A Rice Functional Transcriptional Activator, RISBZ1, Responsible for Endosperm-specific Expression of Storage Protein Genes through GCN4 Motif*

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The GCN4 motif, a cis-element that is highly conserved in the promoters of cereal seed storage protein genes, plays a central role in controlling endosperm-specific expression. This motif is the recognition site for a basic leucine zipper transcriptional factor that belongs to the group of maize Opaque-2 (O2)-like proteins. Five different basic leucine zipper cDNA clones, designated RISBZ1–5, have been isolated from a rice seed cDNA library. The predicted gene products can be divided into two groups based on their amino acid sequences. Although all the RISBZ proteins are able to interact with the GCN4 motif, only RISBZ1 is capable of activating (more than 100-fold expression) the expression of a reporter gene under a minimal promoter fused to a pentamer of the GCN4 motif. Loss-of-function and gain-of-function experiments using the yeast GAL4 DNA binding domain revealed that the proline-rich N-terminal domain (27 amino acids in length) is responsible for transactivation. The RISBZ1 protein is capable of forming homodimers as well as heterodimers with other RISBZ subunit proteins. RISBZ1 gene expression is restricted to the seed, where it precedes the expression of storage protein genes. When the RISBZ1 promoter was transcriptionally fused to the β-glucuronidase reporter gene and the chimeric gene was introduced into rice, the β-glucuronidase gene is specifically expressed in aleurone and subaleurone layer of the developing endosperm. These findings suggest that the specific expression of transcriptional activator RISBZ1 gene may determine the endosperm specificity of the storage protein genes.

Regulated gene expression is mediated by the combinatorial interactions of multiple cis-elements in the gene’s promoter. Specific binding of transcriptional factors to the cognate cis-elements constitute a crucial step in transcription initiation and, in turn, on the spatial and temporal expression of genes.

Seed storage protein genes provide a model system for the study on the regulatory mechanisms of plant genes (1), since their expression is restricted to a specific tissue and stage during seed development. These specific temporal and spatial expression patterns may be explained as the result of regulatory assemblies of several transcriptional activators that recognize the cis-elements implicated in seed-specific expression. Therefore, to understand such molecular mechanisms, characterization of cis-elements and transcription factors has been performed on many storage protein genes of several crop plants (2, 3). Despite numerous studies, the mechanism by which these genes are regulated is poorly understood, since many of the essential cis-elements have not been identified. This is especially true in the case of monocot plants, where many of the promoter analyses of cereal storage protein genes have carried out by transient assays using particle bombardment or heterologous transgenic tobacco system (4–6). Dissection analyses of promoter using homologous stable transgenic plant have been carried out only on glutelin genes of the rice (7–9).

Endosperm-specific expression of cereal storage protein genes is regulated by the combinatorial interactions of several cis-elements (3). Prolamin box (TGTAAAG), GCN4 motif (TGA/G/C/TCA), AAC motif (AACAAAA), and ACCTG motif, which are conserved in many promoters of cereal seed storage protein genes, have been characterized as cis-elements involved in endosperm-specific expression by loss-of-function and gain-of-function experiments (3).

The GCN4 motif is widely distributed in many promoters of not only seed storage protein genes but also genes that code for metabolic enzymes (4). It has been recently demonstrated that it acts as a key element controlling the endosperm-specific expression. Multimers of the rice glutelin GCN4 motif can direct endosperm-specific expression in stable transgenic rice. In contrast, deletion or base substitution of the GCN4 motif in the rice glutelin promoter reduces promoter activity and alters gene expression pattern (8). The GCN4 motif is often linked together with the prolamin box (TGTAAAG), which are separated by a few nucleotides. The two motifs collectively constitute the so called bifactorial endosperm box found in nearly all cereal prolamin genes such as wheat glutenin, barley hordein, rye secalin, sorghum kafirin, and Coix coixin (4, 10, 11). In most rice glutelin genes, these two motifs are also associated with the AACCA motif (12). Gain-of-function experiments showed that combinations of two motifs (GCN4 motif and prolamin box, GCN4 motif and AACCA motif) are not sufficient to confer endosperm-specific expression, suggesting that participation of additional element is required to form a functional complex of trans-acting factors (6, 13). It has been recently demonstrated in the rice glutelin GluB-1 gene that at least three cis-elements containing the GCN4, ACCTG, and AACCA motifs within the
−197 bp proximal promoter are required as minimal elements to direct endosperm-specific expression (8, 9).

Maize Opaque-2 (O2) is an endosperm-specific transcription factor belonging to the basic leucine zipper (bZIP) family that has been shown to bind to the ACCT motif of maize 22-kDa α-zein promoter and activates transcription (14). It has been also reported to be involved in activation of endosperm-specific transcription of b32 ribosome-inactivating protein gene via a distinct cis-sequence (G/aT/GAPyPuyTGPu) (15), thus indicating that the O2 has broad binding specificity. The GCN4 motif has been reported to be recognized by the O2, resulting in the activation of transcription (8, 10, 15). In vivo footprinting of the promoter region of wheat low molecular weight glutenin (16) and maize γ-zein genes (6) in developing endosperm revealed that the region covering the GCN4 motif and prolamin box are occupied by nuclear proteins from maturing seeds. In vitro DNase I footprinting with nuclear proteins from rice maturing seeds and GST-O2 fusion protein also showed that the sequence covering the GCN4 motif in the promoter of glutelin genes is specifically protected from DNase I digestion (8, 17). These findings suggest that O2-like transcription factor may exist and participate in controlling the endosperm-specific expression of many storage protein genes in cereal seed through the GCN4 motif.

The cDNA clones encoding transcription factor recognizing the GCN4 motif have been recently isolated and characterized in wheat (5) and barley (18, 19). These transcription factors, designated SPA, BLZ1, and BLZ2, transcriptionally activate expression of the storage protein genes by interacting with the GCN4 motif in the wheat low molecular weight glutenin and barley B1-hordein promoters. It is interesting to note that they are expressed in seed-specific manner. cDNA clones encoding primary sequences showing high homology to the basic domain of O2 have been also isolated from rice (20, 21), although it has not yet been examined whether they are involved in transcriptional activation of storage protein genes through the GCN4 motif. The RITA-1 gene is highly expressed in aleurone and endosperm tissue during seed maturation (20). The REB cDNA, which shares homology to maize Opaque-2 heterodimerizing protein (OH) (22) and barley BLZ1 (18), specifically binds to the GCCAGCTGTA/AG sequence, designated G/C and A/G hybrid box, in the α-globulin promoter (21).

It has recently been reported that maize O2, barley BL2Z, and wheat SPA interact in vitro with another endosperm-specific transcription factor of the Dof class containing a single highly conserved zinc finger DNA binding domain that recognizes the prolamin box motif (11, 23, 24). Such an interaction has been suggested by the in vivo footprinting of low molecular weight glutenin promoter and maize γ-zein promoter (6, 24).

In this paper, we screened for cDNA clones coding for bZIP transcription factors from rice seed cDNA library to determine the trans-factor responsible for transcriptional activation of storage protein genes through the GCN4 motif. Five types of bZIP proteins were isolated and characterized, two of which are completely identical with the RITA-1 (20) and REB (21) transcription factors. All of the transcription factors are able to bind to the GCN4 motif in the sequence-specific manner, but only RISBZ1 exhibits high levels of transcriptional activation through the GCN4 motif using transient expression assays.

The role of RISBZ1 as an important transcriptional factor in endosperm-specific regulation of storage protein genes is further supported by the analyses of stable transgenic rice containing chimeric genes consisting of the RISBZ1 promoter and GUS reporter gene. We also show that the N-terminal proline-rich domain of RISBZ1 is required for transcriptional transactivation.

EXPERIMENTAL PROCEDURES

Plant Materials—Rice (Oryza sativa cv. Mangetsumochi) seeds were germinated in tap water, and 14-day-old leaves and roots were frozen in liquid nitrogen and stored at −80°C until used. Maturing seeds were harvested from rice grown in the field.

Screening of a Rice Seed cDNA Library—Poly(A)⁺ RNA was isolated from maturing rice seeds harvested 6–16 days after flowering (DAF), as described previously (25). Single-stranded cDNA was synthesized at 1 h at 42°C with SuperScript™ RNase H⁻ reverse transcriptase (Life Technologies, Inc.) using oligo(dT)₁₇ as a primer. This cDNA was used as the initial template in a PCR using a forward primer (TCCAG/C/TX/A/C/GIGA/A/G)(A/AT/CIGC) and a reverse primer (GTTCCTC/G/TGC/CATCTTCACCCTT), sequences conserved in the basic domain of bZIP transcription factors expressed in cereal seeds. The PCR conditions were 94°C for 5 min, followed by three cycles of 1 min at 94°C, 1 min at 66°C, and then 30 cycles of 1 min at 94°C, 1 min at 62°C, and then 2 min at 72°C. Amplified fragments were inserted into the TA cloning vector (pCR™ 2.1; Invitrogen) and sequences determined by a ABI Prism™ dye terminator cycle sequencing kit using the ABI Prism™ 310 genetic analyzer (PE Applied Biosystems). Sequence analyses and data bank searches were carried out using GENETYX (Software Development Co., Ltd.) and the BLAST algorithms, respectively.

Rice cDNA library constructed from poly(A)⁺ RNA of maturing seed (6–16 days after flowering) by ZAP cDNA® synthesis kit (Stratagene) were screened by five types of PCR fragments under high stringent condition. These RNA fragments were labeled by Megaprime™ DNA labeling system (Amersham Pharmacia Biotech). Prehybridization was carried out at 42°C in 5× SSC, 5× Denhardt's solution, 0.1% SDS, 50% formamide, and 100 µg/ml salmon sperm DNA. The filters were washed twice at 55°C in 2× SSC and 0.1% SDS and twice at 55°C in 0.1× SSC and 0.1% SDS.

Cloning of Genomic Sequence of RISBZ1 Gene—To determine the genomic sequence coding for full length of RISBZ1 cDNA region, four primers were devised based on RISBZ1 cDNA sequence. The primers (RIS1f, 5'-ATGGTGTTGCGTATGCAGCT-3'; REL5, 5’-TGTCGGTGGCGATCAGCTG-3'); RELr4, 5’-TTCGATCAAATGC-3') were used to amplify the 5’ portion of the exon/intron region. Two other primers (REL2b, 5’-GAGGATCCGGCCTAT-3'; RIS1r, 5’-TCTGTAATATTTCTAGACACA-3') were used for amplification of the 3’ portion of the gene. Amplification reactions were performed using Takara LA Tag (Takara) for 30 cycles at 98°C for 10 s, at 56°C for 30 s, and 85°C for 3 min after incubation at 94°C for 5 min.

The promoter region of RISBZ1 gene was amplified by thermal asymmetric interlaced PCR according to the method of Liu et al. (26). Three oligonucleotides (tail-1, 5’-TCCCTCATTGCGCTCTGAGCAG; tail-2, ATGAATTCGCGAGGGTTTTCGA; tail-3, GTCCTC(C/T)GC-TTCGATCAAATGC) were used to amplify the 5’ portion of the gene in vitro. Amplification reactions were performed using Takara LA Tag (Takara) for 30 cycles at 98°C for 10 s, at 56°C for 30 s, and 65°C for 3 min after incubation at 94°C for 5 min.

The 5’ flanking region of RISBZ1 gene was amplified by thermal asymmetric interlaced PCR according to the method of Liu et al. (26). Three oligonucleotides (tail-1, 5’-TCCCTCATTGCGCTCTGAGCAG; tail-2, ATGAATTCGCGAGGGTTTTCGA; tail-3, GTCCTC(C/T)GC-TTCGATCAAATGC) were used to amplify the 5’ portion of the gene in vitro. Amplification reactions were performed using Takara LA Tag (Takara) for 30 cycles at 98°C for 10 s, at 56°C for 30 s, and 65°C for 3 min after incubation at 94°C for 5 min.

Northern Blot Analysis—Total RNA from different organs (roots and seedlings) and developing seeds (from 5 to 30 DAF) was isolated as described (25). Blots were probed using cDNA-specific region spanning from downstream of the leucine zipper region to 5′-untranslated regions of individual RISBZ cDNA clones. Hybridization was carried out at 45°C in 50% formamide, 5× SSC, 0.1% SDS, and 5× Denhardt's solution, and then filters were washed four times at room temperature in 2× SSC, 0.1% SDS, and twice at 55°C in 0.1× SSC and 0.1% SDS.

Production of Transgenic Rice Plants—Transgenic rice plants (O. sativa cv. Kitaake) were generated by Agrobacterium tumefaciens-mediated transformation as described previously (28). The 5’-flanking region of the RISBZ1 gene, located between positions −1674 and +4 and between positions −1674 and +213, were amplified by PCR using

1 The abbreviations used are: bp, base pair(s); GUS, β-glucuronidase; PCR, polymerase chain reaction; DAF, days after flowering; GST, glutathione S-transferase; CaMV 35 S, cauliflower mosaic virus 35 S RNA gene; EMSA, electrophoretic mobility shift assay; −46 CaMV, −46 base pair core promoter of CaMV 35 S; 35 S, CaMV 35 S promoter; ORF, open reading frame; bZIP, basic leucine zipper; NLS, nuclear localization signal.
a forward and a reverse primers containing overhanging 
*Pst*I and *Bam*HI recognition sites, respectively. The PCR fragments were di-
gested with *Pst*I and *Bam*HI and then introduced into the correspond-
ning sites of pBl 201. After digestion with *Pst*I and *Sal*I, the chimeric 
genes composed of RISBZ1 promoter and GUS reporter gene was cloned 
into the respective sites of the construct pGUS-Blunt, containing 
CaMV 35 S promoter/hygromycin phosphotransferase gene.

**Expression of the GST Fusion Protein in Escherichia coli**—The coding 
sequences for the five RISBZ cDNAs were amplified by PCR using 
forward and reverse primers that yielded the following restriction sites at 
the termini: RISBZ1, BamHI-bluend; RISBZ2, BamHI-XhoI; 
RISBZ3, BamHI-SalI; RISBZ4, BamHI-SalI; and RISBZ5, BamHI- 
XhoI, respectively.

After digestion, these cDNAs were inserted into the corresponding 
sites of pGEX- 4T-3 GST fusion expression vector (Amersham Pharmacia 
Biotech). The GST-RISBZ fusion proteins were expressed and iso-
lated as described (29). After purification by affinity chromatography, 
GST fusion proteins were dialyzed overnight against 20 mM HEPES- 
KOH, pH 7.9, 50 mM KCl, 1 mM EDTA, and 10% glycerol. 

Endosperm-specific Transactivator Binding to the GCN4 Motif

**Isolation of cDNA Clones Encoding bZIP Transcription Fac-
tors from Rice Seed cDNA Library**—Two degenerate primers, 
designed based on the highly conserved amino acid sequences, 
SNRESA and KVKAED of the basic region of various O2-like 
poz protein transcription factors, were used for reverse-PCR using 
poly(A)+ RNA isolated from developing seeds. The resultant 
PCR products were cloned into TA cloning vector (pCR™ 2.1), 
and insert sequences of more than 50 TA clones were deter-
mined by DNA sequencing gel containing 7M urea in parallel with a DNA se-
quencing ladder.

Methylation Interference Experiments—Methylation interference ex-
periments were carried out as described by Weinberger et al. (30). The 
5'-flanking region between −245 and +18 was digested with *Sall* and 
*Bam*HI and then end-labeled by fill-in reaction with Klenow fragment 
and [α-32P]dCTP, and then methylated by dimethylsulfate. This frag-
ment was incubated with the GST-RISBZ1 fusion protein and fraction-
ated by electrophoresis on 5% native polyacrylamide gel for 20 min at room temperature. For competition assays, 100-fold molar 
excess of unlabeled competitor DNA was added to the binding reac-
tion. The mixture was loaded onto 5% native polyacrylamide gel in 
0.25X TBE (1X: 89 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA) 
buff er at room temperature.

**RESULTS**

Isolation of cDNA Clones Encoding bZIP Transcription Fac-
tors from Rice Seed cDNA Library—Two degenerate primers, 
designed based on the highly conserved amino acid sequences, 
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mined by DNA sequencing gel containing 7M urea in parallel with a DNA se-
quencing ladder.

In Vivo Translation—Full-length coding region and truncated 
cDNAs covering the basic domain of RISBZ1, RISBZ2, and RISBZ3 
were amplified by PCR using a forward primer containing a 5′ NcoI site 
and a reverse primer containing a termination codon and BamHI site. 
After digestion with NcoI and BamHI, the amplified DNA fragments 
were inserted into a pET-8c vector (Novagen). Translation products were 
prepared by coupled *in vitro* transcription/translation system 
(TNT™ coupled wheat germ extract system, Promega) according to 
the manufacturer’s instructions and used for gel mobility shift assays. 

One microgram of amplified DNA was used to produce full-length 
RISBZ proteins and truncated RISBZ proteins. For EMSA, 4 µl of the 
in vivo translation products were used in the binding reactions.

Construction of Effector Plasmids—A progressive deletion series of the 
RISBZ1 protein from the N terminus was generated by PCR-based 
construction. Forward primers corresponding to positions 41, 81, 121, 
161, 201, and 235 residues from the N terminus and a reverse primer 
corresponding to the C terminus were used for the PCR. These primers 
contained an overhanging NcoI (to restore the AUG initiation codon) 
and BamHI site at the 5′ ends. After digestion with NcoI and BamHI, 
PCR fragments were purified by agarose gel electrophoresis. Resulting 
DNA fragments were cloned in pET100 (31). To construct fusion plas-
mds with the GAL4 DNA-binding domain (positions 1–147), various 
DNA fragments were cloned in pRT100 (31). To construct fusion plas-
mds with successive mutations by three bases were synthesized and 
inserted into the corresponding sites of the construct pGEX-Blunt, 
containing CaMV 35 S promoter/hygromycin phosphotransferase gene.

amino acid positions 1 and 57 were amplified by PCR and inserted 
downstream of the GAL4 DNA binding domain.

**Construction of Reporter Plasmid—** 1 × 21-bp, 3 × 21-bp, 5 × 21-bp 
normal GCN4 and 3 × 21-bp mutant GCN4/GUS reporter genes 
were constructed as described (8). For construction of chimeric 
GUS reporter, normal GCN4 (GCTGAGTCATGA) and mutant 
GCN4 (GCTGAGTCATGA), double-stranded oligonucleotides were 
generated by annealing the 12- and 48-base complementary oligonucleotide 
pairs containing the sticky AGGT sequence at the 5′ end of the sense 
strand, respectively. These double-stranded oligonucleotides were 
inserted into the *Not*I and *Sac*I sites of ~ 46 CaMV/GUS reporter gene. 

**Transient Expression Analysis**—Transient expression in rice callus protoplasts was performed by electroporation as 
described previously (8). GUS activities were measured by fluorometric 
quantification of 4-methylumbelliferone produced from the glucuronide 
precursor according to Jefferson (33). Protein concentration was deter-
mined by Bradford method using a Bio-Rad kit using bovine serum 
albumin as a standard.

Histochemical analysis of GUS gene expression was carried out as 
described previously (8).
The CLUSTAL X program was used to determine their degree of relatedness and their evolutionary relationships among different RISBZ and O2-like bZIP proteins. The matrix of the primary sequences was calculated using the CLUSTAL X program.

The N-terminal regions of RISBZ1 and RISBZ2 proteins are rich in prolines (Fig. 2). The N-terminal region (residues 1–60) and the central part upstream of the basic domain of RISBZ1 and -2 are enriched in acidic amino acids (Fig. 2). These acidic and proline-rich regions are found in the other O2-related bZIP proteins. These acidic and proline-rich domains have been implicated in having a role in transcriptional activation of many transcription factors.

A putative serine-rich phosphorylation site (SGSS), a potential target sequence of casein kinase II (34), is found between positions 207 and 210 of the RISBZ1 (Fig. 2). Such a sequence is also found in the corresponding region of the RISBZ2 (SSSS), but is deleted in the other RISBZ primary sequences (Fig. 2). It has recently been reported that the corresponding site of the maize O2 is phosphorylated, which results in a loss of DNA binding activity. Interestingly, the extent of O2 phosphorylation level changes during the day-night cycle (35). Thus, RISBZ1 and -2 may be phosphorylated by a similar mechanism and covalent modification of these O2-like proteins may be implicated in the regulation of target genes.

It has been reported that two nuclear localization signals (NLS), designated NLS A (SV40-like motif) and NLS B (bipartite motif), are responsible for the nuclear localization of the maize O2 (36). Similar nuclear localization sequences are found at the corresponding positions, 118–135 and 238–257 in the RISBZ1 and 118–135 and 234–254 in the RISBZ2 sequences. Given that RISBZ2, -3, -4, or -5 protein could heterodimerize at the corresponding positions, 118–135 and 238–257 in the maize O2, the rice O2-like bZIP proteins may be implicated in the regulation of target genes.

In the 5'-flanking region, a putative TATA box is located between positions -30 and -35 from the site of transcription initiation. Three ACCT motifs are found at -63, -123, and -198 from transcription initiation site, but candidate cis-elements for other types of transcription factors such as GCN4 motif or AACA motif involved in seed-specific expression are not detected. In contrast, Dof recognition sequences (AAAG) are found at many sites. Thus, these motifs may be responsible for the spatial and temporal specific expression of RISBZ1 gene. Given that these ACCT motifs are target sites of RISBZ1 activator, it is suggested that transcription of the RISBZ1 is autoregulated. However, when the chimeric gene consisting of RISBZ1 promoter and GUS reporter gene was cotransfected into rice protoplasts with a CaMV 35 S promoter/RISBZ1 as an effector, there was no significant transcriptional activation (data not shown). These results suggest that the ACCT motifs found in the RISBZ1 promoter may not be the target sequence of RISBZ1. Thus, a possible autoregulation of the RISBZ1 by its own product may be excluded. Overexpression of rice prolamin box binding factor recognizing the AAAG sequence gave rise to enhancement of transcription of RISBZ1 promoter/GUS reporter gene. Dof motif (AAAG) may be implicated in this specific regulation (data not shown).

**Tissue Specificity of RISBZ1 mRNAs**—Northern blot analysis was carried out to examine the expression pattern of these genes during plant development. Total RNA was prepared from leaves, roots, and seeds (5, 10, 15, 20, and 30 DAF), electrophoresed on agarose gel, and transferred to membrane. DNA fragments covering the gene-specific 3'-untranslated region and C-terminal region downstream of the leucine zipper region of individual cDNA was used as probes. As shown in Fig. 4, the RISBZ1 gene is specifically expressed in maturing seeds but is undetectable in other tissues. The expression of mRNA reaches a maximum level at 5–10 DAF. Such high level of mRNA continues to 15 DAF and then drops off toward seed maturation. The temporal expression pattern of the RISBZ1 is slightly different from that of the major storage protein glutelin genes whose accumulation occurs later during seed development. The glutelin mRNA is detected from 5 DAF and reaches a maximum level at 15 DAF and then gradually decreased (Fig. 4).

When accumulation levels of other RISBZ mRNAs were examined among different tissues and during seed development, it was shown that the RISBZ2 is ubiquitously expressed in all tissues examined, although the signal is weak in roots and
leaves (Fig. 4). RISBZ3 and RISBZ4 were specifically expressed in the late stages of maturing seeds (Fig. 4). Their mRNA levels gradually increase and reach a maximum level at 20 DAF, and then decrease.

RISBZ5 was weakly expressed during seed maturation compared with the other O2-like genes (Fig. 4). Its mRNA level is highest at 10 DAF and then declines.

Expression Pattern of the RISBZ1 Promoter/GUS Reporter Gene in Transgenic Rice—To assess the expression pattern of the RISBZ1 gene, flanking region between 2167 and 1213 from the site of transcription initiation was transcriptionally fused to a GUS reporter gene (Fig. 5A). This chimeric gene was introduced into the rice genome by Agrobacterium-mediated transformation. As shown in Fig. 5B, high levels of GUS activity were detected in aleurone and subaleurone layers of maturing seeds and not in the embryo tissues. Highly sensitive fluorometric assays also showed that GUS activity was not
detected in roots, leaf, stem, and anthers (data not shown). These findings indicate that the expression of the RISBZ1 gene is specified to the aleurone and subaleurone tissues. This expression pattern is in contrast with that observed for the RITA-1 (RISBZ3) gene, in which GUS activity is also detected in vascular bundles of stem and anthers, in addition to its dominant expression in aleurone and subaleurone layers of maturing seeds (20).

The function of both the translated open reading frame in the 5'-untranslated region and the 5'-untranslated sequence was examined by comparing GUS activities directed by the construct between 21674 and 14 lacking the ORF in the 5'-untranslated region as described in Fig. 5A. Irrespective of expectation, loss of uORF sequence caused 5–10-fold reduction of promoter activity without changing the expression pattern (Fig. 5C), suggesting a role of the 5'-untranslated region in quantitative regulation. This response contrasts to that observed in the maize O2, where the presence of the uORF suppresses expression (40). To examine the biological function of uORF in the RISBZ1 directly, further frameshift mutational studies of the uORF will be required.

**RISBZ1 Protein Only Activates Transcription through GCN4 Motif**—The ability of the five RISBZ proteins to activate expression from a target GCN4 motif sequence was examined by transient assays. Protoplasts derived from rice calli were transformed with each of the constructs separately or cotransformed with both constructs and GUS activity was measured. A construct consisting of the CaMV 35 S promoter fused to the individual RISBZ and maize O2 coding region was used in combination with the one and four copies of 12-bp GCN4 motif/GUS or one, three, and five copies of 21-bp GCN4 motif/GUS reporters as a positive control. As a negative control reporter gene, 4 × 12-bp or 3 × 21-bp mutagenized GCN4 motif/GUS was used. The individual 12-bp (GCTtc(C/G)TCATGA) or 21-bp (GTTTTGTCATGGCTtc(C/G)TCATG) mutagenized GCN4 differs from the normal GCN4 by two nucleotides, which is located within the RISBZ1 or O2 target site (TGA(G/C)TCA). Transfection of the reporter plasmid DNA or of the effector plasmid DNA alone resulted in weak reporter gene activity (data not shown). As shown in Table I, part A, high levels of transactivation of the reporter gene was observed only in the presence of RISBZ1 or O2 as a positive control. Introduction of mutation into the GCN4 motif of the reporter gene led to drastic reduction of transactivation activities (background level or 4% of native one). These results indicate that the RISBZ1 gene product is able to activate the reporter gene through binding to the GCN4 motif. The activation level exhibited similar or slightly higher than that of the maize O2, and increased in accordance with the copy number of GCN4 motifs. When the activation level was examined using 1–12 copies of the 21 bp GCN4 motif, GUS activity increased linearly up to 9 copies of the GCN4 motif (data not shown).

However, when other four RISBZ coding regions were expressed under the control of CaMV 35 S promoter and used as effector, they only gave rise to <5% level of that of RISBZ1 and O2 as shown in Table I, part B. It remains possible that very low transcriptional activity of these four RISBZs is due to poor protein expression. It is necessary to confirm protein expres-
sion in rice protoplasts by Western analyses. However, it should be noted that their transactivation abilities are significantly higher than the levels of negative control (reporter gene only). These results suggest that only RISBZ1 acts as a functional activator in the family of RISBZ transcription factors.

Identification of Binding Sites of the RISBZ Transcription Factors—We previously showed that the maize O2 protein recognizes the TGAGTCA GCN4 motif between positions −165 and −160 of the glutelin GluB-1 promoter (8). Using methylation interference technique, the recognition site of the rice RISBZ1 in the GluB-1 promoter was determined. As shown in Fig. 6, the GST-RISBZ1 protein protects a region between −165 and −160, indicating that this footprinted sequence is identical to the region protected using the maize O2 protein (8). There is no any other protected sites in the region between −197 and −18, although the ACGT motif (A/G hybrid box) between −79 and −76 is expected as a candidate of target site of bZIP protein.

We also examined by EMSAs whether the GCN4 motif is specifically recognized by the RISBZ1 protein. Binding of GST-RISBZ1 fusion protein to the 21-bp fragment containing the GCN4 motif was detected as a retarded band (Fig. 7B). As shown in Fig. 7A, 21-bp GCN4 motif was sequentially mutated by three bases, and then used as competitors (100-fold molar excess) in EMSAs. Retarded band was completely abolished by the presence of a 100-fold molar excess of wild type fragment. It was revealed that mutated nucleotides introduced into any parts of the GCN4 core motif (TGAGTCA) as a competitor had little or no effect on the binding of the native fragment, whereas introduction of mutations flanking the GCN4 motif led to loss of binding (Fig. 7, B–F). Taken together, the GCN4 core sequence, TGAGTCA, is critical for the binding activity of RISBZ1, and that RISBZ1 interacts with the GCN4 motif in sequence-specific manner.

Similar work was carried out with the other RISBZ proteins to examine whether they specifically recognize the GCN4 motif in the same way as the RISBZ1. As shown in Fig. 7 (B–F), the binding affinity of individual RISBZ proteins to the GCN4 motif differs slightly from one another. There was little or no competition with GCN4 oligonucleotides containing mutations in the core motif. It is interesting to note that oligonucleotides containing mutations outside of the GCN4 motif did not abolish perfectly the binding of the RISBZ2 and RISBZ5 to the native fragment (Fig. 7, C and F).

Taken together, these results indicate that all of the RISBZ proteins recognized the GCN4 motif, although with different binding affinities.

RISBZ1 Can Bind the GCN4 Motif as a Homodimer and a Heterodimer—The preceding results indicated that these five bZIP proteins interacted with the GCN4 motif. Since bZIP proteins interact with DNA as a dimer, in vitro protein dimerization assays were performed. Different portions of three cDNAs, corresponding to full-length or truncated versions of RISBZ1, RISBZ2, and RISBZ3 were transcribed and translated in vitro using a wheat germ extract (Fig. 8A). The translation products were employed in DNA binding studies. Electrophoretic mobility shift assay was utilized to discriminate homodimeric from heterodimeric complexes bound to the target 21-bp GCN4 fragments. As shown in Fig. 8B, when full-length RISBZ1 and truncated RISBZ2 or RISBZ3 were used for this experiment, they exist not only as homodimers but also as heterodimers with intermediate mobility appearing as new retarded bands. These results indicate that RISBZ1 protein heterodimerize with one or more members of the RISBZ family.

The N-terminal Region of the RISBZ1 Is Involved in Transcriptional Activation—To characterize the transcriptional activation domain of RISBZ1, transient expression system in rice callus protoplasts was used. Different portions of the RISBZ1 gene, expressed under the control of CaMV 35 S promoter, were tested for their ability to transactivate a reporter plasmid consisting of three copies of 21-bp GCN4 motif and the −46 core promoter of CaMV 35 S promoter fused to GUS reporter gene.

A progressive series of deletions in 40 amino acid increments from the N terminus to the basic domain (position 235) of the RISBZ1 protein were generated by PCR amplification. The resulting six DNA segments encoding truncated proteins, 41–436, 81–436, 121–436, 161–436, 201–436, and 235–436 were cloned into pRT100. The transactivation obtained with the RISBZ1 wild type construct was used as reference and was set as a relativity of 100%. Deletion of the first 40 amino acids from the N terminus led to a significant reduction in transactivation (21% of the intact RISBZ1), suggesting the presence of an activation domain in this region (Fig. 9). An additional three deletions to position 161 resulted in gradual decrease in activities (5% of the intact RISBZ1). When deleted to position 235 upstream of the DNA binding domain (basic domain), the activation activities were recovered to some extent (17% of the intact RISBZ1) (Fig. 9). These results suggest that the activation domain occurs mainly within the N-terminal 40-amino acid sequence.

To further examine whether the N-terminal region between positions 1 and 40 is mainly responsible for transactivation of the RISBZ1 gene expression, we carried out gain-of-function experiments by fusing the various regions of RISBZ1 to the DNA binding domain of the yeast transcriptional activator GAL4. As shown in Fig. 10, these chimeric plasmid DNA were expressed under the control of CaMV 35 S promoter and used as effector plasmids. These plasmids were cotransfected into rice protoplasts with a reporter plasmid that contained nine copies of a GAL4-binding site and the −46 core promoter of CaMV 35 S fused to GUS reporter gene. Even when the region between positions 1 and 234 was progressively deleted to position 27 from the C terminus as shown in Fig. 10, these chimeric genes still conferred high levels of activation similar to that
obtained with the longer peptide containing amino acids 1 and 234. Further deletion of 7 amino acids to position 20 resulted in background level of activity (2.5%). Furthermore, removal of 8 amino acids from the N terminus between positions 1 and 57 abolished the activity (Fig. 10). In contrast, when the C-terminal regions (positions 27–57, positions 81–234, positions 161–234, positions 235–436) were fused to the DNA binding domain of GAL4, no enhancing effect on transcription of reporter gene was evident. These results suggest that the proline-rich domain between positions 1 and 27 rather than the acidic domains is mainly implicated in transactivation of the RISBZ1.

**Difference in Activation Abilities between the RISBZ1 and the Others—** High level of transactivation ability was observed only for RISBZ1. The remaining four RISBZ proteins were not capable of transactivating the reporter gene although they were able to bind the GCN4 motif. To address the molecular mechanisms responsible for differences in transactivation abilities of the various RISBZ proteins, domain-swap experiments were carried out between the RISBZ1 and the RISBZ2 or RISBZ3. The N-terminal region of the RISBZ1 (positions 1–299) upstream of DNA binding basic domain was exchanged with the corresponding regions of RISBZ2 (positions 1–229) and RISBZ3 (positions 1–137). When the N-terminal region of the RISBZ1 was linked to the DNA binding domain of the nonfunctional RISBZ2 or RISBZ3, the transactivation activity levels increased to about 15% and 38% for that obtained for the intact RISBZ1 (Fig. 11). In light of the fact that RISBZ2 and RISBZ3 have little transactivation ability, the gain of activation ability by fusing the N-terminal region of the RISBZ1 is significant. On the other hand, when the N-terminal region of the RISBZ1 was replaced with the corresponding regions of RISBZ2 or RISBZ3, the high transactivation ability of the intact RISBZ1 was reduced to slightly higher levels (25–27% of the intact RISBZ1) than the background level (16% of the intact) obtained by the basic domain of the RISBZ1 alone (Fig. 11). The activation domain of the RISBZ1 is mainly attributed to the N-terminal region upstream of the basic domain. These results suggest that the N-terminal region is mainly responsible for activation of the expression. The low transactivation ability observed in the RISBZ2 and RISBZ3 may be accounted for by the deletion or mutations in the activation domain corresponding to that of the RISBZ1. One explanation for low activation ability of RISBZ3 is that it completely lacks the region corresponding to the proline-rich activation domain.

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**Figure 5.** Histochemical analysis of the RISBZ1 promoter/GUS reporter gene in transgenic rice seed. **A,** schematic presentation of the promoter structure of RISBZ1 fused to the GUS reporter gene. a and b, the regions between −1674 and +4 and between −1674 and +213 containing the 5′-untranslated open reading frame were transcriptionally fused to the GUS reporter gene in a binary vector and introduced into rice via Agrobacterium transfection. c, GluB-1 promoter (−245 to +18) was fused to a GUS reporter gene and introduced into rice via electroporation. **B,** histochemical analysis of GUS expression in maturing seed. a, RISBZ1 promoter (−1674 to +213); b, RISBZ1 promoter (−1674 to +4); c, glutenin GluB-1 promoter (−245 to +18) as a reference. Seeds from transgenic plants at 15 DAF were longitudinally cut and incubated in X-Gluc solution at 37 °C for 30 min for the construct a and 3 h for the constructs b and c. EN, endosperm; EM, embryo. **C,** GUS activity in extracts from maturing seeds of independent transgenic rice lines. The analysis was performed on seeds collected at 15 DAF. Promoter constructs are described in panel A (parts a and b). Vertical bars indicate mean value. MU, 4-methylumbelliferone.
found in the N-terminal region of RISBZ1 protein. Similar situation is also applicable to the RISBZ4 and RISBZ5. Another explanation is that RISBZ2 and RISBZ3 proteins have lower binding affinity to the GCN4 motif, although results of electrophoretic mobility shift assay indicate otherwise.

Although the proline-rich domain of RISBZ2 protein shares high homology to the RISBZ1 domain, RISBZ2's transactivation ability is much lower than that of RISBZ1. When the corresponding region of RISBZ2 (positions 1–27) was fused to the DNA binding domain of yeast GAL4 and cotransfected into protoplasts with GCN4 reporter gene, it had little ability to enhance transcription of the reporter gene.

To address what amino acids in this region contribute to transactivation ability, individual amino acids of the RISBZ1 protein from positions 1 to 27, which were different from the RISBZ2 protein, was substituted with corresponding amino acid from RISBZ2 protein and the region from positions 1 to 57 was fused to the GAL4 DNA binding domain. As shown in Fig. 12, only eight amino acid differences are seen in the region from positions 1 to 27 between them. These mutations with the exception of the change at position 7 (M5) resulted in loss of transcriptional activation. It should be noted that seven different amino acids cause to alternation of the hydrophobicity pattern, when analyzed by the method of Kyte and Doolittle (41) (data not shown). Taken together, it is suggested that a unique tertiary structure is required for activation ability and the alternation of structure may cause to severe effect on activation ability.

**DISCUSSION**

RISBZ1 Recognizing the GCN4 Motif Is a Functional Transcription Factor Required for Endosperm-specific Expression of Storage Protein Genes—The GCN4 motif is widely distributed in many promoters of cereal seed storage protein genes such as wheat glutenin, rye secalin, and barley hordein, as well as rice glutelin (10, 11). It was first found that the GCN4 motif functions as a seed-specific element, since multimers of GCN4 from the pea lectin gene directed seed-specific expression in transgenic tobacco (42). Involvement of GCN4 motif in endosperm-specific expression or nitrogen response has been reported for the barley hordein (4, 19), wheat low molecular weight glutenin (5), and maize 27-kDa γ-zein genes (6).

Several lines of evidence indicate that the GCN4 motif acts as a central role in determining endosperm-specific expression of rice storage protein glutelin genes (8). (a) Progressive 5' deletion of the glutelin GluB-1 gene showed that loss of the GCN4 motif resulted in background levels of promoter activities. (b) Substitutive mutation of the GCN4 motif in the minimum native promoter gave rise to remarkable reduction of promoter activities and alternation of expression pattern. (c) Multimers of a 21-bp fragment containing the GCN4 motif conferred aleurone- and subaleurone-specific expression in maturing seeds of transgenic rice, when fused to the −46 core promoter of CaMV 35 S linked to the GUS reporter gene.

The GCN4 motif is recognized by maize O2 (8, 10, 43), wheat SPA (5), and barley BLZ1 (18) and BLZZ2 (19) transcription factors. Activation of transcription by these transcriptional factors through the GCN4 motif was demonstrated in both in vitro using transient assays as well as in planta using transgenic plants. EMSAs and methylation interference experiments showed that the O2 binds to the GCN4 motif in a sequence-specific manner (8, 10). These results suggest that a bZIP protein functionally similar to O2 may exist in rice and participate in controlling the endosperm-specific expression of glutelin genes through binding to the GCN4 motif. The presence of transcription factor binding to the GCN4 motif in the rice maturing seed has been suggested by Kim and Wu (17).

Izawa et al. (20) first isolated a cDNA clone coding for a bZIP protein, RITA-1, from rice, which is significantly expressed in aleurone tissue during seed maturation. Although its expression pattern is similar to that observed for the seed storage protein genes, RITA-1 (RISBZ3) is unlikely to be involved in the expression of the glutelin genes, since its capacity to transactivate a GCN4 motif-containing reporter gene was much lower than that obtained with O2 (Table I). A cDNA clone encoding a novel bZIP protein, REB, has also been isolated from rice maturing seed cDNA library (21). Nakase et al. (21) showed that the GCCACGTc/aAG sequence in the 26-kDa α-globulin promoter, designated G/C and A/G hybrid box, is recognized by the GST-REB protein by gel retardation assays. As shown here, it should be noted that very low levels of transactivation via the GCN4 motif were also observed for this transcription factor, even when it is used as an effector (Table I, part B). Taken together, these results suggest that these two rice bZIP proteins may not be candidates for functional transactivators of expression of glutelin genes, thus suggesting that there may be other unidentified other O2-like bZIP proteins in rice maturing seeds, which may be implicated in the transcriptional activation.

Using the conserved basic domain sequences characteristic of the O2-like bZIP proteins, we screened a rice maturing seed cDNA library to isolate functional bZIP protein sharing high transactivation ability. Of O2-like bZIPs isolated, only RISBZ1

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Fig. 6. Methylation interference experiments of RISBZ1 binding to the GCN4 motif in GluB-1 promoter. The GluB-1 promoter fragment from −245 to +18 was labeled on both strands (top and bottom). The both strands were partially methylated and incubated with GST-RISBZ1 protein. Free and retarded protein-DNA complex fragments were separated by polyacrylamide gel electrophoresis. These fragments were eluted from the gel and then chemically digested with piperidine and applied on sequencing gel in parallel run with the sequencing ladder of this fragment. The sequence around the protected region is shown.
functions as a transcriptional activator at comparable or slightly higher levels of transcriptional activation via the GCN4 motif than the O2 (Table I, part A). This result suggests that the RISBZ1 is involved in gene regulation of many storage proteins. This transcriptional activator constitutes a small multigene family consisting of at least five copies per haploid genome, which includes the RITA-1 (RISBZ3) and the REB (RISBZ2). The members of this RISBZ family can be clearly classified into two groups based on their sequence similarities. It is interesting to note that the RISBZ1 protein shares high amino acid sequence homology to the REB (RISBZ2), irrespective of difference in activation ability.

When the binding site of the RISBZ1 was examined in the 5'-flanking region of the glutelin GluB-1 gene between −175 and −118 using a methylation interference experiment, it was shown that the G residue of both strands covering the GCN4 motif (TGAGTCA) between −165 and −160 was specifically protected. It is noteworthy that the ACGT motif (GTACGTGC) between −81 and −75 was not recognized by RISBZ1. This motif (A/G hybrid box), which is involved in quantitative regulation of glutelin gene (9), likely binds a different bZIP transcription factor than RISBZ1 protein. The footprinted sequence of RISBZ1 is identical to the region protected by maize O2 protein (8). Taken together, the RISBZ1 specifically binds to this site and transactivates the expression of glutelin GluB-1 gene.

Binding specificity was further confirmed by gel retardation experiments and transient expression assays using rice protoplasts. Nucleotide substitutions in the GCN4 motif not only abolish the binding, but also its response to transactivation by RISBZ1 in transient assays. Taken together, these findings indicate that the GCN4 motif is recognized by the RISBZ1 in a sequence-specific manner.

It has been studied that the maize O2 protein has a broad binding specificity (20). It can activate the expression of 22-kDa α-zein (14), 14-kDa β-zein (44), b32 (15), and cytosolic pyruvate orthophosphate dikinase (45) by interacting with the TC-CACGT(a/c)R(a/t) and GATGYRTGG sequences of their promoters. When it was examined whether these O2 target sequences are recognized by the rice RISBZ1 protein, it also highly activated the expression of 22-kDa α-zein and b32 genes.
through binding to these target sequences (data not shown). Furthermore, the RISBZ1 protein preferentially binds to G/C and A/G hybrid boxes and activates transcription (data not shown). These results indicate that RISBZ1 exhibits broad binding affinity similar to that displayed by maize O2. It is noteworthy that the RISBZ1 protein is involved in transcriptional activation of not only the glutelin genes but also other storage protein genes such as 13-kDa prolamin and 26-kDa α-globulin genes (data not shown). These rice storage protein genes contain a A/G or G/C hybrid box, which is recognized by RISBZ1 (data not shown). Participation of the RISBZ1 in regulation of several genes is comparable to that of the maize O2. Taken together, these data suggest that these activators play a general role in expression of several genes during seed maturation.

Characterization of the DNA-binding Domain—Plant bZIP proteins associate with DNA binding site as either a homodimer or heterodimer. The dimerization domains are α-helical structures characterized by a periodic repeat of leucine every seventh amino acid, which forms a parallel coiled-coil structure (46). It has been demonstrated that heterodimers between Fos and Jun (47) or between Myc and Max (48) bind their target sequence with higher affinities than either homodimeric complex. It has been demonstrated that heterodimer formation generates an expanded repertoire of regulatory potential for gene expression.

We examined whether RISBZ1 binds to its target sites in vitro as either a homodimer or heterodimer. The dimerization domains are α-helical structures characterized by a periodic repeat of leucine every seventh amino acid, which forms a parallel coiled-coil structure (46). It has been demonstrated that heterodimers between Fos and Jun (47) or between Myc and Max (48) bind their target sequence with higher affinities than either homodimeric complex. It has been demonstrated that heterodimer formation generates an expanded repertoire of regulatory potential for gene expression.

We examined whether RISBZ1 binds to its target sites in vitro as either a homodimer or heterodimer as reported in other members of the bZIP proteins. We showed that RISBZ2 (REB) or RISBZ3 (RITA-1) is able to form heterodimer complexes with RISBZ1 (Fig. 8). The dimerization capability of these transcription factors may result from a lower negative charge distribution in the zipper region as suggested by Menkes and Cashmore (49), showing that a high negative charge distribution at the "g" position close to leucines (position d) prevented the subunits from forming a heterodimer. It is noted that amino acid residues at positions d and g in zipper regions are highly conserved among RISBZ1, RISBZ2, BLZ1, OHP1, and O2. Given that most of RISBZ proteins are implicated in the regul-
Heterodimers among members of RISBZ family with different transactivation of genes may be modulated by the formation of OHP1 (32). One explanation for heterodimer formation is that shown). A similar situation has been observed for the maize no effect on activation level through the GCN4 motif (data not presented). DNA fragments encoding intact, truncated, and chimeric RISBZ proteins were cloned into pRT100 (31). pRISBZ1, pRISBZ2, and pRISBZ3 encode full-length RISBZ1, RISBZ2, and RISBZ3. pRISBZ1, pRISBZ2, and pRISBZ3 encode C-terminal region of RISBZ1 (amino acid positions 235–436), RISBZ2 (231–425), and RISBZ3 (140–298), which contains DNA binding domain. pRISBZ1–2 or pRISBZ1–3 encode N-terminal region of RISBZ1 (1–234) fused to C-terminal region of RISBZ2 (231–425) or RISBZ3 (140–298). pRISBZ1–2 or pRISBZ1–3 encode C-terminal region of RISBZ1 (235–436) fused to N-terminal region of RISBZ2 (1–230) or RISBZ3 (1–139). B and C, each of the effector constructs was transfected into rice callus protoplasts together with the 5 × 21-bp GCN4 motif/GUS reporter gene. All constructs were analyzed three times in separate experiments with similar results.

Fig. 11. Transient expression assay of chimeric RISBZ proteins between the RISBZ1 and RISBZ2 or RISBZ3. A, schematic presentation of the structure of effector plasmids. DNA fragments encoding intact, truncated, and chimeric RISBZ proteins were cloned into pRT100 (31). pRISBZ1, pRISBZ2, and pRISBZ3 encode full-length RISBZ1, RISBZ2, and RISBZ3. pRISBZ1, pRISBZ2, and pRISBZ3 encode C-terminal region of RISBZ1 (amino acid positions 235–436), RISBZ2 (231–425), and RISBZ3 (140–298), which contains DNA binding domain. pRISBZ1–2 or pRISBZ1–3 encode N-terminal region of RISBZ1 (1–234) fused to C-terminal region of RISBZ2 (231–425) or RISBZ3 (140–298). pRISBZ1–2 or pRISBZ1–3 encode C-terminal region of RISBZ1 (235–436) fused to N-terminal region of RISBZ2 (1–230) or RISBZ3 (1–139). B and C, each of the effector constructs was transfected into rice callus protoplasts together with the 5 × 21-bp GCN4 motif/GUS reporter gene. All constructs were analyzed three times in separate experiments with similar results.

Fig. 12. Site-directed mutagenesis of the RISBZ1 transactivation domain. The effector constructs contain the GAL4 DNA binding domain fused to the normal or mutagenized activation domain of the RISBZ1 between positions 1 and 40. Each effector construct was transfected into rice callus protoplasts together with the GAL4/GUA reporter gene. Amino acid residues of the RISBZ1 different from those of the RISBZ2 are underlined.

DNA binding properties. This scenario is likely to occur because all RISBZ proteins are expressed in the endosperm and can bind to the GCN4 motif. It has been demonstrated that the heterodimeric complex may have a significantly different affinity for binding site than either protein as a homodimeric complex. Heterodimerization with RISBZ1 may demonstrate the complex nature of the transcription factor interactions at the C box, G box, or A/C box element as well as GCN4 motif and contribute to the complexity of the regulatory network of expression of seed storage protein genes. Furthermore, heterodimerization between RISBZ1 and the other RISBZ proteins may contribute to the spatial and temporal expression of storage protein genes by attenuating the activation ability through heterodimeric complex formation. For instance, expression of RISBZ3 and -4 during the late stages of seed development (Fig. 4), and their dimerization with RISBZ1 may be responsible for decreased transcription of the glutelin genes during this period.

RISBZ1 Protein Has a Unique Activation Domain That Is Distinct from Other Plant bZIP Proteins—Transcriptional activation domains have been classified into acidic, Gln-, Pro-, and Ser/Thr-rich domains based on their amino acid compositions (50). In contrast to the DNA binding domains, the amino acid sequences comprising these activation domains are not conserved. The domains responsible for transcriptional activity have been defined in detail for the maize O2 (51). A single acidic domain between amino acids 63 and 74 is the main contributor for transcriptional activation. The activation domains of the barley BLZ1 and BLZ2 are also located in the N-terminal region, although not identified to the amino acid level (18, 19). Although sequences rich in acidic amino acids are found at the corresponding regions in all the RISBZ proteins, our gain-of-function assays using the GAL4 DNA binding domain clearly indicated that these regions are not involved in gene activation. In contrast, it was demonstrated that the proline-rich domain in the N-terminal 27 amino acids of RISBZ1 protein functions as transcriptional activation domain. However, it is interesting to note that the corresponding region of the RISBZ2 is not able to transactivate transcription, although it shares high degree of homology to that of the RISBZ1 protein (conservation of 19 out of 27 amino acids). Substitutive mutational analysis of the eight amino acids that differ between RISBZ1 and RISBZ2 revealed that changes of seven of the eight amino acids completely abolished transactivation ability, suggesting that overall conformation (tertiary structure of this region) is required for function (Fig. 12). The requirement of this whole domain is further confirmed by loss of activation ability by deletion of an 8-amino acid N-terminal peptide (positions 1–8). Furthermore, it is noteworthy that
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