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The Rice bZIP Transcriptional Activator RITA-1 Is Highly Expressed during Seed Development

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Systematic protein–DNA binding studies have shown that plant basic leucine zipper (bZIP) proteins exhibit a differential binding specificity for ACGT motifs. Here, we show that the rice transcription activator-1 (RITA-1) displays a broad binding specificity for palindromic ACGT elements, being able to bind A-, C-, and G-box but not T-box elements. By using gel mobility shift assays with probes differing in sequences flanking the hexameric core, we identified high-affinity A-, C-, and G-box binding sites. Quantitative and competition DNA binding studies confirmed RITA-1 specificity for these sites. Using rice protoplasts as a transient expression system, we demonstrated that RITA-1 can transactivate reporter genes possessing high-affinity but not low-affinity RITA-1 binding sites. Our results established a direct relationship between in vivo transactivation and in vitro binding activity. Transient expression assays that demonstrated the ability of RITA-1 to transactivate a construct containing *rita-1* 5′ flanking sequences suggest that the factor may be autoregulated. Histochemical analysis of transgenic rice plants showed that a *rita-1*–β-glucuronidase transgene is expressed in aleurone and endosperm cells of developing rice seeds. We propose that RITA-1 plays a role in the regulation of rice genes expressed in developing rice seeds.

INTRODUCTION

In plants, basic leucine zipper (bZIP) proteins defined by a structural motif composed of a leucine zipper dimerization domain and a positively charged DNA interface comprise the largest known family of DNA binding proteins (Harrison, 1991). To date, at least 22 cDNA clones encoding bZIP proteins have been isolated from no fewer than five different plant species (Foster et al., 1994). The homodimeric form of these recombinant proteins exhibits differential binding specificity for DNA sequence motifs with a symmetrical dyad ACGT core. Systematic protein–DNA binding studies have shown that nucleotides flanking the ACGT core affect bZIP protein binding specificity and affinity (Izawa et al., 1993). Based on these studies, we have defined nomenclature for ACGT elements according to the nucleotide present at position +2 for each half site of the symmetrical dyad sequence motif (Foster et al., 1994). Following the positional nomenclature adopted for the yeast GCN4 binding site (Oliphant et al., 1989), the central two nucleotides, C and G, flanking the axis of symmetry are designated −0 and +0, respectively. For example, the palindromic binding site for the Arabidopsis GBF, CACGTG, is a G-box, whereas the nonpalindromic Opaque2 (O2) binding site in the 22Z-4 zein gene, CACGTA, is a G/A hybrid composed of 5′ G-box and 3′ A-box half sites.

Three classes of ACGT elements, A-boxes, C-boxes, and G-boxes, can productively interact with 10 different plant bZIP proteins (Izawa et al., 1993). Based on qualitative and quantitative protein–DNA binding studies, these factors can be classified according to their preferences for high-affinity C-box and G-box binding motifs. Group 1 factors, which include EmBP-1 (Guiltinan et al., 1990), histone DNA binding protein-1a (HBP-1a; Tabata et al., 1989), common plant regulatory factor-1 (CPRF-1), CPRF-3 (Weisshaar et al., 1991), TAF-1 (Oeda et al., 1991), and octopine synthase element binding factor-1 (OCSBF-1; Singh et al., 1990), bind both G-box and C-box elements but prefer the G-box motif; group 2 members CPRF-2, O2 (Hartings et al., 1989; Schmidt et al., 1990), and TGACG sequence–specific binding protein-1b (TGA1b; Katagiri et al., 1989) bind both G-box and C-box motifs with similar binding affinity; and TGA1a (Katagiri et al., 1989), the only group 3 factor, prefers the C-box element. Notwithstanding their differential binding affinity for these elements, the factors exhibit a similar binding specificity (i.e., all 10 factors preferred G-box and C-box elements with the same flanking nucleotides at positions −4/+4 and −3/+3).

Although these studies have defined the in vitro DNA binding affinity and specificity for 10 factors, the functional relevance of the binding sites in vivo has not yet been evaluated. To date, only five plant bZIP factors have been shown to activate transcription of fusion genes possessing ACGT binding sites in their 5′ flanking regions. For example, the tobacco TGA1a

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protein can function as a transcriptional activator to increase the number of active preinitiation complexes in cell-free assays (Katagiri et al., 1990; Yamazaki et al., 1990). Likewise, TAF-1, O2, GBF1, and PosF21 have been shown to transactivate cotransfected reporter constructs in transient expression assays (Aeschbacher et al., 1991; Lohmer et al., 1991; Oeda et al., 1991; Schindler et al., 1992c; Schmidt et al., 1992; Ueda et al., 1992). In all these studies, however, only one or two binding sites were used; consequently, it was not known whether all binding sites are active in vivo and whether there is a correlation between binding affinity and transcriptional activation.

Considering that transcription factors are functional only in those cell types in which they are expressed, the spatial and temporal expression pattern for a particular bZIP protein should provide valuable insight concerning potential target genes. Although RNA gel blot analyses have been performed with TGA1a, OCSBF-1, GBF3, and TAF-1 (Katagiri et al., 1989; Singh et al., 1990; Oeda et al., 1991; Schindler et al., 1992b), O2 is the only bZIP protein for which tissue-specific expression has been described. Immunochemical studies have shown that the O2 protein is present in endosperm and subaleurone cells of maize seeds reaching a maximum at 18 days after pollination (Varagona et al., 1991). Coupled with genetic analyses, protein–DNA binding experiments, and transient expression assays, these studies have firmly established a functional correlation between O2 and the expression of 22-kD zein storage protein genes, suggesting that this factor plays a role in maize seed development.

In this paper, we describe the isolation and characterization of a novel rice bZIP transcription factor, RITA-1. Quantitative and qualitative protein–DNA binding experiments showed that RITA-1 has a broad binding specificity for ACGT elements. Using transient expression assays, we demonstrated a direct relationship between high-affinity ACGT binding sites and the level of transcriptional activation mediated by these sites. Moreover, the ability of RITA-1 to transactivate a reporter gene linked to *rita-1* 5′ flanking sequences suggests that transcription of the *rita-1* gene is autoregulated. Analysis of transgenic plants carrying a *rita-1*–β-glucuronidase (*GUS*) transgene showed that *rita-1* is expressed in developing endosperm, especially in aleurone layer cells, suggesting that this factor may be involved in the regulation of rice genes expressed during seed development.

RESULTS

Cloning the RITA-1 cDNA

The isolation of more than 22 bZIP proteins from a wide array of plant species suggests that this prominent family of DNA binding proteins plays an essential role in the regulation of plant gene expression. To investigate how these factors may function in regulating genes in the agronomically important crop rice, we screened a rice cDNA library with an oligonucleotide probe derived from the basic region of the wheat EmBP-1 bZIP protein (Guiltinan et al., 1990). Two overlapping cDNA clones encoding a bZIP protein were isolated; this protein was designated RITA-1. Figure 1 shows the consolidated nucleotide sequence of the cDNA clones and the encoded amino acid sequence of RITA-1, which has an M_r of 31,700.

The primary amino acid sequence of the RITA-1 bZIP domain shows striking sequence homology with those of other plant bZIP proteins. For example, the RITA-1 basic domain, amino acids −26 to −1 (N terminus distal to the first leucine of the zipper region), has 19 of 26 amino acids identical to that of the parsley CPRF-2. RITA-1, like group 1 and group 2 bZIP proteins, has a serine at residue −15 of the basic domain. Protein–DNA binding studies have shown this residue to be important for plant bZIP DNA binding specificity (Suckow et al., 1993). In the leucine zipper region, distinct homology other than the heptad hydrophobic repeats can be noted between RITA-1 and three other bZIP proteins: 63% identity with O2

GGGCAGCTCAGCTTTGTGGGGGGCGAAAAAGAGGAGCTTTCCCTTCTCCTTCTTCTCCTT 60

CTCCTCCCTCGGTGATCTGCCTCGCTGGGGCATCTCTAAAAGCATCCGACTCCGACGCCA 120

CCACCACCAGTTCACCACCTCTGGAAGAATCAGCAGCAGCAGCAGAGACCACACGAGGAG 180

ATGAAGAAGTGCCCGTCGGAGCTGAACTTCGAGGCGTTCTTCCACGGCGAGAGGGGGGAG 240
M K K C P S E L N F E A F F H G E R G E 20

GACGACGCCGACGCCGCCGCCGACCAGAAGCCCGGCGGCGGCCCGCACCCGCCGCCGTTC 300
D D A D A A A D Q K P G G G P H P P P F 40

GCCATGTTCTCCGCCGCCGACCTCTCCAGCTTCGGCTTCGCGGACTCGGTGACGAGCACC 360
A M F S A A D L S S F G F A D S V T S T 60

ATCACTGGGGTCATTCCCAATCACATATGGCCCCAGTCCCAGAGCCTCAACGCACGGCAT 420
I T G V I P N H I W P Q S Q S L N A R H 80

CCTGCAGTCTACACAATTGAGTCGCAATCATCAATCTGTGCAGCAGCAAGTCCCACGTCA 480
P A V Y T I E S Q S S I C A A A S P T S 100

GCTACCACTCTGAACATGAAGGAGAGCCAAACTCTGGGAGGCACAAGTGGTTCAGATTCT 540
A T T L N M K E S Q T L G G T S G S D S 120

GACAGTGAGTCGCTGTTGGATATAGAGGGCGGTCCATGCGAACAAAGCACAAACCCACTG 600
D S E S L L D I E G G P C E Q S T N P L 140

GACGTTAAGAGAATGAGAAGGATGGTTTCCAACCGGGAGTCTGCTCGTCGATCAAGGAAG 660
D V **K R M R R M V S N R E S A R R S R K** 160

AGAAAGCAAGCTCACTTAGCTGATCTCGAGACGCAGGTTGACCAGCTCCGGGGCGAAAAC 720
R K Q A H L A D **L** E T Q V D Q **L** R G E N 180

GCATCGCTTTTCAAGCAGTTGACAGATGCCAATCAGCAATTCACGACGGCGGTCACGGAC 780
A S **L** F K Q L T D **A** N Q Q F T T **A** V T D 200

AACAGAATCCTCAAATCAGACGTGGAGGCCCTCCGGGTCAAGGTGAAGATGGCGGAGGAC 840
N R I **L** K S D V E A **L** R V K V K M **A** E D 220

ATGGTGGCGCGGGGGGCGCTGTCGTGCGGCCTCGGCCACCTGGGCGGGCTGTCGCCGGCG 900
M V A R G A L S C G L G H L G G L S P A 240

CTGAACCCCCGGCAGGGGGCGTGCCGCGTCCCCGACGTGCTCACCGGGCTGGACTACGCC 960
L N P R Q G A C R V P D V L T G L D Y A 260

GGCGACGACCCCTTCACCGGGCTGTCCCCGCCGGAGCAGGTGCAGATGCCCGGCGGTGGC 1020
G D D P F T G L S P P E Q V Q M P G G G 280

GAGGTGGGTGACGCCTGGGGCTGGGACAACCACTCCAATGGCGCCATGTCCAAGTGAAAA 1080
E V G D A W G W D N H S N G A M S K 298

CTACTTCTTACTGCTTTGTCAGCTCAGCTACGTATAAAAATGTGATGTCCCAAGTGAACG 1140

GACTTGAGTTTTTCAGAGTCCTCGTGTCGTGTCAAGTGTCGTCCTGTAATAGAACTGACT 1200

AGCTATATATAGTACACAGTATTTGG 1226

Figure 1. Primary Structure of the RITA-1 cDNA.

The cDNA encodes an open reading frame of 298 amino acids. The basic region and the heptad leucine repeats of the bZIP region are shown in boldface letters and underscored. Sequence data has been submitted to GenBank as accession number L34551.

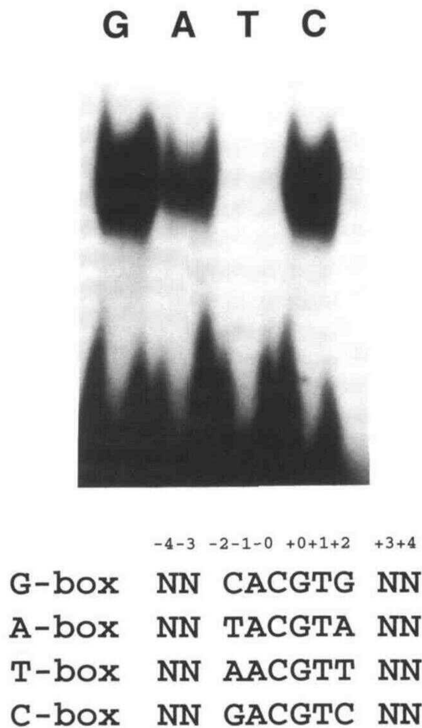

Figure 2. RITA-1 Binds G-Box, A-Box, and C-Box ACGT Elements.

Results of gel mobility shift analyses using recombinant RITA-1 and G-box, A-box, T-box, and C-box oligonucleotide probes are shown. Sequences of G-box, A-box, T-box, and C-box oligonucleotide probes are given below the gel. Numbers above the sequences of the oligonucleotide probes designate nucleotide positions of the symmetrical dyad ACGT motif relative to that described for the yeast GCN4 factor (Oliphant et al., 1989). N indicates random nucleotides— G, A, T, or C.

heterodimerizing protein 1 (OHP1; Pysh et al., 1993), 62% identity with CPRF2, and 49% identity with O2. However, RITA-1 does not exhibit significant homology with any other reported plant bZIP protein outside the bZIP region, suggesting that this factor is not homologous to any of the plant bZIP proteins isolated to date. Genomic DNA gel blot analysis using different restriction enzymes detected only one or two major bands, suggesting that RITA-1 is encoded by a single gene (data not shown).

RITA-1 in Vitro DNA Binding Specificity

As an initial step toward defining potential RITA-1 target genes, we sought to identify ACGT elements for which this factor exhibits a strong binding affinity. To delineate RITA-1 binding specificity, we investigated the effects of nucleotides at positions −4/+4, −3/+3, and −2/+2 on binding affinity. To test positions −2/+2, we designed oligonucleotide probes that contained a common ACGT central core, one of the four possible nucleotide pairs at position −2/+2, and degenerate nucleotides at positions −4/+4 and −3/+3. Because nucleotides at positions −4 and −3 of the G-box motif affected binding activity (Williams et al., 1992), degeneracy at these positions would assay all possible nucleotide combinations. Figure 2 shows that RITA-1 can interact with the G-box, A-box, and C-box probes, but not with the T-box probes.

To systematically analyze positions −4/+4 and −3/+3, we used a battery of palindromic G-box, C-box, and A-box elements containing all possible nucleotide combinations at these flanking positions. Figure 3A shows that of the 16 different G-box oligonucleotides tested, the RITA-1 protein exhibited the strongest binding affinity for GGC and GTC probes and a somewhat weaker affinity for GTA and GTT (oligonucleotide probes are designated by the nucleotides at positions +2, +3, and

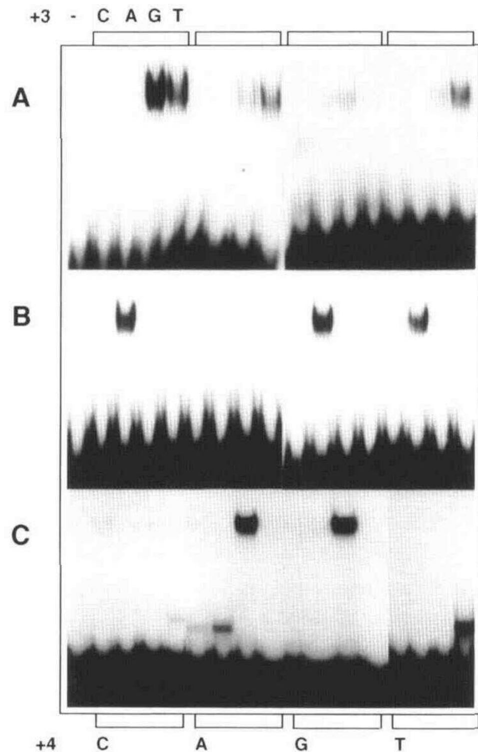

Figure 3. Nucleotides Flanking G-Box, C-Box, and A-Box Hexameric Core Sequences Affect RITA-1 Binding Activity.

(A) G-box probes.
(B) C-box probes.
(C) A-box probes.
Gel mobility shift assays of RITA-1 were conducted with 16 different palindromic G-box, C-box, and A-box probes. The probes were constructed such that all possible nucleotide pairs filled positions −4/+4 and −3/+3. The nucleotide at position +3 is indicated at top and that for position +4 at bottom. For example, the first G-box probe used in **(A),** GCC, has nucleotides CC in positions +3/+4, and the sequence of the entire G-box motif is GG CACGTG CC. The first lane at left, which is designated (−), employed the mutant ACGT probe GC CAATTG GC.

+4). Much weaker protein–DNA complexes were detected with the GGA, GAG, GGG, and GGT probes. RITA-1 binding specificity, however, was much more stringent for the battery of C-box and A-box palindromic probes. Of the 16 C-box and A-box probes tested, RITA-1 could only productively interact with three C-box (Figure 3B) and two A-box probes (Figure 3C): CAC, CAG, and CAT and AAG and AGG, respectively. For C-box binding, an adenine at position +3 (T at −3) is absolutely required, whereas A-box binding shows a clear preference for guanine and adenine at the position +3 (C and T at −3) and an exclusive preference for guanine at the position +4 (C at −4). Taken together, these results indicate that the RITA-1 protein can interact with G-box, C-box, and A-box elements flanked by specific sequences at positions −4/+4 and −3/+3.

Qualitative competition gel mobility shift assays and quantitative protein–DNA binding studies were undertaken to evaluate RITA-1 binding affinity for different palindromic and nonpalindromic ACGT elements. Figure 4 shows that RITA-1 displayed a strong binding affinity for the palindromic GGC G-box and the CAC C-box elements and the nonpalindromic G/A hybrid element and a somewhat weaker binding affinity for the palindromic AAG A-box element and the nonpalindromic G/C and A/C hybrid motifs. Saturating gel shift assays, in which a constant amount of protein extract was titrated against an increasing amount of DNA probe (Izawa et al., 1993), were performed to determine RITA-1 binding affinity for the GGC, CAC, and AAG probes. We found that RITA-1 was able to interact most strongly with the GGC probe, with an apparent dissociation constant of 27 nM. A somewhat lower binding affinity was observed for both the high-affinity CAC C-box and AAG A-box probes, with dissociation constants of 97 and 86 nM, respectively (data not shown). Overall, these results indicate that the RITA-1 protein can interact with hybrid ACGT elements comprised of high-affinity half sites with an affinity similar to that for palindromic binding sites.

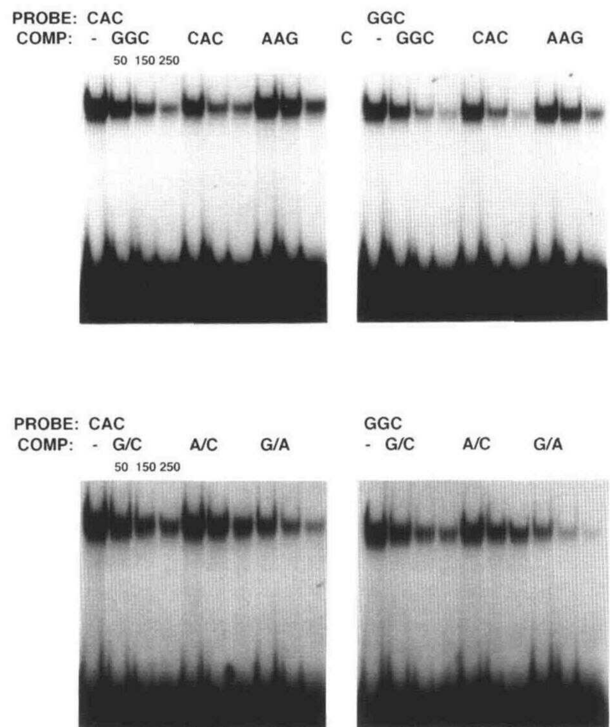

Figure 4. RITA-1 Shows a Strong Binding Affinity for High-Affinity Palindromic and Nonpalindromic ACGT Elements.

The probes and competitors are designated by nucleotides at positions +2, +3, and +4. GGC represents a palindromic G-box oligonucleotide, GCCACGTGGC, CAC a palindromic C-box, GTGACGTCAC, and AAG, a palindromic A-box, CTTACGTAAG. The nonpalindromic competitors are composed of combination half sites with the following sequences: G/C, GCCACGTCAC; A/C, CTTACGTCAC; and G/A, GCCACGTAAG. The left-hand lane of each gel shows the protein–DNA complex formed with the probe in the absence (−) of competitor, whereas the successive three lanes represent those upon the addition of 50-, 150-, and 250-fold molar excess of competitor DNA. Probe, competitor (COMP), and fold molar excess are depicted above the gels.

In Vivo Transactivation of High-Affinity ACGT Motifs by RITA-1

Although many recombinant plant bZIP proteins have been shown to interact with more than one ACGT element in vitro, the transcriptional activation properties of these different DNA motifs in vivo have not been addressed. We have employed a protoplast transient expression assay to examine whether ACGT elements that exhibit high in vitro binding affinity for RITA-1 can mediate transcription in response to the factor in vivo. Rice protoplasts derived from the undifferentiated Oc cell line (Baba et al., 1986) were cotransformed with *GUS* reporter and RITA-1 effector constructs. Reporter plasmids were constructed by ligating tetramers of high- and low-affinity RITA-1 ACGT binding sites to the minimal cauliflower mosaic virus (CaMV) −46 35S promoter fused to a *GUS* reporter gene (Benfey et al., 1989; Tanaka et al., 1990). The effector construct consisted of the full-length CaMV 35S promoter fused to the RITA-1 cDNA. Table 1 shows that the RITA-1 effector was able to activate reporter plasmids carrying high-affinity palindromic C-box, G-box, and A-box motifs as well as nonpalindromic G-box/C-box, C-box/A-box, and A-box/G-box elements. By contrast, reporter constructs that contained mutated or low-affinity ACGT elements were not responsive to RITA-1. Of the reporter constructs tested, the palindromic GGC G-box and CAC C-box elements and the nonpalindromic C/A hybrid element were the most responsive to the RITA-1 effector, whereas the palindromic A-box and nonpalindromic A/G motif were the least responsive. These results clearly demonstrate that RITA-1 is a transcriptional activator that can activate the expression of reporter genes whose 5′ flanking regions possess high-affinity binding sites for this factor. Because fusion genes that contained high-affinity RITA-1 binding sites were the most

responsive to RITA-1, there appears to be a direct correlation between in vitro binding affinity and in vivo transcriptional activation.

Having established a direct relationship between in vitro binding activity and in vivo transcriptional efficiency, it should be possible to identify putative target genes for RITA-1. To this end, we examined the effect of a RITA-1 effector construct on reporter genes containing promoters from two bona fide rice genes, *rita-1* and *waxy*. Table 2 shows that the 5′ flanking region of the *rita-1* promoter used contains three G-box motifs, whereas the *waxy* 5′ sequences contain a single C/G hybrid ACGT element. Our in vitro DNA binding studies predict that RITA-1 would bind two of the *rita-1* G-box motifs, those situated at −305 and −36 nucleotides upstream of the transcription initiation site, but not the one at position −222 nor to the *waxy* C/G hybrid ACGT element. The predictions for the *rita-1* G-box motifs were confirmed by in vitro binding assays, showing that RITA-1 can bind the two predicted high-affinity G-box elements within the *rita-1* 5′ flanking sequence but not to their mutant derivatives (data not shown). Moreover, transient transfection studies in which rice Oc protoplasts were cotransfected with reporter genes containing promoter sequences from the *rita-1* and *waxy* genes and the RITA-1 effector construct indicated that RITA-1 was able to transactivate the *rita-1* reporter construct by about fourfold but not the *waxy* fusion gene (Table 2). Overall, these studies suggest that high-affinity RITA-1 binding sites may

Table 1. Transactivation of Reporter Genes Possessing ACGT Tetramers by RITA-1 in Rice Oc Protoplasts

			GUS Activity[a]		
Reporter[b]	ACGT Sequence	Exp.	−RITA-1	+RITA-1	Fold Induction
MU[c]	GC CAATTG GC	1	0.76	1.72	2.3
		2	1.68	3.12	1.9
		3	2.65	2.62	1.0
CTC (Low-affinity C-box)	GA GACGTC TC	1	33.6	61.4	1.8
		2	41.8	76.8	1.8
		3	108.5	154.1	1.4
CAC (High-affinity C-box)	GT GACGTC AC	1	13.7	220.1	16.1
		2	5.6	91.0	16.2
		3	5.5	163.3	29.6
GGC (High-affinity G-box)	GC CACGTG GC	1	1.8	45.9	26.0
		2	2.3	35.1	15.0
		3	2.5	40.7	16.3
AGG (High-affinity A-box)	CC TACGTA GG	1	0.29	3.3	11.5
		2	0.52	3.0	5.8
		3	1.43	4.2	2.9
MU2[c]	GC CGCGTG GC	4	55.0	59.4	1.1
		5	47.3	78.7	1.7
		6	44.7	71.1	1.6
hex-1[d] (G/C hybrid)	GC CACGTC AC	4	41.1	465.3	11
		5	52.1	472.5	9.1
		6	63.1	921.1	15
Distal ACGT motif[e] of the activator element sequence-1 (C/A hybrid)	CT GACGTA AG	4	5.4	251.1	47
		5	9.3	213.1	23
		6	7.0	185.6	27
Motif I[f] (A/G hybrid)	GG TACGTG GC	4	39.5	206.8	5.2
		5	40.3	181.8	4.5
		6	34.0	215.4	6.3

[a] GUS activities are given in picomoles of 4-methylumbelliferone per minute per milligram of soluble protein.
[b] For reporter genes, tetramers of the described ACGT elements were ligated upstream of a minimal CaMV 35S promoter (−46 to +8) fused to the *GUS* coding sequence.
[c] MU and MU2 represent mutant ACGT elements. MU2, a nonpalindromic point-mutated GGC G-box element, is found in the 5′ flanking region of the rice *rab16B* gene.
[d] The hex-1 site found in the 5′ flanking region of the wheat histone H3 gene is comprised of G-box and C-box half sites.
[e] The CaMV 35S *as-1 cis*-element is composed of two ACGT motifs.
[f] Rice *rab16A* to *rab16D* genes contain motif I sequences.

Table 2. Transactivation of Rice Promoters by RITA-1 in Rice Oc Protoplasts

Promoter	ACGT Motifs	Fold Induction[a]
rita-1[b]	AC CACGTG GC −305 CG CACGTG AC −222 CG CACGTG GC −36	3.86 ± 1.02
waxy	CT GACGTG CG −163	0.4 ± 0.21
No reporter		0.81 ± 0.32

[a] Fold inductions, determined from the ratio of activity in the presence and absence of the RITA-1 effector. Standard deviations for each promoter construct represent the means of a minimum of 13 individual transfection trials. GUS activity is presented as picomoles of 4-methylumbelliferone per minute per milligram of soluble protein.
[b] The *rita-1* and *waxy* reporter genes were constructed as described in Methods.

function in vivo and suggest that the *rita-1* gene may be autoregulated in a positive manner.

The *rita-1* Gene Is Expressed in Aleurone Cells

In addition to their requirement for high-affinity RITA-1 binding sites, putative RITA-1 target genes should be transcriptionally active in cell types that express the RITA-1 protein. To determine whether the *rita-1* gene is expressed in a cell-type–specific manner, we examined the temporal and spatial expression pattern directed by its promoter. Transgenic rice plants carrying a *rita-1–GUS* transgene were generated by protoplast transformation (Shimamoto et al., 1989). Gel blot analysis of genomic DNA confirmed two independent transgenic plants, 3-4 and 10-1 (data not shown). Table 3 shows that the *rita-1–GUS* transgene was expressed at a low level in both leaves and roots but at a much higher level in seeds. By contrast, very low levels of GUS activity were detected in all three organs of a nontransgenic control plant, 4-1. RNA gel blot analysis indicates that *rita-1* is expressed in all plant organs, although the level of mRNA accumulation is somewhat higher in seeds (data not shown).

To ascertain which cells of the developing rice seeds expressed the transgene, GUS was localized by histochemical staining (Jefferson et al., 1987). Figures 5A, 5C, 5D, and 5E show that significant GUS activity was observed in aleurone cells at 2 weeks after pollination. Thereafter, GUS activity gradually appeared in the subaleurone layer as the endosperm matured (Figures 5B and 5F). Some GUS activity was also detected in pericarp vascular bundles (Figure 5C). In mature seeds, a low level of GUS expression was detected in most parts of the seed, including the endosperm and scutellum but not in other regions of the embryo (Figures 5B and 5F). At this stage of development, only a very low level of the GUS activity is present in the aleurone layer. In some tissue samples of line 3-4, weak GUS staining was also observed in vascular bundles of the anther and lodicle (data not shown). A similar pattern of GUS expression was detected in the other transgenic line, 10-1, where weak staining was also seen in the vascular bundles of stem at the bottom node. No GUS staining was observed in any tissue at any developmental stage of the nontransgenic line 4-1. RNA gel blot analysis indicated that the temporal expression pattern of the endogenous *rita-1* gene in developing rice seeds is very similar to that of the *rita-1–GUS* transgene. Figure 6 shows that maximal RITA-1 mRNA levels were detected 6 days after pollination. Collectively, these studies clearly demonstrate that the *rita-1* gene is expressed in aleurone and endosperm cells of developing rice seeds and that this bZIP protein is developmentally regulated.

DISCUSSION

RITA-1 ACGT Binding Specificity

In this study, we describe the isolation and characterization of a novel rice bZIP transcriptional activator, RITA-1. Protein–DNA binding studies have shown that RITA-1 possesses a broad binding specificity displaying a strong binding affinity for A-box, C-box, and G-box palindromic and nonpalindromic hexamers. Ten plant bZIP factors have been categorized according to their binding specificity for high-affinity G-box and C-box elements (Izawa et al., 1993): those that prefer G-box elements (group 1), those specific for C-box elements (group 3), and a cross-over group that can interact with both classes of ACGT elements (group 2). Of the bZIP factors examined, only O2, CPRF-2, and TGA1a were able to interact with A-box elements. Compared to these 10 bZIP proteins, RITA-1 DNA binding specificity most closely resembles that of O2 and CPRF-2, both belonging to group 2.

Table 4 compares RITA-1, CPRF-2, and O2 binding sites. Like O2 and CPRF-2, RITA-1 shows a strong binding affinity for the same two palindromic G-box elements, GGC and GTC. However, it differs from these two factors with respect to low-affinity G-box binding sites. Whereas RITA-1 shows a moderate binding affinity for GTT and a weaker affinity for GGA, O2 and CPRF-2 display a reciprocal preference. RITA-1, like the

Table 3. GUS Activity in Transgenic Plants

Line	Root	Leaf	Seed
4-1	0.87	0.26	1.93
3-4	16.5	40.9	772
10-1	35.1	87.3	250

GUS activities in picomoles of 4-methylumbelliferone per minute per milligram of soluble protein from three independent transgenic rice plants are shown. Average values of two independent measurements are presented. Line 4-1 is a transgenic negative control.

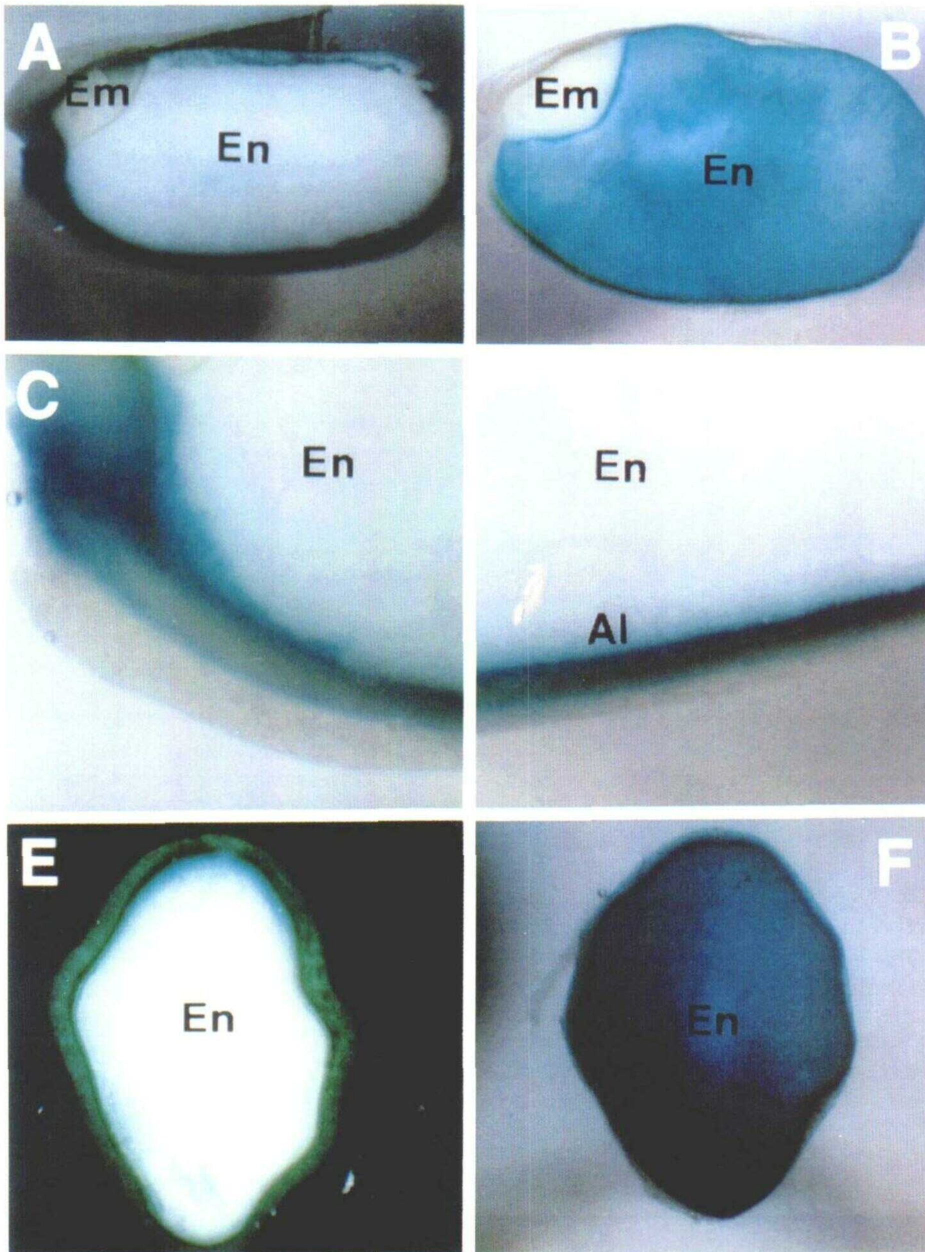

Figure 5. Histochemical Localization of *rita-1–GUS* Expression in Seeds of Transgenic Rice Plants.

The transgenic rice plants carried a fusion gene comprised of *rita-1* 5′ flanking sequences and the *GUS* coding sequence.

(A) Longitudinal section of an immature seed 2 weeks after pollination.

(B) Longitudinal section of a mature seed 4 weeks after pollination.

(C) Magnification of the region just below the embryo shown in **(A)**.

(D) Magnification of the aleurone layer shown in **(A)**.

(E) Cross-section of an immature seed at 2 weeks after pollination.

(F) Cross-section of a mature seed at 4 weeks after pollination.

Immature seeds are shown 5 hr after GUS staining, whereas those of mature seeds are shown 16 hr after GUS staining. En, endosperm; Em, embryo; Al, aleurone layer.

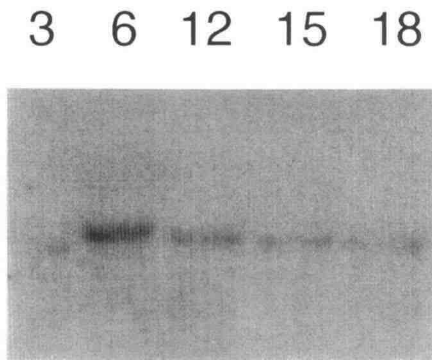

Figure 6. RNA Gel Blot Analysis of RITA-1 mRNA in Developing Labelle, an Indica Cultivar, Rice Seeds.

Total RNA (40 μg) was hybridized with the full-length c61 RITA-1 cDNA. Numbers above each lane indicate days after flowering.

10 other plant bZIP proteins examined, is specific for class I G-box elements (Foster et al., 1994). Of the 16 possible palindromic C-box elements, only three, CAC, CAG, and CAT, are able to productively interact with RITA-1, CPRF-2, and O2. A situation similar to that observed for G-box elements exists for A-box binding specificity. Whereas RITA-1, CPRF-2, and O2 show a strong binding affinity for a common A-box element, AAG, only RITA-1 can recognize an additional A-box motif, AGG. It should be noted, however, that quantitative DNA binding studies have shown that O2 has a stronger binding affinity for AAG than CPRF-2 does (R. Foster, unpublished data). Thus, although the RITA-1 basic region is more homologous with that of CPRF-2 than O2, RITA-1 binding affinity, with respect to A-box elements, is more similar to O2. It may be that amino acids outside the basic region can also influence bZIP binding affinity.

Although plant bZIP proteins differ in their binding specificity and affinity for A-box, C-box, and G-box elements, they prefer palindromic A-box, C-box, and G-box motifs with the same flanking sequences at positions −4/+4, −3/+3, and −2/+2. Thus, it would appear that the first order of plant bZIP protein specificity for ACGT elements is manifested at the level of binding affinity for that element. However, because the specificity of these factors for low-affinity G-box and A-box binding sites differs, it may be possible that these differences in binding specificity reflect in vivo function. Moreover, it is possible that the status of low-affinity binding sites can be elevated to high-affinity sites by other cellular mechanisms (i.e., heterodimerization and post-translational modification). Although CPRF and GBF bZIP proteins have been shown to heterodimerize, the effects upon binding specificity and affinity have not yet been examined (Armstrong et al., 1992; Schindler et al., 1992a, 1992b).

RITA-1 Can Transactivate Fusion Genes That Possess High-Affinity Binding Sites

Transient expression assays have shown that RITA-1 can function as a transcriptional activator. Fusion genes containing tetramers of either high-affinity palindromic or nonpalindromic RITA-1 binding sites were responsive to the cotransfected RITA-1 effector. By contrast, constructs containing a low-affinity C-box element or mutated G-box elements that lack ACGT cores were not active. Previous studies that have addressed the transactivation potential of TGA1a, TAF-1, O2, and PosF21 bZIP proteins had used fusion genes that contained only one type of ACGT element (Katagiri et al., 1990; Yamazaki et al., 1990; Aeschbacher et al., 1991; Lohmer et al., 1991; Schmidt et al., 1992; Ueda et al., 1992). Our results here establish a direct relationship between in vitro DNA binding affinity and in vivo transactivation efficiency.

Although all reporter constructs containing high-affinity RITA-1 binding sites were transactivated by RITA-1, as represented by the fold induction ratio, variable levels of expression were observed in the absence of the effector construct. This variation may be a result of interference from endogenous bZIP proteins present in Oc protoplasts that can recognize and transactivate specific reporter constructs. Schindler et al. (1992c) have reported that a construct containing ribulose bisphosphate carboxylase small subunit (*rbcS*) G-box elements exhibited a high level of expression in transient transfected soybean protoplasts in the absence of the GBF1 effector construct. Because bZIP proteins can form heterodimers (Armstrong et al., 1992; Schindler et al., 1992a, 1992b; Pysh et al., 1993), it is also possible that the transactivation potential of RITA-1 is modulated by the formation of heterodimers with endogenous Oc bZIP proteins. This would explain the ability of RITA-1 to transactivate the *rab16A* motif I, a relatively low-affinity RITA-1 binding site for the RITA-1 homodimers. Weisshaar et al. (1991)

Table 4. Comparison of ACGT Elements that Bind RITA-1, CPRF-2, and O2

Elements	RITA-1	O2/CPRF-2
G-box	GC CACGTG GC	GC CACGTG GC
	GA CACGTG TC	GA CACGTG TC
	TA CACGTG TA (m) [a]	TA CACGTG TA (m)
	AA CACGTG TT (m)	TC CACGTG GA (m)
	TC CACGTG GA (w)	AA CACGTG TT (w)
	CT CACGTG AG (w)	CT CACGTG AG (w)
	CC CACGTG GG (w)	CC CACGTG GG (w)
	AC CACGTG GT (w)	
C-box	GT GACGTC AC	GT GACGTC AC
	CT GACGTC AG	CT GACGTC AG
	AT GACGTC AT	AT GACGTC AT
A-box	CT TACGTA AG	CT TACGTA AG
	CC TACGTA GG	AT TACGTA AT (m)
		CA TACGTA TG (m)
		CC TACGTA GG (m)
		GC TACGTA GC (m)
		GT TACGTA AC (m)

[a] All ACGT elements presented except those designated m (medium affinity) or w (weak affinity) are high-affinity binding sites. Data for O2 and CPRF-2 were taken from Izawa et al. (1993), whereas that for RITA-1 was determined from this work.

have shown that parsley CPRFs can interact with endogenous nuclear proteins, which are likely to be other bZIP factors. It should be pointed out, however, that the artificially high concentration of RITA-1 in these assays may compensate for the weak affinity of the RITA-1 homodimer for motif I.

Comparison of the GUS activity generated by reporter constructs containing tetramers of G/C, C/A, and A/G RITA-1 binding sites to that by the *rita-1* promoter indicates that the ACGT tetramers are much more responsive to RITA-1. Because the former contain four functional binding sites as compared to two active G-box elements present in the *rita-1* promoter, synergism between a greater number of high-affinity ACGT elements alone may account for the higher transactivation levels. Moreover, because these sites are in tandem, whereas those in *rita-1* are ~270 bp apart, spacing of *cis* elements may also play a role in transactivation efficiency. Spacing between GT-1 binding sites within the light-responsive elements of the pea *rbcS* promoter is critical for transcriptional activity (Gilmartin and Chua, 1990).

The observation that RITA-1 can bind to G-box elements in its own promoter and transactivate a construct fused to *rita-1* 5′ flanking sequences in Oc protoplasts indicates that *rita-1* is autoregulated. In plants, two other bZIP DNA binding factors, O2 and PosF21, have been shown to be autoregulated (Aeschbacher et al., 1991; Lohmer et al., 1991). Because the ACGT elements in the *O2* promoter differ from those in albumin *b-32* 5′ flanking sequences, other cellular mechanisms putatively mediated by differential DNA binding specificity may be involved in plant bZIP autoregulation (Schmidt et al., 1992). Alternatively, the transactivation potential of O2 may be regulated at the translational level by upstream open reading frames contained in its unusually long 5′ leader sequence (Lohmer et al., 1993). Whether other bZIP proteins are subject to post-transcriptional regulation is not known.

Spatial Expression of the *rita-1* Gene

Quantitation of GUS expression levels in different organs of transgenic rice plants and RNA gel blot analysis showed that the *rita-1–GUS* transgene and the endogenous *rita-1* gene, respectively, are expressed in developing rice seeds. Histochemical analysis of developing transgenic seeds localized the GUS activity to the aleurone cell layer and endosperm cells. By immunochemical methods, Varagona et al. (1991) have shown that the maize O2 protein is localized to the nuclei of subaleurone and endosperm cells of seeds of developing maize kernels. This similarity between the spatial and temporal expression pattern of RITA-1 and O2 suggests that these two bZIP proteins regulate the expression of genes expressed during seed development. Rice genes, e.g., the starch branching enzyme (*sbe-1*) gene (Kawasaki et al., 1993), that have a temporal and spatial expression pattern similar to *rita-1* in developing rice seeds and contain high-affinity RITA-1 binding sites (see Table 4) may be potential targets for RITA-1 regulation. Because the expression of RITA-1 reaches its highest level 14 to 20 days after pollination, which is a few days before that of the *sbe* gene whose mRNA accumulation reaches its maximal level of expression at 20 days after flowering, we speculate that RITA-1 may play a role in the regulation of starch synthesis during rice embryogenesis. Future studies will be required to establish whether RITA-1 does indeed regulate starch biosynthesis in vivo in developing rice seeds.

METHODS

Isolation of RITA-1 cDNA and Its Genomic Clone

A rice cDNA library (Stratagene) was screened with an oligonucleotide probe that encodes the basic leucine zipper (bZIP) domain of the wheat EmBP-1 protein (Guiltinan et al., 1990). The probe was prepared by annealing two 45-mer oligonucleotides complementary for the central 30 bp and end filling in the presence of α-^{32}P-deoxynucleotide triphosphates and the Klenow fragment of DNA polymerase I (Boehringer Mannheim). After screening 10^6 plaques, several positive clones were isolated. A full-length rice transcription activator (RITA-1) cDNA was obtained by combining two different cDNA clones, designated c61 and c14. A genomic clone encoding the *rita-1* gene was isolated from a rice genomic library using a random-primed c61 clone as a probe.

Plasmid Constructions

Using polymerase chain reaction technology, a BamHI restriction endonuclease site was created at the 5′ distal region of the RITA-1 coding region. The pRITA-1 construct, the source of recombinant RITA-1 protein for gel mobility shift experiments, was synthesized by cloning the BamHI-EcoRI fragment encompassing the entire RITA-1 coding region in frame with the *lacZ* gene of the pBluescript SK− vector (Stratagene). The RITA-1 effector construct used for the transient expression assays was prepared by cloning the BamHI-EcoRI RITA-1 fragment into the pBI221 vector (Clontech, Palo Alto, CA). For reporter constructs, annealed oligonucleotides representing the various ACGT elements and possessing HindIII and XhoI termini were cloned into the pIG221 plasmid (Tanaka et al., 1990) upstream of a truncated minimal cauliflower mosaic virus (CaMV) 35S promoter (Benfey et al., 1989). The pIG221–β-glucuronidase (*GUS*) expression vector includes the first intron of the mung bean catalase gene. Translational fusion genes for the *rita-1* and *waxy* reporter constructs were generated by cloning 3 kb of *rita-1* and 1.6 kb of the *waxy* 5′ flanking sequence, which included 280 bp of the promoter and the 1280-bp first intron (Okagaki and Wessler, 1988; M. Nakajima, unpublished data) upstream of the *GUS* gene of a pIG221 derivative in which the full-length 35S promoter was removed.

Gel Mobility Shift Assays

Protein extracts for protein–DNA studies were prepared from BL21(DE3)/pLysS bacterial cells transformed with the pRITA-1 construct. Oligonucleotide A-box, C-box, G-box, and T-box probes were prepared as described previously (Izawa et al., 1993). Binding reactions were performed essentially as outlined by Williams et al. (1992). Dissociation constants were calculated as described by Izawa et al. (1993) using the least-squares method for line fitting.

Transient Expression in Rice Protoplasts

Rice Oc protoplasts were isolated by the method of Kosugi et al. (1990). Protoplasts (2×10^6 per 0.5 mL) were mixed with 10 μg of the reporter plasmid and 40 μg of the effector plasmid (cesium chloride–purified) or salmon sperm DNA in 70 mM KCl, 5 mM $MgCl_2$, 0.1% Mes, pH 5.8, and 0.4 M mannitol and incubated on ice for 20 min. After electroporation (X-cell 450, Promega; 600 V/cm, 20 msec, and 1000 μF) and 10-min incubation on ice, 4.5 mL of R2P medium (Kosugi et al., 1990) was added, and the protoplasts were cultured at 30°C by the Millicell method using Oc nurse cells (Baba et al., 1986). After a 16-hr incubation, protoplasts were harvested and lysed by sonication in GUS extraction buffer. After centrifugation, the soluble protein concentration was determined using the Bradford assay (Bradford, 1976). GUS activity was determined by the method of Kosugi et al. (1990) using the least-squares method for line fitting.

Histochemical GUS Analysis

Transgenic rice carrying a *rita-1–GUS* transgene was prepared as described previously (Shimamoto et al., 1989). Histochemical analysis of GUS activity was performed as described by Jefferson et al. (1987). The X-gluc solution was modified to include 20% methanol to reduce the background activity. Tissue samples were incubated with X-gluc for 6 hr at 37°C except for immature seeds, which were incubated for only 5 hr.

RNA Gel Blot Analysis

Total RNA prepared from developing Labelle rice seeds using the phenol-chloroform method (Watanabe and Price, 1982) was separated in formaldehyde gels and transferred to Hybond N+ membranes (Amersham Corp.). The filters were hybridized to the RITA-1 c61 cDNA fragment and radiolabeled by the random primer method using standard conditions for RNA gel blot prehybridization, hybridization, and washing (Sambrook et al., 1989).

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