An N-terminal region of a Myb-like protein is involved in its intracellular localization and activation of a gibberellin-inducible proteinase gene in germinated rice seeds

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The expression of the gene for a proteinase (Rep1) is upregulated by gibberellins. The CAACTC regulatory element (CARE) of the Rep1 promoter is involved in the gibberellin response. We isolated a cDNA for a CARE-binding protein containing a Myb domain in its carboxyl-terminal region and designated the gene Carboxyl-terminal Myb1 (CTMyb1). This gene encodes two polypeptides of two distinctive lengths, CTMyb1L and CTMyb1S, which include or exclude 213 N-terminal amino acid residues, respectively. CTMyb1S transactivated the Rep1 promoter in the presence of OsGAMyb, but not CTMyb1L. We observed an interaction between CTMyb1S and the rice prolamin box-binding factor (RPBF). A bimolecular fluorescence complex analysis detected the CTMyb1S and RPBF complex in the nucleus, but not the CTMyb1L and RPBF complex. The results suggest that the arrangement of the transfactors is involved in gibberellin-inducible expression of Rep1.

Key words: intracellular localization; gibberellin; Myb transcription factor; rice; seed germination

Introduction

During the germination of cereal grains including rice, barely, and wheat, gibberellins are synthesized in the embryos. The gibberellins diffuse into the aleurone layers where they induce the expression of hydrolytic enzymes such as α-amylases and proteinases. The hydrolyases are secreted into the endosperm and mobilize nutrient reserves to support seedling growth. Promoter analyses of the α-amylase genes identified three regulatory elements involved in the response to the gibberellins—namely, the gibberellin-responsive element (GARE; TAACAG/AA), the pyrimidine box (C/TCTTTT), and the amylase box (TATCCAT).1) The GARE is essential for gibberellin induction, because it alone can confer gibberellin dependency to the minimal promoter of the cauliflower mosaic virus 35S RNA (CaMV35S).2) Gubler et al.3) have isolated a cDNA clone encoding the gibberellin-regulated R2R3 Myb transcription factor GAMyb that specifically binds to the GARE and transactivates the gene expression of several hydrolases including proteinase, glucanase, and α-amylase.4) Molecular genetic approaches have confirmed that GAMyb is implicated in the α-amylase expression during aleurone and flower development.5) In addition, GAMyb has been shown to be involved in the programmed cell death in the tapetal and aleurone layer cells.6) The multiple roles of GAMyb during the diverse stages of plant growth and development suggest that the germination-specific transcriptional network exists in accordance with the central role of GAMyb in regulating hydrolyase genes expression.

The pyrimidine box is another promoter element that plays an accessory role in the response to gibberelin.7,8) The pyrimidine box is recognized by the DNA binding of one finger (Dof)-class transcription factor, the prolamin box-binding factor (PBF).9,10) PBF was originally identified as the factor that regulates the expression of genes for seed storage proteins in the immature endosperm.11) Extensive analyses have revealed that barley PBF dose activates the promoters of the seed storage protein genes but also represses the
gibberellin-responsive promoter in the germinated seeds.\textsuperscript{12,13} Another DoF protein, scutellum and aleurone expressed DoF transactivate the promoters of both a seed storage protein and an α-amylase gene.\textsuperscript{14,15} Rice prolamin box-binding factor (RPBF) solely activates the expression of the genes for seed storage proteins coordinated by a leucine zipper transcription factor named RISBZ.\textsuperscript{16} In addition, the synergistic interaction between RPBF (originally named OsDOF3) and rice GAMyb (OsGAMyb) regulates the α-amylase promoter.\textsuperscript{9} The DoF proteins bind to similar promoter sequences with an AAAG core and interact with partner proteins, but their effects on gene expression vary.\textsuperscript{7} These observations suggest that the DoF proteins are important for the assembly of the promoter complexes that facilitate the expression of the target genes.

We have analyzed the promoter elements of the gene for a rice cysteine proteinase, Rep1.\textsuperscript{18} Several promoter sequences conserved among the cereal hydrolase genes are found in the Rep1 promoter, including the GARE essential to gibberellin induction. Another regulatory element named the CAACCTC regulatory element (CARE) is also required for gibberellin induction. We found that two copies of the GARE and the CARE conferred gibberellin dependency to the CaMV35S minimal promoter, but four copies of the CARE did not. In addition, a point mutation of the CARE reduced the response of the barley proteinase EPB and rice α-amylase RAmy1A gene promoters to gibberellin. These results suggest that the CARE plays an accessory role in the response to gibberellin. However, the types of proteins that bind to the CARE are unknown.

In this study, we identified a novel class of Myb-like proteins and designated the gene encoding these proteins as Carboxyl-Terminal Myb1 (CTMyb1), confirming their relevant interaction with the CARE of the Rep1 promoter. Our results indicate that CTMyb1 encodes two types of polypeptides, the CTMyb1 long polypeptide (CTMyb1L) and the CTMyb1 short polypeptide (CTMyb1S) that lacks 213 N-terminal amino acids found in CTMyb1L. Transient expression studies showed that CTMyb1L and CTMyb1S influenced the Rep1 promoter differently. Physical and functional interactions between CTMyb1S and RPBF were also observed. Our results indicate that CTMyb1 is an important regulator of the gibberellin response of the gene that mediates the fine-tuning of the promoter-binding complex in germinated rice seeds.

Materials and methods

Plant materials. The embryo-less half-seeds of rice (\textit{Oryza sativa} L. cv. Nipponbare) were sterilized in 1% sodium hypochlorite and 0.05% Tween 20 for 25 min and then washed five times with sterile water. To analyze gene expression, the half-seeds were incubated at 27 °C in shooting buffer (20 mM sodium succinate [pH 5.5], 20 mM CaCl\textsubscript{2}, 10 g/ml chloramphenicol) in the presence or absence of 0.5 μM GA\textsubscript{3}. After the incubation, the seeds were frozen in liquid nitrogen and stored at −80 °C until use. For particle bombardment, the sterilized half-seeds were incubated for 3 days at 22 °C in shooting buffer. The upper and lower ends of the seeds were cut off. The seeds were cut longitudinally and divided into two flat pieces with a razor blade. After the removal of the pericarps, the seed tissue samples were subjected to particle bombardment.

RNA isolation. Total RNA was prepared as described elsewhere.\textsuperscript{18}

Real-time RT-PCR. Total RNA was used for cDNA synthesis with a single strand synthesis kit (Takara Bio, Shiga, Japan). Real-time PCR was performed using an ABI7500 real-time PCR system (Life Technologies, Carlsbad, CA) with a SYBR green system. Ubiquitin (AK121590) was used as an internal reference. Expression levels were displayed relative to the level of each transcript 0 h after imbibition. Primers were designed using Primer Express (Life Technologies) to ensure that the specific transcript would be amplified. PCR was performed with denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing, and extension at 60 °C for 1 min. The mRNA-specific primer pairs were cited in Supplemental Table 1.

One-hybrid system reporter plasmid construction. A synthetic oligonucleotide containing 6 tandem repeats of the cis-element CARE of the Rep1 promoter with EcoRI and XbaI or EcoRI and SalI restriction sites at its 5’ and 3’ ends was cloned into the pHISi and pLacZi plasmid vectors (Matchmaker one-hybrid system; Clontech, Mountain View, CA), respectively, at the corresponding restriction enzyme sites of the plasmid multilinkers; the resulting plasmids were designated pCA6/HISi and pCA6/LacZi, respectively. The two reporter yeast strains were obtained by transforming the yeast strain YM4271 sequentially with the pLacZi and pHISi plasmid constructs. The yeast transformants were subjected to β-galactosidase and 3-amino-1,2,4-triazole control tests to determine the background expression activity. The transformants that could not show lacZ activity and grow in the presence of 30 mM 3-amino-1,2,4-triazole were selected for subsequent use in the cDNA library screening with the one-hybrid system.

cDNA library construction and screening. Total RNA was extracted from the rice half-seeds that had been treated with GA\textsubscript{3} for 24 h. Poly (A)\textsuperscript{+} mRNA was obtained using the Oligotex dT30 mRNA purification kit (Clontech) as directed by the manufacturer. The cDNA was prepared using the Matchmaker library construction and screening kit (Clontech) as directed by the manufacturer, and the library was subsequently cloned into pGADT7-Rec. Screening of the cDNA library was performed as directed by the manufacturer (Matchmaker one-hybrid system; Clontech) in the presence of 30 mM 3-amino-1,2,4-triazole.

The construction of a full-length CTMyb1 open reading frame. The 5’-terminal sequence of the CTMyb1 cDNA insert was elongated and amplified using a
5'-RACE kit (Takara Bio) according to the instructions of the manufacturer. A 685-bp PCR product was cloned. A cDNA clone for CTMyb1L was isolated using RT-PCR based on the expressed sequence tag sequences (AK070394) obtained from the Knowledge-based Oryza Molecular biological Encyclopedia (http://cdna01.dna.afrc.go.jp/cDNA/; accessed October 1, 2011). RT-PCR was performed out using the cDNA library for the screening and ExTaq PCR polymerase (Takara Bio). The following primers were used: forward primer 5'-TCCTTGCGGACATCCTCGGGGC-3' and reverse primer 5'-TGACTATGTCAATCTCCTAAAGTTACG-3'. The amplified PCR products were cloned into a pGEM-T Easy Vector system (Promega, Madison, WI) and sequenced. The translation start sites were determined using a GeneRacer Kit (Life Technologies).

The construction of the effector plasmids. The fragment of the CTMyb1 cDNA (398 to 1711 bp) was generated by RT-PCR to contain BglII sites at the 5' and 3' ends of the fragment. The amplified fragment was cloned into the pGEM-T Easy Vector (Promega). The cDNA cut by BglII was inserted into pAHC18 (Invitrogen) to replace the luciferase (Luc) coding region. This construct, named pHbl:CTMyb1S, was co-bombaraded with the GUS reporter constructs as described below. BglII sites were created at the 5' and 3' ends of the fragment of a CTMyb1L cDNA clone using PCR; the resultant fragment was cut with BglII and inserted into pHbl18 to replace the Luc coding region. This construct was named pHbl:CTMyb1L. Methods for the construction of the effector plasmid, p35S:OsGAMyb (for the over-expression of OsGAMyb), and the reporter plasmids RX-184, pGC2, pGG2, pCC2, and RA-748a have been described by Washio.9 The effector construct, a CaMV35S promoter fused to RPB1 cDNA, has been described by Sutoh and Yamauchi.18 The yeast transformation and β-galactosidase activity assay were performed as described in the Yeast Protocols Handbook (Clontech). The ProQuest two-hybrid system (Life Technologies) was used to examine the interaction between CTMyb1 and OsGAMyb. Using this kit, we constructed the prey plasmids consisting of CTMyb1S cDNA in pDEST22 (p22CTMyb1), OsGAMyb cDNA in pDEST22 (p22OsGAMyb) and the bait plasmids consisting of CTMyb1S in pDEST32 (p32CTMyb1) and OsGAMyb cDNA in pDEST32 (p32OsGAMyb). These plasmids were transformed into the yeast strain MaV203.

Transient expression in embryo-less rice seeds. Gold particles (1 μm diameter; Tokuriki-Honten Co., Ltd, Tokyo, Japan) were coated with DNA by ethanol precipitation. The Luc reporter plasmid pAHC18 was always used together with the DNA constructs, to serve as an internal control. In experiments with an effector construct, 6 μg DNA was conjugated to 1 mg gold particles, and each GUS reporter construct, effector construct, and the internal control (pAHC18) were co-bombaraded at a weight ratio of 5:5:2. Each seed tissue sample was bombarded with 50 μg of gold particles using a biolistic device (IDERA GIE-III, Tanaka Co., Ltd, Sapporo, Japan) according to the manufacturer’s instructions. After bombardment, batches of three seed tissue samples were incubated in 5 ml shooting buffer on a shaker for 20 h at room temperature. The tissue was then homogenized in a mortar in 200 μl extraction buffer (100 mM potassium phosphate pH 7.8, 2 mM DTT, 2 mM EDTA, 5% glycerol). The homogenate was centrifuged at 15,000 × g for 30 min at 4 °C, and GUS and Luc activities were assayed in the supernatant. Luc activity was determined in 20 μl extract using a commercial kit (Pica Gene, Toyoinki, Tokyo, Japan). GUS activity was measured spectrofluorometrically (RF-5000; Shimadzu, Kyoto, Japan) in 50 μl extract that had reacted with 4-methylumbelliferyl-β-d-glucuronide as a substrate, according to Kosugi et al.21 Values of GUS activity were normalized with respect to Luc internal activity.

Subcellular localization analysis. To construct the translational fusions of CTMyb1S and CTMyb1L to the green fluorescent protein (GFP) reporter gene, SalI sites were created at both ends of the corresponding cDNAs by PCR. The amplified fragments were cloned in-frame with the GFP gene in the plasmid containing the CaMV35S promoter fused to sGFP (S65T).22 The constructs were named p35S:CTMyb1SGFP and p35S: CTMyb1LGFP. The transient expression analysis in the onion epidermal cells with the GFP-fused vectors was performed using a Biologic PDS-1000/He System (Bio-Rad, Hercules, CA) with a rupture setting of 1100 psi. After the bombardment, the onion cells were allowed to recover for 18–22 h on a Murashige and Skoog agar plate at 25 °C in darkness. The images of the subcellular localization of CTMyb1 were obtained with a Leica FW4000 Fluorescence Imaging Workstation (Leica Microsystems, Cambridge, UK).
**Bimolecular fluorescence complementation (BiFC) analysis.** The BiFC vectors were prepared using a Gateway system (Life Technologies). The CTMyb1L, CTMyb1S, OsGAMyb, and RBPF open reading frames were amplified by PCR using the following primers: CTMyb1L_Fw, 5′-ATGGAGGTGGGTGGTGTCACAA-3′; CTMyb1_Rv, 5′-CAAGAAGCCGCACTTTCCCGTGA-3′; CTMyb1S_Fw, 5′-ATGGAGGAACCTTCCAGGAACG-3′; GAMyb_Rv 5′-CACCATGTATCGGGTGAAAGAC-3′; GAMyb_Rv 5′-TTTGATTCTTCGACATTCCAGG-3′; RBPF_Fw, 5′-ATGGCGAGCGGCCGCCGCACTATT-3′; and RBPF_Rv, 5′-TGGCCAGAAGATGTTGAGG-3′. The PCR was performed using KOD Plus (Toyobo, Osaka, Japan). To prepare the entry clones for the Gateway system, a TOPO Cloning system (Life Technologies) was used to transfer the amplified CTMyb1L and S fragments to a pCR8/GW/TOPO vector (Life Technologies), the amplified OsGAMyb fragment to a pENTR/D-TOPO (Life Technologies), and the amplified RBPF fragment to a pDONR207 vector (Life Technologies). LR Clonase (Life Technologies) was used to recombine the entry clones with the BiFC vectors pBGN155 and pBCC155, which were provided by Dr Yutaka Kodama (Riken) and modified with a Gateway conversion kit (Life Technologies). The transient expression analysis in the onion epidermal cells with the BiFC vectors and onion cell incubation was performed as described in the section on the subcellular localization analysis. The images for the BiFC assay were captured with a fluorescent microscope (Axio Imager Z1, Carl Zeiss, Oberkochen, Germany).

**Accession numbers.** The cDNA sequences for CTMyb1L and CTMyb1S have been deposited in the DDBJ under the accession numbers AB683452 and AB683453, respectively. The GenBank/EMBL accession numbers mentioned in this paper are as follows: CrBPF1 (AJ251686), RTBPI (AAX97508), OsAlD1 (AY429017), TBP1 (AT5g13820), TRP1 (AT3g59430), TRF1L (At1g46590), TRF2 (At1g07540), TRF3 (At1g17460), TRF4 (At3g53790), TRF5 (At1g15720), TRF6L (At1g72650), TRF7L (At1g06910), TRF8L (At2g37025), TRF9L (AT3G12560), TRF10L (At5g03780), (AK242392), (AK071645).

**Results**

The isolation of a cDNA encoding a CARE-binding protein

The CARE element of the *Rep1* promoter was used as the bait in a yeast one-hybrid transfactor screening. Total RNA was prepared from embryo-less half-seeds treated with Ga$_2$ for 24 h. cDNA was synthesized from the RNAs with an oligo dT primer and cloned as a cDNA library was introduced into a yeast YM4271 derivative containing the DNOR207 vector (Life Technologies). LR Clonase (Life Technologies) was used to recombine the entry clones with the BiFC vectors pBGN155 and pBCC155, which were provided by Dr Yutaka Kodama (Riken) and modified with a Gateway conversion kit (Life Technologies). The transient expression analysis in the onion epidermal cells with the BiFC vectors and onion cell incubation was performed as described in the section on the subcellular localization analysis. The images for the BiFC assay were captured with a fluorescent microscope (Axio Imager Z1, Carl Zeiss, Oberkochen, Germany).

**Comparison of the amino acid sequence of CTMyb1 with other C-terminal Myb domain proteins**

The 1–1 clone lacked the 5′-region corresponding to the cDNA sequence of PcBPF-1. To obtain the 5′-terminus of the CTMyb1 cDNA, 5′-rapid amplification of cDNA ends (RACE) was performed. By combining the cDNA sequences of the 1–1 clone and the RACE product, we determined that the length of the CTMyb1 cDNA is approximately 2.0 kb, and it contains an open reading frame encoding 621 amino acids (Fig. 1(A)). The C-terminal region of CTMyb1 from residues 499 to 550 shares high sequence similarity with that of Myb-like proteins such as OsPcBPF-1 and OsBPF-1. This Myb domain contains the conserved tryptophan (W position), which plays a critical role in stabilizing the DNA-binding domain of the animal Mybs. Notably, the highly conserved residues of the recognition helix are present (LKDKW; Fig. 1(B)). A phylogenetic analysis of the sequences of the C-terminal Myb domain proteins of *Arabidopsis thaliana*, *Oryza sativa*, and *Catharanthus roseus* revealed that this protein family is classified...
into 2 major groups: I and II (Supplemental Fig. 2). Type I includes AtTRFL1 and AtTRP1 of A. thaliana, which have been shown to bind specifically to plant telomeric DNA.28) Rice RTBP1 is categorized as type I and also binds telomeric DNA.29) These characteristics indicate that the type I proteins are telomeric DNA-binding proteins. Conversely, type II proteins of A. thaliana cannot bind telomeric DNA. Although the roles of the type II Mybs remain unknown, they may be transcription factors that recognize the cognate CARE DNA sequences.

CTMyb1 encodes polypeptides of two different lengths

By surveying public databases, we found that various transcript species were derived from the CTMyb1 gene (Supplemental Fig. 3). Therefore, we examined the 5'-terminus of CTMyb1 cDNA in more detail (Fig. 3). The products from 5'-RACE included one major band at 600 bp and two minor bands at 500 and 300 bp (Fig. 3(A)), and the samples were sequenced (Supplemental Fig. 4). From the sequence of the 600-bp fragments, we determined the +1 position of the transcription start site of the CTMyb1 gene, in which consists of nine exons and eight introns (Fig. 3(B)). Nucleotide sequencing of the 600- and 500-bp fragments indicated that at least two transcription start sites are present within the first exon; the mRNAs transcribed from these two sites encode the same polypeptide, from residues M1 to L621 (Fig. 3(B)). The nucleotide sequence of the 300-bp fragment suggested that an additional transcription start site occurs in the third exon; the resulting transcript

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Fig. 1. The amino acid sequences of CTMyb1 and other C-terminal Myb domain proteins. (A) The amino acid sequence of CTMyb1. The shaded M (214) corresponds to the initial methionine of CTMyb1S. A Myb domain in the sequence is shown in the box. (B) The amino acid alignment of the four C-terminal Myb domain proteins, CTMyb1, CrBPFP, RTBP1, and OsAID1. Identical amino acids are indicated at the bottom of the alignment.
might utilize the first initiation codon in the fifth exon and may theoretically encode a short polypeptide comprising residues M214–L621. In addition, the shorter mRNA was identified in a cDNA clone (accession number AK104892) as reported by the NIAS Rice Full-Length cDNA Project Team (Supplemental Fig. 3). These results suggested that CTMyb1 encodes two polypeptides of two distinctive sizes. The long polypeptide with 213 extra amino acids at the N-terminus was designated Carboxyl-terminal Myb1 long
polypeptide (CTMyb1L), and the short polypeptide was named Carboxyl-terminal Myb1 short polypeptide (CTMyb1S).

Subcellular localization of the CTMyb1 polypeptides

We examined the subcellular locations of CTMyb1L and CTMyb1S using plasmid constructs designed to express CTMyb1L/S fused to a GFP reporter. As shown in Fig. 4, onion epidermal cells bombarded with the CTMyb1L/S-GFP fusion construct exhibited bright GFP fluorescence within the nucleus, suggesting that both CTMyb1L and CTMyb1S are nuclear-localized proteins.

The effect of exogenously applied gibberellin on the expression of CTMyb1

To address the gibberellin responsiveness of CTMyb1S and CTMyb1L, their expression levels were determined by quantitative RT-PCR (Fig. 5) because CTMyb1 mRNA could not be detected by Northern blot analysis. Because the mRNA sequence of CTMyb1S completely overlaps that of CTMyb1L, it was difficult to distinguish the two transcripts by RT-PCR. Therefore, we amplified the 3'-untranslated region to determine the mRNA levels of both CTMyb1L and CTMyb1S, and the 5'-portion was amplified to determine the CTMyb1L mRNA level. The results showed that the OsGAMYb mRNA levels began to increase after incubation with GA3 for 12 h, and the levels increased further at 24 h before decreasing at 48 h. These data are consistent with the results of Northern blot analysis of OsGAMYb mRNA in previous studies. The mRNA levels of the gibberellin-inducible hydrolase genes Rep1 and RAmy1A began to increase 24 h after the treatment with GA3, and the levels were maintained for 48 h. In addition, the mRNA levels of RPBF, which encodes a Dof protein that interacts with OsGAMYb, increased 48 h after the application of GA3. Although the mRNA levels of both CTMyb1L and CTMyb1S increased continuously, gibberellin treatment had a weak depressive effect at 24 h, and the levels in the gibberellin-treated seeds at 48 h were similar to those in the untreated controls. The level of CTMyb1L mRNA was also examined, and it increased slightly 24 h after the application of GA3. Although there were slightly differences in the levels between the gibberellin-treated seeds and untreated controls, these data show that the gibberellin treatment does not markedly affect the expression of CTMyb1.

CTMyb1S transactivates the Rep1 promoter in cooperation with OsGAMYb, but CTMyb1L does not

Because the CTMyb1 binding sites, CAREs, are required for the gibberellin induction of the Rep1 promoter, we thought that CTMyb1 might transactivate the Rep1 promoter. We overexpressed CTMyb1 in rice aleurone cells using a plasmid construct driven by the ubiquitin promoter (pUbi:CTMyb1S/L) and examined β-glucuronidase (GUS) activity, in which was dependent on the concomitant expression of RX-184 containing two pairs of the CARE and GARE (Fig. 6(A)). The GUS activity of RX-184 was increased by overexpression of either CTMyb1S or OsGAMYb (Fig. 6(B)). The effect of CTMyb1S on RX-184 transactivation (5.3-fold) was weaker than that of OsGAMYb (8.5-fold). The co-expression of CTMyb1S and OsGAMYb resulted in 12.7-fold induction, indicating a synergistic effect on gene expression (Fig. 6(B)). These data suggest that...
cooperation between CTMyb1S and OsGAMyb causes the effective transactivation of the Rep1 promoter. In addition, we prepared another Rep1 reporter, mCAAC-12 containing a mutated version of the CARE, to check the CARE-dependence effect of CTMyb1S overexpression driven Rep1 expression. Mutation of the CAREs of the Rep1 promoter drastically reduced the transactivation by CTMyb1S (Supplemental Fig. 5). In contrast to the effects of CTMyb1S overexpression, the overexpression of CTMyb1L had no effect on the GUS activity of RX-184, and no synergism with OsGAMyb was observed (Fig. 6(C)).
The CARE and GARE are important for transactivation by CTMyb1S and OsGAMyb.

To evaluate the synergism between CTMyb1S and OsGAMyb, further, we prepared a chimeric promoter including the CARE and GARE in their combinations and performed the GUS reporter assay in rice aleurone cells (Fig. 7). When two pairs of the CARE and GARE were retained in the chimeric promoter (pGC2), GUS activity was increased by the overexpression of either CTMyb1S or OsGAMyb. The co-expression of CTMyb1S and OsGAMyb prolonged these effects, demonstrating the synergism between CTMyb1S and OsGAMyb. When the two CAREs were replaced with GAREs, the chimeric promoter including four GAREs (pGG2) was effectively transactivated by OsGAMyb, but not by CTMyb1S. This result showed that OsGAMyb transactivates the pGG2 promoter through GARE binding. When the two GAREs were converted to CAREs (pCC2), the chimeric promoter with four CAREs was not transactivated by the overexpression of either CTMyb1S or OsGAMyb. However, the co-expression of CTMyb1S and OsGAMyb resulted in moderate transactivation of the pCC2 promoter. These results suggest that CTMyb1S is not a direct transactivator of the pCC2 promoter and requires the transactivating ability of OsGAMyb.

Protein interaction between CTMyb1 and RPBF

We investigated the interaction between CTMyb1 and OsGAMyb with a yeast two-hybrid system but could not demonstrate the binding of CTMyb1S to OsGAMyb (Supplemental Fig. 6). Next, we focused on RPBF, a Dof-class transcription factor binding a pyrimidine box in the gibberellin-inducible Raamy1A promoter.9) Previously, we found that CARE is involved in the GA response of the barley proteinase gene EPB1 in addition to two GAREs and one pyrimidine box.18,32) Therefore, it is possible that the pyrimidine box is interchangeable with the CARE and that pyrimidine box-binding protein, RPBF is involved in the gibberellin response of Rep1.

Hence, we examined the physical interaction between CTMyb1 and RPBF in a yeast two-hybrid system. The cDNA clone 1–1 containing the partial CTMyb1S polypeptide (E219–L621; Fig. 1) fused to the AD (activation domain) was used as the prey; RPBF used as the bait. Because RPBF has a transactivation domain active in yeast cells,9) weak β-galactosidase activity was detected in yeast cells into which the RPBF-BD (binding domain) fusion and the AD alone were introduced (Supplemental Fig. 7). In contrast, co-transformation of RPBF-BD and the partial CTMyb1-AD resulted in higher activity in the LacZ reporter assay (Supplemental Fig. 7), suggesting that RPBF can interact with CTMyb1 in yeast cells.

We examined the physical interaction of CTMyb1S/L and RPBF in plant cells using a BiFC assay. Either CTMyb1S or CTMyb1L was fused to the N-terminal portion of GFP, and RPBF was fused to the C-terminal portion of cyan fluorescent protein. Upon co-expression in onion epidermal cells, their interactions, which reconstitute the GFP fluorophore, were monitored. The results showed that the GFP fluorophore was reconstituted and targeted to the nucleus when both the CTMyb1S and RPBF fusion proteins were expressed (Fig. 8). Co-expression of the CTMyb1L and RPBF fusion proteins resulted in reconstituted GFP fluorophores that were excluded from the nucleus and targeted to the perimeter of the cell. These results imply that RPBF can complex with CTMyb1S/L and may affect the subcellular localization of the partner protein. Using this system, we also examined the physical interaction of CTMyb1S/L and OsGAMyb, but we could not detect the GFP fluorophore (data not shown).

RPBF is involved in the transactivation of the Rep1 promoter

The results obtained in the BiFC assay demonstrated interactions between the RPBF and CTMyb1S/L proteins, but the subcellular localizations of the
RPBF-CTMyb1S and RPBF-CTMyb1L complexes differed from each other (Fig. 8). To investigate how the different localizations of CTMyb1S and CTMyb1L influence the transactivation of the Rep1 promoter, RPBF was co-expressed with CTMyb1S/L in rice aleurones. The Rep1 promoter was transactivated by the expression of RPBF alone (Fig. 9(B)). However, no synergistic effect of the co-expression of RPBF, CTMyb1S, and OsGAMyb was observed, except when CTMyb1S and OsGAMyb were co-expressed (Fig. 9(B)). Additionally, the co-expression of RPBF and CTMyb1L also had no synergistic effect on GUS activity (Supplemental Fig. 8).

**Discussion**

CTMyb1 belongs to the family of telomeric repeat binding factor-like proteins

The sequence analysis of CTMyb1 showed that it has a C-terminal Myb domain. C-terminal Myb domain proteins are conserved in plants and are structurally related to human telomere repeat binding factors. These Myb proteins make up the telomeric repeat binding factor-like (TRFL) family, which is divided into 2 types. CTMyb1 belongs to the type II group (Supplemental Fig. 2), the members of which are unable to interact with telomeric DNA. A few typeII proteins are known to bind sequences within 5'-upstream regulatory
The transcription start sites determine both the length and the function of the CTMyb1 protein

We found that the transcription start sites of CTMyb1 determined the length of the CTMyb1 polypeptide (Fig. 3). The Rep1 promoter can be activated by the shorter polypeptide, CTMyb1S (408 amino acids), but not by the longer polypeptide, CTMyb1L (621 amino acids; Fig. 6). Using an expression system with GFP protein fused to CTMyb1S/L, we found that both proteins localized to the nucleus (Fig. 4). We found that CTMyb1 interacts with RPBF. The result of the BiFC analysis showed that the complex of CTMyb1S and RPBF localizes to the nucleus, whereas the complex of CTMyb1L and RPBF was found in the cytoplasm (Fig. 8). A transient expression system in rice aleurone cells revealed that RPBF activated the Rep1 promoter, as did CTMyb1S and OsGAMyb, but the co-expression of RPBF with either CTMyb1S or CTMyb1L had no synergistic effect on the reporter’s activity (Fig. 9). These results suggest that CTMyb1L may be transported from the nucleus to the cytoplasm in complex with RPBF and is thus unable to activate the Rep1 promoter directly. Because the CTMyb1S complex with RPBF is localized in the nucleus, CTMyb1S is capable of interaction with the Rep1 promoter. RPBF is known to interact with OsGAMyb. Therefore, a complex consisting of CTMyb1S, RPBF, and OsGAMyb may interact with the Rep1 promoter and activate gene transcription. The acidic amino acid-rich N-terminal region of CTMyb1L (213 amino acids), which CTMyb1S lacks, may determine the intracellular localization of the complex of CTMyb1L and RPBF. The GAL4 activation domain, which is also rich in acidic amino acids, interacts with a repressor. Therefore, the amino acids in the N-terminal domain of CTMyb1L may be involved in the determination of CTMyb1L localization by interacting with unknown proteins in this domain.

The interaction of CTMyb1 and RPBF

We were unable to detect a physical interaction between CTMyb1S and OsGAMyb (Supplemental Fig. 6), although RPBF interacts with both of CTMyb1S and OsGAMyb (Fig. 8). The Rep1 promoter includes no pyrimidine box to which RPBF binds. These data indicate that RPBF mediates or supports the interaction between OsGAMyb and CTMyb1. Thus, we presume that gibberellin induces the formation of a complex containing CTMyb1S, RPBF, and OsGAMyb to activate gibberellin-inducible promoters. We have shown that the CARE is required for the gibberellin induction of the promoter of a barley protease gene, EPB1. In addition, it has been reported that the two GAREs (nucleotide positions −142 and −156), the pyrimidine box at −120, and the region from −409 to −359, are involved in the gibberellin response. The set of CARE and GARE together with a set of GARE and the pyrimidine box likely mediates the gibberellin induction of the EPB1 promoter synergistically.

Interaction of the OsGAMyb complex with the Rep1 promoter

Our results suggest that the formation of a complex between CTMyb1S, RPBF, and OsGAMyb is required to activate the Rep1 promoter. Because co-expression of CTMyb1S and OsGAMyb activated a chimeric promoter containing two sets each of the GARE and CARE (Fig. 7), the complex including the two proteins is important. In contrast, the co-expression of the two proteins exhibited no synergistic effect on the chimeric promoter with four copies of the GARE. Our previous data have indicated that this promoter does not respond to exogenously applied GA3. The promoter including
four copies of the CARE was activated by the co-expression of CTMyb1S and OsGAMyb, but its activation was lower than that of pGC2 (Fig. 7). Therefore, efficient activation by co-expression requires two sets of the GARE and CARE. This result indicates that the binding of both cis-elements by CTMyb1S and OsGAMyb is necessary for high Rep1 expression levels and that these transcription factors can complex with RPBF.

To determine whether CTMyb1 is involved in the expression of gibberellin-inducible genes, we examined the effect of CTMyb1S/L and OsGAMyb overexpression on activation of RAmy1A. Both CTMyb1S and CTMyb1L activated the promoter, and no synergistic effect between CTMyb1S and OsGAMyb was found (Supplemental Fig. 9). Because it is known that RAmy1A is activated by MybS binding the amylase box together with OsGAMyb and RPBF,9,33) the function of CTMyb1S may be limited to a partial involvement in activation of the gene.

RPBF has two functions for activation of the Rep1 promoter

Our results indicated that CTMyb1L alone is localized in the nucleus (Fig. 4), but co-expression of the protein with RPBF changed its cellular localization; the CTMyb1L–RPBF complex was found in the cytoplasm (Fig. 8). In contrast, the CTMyb1S–RPBF complex was located in the nucleus. Additionally, the expression of RPBF alone activated the Rep1 promoter, and the co-expression of RPBF with CTMyb1L did not affect activation of the promoter compared with that of RPBF alone (Supplemental Fig. 9). In fact, an inconsistency between the cellular localization and the transcriptional activation was observed. We presume that RPBF is involved in replacing CTMyb1L with CTMyb1S on the Rep1 promoter. Because CTMyb1L mRNA is present in the absence of gibberellin, CTMyb1L conceivably interacts with the CARE in the nucleus before gibberellin synthesis; after the synthesis of RPBF is induced by gibberellin, RPBF interacts with CTMyb1L, transports it from the nucleus to cytoplasm, and RPBF interacting with CTMyb1S and OsGAMyb binds to the Rep1 promoter for its activation (Fig. 10). Another possibility we consider is that RPBF binds to CTMyb1L in the cytoplasm and prevents the transport of CTMyb1L to the nucleus, but not that of CTMyb1S. As a consequence of CTMyb1L remaining in cytoplasm, CTMyb1S is able to interact with CARE. To verify these hypotheses, it is necessary to examine whether the complex of CTMyb1L and RPBF translocates from the nucleus to the cytoplasm.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.998620.

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