGCAG TCAAGAACA 600
E T V M Q E Y L S I K E T N K Q I K E Q
 166 GCCACAGCAT CATCTTTCG TTTCTGTTT CTAATTAAT TCTTATAGT ATAATCTTCT 660
 186 A Q W H L S L S L F
 TCTTTTGT TCTCTTTC TACAATAA TTATCTCTC TACATCTG AGGCCAAGA 720
 GAGCCCAAGA GAATCTACT TACTCTACT TACATGTC CAAATCCA ACCTCCAGT 780
 ACTTAACTC TAAATCTCT TCTTCTTTA CTGTACTAC AAATTAAGT AAGTACCAT 840
 CGCTTTAAG GCCAAATTA AGAATCTA CTAGCATGTT CTTCCATTG CCAACTACT 900
 GACTTACT CTGCTACTG GCTCATGAG TGCTCAATC AATTTGGCCA TTGCTGTAGC 960
 GCTGTAGTA CTTGCTGGG AITTTGGAAC TGAATAATG TTTATGGAT GCTCTGCGAC 1020
 TTGTGACTG CTGTGTATG GCCAACCTGA CATCTGTAAT GGATATAACC ATTTTCTCA 1080

FIG. 1. Nucleotide sequences of three rice cDNAs. Nucleotide sequences of the *osZIP-1a* (A), *osZIP-2a* (B), and *osZIP-2b* (C) genes are

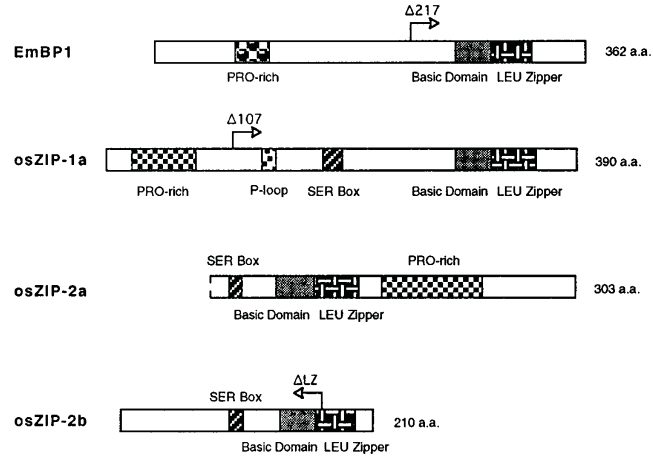


FIG. 2. Schematic representation of the EmBP-1, *osZIP-1a*, *osZIP-2a*, and *osZIP-2b* polypeptides. Boxes represent the positions of regions rich in prolines or serines residues, the P-loop, the basic DNA-binding domain, and the leucine zipper dimerization domain. The arrows represent the extent of the deletion mutants used in the gel shift and resin-binding assays.

used to screen a rice cDNA expression library (30). The sequence of two clones whose products interacted specifically with EmBP-1, pE3-1 and pE4-1, revealed that both were partial cDNAs encoding polypeptides with homology to transcription factors of the bZIP class. The gene encoded by pE4-1 was named *osZIP-1a*, while the one corresponding to pE3-1 was named *osZIP-2a*. Using the 5' region of each cDNAs as hybridization probes, several clones with longer inserts were isolated from the rice cDNA library.

The sequence of the longest *osZIP-1a* clone contained a 1755-bp cDNA with a 1173-bp open reading frame (ORF) that encoded a 41-kDa polypeptide (Fig. 1A). None of the three *osZIP-1a* cDNAs that were analyzed in detail had the same polyadenylation site, resulting in 3'-untranslated regions of 121, 316, or 350 bp. The 5'-untranslated region of *osZIP-1a* contains three small ORFs of 54, 24, and 9 bp. The presence of three in-frame stop codons upstream of a start codon in the proper sequence context for translational initiation (42) indicated that the entire coding sequence was present. The carboxyl-terminal half of the *osZIP-1a* polypeptide contained a basic DNA-binding domain and a leucine zipper dimerization domain (Figs. 1 and 2). The bZIP domain of *osZIP-1a* was similar to the one described for EmBP-1 and other plant GBFs (Fig. 3). Outside of its bZIP domain, *osZIP-1a* shows 46% amino acid homology with the amino-terminal domains of the tomato GBF9 (48) and 41% homology with the NH₂ terminus of the wheat HBP-1a(17) and HBP-1a(c14) factors (43, 49). The amino acid region between 72 and 106 contained 40% proline residues, while the region between amino acids 180 and 195 was very acidic and composed almost exclusively of serine and glutamic acid residues. Similar elements in other transcription factors have been shown to act as transcriptional activators (13, 50, 51). Interestingly, a domain between amino acids 130 and 137 fits the P-loop consensus sequence, (A/G)₄GK(S/T) (where X represents any amino acid), characteristic of ATP/GTP binding domains (52).

shown along with the encoded amino acid sequence. Underlined in the nucleotide sequences are the start and stop codons as well as all identified polyadenylation sites. In the amino acid sequences, the basic DNA-binding domains are boxed, circles denote the leucine repeats in the dimerization domains, while shaded areas represent putative domains such as the P-loop in *osZIP-1a* and a serine box found in all three factors.

		Group
TsEmBP1	DERELKRERRKQSNRESARRSRLRKKQCECELAQKVSELTAAANGTIRSELDTLKKDKCTMETENKRLMGKIL	1
OsZIP-1a	-----q-----a-----raev-kqe-Ts--d-vnrir-eYDeLlLsK-ss-Ke-lE	1
HBP1a(c14)	---v-kqk-----a-W--V-SradI-kqe-ss-ke--K--qEK-DnI ts--ts--he-lK	1
TAF1	n-----k-----a-A--I-r-Qs--e-M--k--ink-MEnsEKlKL--AA--erlK	1
GBF2	n-k-v--k-----a-T-c--sv--da--v-e-Ms--k-g--mnesEKlRL--EAldqIk	1
RITA-1	nPLdv--M--mV-----Kk--ahLAc--eTq-dc--Rge-as-Fkq-tcAnqgFT--AV-d-r-I--Ksdve	2
O2	MPT-ErVRk-Es-----R--ahlKe--dqv-c--la-n-c--L-rIaa-LqkyncANvdn-v--RadmE	2
OHP1	nPVQQRlQ-----S--aahLn--eaq-ac--Rve-ss--LrR-acvngKfNeAavd-rv--KaDvE	2
lip19	GGADERkRk-mL-----A---RL--IaeeaR--Q-e-aRveaqigayAGeLsKvDg--AV--RarHG	?
OsZIP-2a	A-k-A--L--VLa-----k(I)--r-aIRd--r--ac--Ssq-E--Mkk-K-VvMqeYLSlKET--C--KEQvA	?
OsZIP-2b	e-k-A--L--VLa-----qt(I)--r-aIRd--r--ac--Stq-Esmkk-RelvMqeYLSlKET--C--KEQAQ	?
TGA1a	SKPVEKVL--LaQ--a--k----kaYVqc--ensKlK--iqLeqe--EraRK--GMCVGGGvqASQLS YS-TAS	3
HBP-1b(c1)	-kLdH-sL--LaQ--a--k----kaYIqr--eSsRLK--qLeqe--qraRq--GIFIsSSGdQSQsASNGAV	3
OBf3.1	-kLdQ-TL--LaQ--a--k----kaYIqr--eSsRLK--qLeqe--hQtRq--GIFIsTSsGdQPQsTSNGA-	3

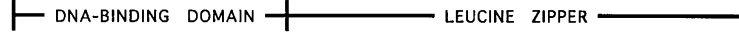


FIG. 3. Amino acid homology between plant bZIP factors. Amino acid homologies between the bZIP domains of EmBP-1, osZIP-1a, osZIP-2a, and osZIP-2b along with the two other rice bZIP factors and representative members of each group of plant bZIP factors (12). A dash corresponds to an amino acid identity to EmBP-1, while conservative substitutions are represented in lowercase. The unusual Arg → Ile mutations in the DNA-binding domains of the osZIP-2 factors are circled. Black boxes show the position of the conserved leucine residues in the leucine zipper domain. References are as follows: EmBP-1 (10), HBP-1a(c14) and HBP-1b(c1) (43), TAF1 (14), GBF2 (44), RITA-1 (17), O2 (16), OHP1 (45), lip19 (26), TGA1a (46), and OBf3.1 (47).

All the clones that were isolated with the osZIP-2a fragment encoded a similar but different gene that was named *osZIP-2b*. In Fig. 1 (B and C), the sequence for both of the *osZIP-2* genes are given. As was the case for *osZIP-1a*, the *osZIP-2b* gene has at least two polyadenylation sites resulting in 3'-untranslated regions of 221 or 445 bp. The largest osZIP-2b cDNA started with a 633-bp ORF encoding a 22.5-kDa protein. This cDNA may not be full-length, since the largest internal ORF started at the second ATG and this start codon was not in a favorable sequence context (42). As illustrated in Fig. 2, the main difference between the two osZIP-2 polypeptides is a large proline-rich region downstream of the osZIP-2a bZIP domain. At the nucleotide sequence level, the two *osZIP-2* genes are 82% identical in the regions encoding a serine-rich box and the bZIP domains (results not shown). In addition, an alignment of the osZIP-2 bZIP domains along with representative members of all groups of plant bZIP factors has been presented in Fig. 3. In particular, an arginine residue in the putative DNA-binding domain, which appears to be conserved in almost all bZIP factors described to date (53), was replaced by an isoleucine (circled in Fig. 3). The leucine zipper domains of osZIP-2a and osZIP-2b also have a few unusual features. In the third and fourth heptad repeats, the leucine residues are replaced by a methionine and valine residues respectively. From such an analysis, it was clear that the osZIP-2 DNA-binding domains and leucine repeats were unique, with none of the characteristic patterns of the other plant bZIP groups.

osZIP Gene Families and Their Expression—Blots of rice genomic DNA were probed at high stringency with the osZIP-1a and osZIP-2b cDNAs. The osZIP-1a probe hybridized to one major band in each lane and a few minor bands (Fig. 4A). The osZIP-2b probe hybridized to 3–5 bands in all lanes even with high stringency washes (Fig. 4A). Preliminary experiments showed that, under these washing conditions, no cross-hybridization between the osZIP-1a and osZIP-2 cDNAs was observed. This result, and our isolation of two homologous genes, demonstrated that the osZIP-2 proteins were encoded by a multigene family that may show substantial homology at the DNA sequence level.

To determine the expression pattern of the new bZIP factors, total RNA was isolated from rice suspension cells incubated in the presence or absence of 100 μ M ABA. The osZIP-1a cDNA hybridized to a 1.8-kb transcript whose abundance was significantly reduced in the presence of ABA. The osZIP-2b probe hybridized to a major transcript of about 900 bases, as well as three minor transcripts of 1.5, 1.8, and 2 kb. The addition of

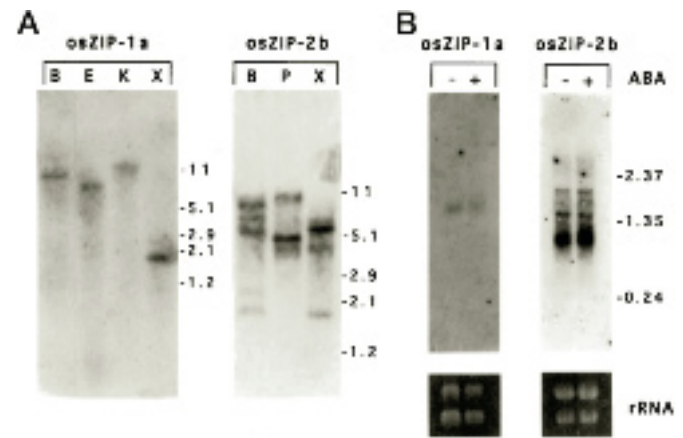


FIG. 4. Analysis and expression of the osZIP-1a and osZIP-2 gene families. A, Southern blot of rice genomic DNA digested with *Bgl*II (B), *Eco*RI (E), *Kpn*I (K), *Pst*I (P), or *Xmn*I (X) and probed at high stringency with the osZIP-1a or osZIP-2b cDNAs. Mobility of molecular weight markers (in kb) is indicated to the right of each blot. B, 10 μ g of total RNA, extracted from rice suspension cells treated with or without ABA, were probed with full-length osZIP-1a or osZIP-2b cDNAs and washed at high stringency. To confirm the accuracy of loading, the gels were stained with ethidium bromide and the rRNAs were photographed prior to blotting. Mobility of an RNA molecular mass ladder (in kb) is also indicated to the right. Both Northern blot filters were exposed for 3 days at -70° C with an intensifying screen.

ABA had no effect on the abundance of any of the osZIP-2 transcripts (Fig. 4B).

DNA-binding Specificity—A truncated osZIP-1a polypeptide containing amino acids 107–390 was purified from *E. coli* as a fusion with MBP (MBP-osZIP-1a Δ 107; see Fig. 2). The electrophoretic mobility shift assay demonstrated that MBP-ZIP1a Δ 107 can efficiently recognize the ABA response element of the Em promoter (Fig. 5A, lanes 1 and 2). In order to determine the relative affinity of this transcription factor for the putative regulatory elements of the Em promoter, we added a 100-fold excess of various unlabeled competitors to the binding reactions (Fig. 5B, lanes 3–8). These competitors (see Fig. 5b) included the Em1a, Em1b, and Em2a elements found in the ABRE, as well as Em1c and Em1d, two other ACGT elements located further upstream in the Em promoter. Hex, a particular type of ACGT element (a G/C-box hybrid), was recognized by plant bZIP factors categorized in Groups 2 or 3 (2). The competition pattern was very similar to the one observed with

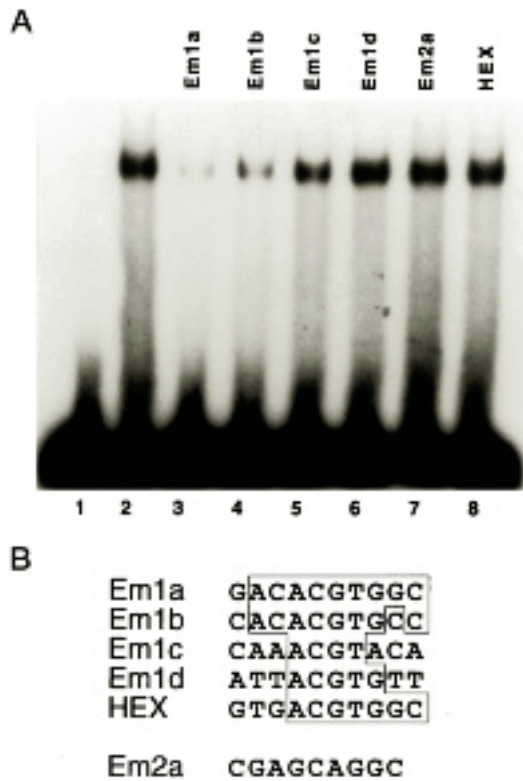


FIG. 5. DNA-binding specificity of osZIP-1a. *A*, gel mobility shift assay of the ABA response element of the wheat Em promoter alone (lane 1) or preincubated with 20 ng of MBP-osZIP-1aΔ107 (lane 2). In lanes 3–8, a 100-fold excess of unlabeled competitors, whose sequence homologies are given in *B*, were added along with the probe.

bacterially purified EmBP-1,² with Em1a being the best competitor followed by Em1b and Em1c. The Em1d and Em2a elements were not recognized by MBP-osZIP1aΔ107. The addition of unlabeled Hex competitor had no effect on the binding activity of MBP-osZIP1aΔ107, demonstrating that this factor had no affinity for this element. Hence, osZIP-1a was classified as a Group 1 bZIP factor as determined by its binding specificity, the sequence of the DNA-binding domain, and the location of the bZIP domain at the carboxyl-terminal end of the protein (see Figs. 2 and 3).

We were unsuccessful in purifying the osZIP-2 polypeptides as MBP fusions. Instead, these were synthesized by *in vitro* translation. In contrast to *in vitro* translated EmBP-1 and osZIP-1a, neither osZIP-2a or osZIP-2b had any affinity for the Em1a element (results not shown and Fig. 8). We also tested the binding of both osZIP-2 factors to a degenerate probe in which an ACGT core is flanked by four random nucleotides and did not observe any additional retarded complexes (results not shown).

Dimerization Specificity—To determine the relative homo- and heterodimerization efficiencies of EmBP-1 and osZIP-1a, equal amount of MBP-EmBP-1 or MBP-ZIP1aΔ107 were applied to nitrocellulose and probed with either biotinylated MBP-EmBP-1 or biotinylated MBP-osZIP-1aΔ107. When EmBP-1 was used as the probe, the strongest signal was detected with osZIP-1a, whereas, if osZIP-1a was used as the probe, EmBP-1 became more efficiently labeled (Fig. 6A). Equivalent binding to the targets was observed with antibodies to MBP.

Preferential heterodimerization was also observed when the proteins were bound to the Em1a element. We produced a

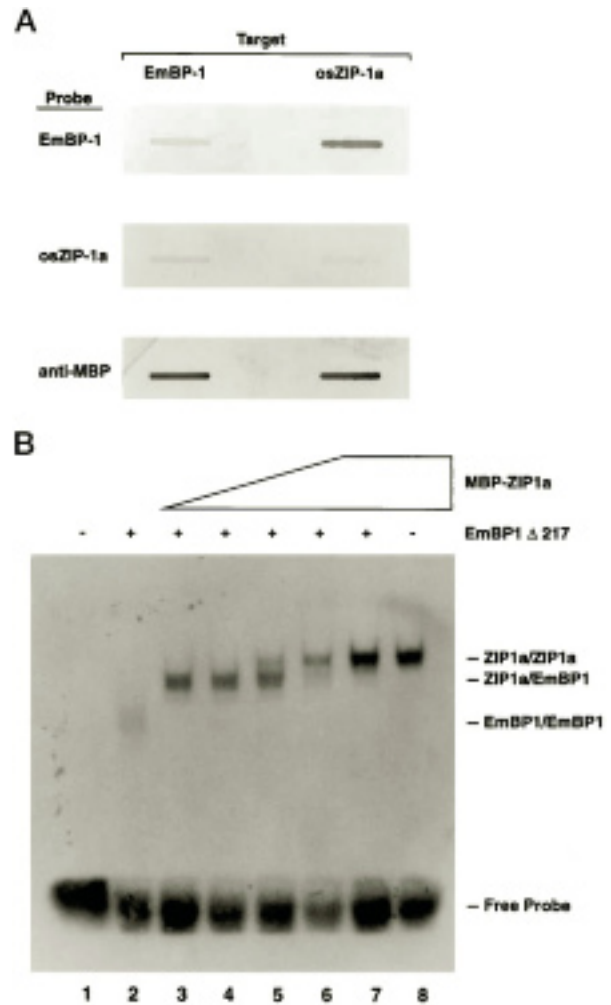


FIG. 6. Preferential heterodimerization of EmBP-1 and osZIP-1a. *A*, slot blots loaded with 30 ng of either MBP-EmBP-1 or MBP-osZIP-1aΔ107 (targets) were probed with biotinylated MBP-EmBP-1, with biotinylated MBP-osZIP-1aΔ107, or with an antiserum to MBP. Filters probed with the anti-MBP were developed in NBT/BCIP for 5 min, while the ones probed with biotinylated proteins were developed overnight. *B*, electrophoretic mobility shift assay of a labeled Em1a probe incubated alone (lane 1) or with 50 ng of Factor Xa-cleaved MBP-EmBP-1Δ217 (lanes 2–7) and 25 ng (lane 3), 50 ng (lane 4), 100 ng (lane 5), 200 ng (lane 6), or 400 ng (lanes 7 and 8) of MBP-osZIP1aΔ107. Markers indicated the mobility of the three possible protein-DNA complexes.

truncated version of EmBP-1 (EmBP-1Δ217, see Fig. 2), whose mobility in a gel shift assay is much faster than the one obtained with MBP-ZIP1aΔ107 (Fig. 6B, lanes 2 and 8). In lanes 3–7, we added increasing concentrations of MBP-ZIP1aΔ107 to a constant amount of EmBP-1Δ217. DNA binding by an EmBP-1/osZIP-1a heterodimer resulted in the formation of a protein-DNA complex of intermediate mobility. When the amounts of each bZIP factor were equivalent (lanes 3 and 4), formation of the intermediate complex was greatly favored compared with the homodimeric complexes.

We used a resin-binding assay to determine if the osZIP-2 factors could dimerize with both of our Group 1 bZIP factors. EmBP-1, osZIP-2a, and osZIP-2b were labeled with [³⁵S]methionine by *in vitro* translation and incubated with amylose resin containing MBP-LacZ, MBP-EmBP-1, or MBP-osZIP-1aΔ107. The resins are then washed several times and bound proteins detected by SDS-polyacrylamide gel electrophoresis, followed by fluorography. This assay allowed us to observe that the labeled EmBP-1 bound to both MBP-EmBP-1 and MBP-osZIP-1aΔ107, while none of the labeled product was retained when

² A. Hill and R. S. Quatrano, unpublished observations.

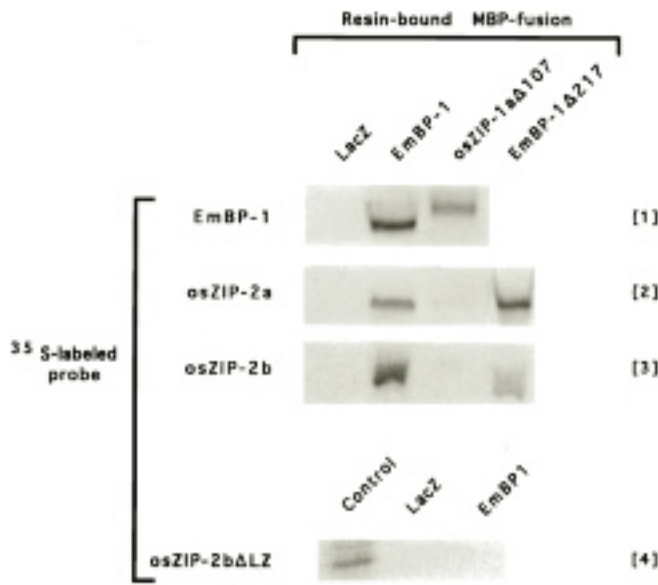


FIG. 7. **Dimerization specificity of the bZIP factors.** Fluorogram of an SDS-polyacrylamide gel showing the binding of *in vitro* translated and ³⁵S-labeled EmBP-1, osZIP-2a, osZIP-2b, and osZIP-2bΔLZ to amylose resin loaded with either MBP-LacZ, MBP-EmBP-1, MBP-osZIP-1aΔ107, or MBP-EmBP-1Δ217. In the osZIP-2bΔLZ control lane, 2 μl of *in vitro* labeled product was loaded directly in the gel.

the resin was loaded with only MBP-LacZ (Fig. 7, [1]). The slight changes in mobility occasionally observed with EmBP-1 and some of the other proteins were caused by comigration of the labeled proteins with a large amount of unlabeled MBP fusions. Although neither of the osZIP-2 factors were retained by MBP-LacZ, both were efficiently bound with the MBP-EmBP-1 resin (Fig. 7, [2] and [3]). Interestingly, neither of the osZIP-2 factors were efficiently retained by MBP-osZIP-1aΔ107 (although a small amount of bound osZIP-2 can be detected if the gel is greatly overexposed).

Our use of a partial osZIP-1a construct might explain the reduced binding by the osZIP-2 factors. Therefore, we repeated the binding reaction using resin loaded with MBP-EmBP-1Δ217, an amino-terminal deletion of EmBP-1 (containing amino acids 217–362) similar to the truncated MBP-osZIP-1aΔ107 (see Fig. 2). Both osZIP-2a and osZIP-2b were efficiently bound by the partial EmBP-1 construct (see Fig. 7, [2] and [3]). Hence, the amino-terminal half of EmBP-1 (and presumably osZIP-1a also) was not involved with its interaction with the osZIP-2 factors. In order to confirm the site of interaction between EmBP-1 and osZIP-2b, we also constructed a carboxyl-terminal deletion of osZIP-2b (osZIP-2bΔLZ, containing amino acids 1–155), which was missing four of the six heptad repeats in its leucine zipper domain (see Fig. 2). This construct showed no capacity for interaction with the MBP-EmBP-1 resin (see Fig. 7, [4]).

The ability of the EmBP-1, osZIP-1a, and osZIP-2b transcription factors to dimerize through their bZIP domains was confirmed *in vivo* using the yeast two-hybrid assay (37). In this system, one protein is fused to the LexA DNA-binding domain, while the other is fused to an acidic transcriptional activator. If the two proteins interact, the complex becomes a functional transcription factor, which is capable of binding to LexA operators and activate transcription of *LEU2* and β -galactosidase reporter genes. As seen in Table I, the bZIP domains of EmBP-1 and osZIP-1a can form homo- and heterodimers *in vivo*. As was seen with the resin-binding assay, we also observed an interaction between the full-length osZIP-2b and the bZIP domain of osZIP-1a but this interaction seemed weaker than the one

TABLE I

Interactions between the bZIP factors in a yeast two-hybrid assay

The full-length osZIP-2b or the bZIP domains of EmBP-1 and osZIP-1a were fused either to LexA (in pEG202) or to an acidic transcriptional activator (in pJG4–5). Yeast EGY48 strain cells, transformed with various combinations of plasmids, were tested for their ability to grow on media lacking leucine: the plus signs represent the appearance of colonies after 24 (++) or 72 (+) h. ND, not determined due to deleterious effect on cell growth.

pEG202	pJG4–5			
	Empty	EmBP-1	ZIP-1a	ZIP-2b
Empty	–	–	–	–
EmBP-1	–	+	+	++
ZIP-1a	–	+	+	+
ZIP-2b	–	ND	ND	–

observed between osZIP-2b and the EmBP-1 bZIP domain. In the latter case, growth on media lacking leucine was much faster than any of the other combinations. This higher affinity was confirmed in a quantitative β -galactosidase assay in which binding of osZIP-2b to the EmBP-1 bZIP domain had a 21-fold higher activity than the osZIP-2b/osZIP-1a interaction (Table II). Strangely, expression of a LexA-osZIP-2b fusion was found to have toxic effect on cell growth when co-expressed with acidic domain fusions of the EmBP-1 and osZIP-1a bZIP domains. Finally, no homodimerization was observed between the full-length osZIP-2b factors (Table I).

The osZIP-2s Can Inhibit the DNA Binding Activity of EmBP-1—Since the osZIP-2s and EmBP-1 can heterodimerize but differ in the sequence of their DNA-binding domains, we determined the effects of heterodimerization on the affinity of EmBP-1 for the Em1a element. The DNA binding activity of *in vitro* translated EmBP-1 was greatly reduced following preincubation with an equal amount of *in vitro* translated osZIP-2b (Fig. 8, lanes 3 and 4). The same results were obtained when osZIP-2a was substituted for osZIP-2b (results not shown). One possible mechanism for this DNA binding inhibition is the formation of inactive heterodimers between EmBP-1 and the osZIP-2 factors. We preincubated EmBP-1 with the osZIP-2bΔLZ protein (lacking most of its leucine zipper domain, thus preventing heterodimerization; see Figs. 2 and 7) and noted that the addition of this partial protein had no effect on the DNA binding activity of EmBP-1 (see Fig. 8, lane 6).

We also knew from the resin-binding and two-hybrid assays that the osZIP-2 factors dimerized less efficiently with osZIP-1a. Probably because of this inefficient heterodimerization, the DNA binding activity of *in vitro* translated osZIP-1aΔ107 was not affected by the addition of either osZIP-2a or osZIP-2b (Fig. 8, lanes 6–8). Unlike MBP-osZIP-1aΔ107, *in vitro* translated osZIP-1aΔ107 produced two complexes in the gel shift assay. These might result from binding by partial proteins produced through degradation or incomplete translation as had been observed before with other plant bZIP factors (54).

Overexpression of EmBP-1 and osZIP-1a in Rice Protoplasts—Finally, we were interested in determining if either EmBP-1 or osZIP-1a have a role to play in the induction of the *Em* gene promoter by ABA. Genes for both of these bZIP factors were fused to the constitutive CaMV 35S-promoter and introduced into rice protoplasts by polyethylene glycol-mediated transfection. We measured the effects of EmBP-1 and osZIP-1a overexpression on the activity of two promoters that were fused to a GUS reporter gene: the wheat *Em* gene promoter (9) and the rice promoter of the Act-1D actin gene (40). As can be seen in Fig. 9A, overexpression of either bZIP factors had no effect on the activity of the *Em* promoter in the absence of ABA. When protoplasts were incubated with exogenous ABA, the *Em* promoter was activated 25-fold, but its expression was not affected by overexpression of EmBP-1 ($p > 0.4$). However,

TABLE II
Interaction of *osZIP-2b* with the other bZIP factors

Table gives results of β -galactosidase assays on some of the two-hybrid plasmid combinations described in Table I. Units are in A_{420} of *o*-nitrophenol/(A_{600} of culture \times ml of culture \times min) \pm standard deviation ($n = 2$).

pEG202	pJG4-5	
	Empty	ZIP-2b
Empty	2.6 \pm 1.8	4.6 \pm 3.6
EmBP-1	3.8 \pm 1.2	2083 \pm 673
ZIP-1a	5.4 \pm 2.0	99 \pm 13
ZIP-2b	7.7 \pm 4.6	11 \pm 10

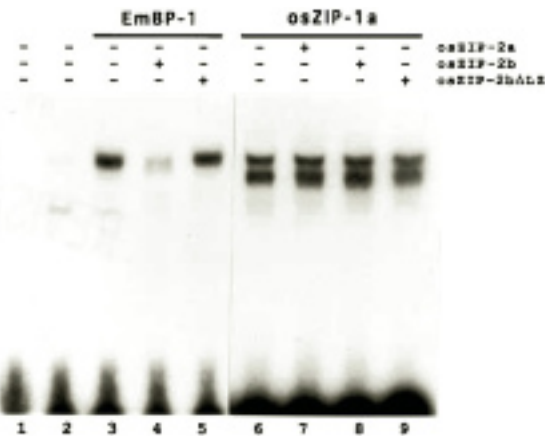


FIG. 8. Inhibition of EmBP-1 DNA binding activity by the *osZIP-2* factors. Electrophoretic mobility shift assay of an Em1a probe incubated alone (lane 1) with 4 μ l of reticulocyte lysate (lane 2), with 2 μ l of *in vitro* translated EmBP-1 (lanes 3–5) or with *in vitro* translated *osZIP-1a* Δ 107 (labeled *osZIP-1a*; lanes 6–9). The samples were also preincubated with 2 μ l of unprogrammed lysate (lanes 2, 3, and 6) or lysate programmed with *osZIP-2a* (lane 7), *osZIP-2b* (lanes 4 and 8), or *osZIP-2b* Δ LZ (lanes 5 and 9) mRNA.

overexpression of *osZIP-1a* leads to an additional 2-fold activation of Em promoter activity. Although Fig. 9 only represents a single representative series of experiments, a variance analysis (Table III) from 20 independent transfection experiments using five different protoplast preparations and three different plasmid stocks confirms that the observed increase in Em-GUS activity, following *osZIP-1a* overexpression, is statistically significant ($p < 10^{-10}$) and was consistently observed in all of our active protoplast preparations ($p < 10^{-9}$). The actual levels of *osZIP-1a* transactivation show a slightly higher variability (between 1.7- and 2.3-fold), but these are still statistically significant ($p < 0.02$) and most probably result from the use of different plasmid preparations. The transactivation by *osZIP-1a* was specific to the Em promoter, since the activity of the actin promoter remains constant both in the absence or the presence of ABA, and whether EmBP-1 or *osZIP-1a* were overexpressed (Fig. 9B). These results are consistent with the hypothesis that *osZIP-1a*, but not EmBP-1, can bind and activate the Em promoter. Finally, several experiments failed to observe any effects from *osZIP-2a* or *osZIP-2b* overexpression on the activity of the Em promoter (results not shown).

DISCUSSION

In this paper, we describe the characterization of three rice bZIP factors, *osZIP-1a*, *osZIP-2a*, and *osZIP-2b*. *osZIP-1a* and *osZIP-2a* were isolated through an interactive screen using biotinylated EmBP-1 (30), while *osZIP-2b* was isolated by DNA hybridization using *osZIP-2a* as a probe. The structure and DNA-binding specificity of *osZIP-1a* were very similar to EmBP-1, making *osZIP-1a* the first Group 1 GBF isolated from rice. The amino-terminal domain of *osZIP-1a* contained a P-

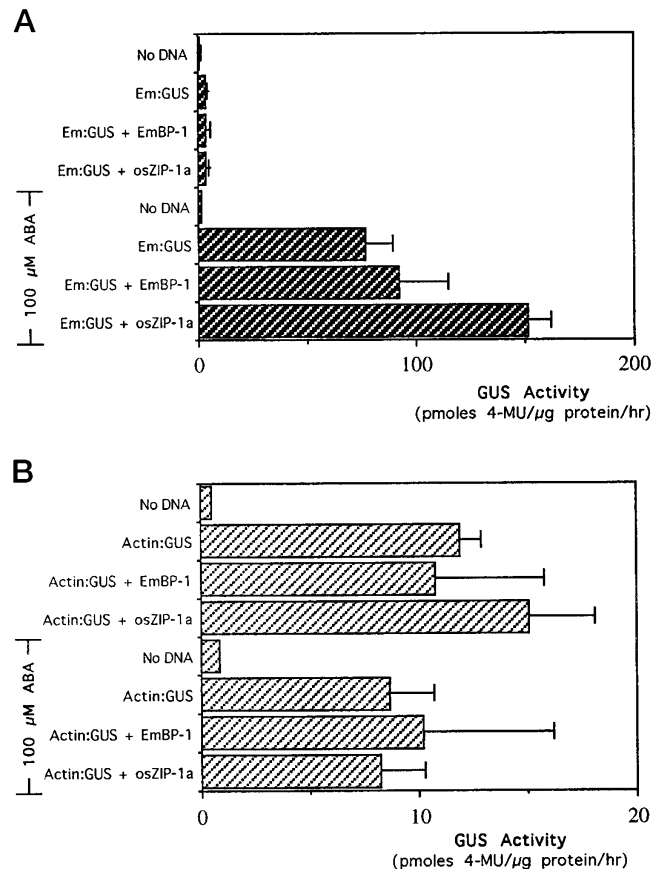


FIG. 9. ABA-dependent transactivation of the Em promoter by *osZIP-1a* in rice protoplasts. Effects of the overexpression in rice protoplasts of the EmBP-1 or *osZIP-1a* bZIP factors on the activity of the wheat Em (A) or the rice Act-1D (B) promoters as measured by the resulting GUS activity. After transfection, cells were incubated overnight in the absence or the presence of 100 μ M ABA. Each column represents the average of four for Em (A) or three for actin (B) independent transfections with 5 μ g of effector and 2 μ g of reporter plasmids. The standard deviation of each group of samples is represented as error bars.

loop, which is a putative ATP/GTP-binding domain. Of all published plant bZIP sequences, only HBP-1a(17) from wheat shares this consensus site (55). P-loops have been found in kinases and G-proteins, but these proteins often contain other consensus sequences that are not found in *osZIP-1a*. If the P-loop is functional in *osZIP-1a*, it may play a key role in some signaling cascade. Additionally, the amino-terminal domain of *osZIP-1a* contained all of the peptide motifs observed in the Class B GBFs (48), making it a probable homolog of the tomato GBF9, wheat HBP-1a(17), and HBP-1a(14) bZIP factors (43, 48, 49).

It appeared from Southern analysis that *osZIP-1a* was a single-copy gene. However, other GBF genes may be present in the rice genome but are not detected, since DNA sequence homology among members of the GBF family is usually very low outside the DNA-binding domain. *osZIP-1a* appeared to be a full-length clone based on sequence characteristics and because it hybridized to a single 1.8-kb transcript, the same size as the largest *osZIP-1a* cDNA isolated. Reduced abundance of the *osZIP-1a* transcript in the presence of ABA might be part of an attenuation mechanism, although there are no reported studies on the attenuation of the ABA response. Additionally, the presence of three small ORFs in the 5'-untranslated region of the *osZIP-1a* gene could play a regulatory role by effecting translation efficiency, as has been suggested in the regulation of translation of the maize *Opaque-2* mRNA (56). We have also

TABLE III
Statistical analysis of the effect of *osZIP-1a* overexpression

Activity of the ABA-induced *Em-GUS* reporter gene following independent transfections ($n = 4$) of five different protoplast preparations with either an empty pDH51 vector or one that contains an *osZIP-1a* cDNA. These results were then used in an analysis of variance to confirm the statistical significance of the *Em-GUS* activation by overexpressed *osZIP-1a*.

Protoplast stock	GUS activity (transfected gene)		Average activation
	None	<i>osZIP-1a</i>	
	<i>pmol 4-MU / μg protein / h</i>		<i>-fold</i>
12/9	201.78	512.94	1.9
	190.11	290.99	
	220.65	430.98	
	209.75	358.10	
1/19	109.12	391.17	2.3
	77.46	345.89	
	210.13	375.26	
	235.75	338.65	
1/27	81.51	141.23	2.3
	45.71	182.37	
	98.44	204.31	
	78.50	188.09	
2/1	109.22	200.59	1.7
	121.01	224.10	
	151.44	232.07	
	154.08	271.28	
2/8	94.57	148.63	2.0
	63.39	152.17	
	71.66	164.62	
	76.87	140.51	

Analysis of variance ($\alpha = 0.05$): two-factor with replication			
Source of variation	df	F	p value
Protoplast stock	4	28.9	6.4^{-10}
Transfected gene	1	97.40	6.2^{-11}
Interaction	4	3.8	0.0127

demonstrated that EmBP-1 and *osZIP-1a* will preferentially heterodimerize *in vitro* whether bound to the Em1a element or not. Preferential heterodimerization had also been reported between the maize Opaque-2 and OHP-1 factors. In this last case, a gel shift assay in which dimer exchange was allowed to reach equilibrium resulted in only the intermediate mobility shift (45). Whether this preferential heterodimerization has any role to play in the *in vivo* regulation of the *Em* gene promoter remains to be determined.

The *osZIP-2a* and *osZIP-2b* genes appear to be members of a novel multigene family. The *osZIP-2b* cDNA hybridized to multiple bands in a genomic Southern, as well as to several transcripts of different size whose abundance were not affected by ABA. The size of the most abundant transcript was similar to our two largest *osZIP-2b* cDNAs, indicating that these might be close to full-length. The additional transcripts may be encoded by different *osZIP-2* genes, including the one that codes for *osZIP-2a*, or they may arise through alternative splicing. The expression levels of the *osZIP-2* genes was particularly high, especially when compared with the levels of *osZIP-1a* transcripts.

An analysis of the amino acid sequence of the *osZIP-2* basic domains also revealed an interesting feature. The DNA-binding domains of most bZIP factors isolated from plants, mammals, or yeast share the consensus sequence $NX_2AAX_2(C/S)R$, where X is any amino acid (53). This was especially true for the asparagine and arginine residues, which appeared to be conserved in all bZIP factors with two exceptions, the yeast Met4 protein (which has a unique DNA-binding domain; Ref. 57), and the *Arabidopsis* posF21 factor (which carries a conservative Arg \rightarrow Lys substitution; Ref. 24). We described another exception to this consensus, since in both of the *osZIP-2*

polypeptides this conserved arginine was replaced by an hydrophobic isoleucine residue (see Fig. 3). Would the unusual Arg \rightarrow Ile mutation in the DNA-binding domain of the *osZIP-2* factors be expected to affect their DNA-binding specificity? Studies of x-ray structure of the GCN4-DNA complex have shown that these amino acids play a major role in DNA-binding specificity (58). Furthermore, one of the maize *opaque-2* mutants has been shown to carry a conservative Arg \rightarrow Lys mutation in its bZIP domain, and even this conservative substitution was sufficient to inhibit its binding to the 22-kDa Zein ACGT element (59). Finally, it was demonstrated that an Arg \rightarrow Ile mutation in the basic domain of the yeast bZIP factor GCN4 completely eliminated its affinity for the AP1 site (60). These results suggested that these novel factors either have a DNA-binding specificity that may be different from that of any other plant bZIP factor, or that they do not bind DNA. To date, we have been unable to observe any DNA binding by the *osZIP-2* factors, although this may be explained by our two-hybrid data, which failed to detect homodimerization of *osZIP-2b*.

Insights into the function of the *osZIP-2* factors came from the study of their dimerization specificity. Both *osZIP-2a* and *osZIP-2b* will heterodimerize very efficiently with EmBP-1, and this interaction is mediated through their respective leucine zipper domains. Additionally, both *osZIP-2* factors prevented EmBP-1 from binding to the Em1a element of the *Em* promoter. Even though it belongs to the same bZIP group as EmBP-1, the *osZIP-1a* factor dimerizes much less efficiently with *osZIP-2a* or *osZIP-2b*. In addition, its DNA binding activity was unaffected by the presence of either the *osZIP-2* factors. This result, and the fact that the dimerization-deficient *osZIP-2b* Δ LZ mutant did not inhibit EmBP-1 DNA binding activity, suggests that the inhibition of the DNA binding activity of EmBP-1 results from the formation of inactive EmBP-1/*osZIP-2* heterodimers. Since *osZIP-1a* did not heterodimerize well with the *osZIP-2* factors, its DNA binding activity consequently remained unaffected. Because the *osZIP-2* factors discriminated between *osZIP-1a* and EmBP-1, we hypothesize that one of their functions may involve the selective inactivation of specific Group 1 bZIP factors. Although this is the first described case of a DNA-binding inhibitor in plants, there exist numerous examples in which this mechanism of gene regulation appears to be occurring in other eukaryotes. Specific inhibitors have been identified that act on such well known transcription factors as MyoD (61), POU (62), C/EBP (63), NF κ B (64, 65), c-Jun (66), and Sp1 (67). It is also possible that the *osZIP-2* proteins might be transcription factors in their own right and what we observed was an example of cross-family dimerization similar to the reciprocal inhibition observed between c-Jun and MyoD (68). In any case, the proposed functions of this novel new class of bZIP proteins, as demonstrated by these *in vitro* experiments, can be tested *in vivo* by using plant protoplasts.

We used a transient expression assay in rice protoplast to determine if overexpression of any of the *osZIP* factors influenced the ABA induction of the *Em* gene promoter. We observed that *osZIP-1a* overexpression transactivated the *Em* promoter, but only in the presence of exogenous ABA (see Fig. 9). The lack of an effect in the absence of ABA raises some very interesting hypotheses to test. For example, could the ABA signal activate a specific reaction that might alter the properties of the overexpressed *osZIP-1a* (e.g. phosphorylation) that would make it more efficient to interact with the transcriptional complex on the *Em* promoter? Additionally, an ABA-inducible nuclear localization of the *osZIP-1a* could also explain these results. A similar mechanism has already been described, in which light was shown to induce nuclear localization of GBF

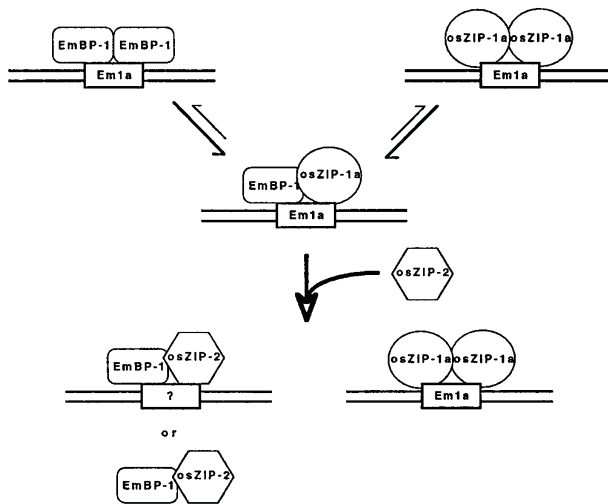


FIG. 10. **A model for osZIP-2 function.** *In vitro* results demonstrate that the Em1a element can potentially be recognized by homodimers or heterodimers made up from EmBP-1 or osZIP-1a subunits. In the presence of the osZIP-2 factors, we propose that EmBP-1 becomes unable to recognize the Em1a element leaving only osZIP-1a to interact with this important element in *Em* gene regulation.

factors in parsley protoplasts (69). Although the 2-fold transactivation levels are lower than the 4–20-fold levels reported with other plant bZIP factors (14, 17, 70), these reduced levels were somewhat expected. Appreciable amounts of osZIP-1a mRNA are already present in the rice protoplasts, and the reporter gene was fused to the full-length *Em* promoter instead of an artificial construct composed of multiple binding sites.

Even though it can efficiently heterodimerize with osZIP-1a, overexpression of EmBP-1 did not have any significant effects on the activity of the *Em* promoter. Although this result was consistent with the possibility that all of the overexpressed EmBP-1 was inactivated by heterodimerization with the large amount of endogenous osZIP-2 factors, other explanations are also possible. For example, the rice cell line might already contain more of its EmBP-1 homolog than is necessary for full activation of the *Em* promoter. Another possibility could be that a species-specific rice co-activator does not recognize the wheat EmBP-1 transcriptional activator domain, although if this were the case, we might expect EmBP-1 overexpression to actually repress *Em* promoter activation through the formation of less active heterodimers with the endogenous osZIP-1a. Finally, we were unable to see any effect from overexpression of either of the osZIP-2 factors.

All of these results are consistent with a model, illustrated in Fig. 10, by which regulation of the *Em* promoter is effected by a particular subgroup of GBFs, such as osZIP-1a, that are not inhibited by the osZIP-2 proteins. If rice cells contain one or more homologs of the wheat EmBP-1 factor, we can assume that the Em1a element could theoretically be occupied by a variety of Group 1 GBF subunits and that one role of the osZIP-2 factors would be to determine which GBF homo- or heterodimers are available to effect *Em* gene expression. Control of repression by the osZIP-2 factors could thus be an additional site of regulation. Also supportive of this model was the observation that osZIP-1a enhanced *Em*-GUS expression, as well as the absence of repressive effects on *Em*-GUS activity from EmBP-1 and osZIP-2 overexpression.

In conclusion, our identification of a novel family of plant bZIP factors, whose function may be to inactivate specific members of the GBF family, allowed the elaboration of a model by which the amount and activity of the osZIP-2 factors can be

used to modulate which GBF factor will be available for interaction with the G-boxes in the promoters of inducible genes.

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