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A Nuclear Factor That Binds to a Dyad-Symmetric Sequence with a CGTCA Motif in the 5′-Upstream Region of the Sweet Potato β-Amylase Gene

Sumie Ishiguro [1], Mihoko Tanaka [1], Akihiro Kojimoto [1], Mariko Kato [1], Masaki Iwabuchi [2] and Kenzo Nakamura [1, 3]

[1] *Laboratory of Biochemistry, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464-01 Japan*
[2] *Department of Botany, Faculty of Science, Kyoto University, Kyoto, 606 Japan*

A nuclear extract from petioles of sweet potato protected several sites in the 5′-upstream region of a gene for β-amylase from DNase I digestion. One of these sites, located at a region around 800-base pairs upstream from the transcription start site, having an imperfect palindromic sequence of CGTCACGTCACG, was designated the R-box. The site contained tandemly duplicated CGTCA sequences, referred to below as 5′- and 3′-CGTCA. Competition experiments in gel mobility shift assays with mutant R-box oligonucleotides indicated that mutations in bases outside the 3′-CGTCA of the R-box do not severely affect the binding. By contrast, single-base substitutions in any one base of the 3′-CGTCA greatly abolished the binding even when the mutated R-boxes contained intact 5′-CGTCA. However, oligonucleotides with mutations in the 3′-CGTCA had the ability to bind the nuclear factor when additional mutations were introduced to create a partially palindromic sequence containing the CGTCA sequence in its 3′-half on the opposite strand. These results indicate that the CGTCA sequence alone is not sufficient for the binding of the R-box binding factor (RBF) and that the RBF binds to the sequence with partial dyad symmetry that contains the CGTCA motif in its 3′-half. The optimum sequence for the binding of the RBF is suggested to be a palindromic octameric sequence TGACGTCA, which is identical to the consensus sequence of the cAMP-responsive element (CRE) of animal genes. Bacterially produced HBP-1b of wheat bound to the R-box, and its binding to mutated R-boxes was similar to that of RBF, suggesting that the RBF belongs to a family of bZIP-type plant nuclear factors that bind to CGTCA-related sequences. However, several differences between the RBF and HBP-1b were also noted.

Key words: β-Amylase gene — CGTCA motif — DNA-binding protein — R-box binding factor.

The activation of transcription of inducible genes is caused by the binding of transcription factors to *cis* regulatory elements of the target genes. The transcriptional regulation of any particular gene is generally mediated by several factors which act synergistically or independently. Therefore, to understand the molecular mechanism of induction of any particular target gene, it is neccessary to characterize the transcription factors that are involved in the regulation of transcription.

β-Amylase is a major protein in tuberous roots of sweet potato and it accounts for about 5% of the total soluble protein in this organ (Yoshida and Nakamura 1991). In contrast to sporamin, which is the most abundant protein in the tuberous root encoded by a multigene family, β-amylase is encoded by a gene that is present as a single copy per haploid genome of the sweet potato (Yoshida et al. 1992). In the petioles or leaves of field-grown plants, expression of the gene for β-amylase and the gene for sporamin is usually not detectable. However, when large amounts of su-

Abbreviations: CaMV, cauliflower mosaic virus; PGA, polygalacturonic acid; RBF, R-box binding factor; CRE, cAMP-responsive element.

[3] To whom correspondence should be addressed.

crose or other metabolizable sugars, such as glucose or fructose, are supplied to them, the expression of these genes is induced with the concomitant accumulation of starch (Hattori et al. 1990, 1991, Nakamura et al. 1991). In addition to sucrose, both polygalacturonic acid (PGA) and chitosan, which are known to induce wound-inducible genes for proteinase inhibitors in *Solanaceae* (Ryan and Farmer 1991), at concentrations much lower than the effective concentration of sucrose, can also induce expression of the β-amylase gene and sporamin genes (Ohto et al. 1992). Unlike induction by sucrose, induction by PGA of these genes is not accompanied by the accumulation of starch.

Analysis of *cis* elements in the 5′-upstream region of gSPO-A1, which encodes A-type sporamin, that can be expressed in leaves of transgenic tobacco plants (Ohta et al. 1991, Morikami, A., Mano, S., Suzuki, S. and Nakamura, K., unpublished results) indicates that inducibility by sucrose of this promoter depends on the synergistic action of several *cis* elements, in addition to the region with the TATA motif, within the 305-bp region upstream of the transcription start site. In case of the promoter of the gene for β-amylase, gβ-Amy (Yoshida et al. 1992), the sequence from −1,267 to −779 relative to the transcription start site has been shown to be important for the sucrose-induced expression of a chimeric β-amylase promoter:β-glucuronidase gene in leaves of transgenic tobacco plants (Hayashi, K., Ishiguro, S. and Nakamura. K., manuscript in preparation).

Previously, we reported a nuclear factor, designated SP8BF, that binds to the 5′-upstream regions of two sporamin genes, gSPO-A1 and gSPO-B1, and to that of gβ-Amy (Ishiguro and Nakamura 1992). In this paper, we describe a nuclear factor, designated RBF, from sweet potato that binds to a dyad-symmetric sequence CGTCACGTCACG, designated the R-box, at the −800 region of gβ-Amy. From an analysis of the specificity of the binding sequence, we determined that RBF binds to a palindromic sequence that contains a CGTCA motif.

Materials and Methods

Plant materials—Sweet potatoes (*Ipomoea batatas* Lam. cv. Kokei No.14) were grown at Nagoya University Experimental Farm. Petioles, with the intact leaf still attached, were cut from plants and these leaf-petiole cuttings were treated with 6% sucrose or with 0.1% PGA for 1 to 2 days in darkness as described previously (Nakamura et al. 1991, Ohto et al. 1992).

Probe and competitor DNAs—Restriction fragments BA1, BA2 and BA3 were prepared by digestion of the 5′-upstream region of gβ-Amy (Yoshida et al. 1992) with *Bam*H I, *Tth*HB8 I and *Hae* III (Ishiguro and Nakamura 1992). BA4, BA5 and BA6 fragments were prepared from the BA1 fragment by digestion with *Sac* II and *Dra* I. The SA1 fragment was prepared from the 5′-upstream region of gSPO-A1 (Hattori and Nakamura 1988) as described previously (Ishiguro and Nakamura 1992). A fragment of the 35S promoter of cauliflower mosaic virus (CaMV), from −414 to +3 relative to the transcription start site, was excised from pCaMVCAT (Fromm et al. 1985) and divided into 35S-A and 35S-B fragments by digestion with *Eco*R V, which cleaves between −90 and −89. These restriction fragments were purified by electrophoresis through a 5% polyacrylamide gel, and used as probes, after 3′-end-labeling with [α-^{32}P]dCTP by the Klenow enzyme, or as competitors.

Oligonucleotides were synthesized on a DNA synthesizer (Model 381A; Applied Biosystems). Complementary oligonucleotides were annealed, and used as competitors. Oligonucleotide R1 (oligo-R1) covering a sequence from −819 to −790 of gβ-Amy was cloned into the *Hinc* II site of pUC119 and an 81-bp *Eco*R I -*Hind* III fragment containing oligo-R1 was excised from the resultant plasmid. This fragment was labeled with ^{32}P as indicated above and used as a probe.

Preparation of plant nuclear extract and bacterial extract—Nuclear extracts were prepared from petiole portions of leaf-petiole cuttings as described previously (Ishiguro and Nakamura 1992), with the exception that desalting by gel filtration was replaced by dialysis and the subsequent column chromatography on heparin-Sepharose was omitted in the initial experiments of gel mobility shift assay.

Escherichia coli strain BL21 (DE3), harboring pAR1b-38, an expression plasmid for HBP-1b, was used to prepare bacterially produced HBP-1b as described previously (Tabata et al. 1991), with the exception that the partial purification by column chromatography on DNA-Sepharose was replaced by column chromatography on heparin-Sepharose.

Gel mobility shift and DNase I footprinting assays—Gel mobility shift assays were performed as described previously (Ishiguro and Nakamura 1992) with slight modifications. For nuclear extracts, the standard binding reaction (20 μl) contained 2 μg poly(dI-dC)·poly(dI-dC), 25 to 60 mM KCl, 25 mM HEPES-KOH pH 7.9, 0.5 mM EDTA, 7.5% glycerol, 1 mM dithiothreitol, and competitor DNA as indicated in legends to Figures. In the case of bacterially produced HBP-1b, 1 mM $MgCl_2$ was added to the reaction mixture. Reactions were pre-incubated at 25°C with 2 to 8 μg protein of nuclear extract or 0.4 to 0.8 μg protein of bacterial extract containing HBP-1b for 3 min, then 4 to 10 fmol ($1–6 \times 10^4$ cpm) of end-labeled DNA probe were added. The binding reaction was continued for 10 min and analyzed by gel electrophoresis. DNase I footprinting assays were performed as described previously (Ishiguro and Nakamura 1992), with the exception that the concentration of KCl in the binding reaction was reduced to 30 mM.

Methylation interference assay—Methylation interference assays were performed as described elsewhere (Boldwin 1988) with the following modifications. The DNA probes (1×10^5 cpm) were partially methylated by treatment with dimethyl sulfate and purified by ethanol precipitation. The binding reactions were carried out as described above, except that the reaction volume was scaled up 5-fold and 20 to 50 μg of nuclear extract or 10 μg of bacterial extract containing HBP-1b was added. The fragments of free and bound DNA were separated on a nondenaturing 5% polyacrylamide gel, and purified by passage through an Elutip-d column (Schleicher & Schuell). The purified DNA fragments were treated with 100 μl of 1 M piperidine for 30 min at 90°C and recovered by ethanol precipitation. Aliquots corresponding to equal amounts of radioactivity was subjected to electrophoresis through an 8% polyacrylamide sequencing gel.

Results

Multiple factors in a nuclear extract from sucrose-treated petioles of sweet potato bind to the 5′-upstream region of the β-amylase gene—In our previous study (Ishiguro and Nakamura 1992), a DNase I footprinting assay was used to characterize the binding of a nuclear factor to restriction fragments from the 5′-upstream regions of gβ-Amy, which encodes β-amylase (Yoshida et al. 1992), and gSPO-A1 and gSPO-B1, which encode A-type and B-type sporamin, respectively (Hattori and Nakamura 1988). During these studies, we noticed that nuclear extracts prepared from sucrose-treated petioles of sweet potato protected multiple sites between −890 and −740 in the 5′-upstream region of gβ-Amy from digestion by DNase I (Fig. 1). One of the protected sites contained an SP8a sequence, $^{-883}$TACACAGT^{-876}, to which SP8BF binds (Ishiguro and Nakamura 1992). Relatively strong protection was also observed at the sequence between −784 and −747. This region contained a sequence $^{-776}$TACACGTGGC^{-767}, which is similar to the G-box sequence that is known to be present in many photoregulated and other plant promoters (see references in Williams et al. 1992). Relatively weaker protection was also observed between −861 and −850 and between −812 and −791. The latter weakly protected site contained the imperfect dyad-symmetric sequence of $^{-810}$CGTCAC·GTCACG^{-799} (the center of symmetry is indicated by the dot). This sequence, designated the R-box, contains two copies of a CGTCA sequence, referred to below as 5′- and 3′-CGTCA, that are directly repeated. CGTCA and related sequences are known to be contained within the *cis* regulatory elements of various promoters that are active in plant cells (Bouchez et al. 1989, Fromm et al. 1989, Lam et al. 1989, 1990a, b), and the $^{-806}$ACGTCA^{-801} sequence is identical to the hexamer sequence motif that was first described as a putative regulatory element of histone genes (Mikami et al. 1987).

Nuclear factor(s) that binds to the R-box—When gel

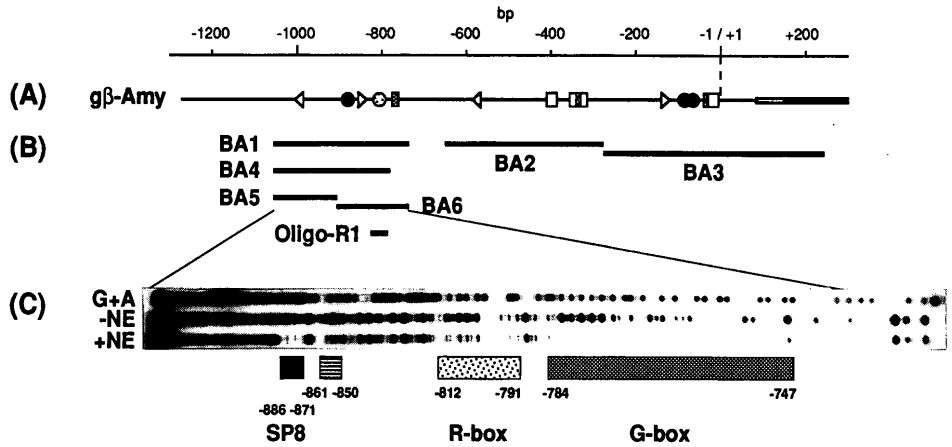

Fig. 1 Structure of the 5′-upsteam region of the sweet potato β-amylase gene gβ-Amy (A), the fragments of gβ-Amy used in this paper (B), and DNase I footprinting of the region from −1,048 to −741 of gβ-Amy with a nuclear extract from sucrose-treated petioles of sweet potato (C). In panel (A), the Box 1 sequence that contains the TATA-like sequence (▨), Box 2 (□), and the Box 3 (◁, ▷) sequence that are found in both the sporamin and β-amylase genes (Hattori and Nakamura 1988, Yoshida et al. 1992) are indicated. The locations of the SP8 sequence (●), the R-box (◎), and the G-box-like sequence (▩) to which nuclear proteins bind are also indicated. The site of initiation of transcription is numbered +1 (Yoshida et al. 1992) and the intron within the coding region is indicated by a thick bar. In panel (C), the result of the DNase I footprinting assay with the nuclear extract from sucrose-treated petioles of sweet potato (+NE) and ^{32}P-labeled BA1 fragment is shown. Lane (G+A) shows the sequencing reaction of the BA1 fragment and lane (−NE) shows a result of a control experiment in which nuclear extract was omitted from the reaction mixture. Protected regions are indicated under the ladders.

mobility shift assays were carried out with the BA1 fragment that covers the sequence from −1,048 to −741 of gβ-Amy (Fig. 1A; Ishiguro and Nakamura 1992) and the nuclear extract in the presence of poly(dI-dC)·poly(dI-dC), a clearly shifted band of BA1 appeared in the gel (Fig. 2A, lane 2). Generation of this shifted band was inhibited by the presence of excess amounts of the BA1 fragment (Fig. 2A, lanes 3 and 4). In contrast, the addition of the BA2 and BA3 fragments from gβ-Amy (Fig. 1B) or the SA1 fragment from gSPO-A1 (Ishiguro and Nakamura 1992) in amounts similar to the amounts of BA1 fragment used for competition did not result in significant competition for binding (Fig. 2A, lanes 5–10).

The BA1 fragment contains the SP8a sequence to which the SP8BF in nuclear extracts binds (Ishiguro and Nakamura 1992). However, the formation of this shifted band was not due to the binding of SP8BF, since the binding of SP8BF to these restriction fragments could not be detected in a gel mobility shift assay under these conditions and the BA3 and SA1 fragments, which also contain the SP8BF-binding sites (Ishiguro and Nakamura 1992), did not compete for the formation of this shifted band. The BA1 fragment was divided into several fragments (Fig. 1B), and each fragment was added as a competitor to the binding reaction with the BA1 probe. Although the BA4 and the BA6 fragment competed for the binding, the BA5 fragment did not affect the binding (Fig. 2B). These results indicate that the shift in mobility of the band of the BA1 fragment after reaction with the nuclear extract was due to the binding of factor(s) to a sequence in a region shared by the BA4 and BA6 fragments, or within the sequence between −906 and −785.

When sweet potato nuclear extract was mixed with a restriction fragment H3 from the promoter for the wheat histone H3 gene that includes the hexamer motif sequence (Mikami et al. 1989a) as a probe, the formation of a shifted band of this probe was observed (data not shown). Furthermore, the formation of a shifted band of the BA1 fragment with the nuclear extract was competed by the 35S-A fragment from the CaMV 35S promoter (Fig. 2C). The 35S-A fragment covers nucleotides −89 to +3 of the 35S promoter and includes two copies of the CGTCA motif sequence (Lam et al. 1989). By contrast, another fragment, 35S-B, from the 35S promoter, which includes nucleotides −414 to −90, competed only weakly for the binding (Fig. 2C). We prepared a synthetic 30-mer double-stranded oligonucleotide R1 (oligo-R1) that covered the sequence from −819 to −790 of gβ-Amy (see Fig. 3). This oligo-R1 abolished the formation of the shifted band of the BA1 probe (Fig. 2D) and the H3 probe (data not shown) after reaction with the nuclear extract. By contrast, the unrelated oligonucleotide oligo-SP8a (Ishiguro and Nakamura 1992) did not compete for the binding (Fig. 2D). These results strongly suggest that the nuclear extract contains a factor(s) that binds to the CGTCA-related sequence within the R-box, causing the formation of the shifted band. The factor that binds to the R-box sequence was designated R-box binding factor or RBF.

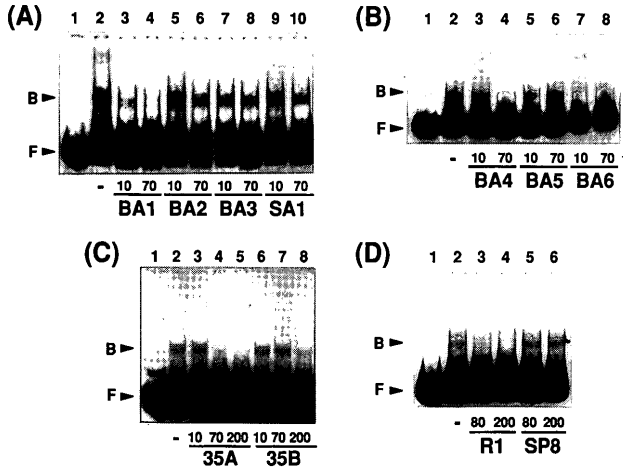

Fig. 2 Competition for binding to a factor(s) in the nuclear extract between the BA1 probe and various fragments. Gel mobility shift assays were carried out with the BA1 probe and nuclear extract from sucrose-treated petioles of sweet potato. Competitor fragments (see Fig. 1B and text) were added in molar excess with respect to the probe, as indicated in the Figure. The bands of free probe (F) and the complexed probe (B) are indicated by arrowheads at the left of each panel. In each panel, lanes 1 and 2 show results of reactions without nuclear extract and those without competitor, respectively. In some preparations of the BA1 probe [lane 1 in (B) and (C)], more slowly migrating minor band were probably generated during preparation of the probe.

The binding sequence-specificity of RBF—We introduced various mutations into the R-box sequence within the oligo-R1 (Fig. 3), and examined the ability of the mutated sequences to compete with the oligo-R1 probe for the binding of RBF (Fig. 4A). Sequence modifications outside the central 8-bp sequence, $^{-808}$TCACGTCA^{-801}, of the R-box (Fig. 3; m1, m3, m6 and m8) did not affect the ability to compete for binding. Furthermore, mutations of $^{-808}$T (m4), $^{-807}$C (m7) and $^{-806}$A (m11) in the oligo-R1 did not affect the ability to compete with the oligo-R1 probe for the binding to RBF (Fig. 4A), indicating that the 5′-CGTCA is not critical for the binding.

The binding of RBF to the oligo-R1 probe was not competed by mutant oligonucleotides with mutations at any single site in the 3′-CGTCA sequence of the R-box (Fig. 3; m10, m2, m13, m14 and m5). It should be noted that, in mutants m10, m2, m13 and m5, the 5′-CGTCA sequence remained intact. These results indicate that although the R-box contains two directly repeated CGTCA sequences, the 3′-CGTCA, and not the 5′-CGTCA, is critical for the binding of RBF. Since the 3′-CGTCA is part of

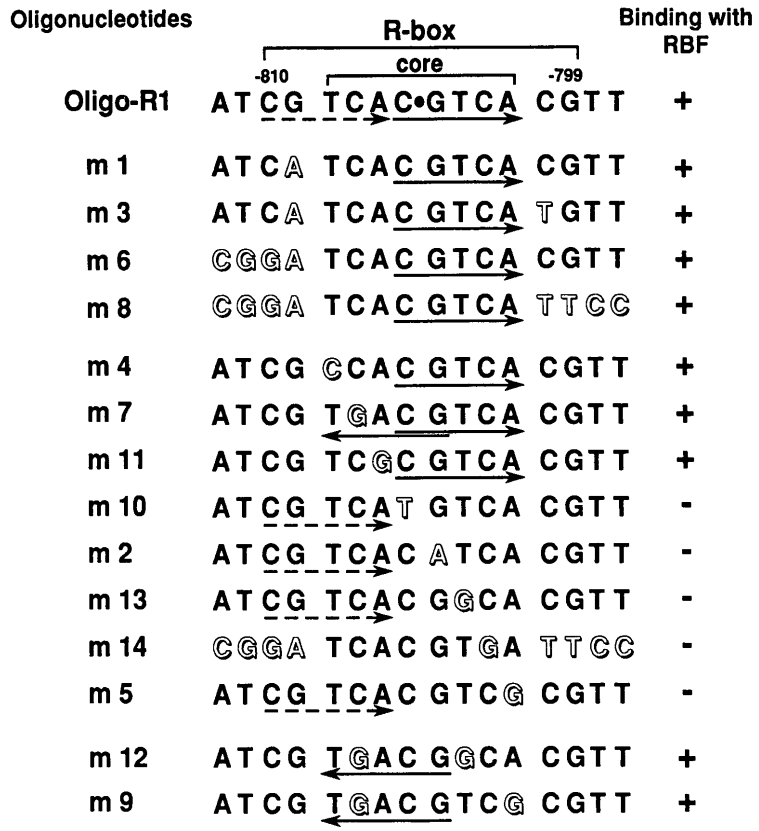

Fig. 3 Nucleotide sequences of the various oligonucleotides used for the competition assay. Mutants were generated by base substitution and/or deletion from the wild-type oligo-R1, which was a 30-bp double-stranded oligonucleotide that corresponded to positions −819 to −790 of gβ-Amy. Only the sequence surrounding the R-box core of the top strand is shown. In the sequence of the oligo-R1, the 5′- and 3′- CGTCA are indicated by a dotted arrow and an arrow, respectively, and the center of symmetry of the R-box core is indicated by a dot. In mutant oligonucleotides, those bases different from the wild-type sequence are indicated by outlined letters. The binding of RBF to oligonucleotides, as judged from the competition assay for which results are shown in Fig. 4, is indicated by + or −.

the imperfect palindromic R-box sequence and the m7 mutant oligonucleotide, in which $^{-807}$C was changed to G to generate a perfect palindromic TGACGTCA sequence, strongly competed for the binding of RBF (Fig. 4A), we tested the hypothesis that the presence of the CGTCA motif in the 3′-half of the palindromic sequence is important for the binding of RBF. We constructed two mutants, m9 and m12, in which a second mutation, $^{-807}$C to G, was introduced into m13 and m5 mutant oligonucleotides, respectively, to create a CGTCA motif in the 3′-half of the palindromic sequence on the opposite strand (Fig. 3). RBF bound to both of these mutant oligonucleotides (Fig. 4A).

Methylation interference assay—In order to identify the guanine residues whose methylation affects the binding of RBF, a partially methylated oligo-R1 probe with a ^{32}P-label at the 3′-end of the bottom strand was incubated with nuclear extract, and free (F) and protein-bound (B) probes were separately isolated from the gel. These DNA probes were analyzed on a sequencing gel after cleavage by piperidine. In the case of DNA fragments in the bound fraction, bands of guanines at positions −805 and −802 were almost completely missing, and bands of guanines at positions −807 and −800 were significantly reduced compared to those in the case of DNA fragments in the free fraction (Fig. 5B). We also used the BA6 fragment (Fig. 1A) to analyze guanine residues in the top strand of the R-box. Binding of nuclear extract to the BA6 probe produced two protein-bound complexes (B1 and B2) on the gel (data not shown), and we analyzed the DNA fragments in these complexes separately. In DNA fragments from the more rapidly migrating B1 complex, the bands corresponding to guanines at positions −809 and −804 were significantly diminished in intensity as compared to those in the free fraction (Fig. 5A). These results indicate that guanine residues whose methylation affects the binding of RBF are located around the center of symmetry of the R-box (Fig. 5D). The results obtained with the DNA fragments in the B2 complex are described below.

Binding of wheat HBP-1b to the R-box—Nuclear extracts from sweet potato showed binding to the H3 frag-

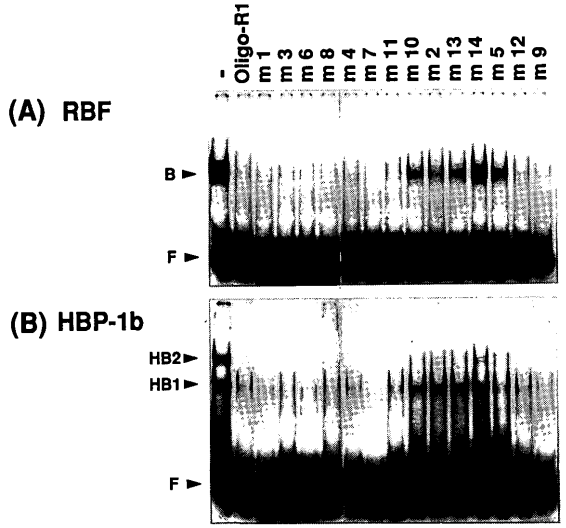

Fig. 4 Competition for the binding of the nuclear factors between the R-box sequence and various mutants of oligo-R1. Competition for binding of the RBF in the nuclear extract (A) or for binding of bacterially produced HBP-1b (B) between the oligo-R1 probe and the various mutant oligonucleotides shown in Fig. 3 was examined. The bands of free probe (F) and the complexed probe (B) are indicated by arrowheads. Two forms of the probe bound to HBP-1b are indicated as HB1 and HB2 (see text).

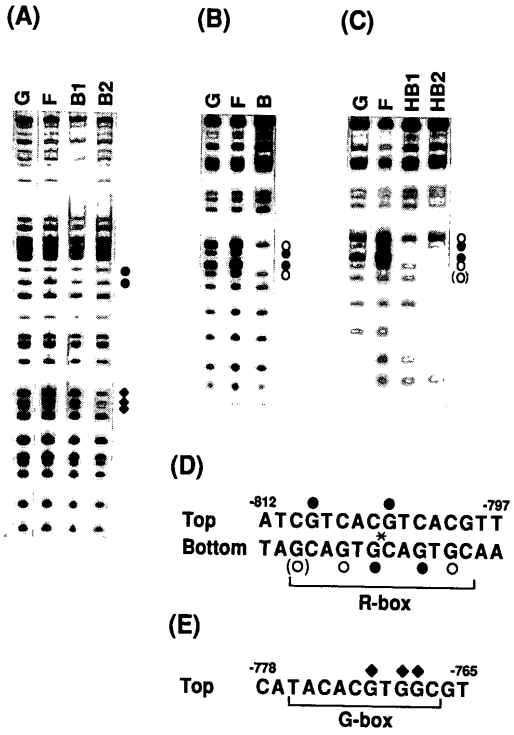

Fig. 5 Methylation interference assays to analyze the binding of RBF and HBP-1b to DNA fragments. (A) The BA6 fragment (see Fig. 1B) with ^{32}P at the 3′-terminus of the top strand was partially methylated and incubated with the nuclear extract from sweet potato. DNA fragments were purified from B1 and B2 complexes and the free fraction (F) was separated on a polyacrylamide gel (see text), cleaved at methylated guanine residues and analyzed by electrophoresis on an 8% sequencing gel. Lanes designated G show guanine sequencing ladders generated by cleavage of the probe without the binding reaction. (B) The oligo-R1 probe with a ^{32}P-label at the 3′-terminus of the bottom strand was mixed with the nuclear extract and analyzed as in (A) but only one bound complex was formed with this probe. (C) Same as (B), with the exception that bacterially produced HBP-1b was used instead of sweet potato nuclear extract and DNA fragments from two bound complex, HB1 and HB2 (Fig. 4B), were analyzed separately. (D) The positions of methylated guanine residues that strongly (●) or weakly (○) interfered with the binding of nuclear factors to the R-box. The center of symmetry of the R-box sequence is indicated with *. (E) The positions of methylated guanine residues (◆) that interfered with the binding of a nuclear factor to the G-box-like sequence.

ment from the histone H3 promoter (data not shown) and to the CaMV 35S promoter (Fig. 2C). Since wheat transcription factor HBP-1b is known to bind to the sequence containing CGTCA motif in both of the histone H3 promoter and the CaMV 35S promoter (Mikami et al. 1989b), we examined the binding of HBP-1b to the oligo-R1 probe and its mutants. The binding of bacterially produced HBP-1b to the oligo-R1 probe resulted in the formation of two shifted complexes (Fig. 4B). The formation of the more slowly migrating HB2 complex was dependent on the amount of HBP-1b added in the binding reaction, and only the more rapidly migrating HB1 complex was formed when the amount of HBP-1b was limiting (data not shown). In the analysis of these two complexes, the HB1 complex was identical in terms of electrophoretic mobility to the complex between RBF and the oligo-R1 probe. Competition by various mutant oligonucleotides with the wild-type oligo-R1 for the formation of the HB1 complex with HBP-1b was similar to that obtained with RBF (Fig. 4B). By contrast, the formation of the HB2 complex was more severely competed by mutant oligonucleotides than was the formation of the HB1 complex. These results suggest that the formation of the HB2 complex is due to weaker protein-DNA interactions than is the formation of the HB1 complex.

We also carried out a methylation interference assay with bacterially produced HBP-1b and the bottom strand of the oligo-R1 probe. Partially methylated DNA fragments were separately isolated from HB1 and HB2 complexes and analyzed. Methylated guanine residues that affected the formation of the HB1 complex with HBP-1b were almost identical to those that affected formation of the complex with RBF (Fig. 5C). In contrast, the sites of methylated guanine residues that affected the formation of the HB2 complex were shifted slightly to the 5′-side of the R-box sequence as compared to those that affected the for-

mation of the HB1 complex (Fig. 5C). The intensities of bands of guanines at positions −810 and −807 of the bottom strand were greatly diminished in the HB2 complex as compared to those in the HB1 complex, while the effect of the guanine residue at position −800 of the bottom strand on the formation of the HB2 complex was not significant as compared to that on the formation of the HB1 complex. The weak interaction of HBP-1b with the 5′-CGTCA sequence of the R-box may explain the formation of the HB2 complex.

A factor that binds to the G-box like sequence—As described above, in the gel mobility shift assay, we observed two shifted complexes with the BA6 probe after incubation with nuclear extract. The methylation interference assay of DNA fragments from the more rapidly migrating B1 complex indicated that this complex contained RBF (Fig. 5A). In the analysis of DNA fragments from the more slowly migrating B2 complex, intensities of bands of guanines at positions −771, −769 and −768, in addition to those of guanine residues in the R-box, were severely diminished as compared to those from the free fraction (Fig. 5A). These guanine residues were localized in the G-box-like sequence $^{-776}$TACACGTGGC^{-767} (Fig. 5E). These results, taken together with the protection of this region in the DNase I footprinting assay (Fig. 1C), indicate that sweet potato nuclear extract contains a factor that binds to the G-box-like sequence in gβ-Amy and that the formation of the B2 complex is due to the binding of this factor to the BA6 probe in addition to the binding of the RBF. Nuclear factors that bind to the G-box element have been identified in several plant species (see references in Williams et al. 1992).

Effects of $MgCl_2$ on the binding of RBF and HBP-1b to DNA—The binding of RBF to restriction fragments of gβ-Amy that could be detected by the DNase I footprinting assay was very weak (Fig. 1C). We examined the effects of $MgCl_2$ on the binding of RBF to the oligo-R1 probe by a gel mobility shift assay. The binding of RBF to the oligo-R1 probe was sensitive to $MgCl_2$, and the intensity of the shifted band was diminished by the addition of $MgCl_2$ even at 1 mM (Fig. 6A). The inhibitory effect of $MgCl_2$ was reversed by EDTA (data not shown). By contrast, the binding of bacterially produced HBP-1b with the oligo-R1 probe, especially the formation of the HB2 complex, were not significantly inhibited by $MgCl_2$ (Fig. 6B).

The binding of RBF, as well as of HBP-1b, to the CaMV 35S probe resulted in the formation of the two shifted complexes (Fig. 6, C and D). The *as-1* site on this fragment contains tandemly duplicated copies of the CGTCA sequence that are separated by 7 bp, and the formation of two shifted complexes by the binding of either one or two nuclear factors to these two sites has been reported previously (Lam et al. 1990a). The binding of RBF to the CaMV 35S probe was also more sensitive to $MgCl_2$ than the binding of HBP-1b to the same probe

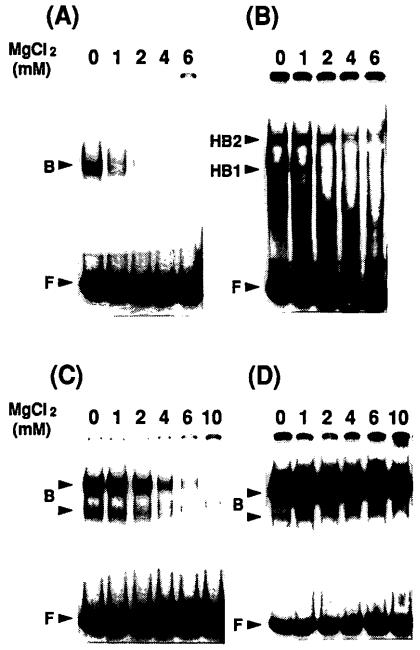

Fig. 6 Effects of $MgCl_2$ on the binding of RBF and HBP-1b to the oligo-R1 probe and the CaMV 35S probe. Gel mobility shift assays with RBF in sweet potato nuclear extract (A and C) or bacterially produced HBP-1b (B and D) and the oligo-R1 probe (A and B) or the CaMV 35S-A probe (C and D) were carried out in the presence of various concentrations of $MgCl_2$, as indicated in the Figure. The bands of free probe (F) and the complexed probe (B, HB1, HB2) are indicated by arrowheads.

(Fig. 6, C and D). Since Mg^{2+} ions are required in the DNase I footprinting assay, this inhibitory effect of $MgCl_2$ on the binding of RBF to the R-box may explain the weak protection of the R-box sequence in the DNase I footprinting assay.

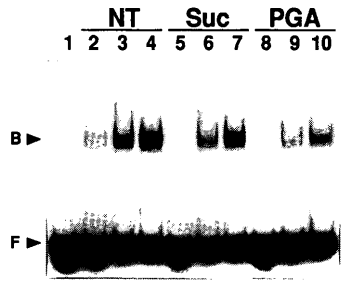

Fig. 7 The RBF activity in extracts from petioles of sweet potato. Nuclear extracts were prepared from non-treated petioles of sweet potato (NT), petioles that had been treated with 6% sucrose for 12 hours (Suc), and petioles that had been treated with 0.1% PGA for 12 hours (PGA). Gel mobility shift assays were carried out with the oligo-R1 probe with 2 μg (lanes 2, 5, and 8), 6 μg (lanes 3, 6, and 9), or 12 μg (lanes 4, 7, and 10) of the nuclear proteins. The bands of free probe (F) and the complexed probe (B) are indicated by arrowheads.

RBF is present not only in sucrose-treated or PGA-treated petioles but also in non-treated petioles—We prepared nuclear extracts from petioles that had been treated with 0.1% PGA for 12 h to induce the expression of the β-amylase gene, and from non-treated petioles in which β-amylase mRNA could not be detected. Increasing amounts of these nuclear extracts were added to the binding reaction with the oligo-R1 probe, and assayed for the binding activity was examined by the gel mobility shift assay. Binding activities similar to that in nuclear extracts from petioles that had been treated with 6% sucrose for 12 h were detected in both of these nuclear extracts (Fig. 7).

Discussion

DNase I footprinting and gel mobility shift assays revealed the presence in nuclear extracts of sweet potato of a factor that binds selectively to the 12-bp imperfect palindromic sequence CGTCACGTCACG, designated the R-box, which is located in the −800 region of gβ-Amy (Figs. 1 and 2). The R-box contains two directly repeated copies of the CGTCA sequence and single-base substitutions at any one site in the 3′-CGTCA greatly abolished the binding (Figs. 3 and 4). However, the results presented in this paper (Figs. 2, 3 and 4) indicate that the mere presence of the CGTCA sequence alone is not sufficient for the binding of RBF. The palindromic nature of the sequence, with its center of symmetry between the C and G residues of the CGTCA motif, seems to be important for the binding of RBF (Fig. 3). The 5′-half of this palindromic sequence can tolerate several mismatches. Moreover, methylated guanine residues that influence the binding of RBF to oligo-R1 are located not only to the 3′-side but also to the 5′-side of the center of symmetry (Fig. 5D). Thus contacts of RBF with the R-box sequence extend to the 5′-side of the indispensable 3′-CGTCA. The binding of RBF to the oligo-R1 probe was subject to strong competition by the mutant oligonucleotide m7 that contained the perfect dyad-symmetrical element TGACGTCA (Fig. 3). This 8-bp palindromic sequence differes from the core octamer of the R-box by only one base, and it is identical to the consensus sequence of the cAMP response element (CRE) of animal genes (Montminy et al. 1986). Mammalian transcription factors that can bind to CRE and related sequence elements are bZIP-type transcription factors (Ziff 1990). These factors contain a basic region for specific binding to DNA that is located immediately on the N-terminal side of the leucine zipper which is required for the formation of homo- or hetero-dimers. The scissors-grip model for the binding of dimeric bZIP proteins to DNA has been proposed (Vinson et al. 1989).

The CGTCA and ACGTCA motifs are contained within the *cis*-regulatory elements of promoters of various plant cellular and viral genes and in those of genes in T-DNAs of *Agrobacterium*, and these motifs have been shown to be the sites of binding of nuclear factors (Bouchez et al. 1989, Fromm et al. 1989, Lam et al. 1989, 1990a, b, Mikami et al. 1987, 1989b, Prat et al. 1989). Several distinct cDNA clones encoding nuclear factors that bind to the CGTCA-related sequences have been isolated even from a single plant species (Armstrong et al. 1992, Katagiri et al. 1989, Singh et al. 1990, Tabata et al. 1989, 1991). These factors appear from their amino acid sequence to belong to the family of bZIP-type transcription factors. Although they bind to sequences with the common CGTCA or related motifs, they are not necessarily similar to one another in regions beyond their bZIP structures. Furthermore, they bind to CGTCA motifs with different surrounding sequences with significantly different affinities (Tabata et al. 1991).

The bacterially produced HBP-1b bound to the oligo-R1 probe, and the effects of various mutations within the R-box on the formation of the HB1 complex with HBP-1b were similar to those observed with RBF (Fig. 4). Furthermore, the methylation interference assay of the binding of HBP-1b to the oligo-R1 probe to form the HB1 complex gave a result that was similar to the result with RBF (Fig. 5). These results suggest that RBF is a member of the family of plant bZIP-type transcription factors that bind to CGTCA-related sequences, being similar to HBP-1b. RBF may also bind to the pallindromic R-box sequence as a dimer, as in the scissors-grip model (Vinson et al. 1989), and the strict recognition of the 3′-CGTCA by one of the subunits could be critical for the binding. However, despite the similarities between RBF and HBP-1b, these two factors differed in the formation of the HB2 complex with the R-box probe (Figs. 4 and 5) and in the sensitivity to $MgCl_2$ of their binding to the CaMV 35S probe (Fig. 6). Since it is not known whether the RBF activity detected in this study was due to single or multiple nuclear factor(s), further characterization of RBF by molecular cloning is required to clarify these issues.

Recent analysis in our laboratory indicates that the sequence from −1,267 to −779 of the 5′ upstream region of the β-amylase gene is important for the sucrose-induced expression of the chimeric β-amylase promoter:β-glucuronidase gene in leaves of transgenic tobacco plants (Hayashi, K., Ishiguro, S. and Nakamura, K., manuscript in preparation). This sequence covers the R-box sequence, but not the G-box-like sequence. The activity of RBF was detected not only in sucrose-treated petioles but also in non-treated petioles, as well as in petioles treated with PGA (Fig. 7). The trans-activation activities of CREB are regulated by phosphorylation by protein kinase A and calmodulin-dependent protein kinase (for review, see Hunter and Karin 1992). HBP-1b can also be phosphorylated (Takase et al. 1991). The activity of RBF in vivo may also be regulated by protein modification, for example, by phosphorylation or

dephosphorylation, after stimulation by sucrose or PGA. It is now necessary to examine the role of the R-box sequence in sucrose- or PGA-induced expression of the β-amylase gene to determine whether RBF plays any role in the signal transduction pathways following the stimulation by sucrose or PGA. Further analyses of the *cis*-regulatory elements in the 5′-upstream region of the β-amylase gene are in progress in our laboratory.

In conclusion, we detected a nuclear factor in extracts of sweet potato, designated RBF, that binds to the dyad-symmetric sequence CGTCACGTCACG, designated the R-box, in the 5′-upstream region of the β-amylase gene. The RBF binds to the sequence with partial dyad symmetry that contains CGTCA motif in the 3′-half and it seems to be a member of the family of plant bZIP-type transcription factors.

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