An Interaction between a MYC Protein and an EREBP Protein Is Involved in Transcriptional Regulation of the Rice Wx Gene*

Ying Zhu, Xiu-Ling Cai, Zong-Yang Wang, and Meng-Min Hong‡

From the National Key Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, People’s Republic of China

We previously demonstrated that a 31-bp nucleotide sequence located upstream of the rice Wx gene played an important role in its expression. We further showed that this cis-acting regulator interacts with nuclear proteins extracted from developing rice endosperm. We used the 31-bp sequence as bait in a yeast one-hybrid system to isolate several cDNA clones from a rice cDNA expression library. One of these cDNAs encodes a MYC protein, designated OsBP-5, which is 335 amino acids long and contains a putative basic helix-loop-helix-ZIP DNA-binding domain. This domain exhibits 50% amino acid sequence identity with the R/B proteins that regulate the expression of genes involved in anthocyanin biosynthesis in plants. The results of electrophoretic mobility shift assays (EMSAs) and Western blots indicate that this protein binds specifically to the CAACGTG motif within the 31-bp sequence. However, by itself, the OsBP-5 protein is unable to trans-activate a lacZ reporter gene controlled by the 31-bp sequence when tested in a yeast expression system. Interestingly, OsBP-5 can trans-activate this reporter gene when another protein, OsEBP-89, a member of the EREBP family of transcription factors, is present. Furthermore, in vitro pull-down experiments show that a protein isolated from developing rice endosperm interacts with the OsBP-5 protein, and Western blots confirm that the interacting protein is OsEBP-89. The formation of a super-shift band in EMSAs also indicates that two proteins interact with each other. Interference of OsBP-5 gene expression by double-stranded RNA reduces the amylose content in mature seed of transgenic rice plants but has no visible effect on their phenotype. These results suggest that the OsBP-5 and OsEBP-89 proteins act synergistically, perhaps as a heterodimer, to regulate the transcription of the rice Wx gene.

Appropriately regulated gene expression is a fundamental aspect of most biological phenomena, including development, differentiation, and an organism’s ability to respond to environmental signals, and some of the most important regulatory mechanisms act at the level of transcription. Indeed, a large number of proteins have been shown to function as trans-acting activators of transcription. These transcription factors bind specifically to cis-acting sequences of the genes that they regulate, and in many cases these DNA-protein interactions are required for the genes to be transcribed. In some instances, DNA-binding proteins have been shown to stimulate RNA polymerase II-catalyzed transcription in vitro. In other cases, heterodimers or larger complexes formed by two or more different proteins must bind to cis-acting elements before a gene can be transcribed. In fact, heterodimers are known to play important roles in regulating the temporal and spatial expression of many genes and in controlling the development of tissues and organs of both animals and plants (1–4).

The DEFICIENS (DEF) and GLOBOSA (GLO) proteins of Antirrhinum are transcription factors that contain a MADS-box, a DNA-binding domain, and a K-box. The results of detailed functional analyses of the floral homeotic gene DEF demonstrated that combinatorial regulatory interactions occur between the DEF and GLO proteins (2). In rice, the OsMADS4 protein, a member of the phosphatidylinositol family, is involved in controlling the development of the second and third flower whorls. Another rice protein, OsMADS16, contains a phosphatidylinositol-derived motif and belongs to the AP3 family. The results of studies on the interactions between OsMADS4 and OsMADS16 and flower development in rice indicate that OsMADS4 and OsMADS16 form heterodimers that play important roles in specifying sepal and petal identities (3). In barley, HvGAMYB is a MYB transcription factor synthesized in barley aleurone cells in response to gibberelin during germination. HvGAMYB has been shown to interact with PB-binding factor BPBF to control the expression of the Hor2 gene during endosperm development (4).

Anthocyanins are a class of secondary metabolites that are widespread in the plant kingdom, and protein heterodimers play an important role in regulating the transcription of genes encoding enzymes involved in anthocyanin biosynthesis. The transcription of these structural genes is coordinately regulated by at least two classes of regulatory genes. One class, the R/B gene family, encodes proteins with a basic helix-loop-helix (bHLH)1 region, a domain present in MYC proteins. The other class is the C1/pl gene family. C1/pl genes encode proteins that have homology to the DNA-binding domains of MYB proteins. The members of these two sets of transcription factors exhibit tissue-specific patterns of synthesis. The observed spatial and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AJ487830. To whom correspondence should be addressed. Tel.: 86-21-64042409/4429; Fax: 86-21-64042385; e-mail: Hongmn@sip.simm.ac.cn.

1 The abbreviation used are: bHLH, basic helix-loop-helix; EMSA, electrophoresis mobility shift assays; dsRNA, double-stranded RNA interference; Ni-NTA, nickel-nitrilotriacetic acid; PVDF, polyvinylidene difluoride; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; IPTG, isopropyl-β-D-thiogalactoside; ADH, alcohol dehydrogenase.
temporal distribution of anthocyanins in maize tissues is caused by the differential expression of the regulatory genes. Interestingly, activation of the genes encoding anthocyanin biosynthetic enzymes requires both an R/B protein and a C1/pl protein. Neither protein can induce the transcription of these genes by itself (5). Genetic studies have demonstrated that the combination of R and C1 is responsible for pigmentation in kernels, whereas the combination of B and pl is responsible for pigmentation in the tissues of mature plants (6). The importance of this type of combinatorial control has also been documented in studies carried out with transgenic tobacco and Arabidopsis plants (7).

Starch is the major constituent and energy reserve in many important foods, including the seeds of cereal grains and the potato tuber. Starch consists of two types of polysaccharides: amylose and amylopectin. Amylose makes up ~0–30% of the starch in the cereal grains. The amylose content varies widely in different plant species and even in different cultivars of the same species. Studies on starch mutants of maize, rice, and barley have shown that the enzyme granule-bound starch synthase, encoded by the waxy (Wx) gene, catalyzes the synthesis of amylose. In these studies, Wx transcripts could be detected only in the endosperm, pollen, and embryo sac, indicating that the expression of the Wx gene is probably regulated at the level of transcription.

We previously cloned and sequenced the Wx gene of rice (8, 9) and demonstrated that the sequences within the first intron of the gene enhance its expression (10). Additional studies revealed that post-transcriptional events play important roles in regulating the concentrations of Wx protein and the amylose in rice seeds (11). We also demonstrated that a sequence (~860 to ~640 upstream (5') from the rice Wx gene is a positive regulator of its expression (12). The results of electrophoretic mobility shift assays (EMSAs) showed that a 31-bp nucleotide sequence (~840 to ~810) located within the ~860 to ~640 region is the binding site for nuclear proteins isolated from developing rice seeds (13). Furthermore, experiments performed on transgenic rice plants containing a GUS reporter gene controlled by the Wx promoter with the 31-bp sequence present synthesized 2–3 times the amount of GUS as plants carrying the reporter gene and a Wx promoter with the 31-bp sequence deleted (14). These results all indicate that the 31-bp sequence plays an important role in the transcriptional regulation of the rice Wx gene.

More recently, we have used a yeast one-hybrid system to identify cis- and trans-acting factors that regulate Wx gene expression. We constructed a one-hybrid system in which rice cDNAs were fused to the GAL4 activating domain (in plasmid pPC86), and the 31-bp Wx promoter sequence was inserted upstream of the lacZ reporter gene (in plasmid p178). After several rounds of selection, three cDNAs (C5, C89, C73) were isolated from a rice cDNA expression library (15). In this paper, we present results showing that cDNA C5 encodes a MYC protein (OsBP-5) that interacts with an EREBP protein (OsEBP-89) to regulate Wx transcription. The heterodimers formed by these two proteins efficiently up-regulate the expression of a lacZ reporter gene in yeast cells transformed with plasmid p178–6×31, which carries a lacZ reporter gene controlled by a mini-promoter and six tandem copies of the Wx 31-bp promoter sequence. In addition, we used double-stranded RNA interference (dsRNA) to decrease OsBP-5 gene expression and observed a correlation between the decreased levels of OsBP-5 mRNA and amylose content in seeds of the transgenic plants carrying the dsRNA construct.

## Materials and Methods

### RNA Gel-blot Analyses

Total RNAs were isolated, and Northern blots were performed as described previously (16). RNA samples (100 μg) from endosperms, roots and leaves of Oryza sativa (ZhongHua 11 cultivar) were separated on 1% agarose gels containing formaldehyde (final concentration: 2.2 M), transferred to nylon membranes (17), and hybridized to 32P-labeled cDNA C5. The cDNA was labeled by using [32P]dATP as a substrate and a random primer DNA labeling kit (TaKaRa).

### Protein Synthesis in Escherichia coli—A 1430-bp SalI-NotI restriction fragment containing the C5 cDNA was inserted into vector pET28C (+) (Novagen) in-frame to construct plasmid pET5, and pET5 was expressed in E. coli strain BL21 (DE3). The resulting His-tagged fusion protein (p5H) was extracted and purified by using a Ni-NTA resin and the protocol provided by the supplier (Qiagen).

### Electrophoretic Mobility Shift Assays and Competition Experiments

- **Both strands of the 31-bp nucleotide sequence (GCAACGTGCTAACGTA and CGCACGCTAACGTGA) and two mutant sequences (L strand, GCAACGTGCCAACGTA and R strand, CGCACGCTAACGTGA) were synthesized, annealed, and inserted into vector pUC18. A plasmid (designated pUC31bp) that contained two tandem copies of the 31-bp nucleotide sequence was used in EMSAs. The DNA fragment containing the 31-bp sequence was digested with EcoRI and BamHI and labeled by using the Klenow fragment of DNA polymerase I to catalyze nick-translation in the presence of [32P]dATP. Both strands of the 31-bp sequence (L strand, GCAACGTGCCAACGTA and R strand, CGCACGCTAACGTGA) and two mutant sequences of the left strand (LM1, GCAACGTGCCAACGTA and LM2, GCAACGTGCCAACGTA) were synthesized, annealed, and labeled using T4 polynucleotide kinase in the presence of [32P]ATP. EMSAs were performed as follows: 10 fmol of labeled probe was mixed with 0.2 μg of Ni-NTA-purified fusion protein, 4 μg of calf thymus DNA, with or without competitor DNA, in a DNA-binding buffer containing 10 mM TrisCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.05% Nonidet P-40. After incubating the DNA-binding mixtures at 25°C for 20 min, they were subjected to electrophoresis on 4% acrylamide gels containing TBE (22.5 mM Tris borate, pH 8.0, 0.5 M EDTA). The gels were stained with ethidium bromide and exposed to x-ray film.

### In Vitro Pull-down Assays

- A yeast one-hybrid system was constructed from the DupLEX-A two-hybrid system (Origene). Recombinant plasmids used in the trans-activation experiments were constructed by inserting cDNAs C5 and C89LH (a full-length cDNA produced from the transcript of the OsEBP-89 gene) into plasmid pEG202 (His3, 2 μm, Ap′, ADH constitutive promoter, LexA DNA-binding domain, and SV40 nuclear localization signal). The resulting plasmids or plasmid pEG202 were individually introduced into cells of yeast strain YEG48 (MATa trp1 his3 ura3 leu2 2:6LexAop LEU2) harboring the reporter plasmid pSH18–34 (URA3, 2 μm, Ap′, LexAop–lacZ). The resulting yeast transformants were grown on SD medium (35) in the presence of tryptophan and uracil, but no histidine or uracil, for 2–3 days at 30°C. The SD medium was then overlaid with a nitrocellulose membrane and incubated on X-gal plates for 12–24 h at 30°C.

### In Vitro Pull-down Assays—Nuclear proteins were isolated from developing endosperm cells by the protocol of Takawa (19), and the P5H fusion protein was synthesized in E. coli strain BL21 (DE3) cells. Total proteins isolated from E. coli BL21/pET5 cells were dissolved in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 10 mM imidazole) and passed through two Ni-NTA-resin columns. One column was washed three times with buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 20 mM imidazole), and the resin-bound proteins were removed with an elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). The nuclear proteins isolated from endosperm cells and P5H fusion protein were then passed through a second Ni-NTA-resin column, washed, and eluted as described above. As a control assay, the same nuclear extracts from developing endosperm cells were added to the third column, washed, and eluted as described above.

The eluted proteins were separated by SDS-PAGE and transferred to PVDF membranes. Western blots were performed as described by Sam-
Transcriptional Regulation of Rice Wx Gene by MYC and EREBP proteins

brook et al. (17) by using a 1:100 dilution of the anti-OsERF-89 rabbit antisera and a 1:3000 dilution of an alkaline phosphatase conjugate of the secondary antibody (Sigma anti-rabbit IgG). Detection was performed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma) as substrate. The protein gels were stained with Coomassie Brilliant Blue as described by Sambrook et al. (17).

**Yeast Two-hybrid System I**—A DupLEX-A yeast two-hybrid system (Origene) was used in the initial experiments. The C5 cDNA was inserted into the BamHI and NotI sites of plasmid pEG202 to produce bait plasmid pEGC5, and cDNAs C89LH, C73, myb12, myb13, and myb14, and REB were each inserted into the EcoRI and XhoI sites of plasmid pJG4–5 (TRPI, 2 μm, Ap’, mini-promoter-lacZ) to form reporter plasmid pJG4–5. The expression of the LacZ reporter gene in plasmid pJG4–5 should, therefore, be driven by the tandemly repeated 31-bp cis-element. The C5 cDNA was then inserted into plasmid pPC86Gal4AD (TRPI, 2 μm, Ap’, GAL4 activation domain) to form plasmid pJC86Gal4AD. The 31-bp sequence of plasmid pPC86Gal4AD was retained in plasmid pEG202 to form the derivative pEG89SX. The 31-bp sequence of plasmid pJG4–5 was co-transformed into yeast cells with either pC5Gal4AD, pEG89SX, or both co-transformants were selected on medium lacking histidine, uracil, and tryptophan, for 2–3 days at 30 °C. The resulting colonies were then overlaid with a nitrocellulose membrane and incubated on X-gal plates for 12 to 24 h at 30 °C.

**Yeast Two-hybrid System II**—A DNA fragment containing six copies of one of these cDNAs (C5) and the protein that it encodes. The C5 cDNA was inserted into the BamHI and NotI sites of plasmid pEG202 to produce bait plasmid pEGC5, and cDNAs C89LH, C73, myb12, myb13, and myb14, and REB were each inserted into the EcoRI and XhoI sites of plasmid pJG4–5 (TRPI, 2 μm, Ap’, inducible Gal4 promoter, B42 activation domain) to produce prey plasmids pJG89LH, pJGC73, pJGmyb12, pJGmyb13, pJGmyb14, and pJGmyb14, respectively. Each of these prey plasmids was transformed into yeast cells harboring both the bait plasmid pEGC5 and the reporter plasmid pSH18–34. The resulting yeast transformants were grown on SD medium containing leucine, but lacking histidine, uracil, and tryptophan, for 2–3 days at 30 °C. The SD medium was then overlaid with a nitrocellulose membrane and incubated on X-gal plates for 12–24 h at 30 °C.

**Transformation of Rice**—Agrobacterium tumefaciens-mediated transformation of rice was performed as previously described (21, 22).

**Results**

The OsBP-5 Gene of Rice Encodes a New MYC Protein Containing a bHLH DNA-Binding Domain—Our previous data demonstrated that a 31-bp nucleotide sequence in the upstream region of the rice Wx gene interacted with nuclear proteins extracted from developing rice endosperm (13). Three cDNA clones (pC5, pC73, pC89) were isolated from a rice cDNA expression library by using the 31-bp nucleotide sequence as bait in a yeast one-hybrid screening protocol (15). In this paper, we present the results of studies on the structure and function of one of these cDNAs (C5) and the protein that it encodes. The C5 cDNA contains 1430 bp and encodes a protein 335 amino acids long. The predicted product of this gene contains a typical bHLH-ZIP domain, which exhibits about 50% amino acid sequence identity and 70% similarity with the R/B proteins of maize. The R/B proteins are MYC-like transcription factors that control anthocyanin accumulation in maize. Outside of the bHLH-ZIP domain, the C5 protein bears little similarity to any known protein, including the R/B proteins. Thus, C5 appears to be a new MYC-like protein. The sequence of the C5 cDNA has been deposited in the GenBankTM/EBI Data Base (accession number AJ487830). The results of Northern blot hybridization experiments demonstrated that the transcript of the OsBP-5 gene is ~1.4 kb in length and is synthesized in several tissues including endosperm, leaves, and roots of rice plants (Fig. 1).

The OsBP-5 Protein Binds Specifically to the CAACGTG Box in the 31-bp Sequence of the Wx Promoter—To confirm that the protein encoded by cDNA C5 can interact with the 31-bp nucleotide sequence, the C5 cDNA was expressed in E. coli to produce a fusion protein (designated P5H) containing six histidine residues at its N terminus. The P5H protein was purified by affinity chromatography and used in Southern blot experiments. The E. coli proteins were separated by SDS-PAGE, renatured, and electroblotted onto PVDF membranes (Fig. 2A). A 32P-labeled 31-bp oligonucleotide (equivalent to the 31-bp sequence in the Wx promoter) was incubated with the bound P5H protein on the membrane. The DNA-protein band present at the P5H position on the gel indicates that P5H does indeed bind to the 31-bp element (Fig. 2B). When unlabeled 31-bp sequence was added to the binding solution as a specific competitor, the resulting DNA-protein band was much lighter (Fig. 2C).

The ability of protein P5H to bind to a sequence-specific manner to the 31-bp sequence was further confirmed by EMSA experiments. In these assays, a strong band shift was produced after incubation of 10 fmol of 32P-labeled probe with 0.2 μg of protein P5H under binding conditions (Fig. 3, lane 2). The band shift gradually disappeared in the presence of increasing amounts of specific competitor-unlabeled 31-bp probe (Fig. 3, lanes 3, 5, and 7). However, a strong band shift was still observed when plasmid pUC18 DNA was added to the binding reactions as a nonspecific competitor (Fig. 3, lanes 4, 6, and 8).

MYC-like proteins are known to bind to E-box sequences (CACCGTG) in DNA. However, studies on the binding of Myc-Mad complexes to random oligonucleotides in vitro revealed that these proteins also bind to a number of other non-canonical sequences with lower affinity (24). One of these non-canonical sequences is CAACGTG. We noticed that both the CAACGTG sequence and the CAACGTG-like sequence CAACGTA are located near the left (5′) end of 31-bp sequence. To determine whether the CAACGTG sequence is a binding site of protein P5H, two fragments (L and R) derived from the left and right halves, respectively, of the 31-bp sequence were synthesized and 32P-labeled separately. Subsequently, variant L fragments (LM1 fragment = TAAACGTG and LM2 fragment = TAACATA) (Fig. 4) were also synthesized and used as
Transcriptional Regulation of Rice Wx Gene by MYC and EREBP proteins

Analysis of the trans-Activation Ability of Protein OsBP-5—We tested whether protein OsBP-5 has trans-activation ability in a yeast one-hybrid system derived from the DupLEX-A yeast two-hybrid system. Fig. 5A shows the structures of the effector and reporter constructs used in the trans-activation assays. Plasmids pEGC89LH and pEG202 were used as positive and negative controls, respectively. Plasmids pEGC5, pEGC89LH, and pEG202 were introduced separately into yeast cells harboring reporter plasmid pSH18–34. All of the transformants grew on SD plates containing leucine and tryptophan, but lacking histidine and uracil (data not shown), indicating that the transformations were successful. When these transformants were transferred to plates containing the same media plus X-gal, only the cells containing both plasmids pEGC89LH and pSH18–34 turned blue. Cells co-transformed with plasmids pEGC5 and pSH18–34 remained white, just like the negative controls (Fig. 5B). Thus, protein OsBP-5 does not seem to have trans-activation ability, unless the OsBP5-LexA fusion protein was, for some reason, not synthesized in the transformed yeast cells. However, please note that the results presented in the next section indicate that the fusion protein is synthesized in yeast cells.

Fig. 2. Southwestern blot analysis of the P5H protein synthesized in E. coli cells performed by using a 32P-labeled 31-bp sequence of Wx promoter as probe. Total proteins were extracted from E. coli BL21 (DE3)/pET5 cells after IPTG induction (lane 1), E. coli BL21 (DE3)/pET5 cells before IPTG induction (lane 2), E. coli BL21 (DE3)/pET5 cells after 2, 4, and 8 h of IPTG induction, respectively (lanes 3, 4, and 5) and were separated, along with purified P5H fusion protein (lane 6), by electrophoresis on 12% SDS-polyacrylamide gels. A, Coomassie Brilliant Blue staining of the SDS-PAGE gel. B, Southwestern blot obtained when the proteins shown in A were transferred to a PVDF membrane, renatured and incubated with the 32P-labeled 31-bp probe. C, same as B but with the incubation performed in the presence of 10× unlabeled 31-bp nucleotide as a specific competitor.

| P5H protein | - | + | + | + | + | + | + |
| Probe | + | + | + | + | + | + | + |
| Competitor | 31bp | pUC | 10X | 100X |

Fig. 3. EMSAs of the P5H protein synthesized in E. coli cells performed with the 31-bp sequence of Wx promoter as probe. EMSAs were performed using no P5H protein (lane 1) or 0.2 μg of P5H protein (lane 2) and 32P-labeled probe. Competition experiments were performed using increasing amounts (1×, 10×, and 100×) of the unlabeled 31-bp sequence (lanes 3, 5, and 7) and plasmid pUC18 DNA (lanes 4, 6, and 8).

Fig. 4. EMSAs of the P5H protein synthesized in E. coli cells performed by using different fragments of the 31-bp sequence of the Wx promoter as probes. EMSAs were performed with no P5H protein (lane 1) and with 0.2 μg of purified P5H protein and the 32P-labeled oligonucleotide probes shown below the photograph (lanes 2–5). The mutated bases in the oligonucleotides are shown using lowercase letters. The E-box-like sequence is shown using italic letters.

Fig. 5. The results of yeast one-hybrid assays indicate that the OsBP-5 protein lacks transcriptional trans-activation activity. A, schematic diagrams of the structures of the effector and reporter plasmids used in the trans-activation assays. The cDNAs C89LH and C5 were fused to the LexA-BD coding region of the yeast expression vector pEG202 to produce recombinant plasmids pEGC89LH and pEGC5, respectively. Abbreviations: LBSs, LexA-binding sites; P_{ADH}, ADH promoter; T_{ADH}, ADH terminator; Mini-P, minimal GAL1 promoter; NLS, nucleus targeting signal. B, assays of the ability of yeast cells carrying the reporter plasmid pSH18–34 and one of the three effector plasmids (pEG202, pEGC5, or pEGC89LH) to grow on SD medium containing tryptophan and leucine but no uracil or histidine.
The individual recombinant prey plasmids were introduced into yeast strain EGY48 harboring reporter plasmid pSH18–34 and bait plasmid pEGC5. All-transformants grew on SD plates containing leucine, but lacking histidine, uracil, and tryptophan (data not shown). After transfer to plates containing the same medium plus X-gal, only the yeast cells co-transformed with plasmids pJGC89LH, pEGC5, and pSH18–34 turned blue, whereas yeast cells co-transformed with pEGC5, pSH18–34, and any one of the other five prey plasmids did not turn blue on the X-gal medium (Fig. 6B). These results indicate that the OsEBP-89 protein (a rice EREBP protein) (25) and protein OsBP-5 together are able to trans-activate the expression of the reporter gene. In addition, these results demonstrate that the OsBP5-LexA fusion protein is indeed synthesized in yeast cells (see the preceding section).

To obtain additional evidence of an interaction between proteins OsBP-5 and OsEBP-89, we performed both super-shift and pull-down assays. Both His-tagged fusion proteins (P89H and P5H) were purified by affinity chromatography from extracts of IPTG-induced E. coli BL21/pET89 and BL21/pET5 cells. 32P-Labeled fragment LM2 (see Fig. 4) was used as probe. The results of the band-shift assays (Fig. 7) showed that protein P5H was able to bind to the LM2 probe (lane 2), whereas P89H did not bind (lane 3). However, when protein P89H was added to the incubation mixture containing the P5H protein-LM2 probe complex, no supershifted band formed (lane 4). In a control in which BSA was added to the incubation mixture containing the P5H protein-LM2 probe complex, no super-shifted band formed (lane 5). These results suggest that proteins OsBP-5 and OsEBP-89 interact to form heterodimers.

In vitro pull-down assays were carried out to determine whether the native form of protein OsEBP-89 is capable of interacting with protein P5H. Total proteins were isolated from developing endosperm cells of rice plants, mixed with proteins OsBP-5 and OsEBP-89, we performed both super-shift and pull-down assays. Both His-tagged fusion proteins (P89H and P5H) were purified by affinity chromatography from extracts of IPTG-induced E. coli BL21/pET89 and BL21/pET5 cells. 32P-Labeled fragment LM2 (see Fig. 4) was used as probe. The results of the band-shift assays (Fig. 7) showed that protein P5H was able to bind to the LM2 probe (lane 2), whereas P89H did not bind (lane 3). However, when protein P89H was added to the incubation mixture containing the P5H protein-LM2 probe complex, no supershift band formed (lane 4). In a control in which BSA was added to the incubation mixture containing the P5H protein-LM2 probe complex, no super-shifted band formed (lane 5). These results suggest that proteins OsBP-5 and OsEBP-89 interact to form heterodimers.

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In vitro pull-down assays were carried out to determine whether the native form of protein OsEBP-89 is capable of interacting with protein P5H. Total proteins were isolated from developing endosperm cells of rice plants, mixed with proteins OsBP-5 and OsEBP-89, we performed both super-shift and pull-down assays. Both His-tagged fusion proteins (P89H and P5H) were purified by affinity chromatography from extracts of IPTG-induced E. coli BL21/pET89 and BL21/pET5 cells. 32P-Labeled fragment LM2 (see Fig. 4) was used as probe. The results of the band-shift assays (Fig. 7) showed that protein P5H was able to bind to the LM2 probe (lane 2), whereas P89H did not bind (lane 3). However, when protein P89H was added to the incubation mixture containing the P5H protein-LM2 probe complex, no supershifted band formed (lane 4). In a control in which BSA was added to the incubation mixture containing the P5H protein-LM2 probe complex, no super-shifted band formed (lane 5). These results suggest that proteins OsBP-5 and OsEBP-89 interact to form heterodimers.
The Interaction of OsEBP-89 with OsBP-5 Enhances Gene Expression—Two rice cDNAs, one encoding protein OsEBP-89 and the other OsBP-5, were isolated by using a yeast one-hybrid screening system with the 31-bp Wx promoter sequence as bait. The results of EMSAs confirmed that these two proteins are able to bind to the 31-bp probe, but the binding of OsEBP-89 to 31-bp was inefficient (25). Our results also showed that these two proteins can interact with each other. Another yeast two-hybrid system (designated II in this paper) was used to determine whether the interaction of these two proteins results in the enhanced expression of genes under the control of the by 31-bp sequence. Three plasmids are used in yeast two-hybrid system II (Fig. 8A). Plasmid pC5Gal4AD− was constructed by inserting cDNA C5 into plasmid pPC86 and deleting the Gal4 activation domain. Plasmid pEG89SX contains cDNA C89SX, which encodes a truncated protein (89SX) lacking the C-terminal region of protein OsEBP-89. The absence of the C-terminal region of protein OsEBP-89 results in an enhanced interaction between OsBP-5 and OsEBP-89 in yeast.2 Plasmid pSH18−34 is the reporter plasmid containing the lacZ reporter gene under the control of the 6×31-bp Wx promoter sequence. The levels of expression of the lacZ gene in yeast cells containing pSH18−34 and co-transformed with pC5Gal4AD− or pEG89SX individually or with both of these plasmids were analyzed. The results shown in Fig. 8B indicate that the C5 fusion protein alone can not trans-activate the reporter gene. Protein 89SX did trans-activate the reporter gene, but the level of expression was low (a light blue color was observed in colonies grown on X-gal plates). However, when proteins C5 and 89SX were both synthesized in yeast cells, the reporter gene was expressed at a much higher (about 3-fold) level.

Interference with OsBP-5 Gene Expression by Double-stranded RNA Results in Reduced Amylose Content in Developing Endosperm Tissues—The rice Wx gene encodes granule-bound starch synthase, the enzyme responsible for the synthesis of amylose in endosperm tissues, and the results described in the preceding sections of this paper are all consistent with a role for the OsBP-5 gene product as a positive regulator of Wx expression. In an attempt to obtain further evidence for the involvement of the OsBP-5 gene product in the regulation of Wx gene expression, we used dsRNA to gene-rate a loss-of-function phenotype in transgenic rice plants. The dsRNA cassette contained a 450-bp gene-specific fragment of the OsBP-5 cDNA in the sense and antisense orientations separated by a GUS coding spacer region and placed under the control of the constitutive Ubi promoter (p13dsC5, Fig. 10A). The GUS gene served both as a spacer separating the sense and antisense sequences of the OsBP-5 gene and as a reporter gene with which to detect the presence of the dsRNA cassette. Agrobacterium-mediated transformation was used to generate 35 independent lines containing the dsRNA, cassette (p13dsC5), and GUS activity was observed in the tissues of T0 plants for all 35 lines (data not shown). Most of the 35 independent transgenic lines exhibited decreased amylose content in T1 seeds when compared with wild-type seeds. The decreases

Fig. 8. Evidence for a specific interaction between OsEBP-89 and 5PH from pull-down assays. Total proteins expressed in BL21 (DE3)/pET5 (lane 1), nuclear proteins extracted from developing rice endosperm (lane 2), or both (lane 3), respectively, were added to columns filled with Ni-NTA resin. The columns were washed three times and then eluted with buffer. The eluted proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels. A, SDS-PAGE gels stained with Coomassie Brilliant Blue. B, Western blot of the proteins shown in A after transfer to a PVDF membrane and incubation with antibodies to protein OsEBP-89.

Fig. 9. trans-Activation of a lacZ reporter gene controlled by a promoter consisting of six tandem repeats of the 31-bp sequence of the rice Wx promoter by proteins OsBP-5 and OsEBP-89 in a yeast two-hybrid system. A, schematic diagrams of effector and reporter constructs used in the experiments. Plasmid pC5Gal4AD− (the Gal4-AD in plasmid pPC86 was deleted) contained the C5 cDNA fused to the ADC1 promoter. Plasmid pEG89SX contained the C89SX cDNA (encoding amino acid residues 1–278) fused to the ADH1 promoter and LexA-AD sequence. The reporter plasmid, p178–6×31, contained six tandem copies of the 31-bp regulatory fragment of the rice Wx promoter. The 6×31 promoter sequence was fused to the minimal Gal1 promoter and the lacZ coding region. B, growth of yeast cells containing plasmids pC5Gal4AD− (transformants 1) and p178–6×31, plasmids pEG89SX and p178–6×31 (transformants 2), and plasmids pC5Gal4AD−, pEG89SX, and p178–6×31 (transformants 3) on SD X-gal medium lacking tryptophan and uracil (transformants 1), histidine and uracil (transformants 2), or histidine, tryptophan, and uracil (transformants 3), respectively. β-Galactosidase activities were measured in a minimum of three independent transformants; the table shows average activities along with ± 1 S.D.

2 Zhu, Y., Wang, Z. Y., and Hong, M. M. (2003) J. Plant Physiol. Mol. Biol. (in Chinese), in press.
in amylose content ranged from over 23% to no change (two lines). The average decrease in the amylose content in T2 seeds produced on T0 plants was 10% with a S.D. of 1.23%. Plants of two independent lines transformed with plasmid p13UGN (see Fig. 10) were used as controls. The amylose content in T2 seeds of both lines was similar to that of wild-type seeds. Differences in transgene copy number and position effects undoubtedly contribute to the observed variation in OsBP-5 transcript level and amylose content in the seeds of individual transgenic dsRNAi lines. Similar variation in the strength of interference (weak, intermediate, and strong) induced by dsRNAi constructs have been observed in transgenic Arabidopsis plants (27).

Because the RNAi cassette has a dominant effect on OsBP-5 gene expression, T2 plants should segregate RNAi-positive and RNAi-negative progeny. Indeed, PCR analyses demonstrated that T2 plants that exhibited GUS activity contained the dsRNAi cassette, whereas segregants with no GUS activity did not carry the RNAi cassette (see examples L25/2 versus L25/4 in Fig. 10B). The results of Northern blot hybridizations showed that the levels of OsBP-5 transcripts in the T2 immature seeds (endosperm tissues) of three independent T1 plants with GUS activity in leaves were reduced when compared with segregants with the no GUS activity and with wild-type plants (Fig. 10C). Among the transgenic lines with GUS activity, lines L32/7 and L35/4 had a significant reduction in OsBP-5 transcript, whereas line L28/3 showed little reduction when compared with line L25/4, which is GUS-negative and wild type. In general, the GUS activity in transgenic plants was correlated with the degree of inhibition of OsBP-5 transcript accumulation. We also measured the amylose content of T2 seeds produced by heterozygous transgenic plants. The results showed that the amylose contents of seeds with embryos exhibiting GUS activity were lower than those of seeds with embryos lacking GUS activity, when both sets of seeds were harvested from the same T1 plant (Fig. 11). Thus, the reduction in the amylose content of mature seeds was positively correlated with the inhibition of OsBP-5 gene expression. All of these results suggest that the OsBP-5 protein is a positive regulator of Wx gene expression.

**DISCUSSION**

bHLH proteins are ubiquitous transcription factors found in virtually all eukaryotes examined to date. The proteins form either homo- or heterodimers and play major roles in cell-type determination, cell growth, and development (28). The bHLH proteins that contain both bHLH and ZIP motifs are called MYC proteins or MYC-like proteins. The presence of both bHLH and ZIP motifs can determine the affinity and specificity of sequence-specific DNA binding or mediate the formation of different types of homo- and heterodimers (29). The MYC-like proteins of animals are involved in tumorigenesis, differentiation, and cell progression or proliferation.

In plants, the best known bHLH proteins are the members of the R gene family. As transcription factors, they play key roles in regulating anthocyanin accumulation in plants. In this paper, we describe a new MYC-like protein, OsBP-5, in rice. Amino acid sequence comparisons showed that the OsBP-5 bHLH domain shares 50% amino acid identity with R protein of maize. We compared the various predicted R proteins from different species and found that the average amino acid sequence identity of the R proteins of monocots is about 84%, with the N- and C-terminal regions exhibiting about 67 and 64% identity, respectively. The amino acid sequence identities of R proteins of monocots and dicots are lower: 60, 50, and 20% for the bHLH, N-terminal, and C-terminal regions, respectively. The OsBP-5 and Ra proteins of rice contain the lowest amino acid sequence identity observed among plant MYC-like proteins, with the bHLH, N-terminal, and C-terminal regions sharing 50, 18, and 5% identity, respectively. The results of the sequence comparisons suggest that the OsBP-5 protein may not be a homolog of the rice R gene but, instead, may represent a new class of MYC-like proteins.

MYC-like proteins can bind specifically to E-box (CACGTG) sequences. Since E-box sequences contain binding sites for both bHLH proteins and bZIP proteins (consensus ACGT), MYC-like proteins recognize E-box sequences with high affinity and specificity (30). Non-canonical DNA binding sequences were identified by determining which random oligonucleotides were bound by using Myc-Max protein complexes in *in vitro* DNA binding experiments (24). One of the non-canonical sequences bound was CAACGTG, a sequence that is present in the 5' region of 31-bp Wx promoter sequence. The results of our EMSA experiments showed that the OsBP-5 protein does indeed bind to CAACGTG in a sequence-specific manner.

For most MYC proteins, an association with a “partner protein” to form heterodimers (in addition to the homodimers formed by the individual proteins) is an important part of the *trans*-activation mechanism. Usually, the heterodimers bind target sequences more efficiently than the respective homodimers. Shifts from the formation of homodimers to heterodimers, or from heterodimers to homodimers, play important roles in transcriptional regulation. In animals, for
example, the overexpression of Max represses transcription because of a shift from Myc-Max heterodimers to Max-Max homodimers. This repressive effect is abrogated by the simultaneous overexpression of Myc, which restores the Max-Myc stoichiometry and thus results in the formation of Myc-Max heterodimers (31).

Plant MYC proteins usually interact with MYB proteins in carrying out their regulatory functions. The regulation of anthocyanin production in maize is one of the best characterized examples of the importance of combinatorial protein interactions in the regulation of transcription in plants. The developmental regulation of anthocyanin synthesis is controlled by the combinatorial interaction between R/B (MYC-like proteins) and C1/PL (a MYB-like protein). R/B or C1/PL alone is not sufficient to trans-activate the target genes (32). In Arabidopsis, the MYC-like rd22BP1 protein interacts with the MYB-like protein ATMYB2 to trans-activate dehydration-responsive genes under drought conditions (33). The Arabidopsis GLA-BRBA3 (GL3) gene encodes a protein with homology to R proteins, and the protein (GL3) regulates trichome development through an interaction with GL1, a MYB-like transcription factor (34).

In this report, we demonstrate that OsBP-5 protein of rice, a new MYC-like protein, interacts with another transcription factor, the OsEBP-89 protein, in yeast cells. Interestingly, the OsEBP-89 protein is a member of the EREBP family of transcription factors and not a MYB-like factor. Among the members of the EREBP family, only the Arabidopsis protein AtEBP has been reported to interact with another transcription factor. AtEBP has been shown to interact with OBFB1, a bZIP protein in Arabidopsis (26). This paper presents the first evidence for an interaction between a MYC-like protein and an EREBP protein.

Both OsEBP-89 and OsBP-5 were originally isolated from a rice cDNA library by using the 31-bp sequence of the rice Wx gene promoter as bait. The OsEBP-89 gene was shown to be a new member of EREBP subfamily in rice. The OsEBP-89 protein contains a single conserved EREBP domain and an activation domain, and it binds strongly to GCC boxes and weakly to GCCAAC sequences. The expression pattern of the OsEBP-89 gene is similar to that of Wx, with high levels of expression in endosperm cells (25). The OsEBP-89 protein can bind to the OsBP-5 protein, which is also synthesized in endosperm cells. Within the 31-bp sequence of the Wx promoter, the GCCAAC binding site of OsBP-5 is followed by the GCCAAC binding site of OsEBP-89 (Fig. 12). In this paper, we have presented evidence indicating that the OsBP-5 and OsEBP-89 proteins interact and that together they are positive regulators of the transcription of the Wx gene. In addition, we observed that reducing the levels of OsBP-5 protein by dsRNA, resulted in lower amyllose content in seeds of transgenic plants. This result also suggests that the OsBP-5 protein plays a role in regulating the expression of the Wx gene. All of these results support a combinatorial model for the regulation of Wx gene expression by these two proteins. In this model, we propose that the OsBP-5 protein binds in a sequence-specific manner to the 31-bp sequence of the Wx promoter but that OsBP-5 lacks an activation domain. We further propose that OsEBP-89 has a strong trans-activation domain but cannot bind efficiently to the 31-bp sequence. Finally, we propose that OsEBP-89/OsBP-5 heterodimers trans-activate Wx transcription more efficiently, because they have both high binding ability and a strong trans-activation domain.

In conclusion, we should emphasize that proteins other than OsBP-5 and OsEBP-89 probably are involved in regulating the transcription of the rice Wx gene as well, and these proteins will have to be identified and characterized before we will have a complete picture of the Wx regulatory circuits. We are currently in the process of identifying additional cis-acting elements and trans-acting factors to understand in detail how Wx gene expression is regulated.

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