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Functional Analysis of RF2a, a Rice Transcription Factor*

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RF2a is a bZIP transcription factor that regulates expression of the promoter of rice tungro bacilliform badnavirus. RF2a is predicted to include three domains that contribute to its function. The results of transient assays with mutants of RF2a from which one or more domains were removed demonstrated that the acidic domain was essential for the activation of gene expression, although the proline-rich and glutamine-rich domains each played a role in this function. Studies using fusion proteins of different functional domains of RF2a with the 2C7 synthetic zinc finger DNA-binding domain showed that the acidic region is a relatively strong activation domain, the function of which is dependent on the context in which the domain is placed. Data from transgenic plants further supported the conclusion that the acidic domain was important for maintaining the biological function of RF2a. RF2a and TBP (TATA-binding protein) synergistically activate transcription *in vitro* (Zhu, Q., Ordiz, M. I., Dabi, T., Beachy, R. N., and Lamb, C. (2002) *Plant Cell* 14, 795–803). *In vitro* and *in vivo* assays showed that RF2a interacts with TBP through the glutamine-rich domain but not the acidic domain. Functional analysis of such interactions indicates that the acidic domain activates transcription through mechanisms other than via the direct recruitment of TBP.

The severe stunting symptoms of rice tungro disease are caused by infection of rice tungro bacilliform virus (RTBV),[1] a double-stranded DNA badnavirus. Understanding the transcriptional regulation of RTBV is an important factor to elucidate the basis of the disease. RTBV carries a single, vascular tissue-specific promoter with several defined DNA *cis*-elements (1–4). Box II, one of the DNA *cis*-elements in the promoter, is essential for phloem-specific expression of the promoter (3, 4). A bZIP type rice host transcription factor, RF2a, was identified by its interaction with Box II (5). Furthermore, overexpression of RF2a in transgenic plants is sufficient to activate expression of RTBV promoter in other than vascular tissues (6).

Temporal and spatial regulation of gene expression relies largely on the function of gene-specific transcription factors and is achieved by the activity of multiple proteins that bind to regulatory elements and with other proteins to alter basal rates of transcription initiation and/or elongation (7–9). A typical gene-specific eukaryotic transcription factor includes a DNA-binding domain and one or more domains that influence the activation or repression of transcription through interactions with general transcription factors, co-factors, chromatin remodeling complexes, and components of RNA polymerase II holoenzyme, among others (7, 10–13). Transacting domains are often characterized as having a high content of specific amino acids, including domains rich in the acidic amino acids, proline or glutamine (14–16). Acidic domains have been reported to possess activation functions that include interactions with TATA-binding proteins (TBP) (13, 17), TBP-associated factors (TAFs) (18), TFIIA (19), TFIIB (20, 21), other general transcription complexes (13, 22), and co-factors (12). Proline-rich and glutamine-rich domains typically act through interactions with TBP, TAFs, and other co-factors (14). Although proline-rich and glutamine-rich domains act as activation domains in most of the cases, they can also function as repression domains (23, 24). RF2a contains three putative transacting domains, namely proline-rich and acidic domains at the N terminus and a glutamine-rich domain at the C terminus (5). However, the function of these domains and their contribution to transcriptional activation by RF2a remain unclear.

We reported previously that RF2a interacts with rice TBP and stimulated RTBV promoter activity in an *in vitro* transcription system (25). Theoretically, each of the three putative functional domains of RF2a has the potential to interact with TBP; however, the role of such interaction(s) in contributing to the transactivating function of RF2a is not clear.

To address these questions, the putative functional domains of RF2a were studied by removing one or more domains from RF2a or by fusing individual domains with 2C7 synthetic zinc finger protein, a DNA-binding domain. The abilities of the mutants of RF2a and of the fusion proteins to regulate gene expression were tested. The data of experiments in BY-2 protoplasts and transgenic tobacco plants demonstrate that the acidic domain is the primary activation domain of RF2a. Interactions between mutants of RF2a and rice TBP, *in vitro* and *in vivo*, demonstrated that the glutamine-rich domain, rather than the acidic domain, of RF2a directly interacts with TBP. These results support the hypothesis that the acidic domain is critical to the activation function of RF2a and activates transcription through mechanisms other than directly interacting with TBP.

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[1] The abbreviations used are: RTBV, rice tungro bacilliform virus; TBP, TATA-binding protein; TAF, TBP-associated factor; GST, glutathione *S*-transferase; DBD, DNA-binding domain; GUS, β-glucuronidase; CaMV, cauliflower mosaic virus.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Plasmid Constructions for Protein Purification—Mutants of RF2a were created through PCR amplification. A *Nde*I restriction site was added to 5′ end of all primers, and the ATG in the restriction site was in frame with the His_6 tag in vector pET28a (Invitrogen) and served as the transcription start codon for plasmids described under "Plant Expression Constructs with RF2a Deletion Mutants." A *Bam*HI site was added to all 3′ primers with a stop codon in front of the restriction site. The primers used for amplification of different fragments of RF2a are listed below. The ΔP fragment was amplified using primers RF2a-ΔP5′ and RF2a3′; ΔQ was amplified using primers RF2a 5′ and RF2a-ΔQ3′; ΔPΔA was amplified using primers RF2a-ΔPΔA 5′ and RF2a3′; and ΔPΔQ was amplified using primers RF2a-ΔP5′ and RF2a-ΔQ3′. All of the fragments were restricted with *Nde*I and *Bam*HI and were cloned into pET28a through the same set of restriction sites. All of the mutations were verified by DNA sequence analysis. The derived plasmids were designated pET-RF2a-ΔP, pET-RF2a-ΔQ, pET-RF2a-ΔPΔA, and pET-RF2a-ΔPΔQ. TBP coding sequence was released from pOsTBP2 (25) through *Nco*I digestion (made blunt with Klenow DNA polymerase) followed by *Xho*I digestion. The released fragment was cloned into pGEX-4T-1 (Amersham Biosciences) through *Bam*HI (made blunt with Klenow DNA polymerase) and *Xho*I sites (pGST-TBP) to produce a GST-TBP fusion protein with a thrombin recognition site between GST and TBP.

Plant Expression Constructs with RF2a Deletion Mutants—The coding sequences for mutants of RF2a were released from pET28a-derived plasmids and cloned into the plant expression vector pMON999 (a gift from Monsanto company) to place each gene downstream of an enhanced CaMV 35S promoter, followed by a nopaline synthase terminator sequence. The resulting constructs are named p35S:RF2a, p35S:RF2a-ΔP, p35S:RF2a-ΔQ, p35S:RF2a-ΔPΔA, p35S:RF2a-ΔPΔQ, and p35S:RF2a-3Δ. The reporter gene construct, pBII-48Ca:GUS, was built using PCR to introduce the Box II sequence element (4) to a minimal CaMV 35S promoter comprising nucleotides −48 to +8 with primers BII-48Ca and GUS 3′ using p35S:GUS plasmid as template. The PCR product was restricted with *Hin*dIII and *Nco*I, and the resulting fragment was inserted into p35S:GUS to replace the 35S promoter.

Constructs with RF2a Domains and 2C7 DNA-binding Domain (DBD) Fusion Proteins—To create effectors with RF2a domains fused to the N terminus of 2C7 DBD (26), coding sequences for the acidic domain (A), proline-rich domain (P), glutamine-rich domain (Q), and proline-rich plus acidic domains were amplified using primer pairs A-2C7 5′/A-2C7 3′, P-2C7 5′/P-2C7 3′, and Q-2C7 5′/Q-2C7 3′, respectively, with pET-RF2a as template. *Bgl*II and *Bam*HI restriction sites were introduced in the 5′ and 3′ primers. The PCR products were restricted with these set enzymes and cloned into pMON999 through *Bgl*II and *Eco*RI along with the DNA fragment that encodes the 2C7 DNA-binding domain. The latter DNA fragment was released from p35S:2C7 using *Bam*HI and *Eco*RI (26). The plasmids were designated as p35S:A-2C7, p35S:P-2C7, and p35S:Q-2C7. For effectors with RF2a domains at the C terminus of the 2C7 DBD, coding sequences for A, P, Q, and P plus A (PA) domains were released from pET-RF2a-A, pET-RF2a-P pET-RF2a-Q, and pET-RF2a-PA[2] using the enzymes *Xba*I and *Eco*RI and cloned into p35S:2C7-VP16 (26) to replace the VP16 domain with the same restriction sites. The resultant plasmids were named p35S:2C7-A, p35S:2C7-P, p35S:2C7-Q, and p35S:2C7-PA.

Plasmid Constructs for Agrobacterium-mediated Transformation—The fusion genes described under "Plant Expression Constructs with RF2a Deletion Mutants" were released from pMON999-derived plasmids using *Not*I (blunted) and cloned into the binary vector pGA-E:GUS (6) using the blunt *Hin*dIII site. The final plasmids were named pGA-E:GUS/P-35S:ΔP, pGA-E:GUS/P-35S:ΔQ, pGA-E:GUS/P-35S:ΔPΔA, and pGA-E:GUS/P-35S:ΔPΔQ. The primers were: RF2a 5′, GCCGCCCATATGGAGAAGATGAACAGGGAGAAATCC (*Nde*I); RF2a 3′, 5′-CGCGGATCCTCAGTTGCCGCTGCTTCCTGA-3′ (*Bam*HI); RF2a-ΔP 5′, 5′-GCCGCCCATATGGAGAAGATGGGCCACAGGCGC3′ (*Nde*I); RF2a-ΔPΔA 5′, 5′-GCCGCCCATATGGAGAAGATGTCCGCCGCCGCC3′ (*Nde*I); RF2a-ΔQ 3′, 5′-CGCGGATCCTCAGTGTGGCATGCCACCGAA-3′ (*Bam*HI); BII-48Ca 5′, TGATCAAAGCTTCCAGTGTGCCCCTGGTCGCAAGACCCTTCCTC (*Hin*dIII); GUS 3′: GATTTCACGGGTTGGGGTTTCTA; P 3′, CGCGGATCCTCAGGGGTTCCTCGTCGGGAA (*Bam*HI); A 3′, CGCGGATCCTCATCCATGGGCGGCGGCGGA (*Bam*HI); A-2C7 5′, GCCAGATCTATGGGCCACAGGCGCGCCC (*Bgl*II); A-2C7 3′, CGCGGATCCTCCATGGGCGGCGGCG (*Bam*HI); P-2C7 5′, GCCAGATCTATGAACAGGGAGAAATCCCC (*Bgl*II); P-2C73′, CGCGGATCCGGGGTTCCTCGTCGGGAA (*Bam*HI); Q-2C7 5′, GCCAGATCTATGCAATTCGGAGGCAACCAGC (*Bgl*II); and Q-2C7 3′, CGCGGATCCGTTGCCGCTGCTTCCTGA (*Bam*HI).

Protein Purification

The pET28a-derived plasmids were transformed into *Escherichia coli* strain BL21(DE3)pLysS for protein expression. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 3 h after the cell density reached A_{600} of ~0.6. His-tagged proteins were purified according to procedures provided by Novagen using nondenaturing conditions. The purified recombinant proteins were dialyzed in 1× phosphate-buffered saline with 20% glycerol to remove imidazole and stored at −70 °C. GST-TBP fusion protein was purified using glutathione-cross-linked agarose beads (Sigma). After extensive washing with 1× phosphate-buffered saline plus 0.1% Tween 20, GST-TBP fusion protein was eluted with 10 mM reduced glutathione and dialyzed against 1× phosphate-buffered saline with 20% of glycerol. In some cases, TBP was released from the beads using thrombin (Sigma) digestion.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were carried out essentially as described by Yin and Beachy (2). 100 ng of proteins purified from *E. coli* were incubated with ^{32}P-labeled Box IIm1 DNA probe followed by electrophoresis in a 5% acrylamide gel (4). For supershift assays, various amounts of TBP were added to the reactions as indicated.

Transfection of Tobacco BY-2 protoplasts

The protoplasts were isolated from tobacco cell line BY-2 as described by Watanabe *et al.* (43). Approximately one million protoplasts were transfected by electroporation with 20 μg of effector DNA, 15 μg of herring sperm DNA, 2.5 μg of reporter gene DNA, and 15 μg of pCat-GFP DNA (constructed by C. Reichel). In samples with reporter gene alone, the total amount of DNA was adjusted by adding 20 μg of herring sperm carrier DNA. The electroporation parameters used were 300 V and 250 microfarads with the Bio-Rad electroporation system. Protoplast samples were cultured in Murashige and Skoog medium with 0.4 M mannitol, pH 5.8, at 28 °C. The protoplasts were collected 24 h after electroporation.

Quantitative Analysis of GUS Activity and GFP

Protein samples from protoplasts were prepared using protein extraction buffer (27) and quantified using DC protein assay kit (Bio-Rad). GUS activity was measured using 4-methylum-belliferyl-β-D-glucuronide as substrate as described by Jefferson *et al.* (27). GFP was quantified by spectrometry with excitation at 460 nM and emission at 510 nM.

Tobacco Transformation

pGA482-derived plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 and used for tobacco transformation. Leaf discs from *Nicotiana tabacum* cv. Xanthi NN were used following the protocol of Horsch *et al.* (28). At least 15 independent transgenic lines were produced with each gene construct. Transgenic plants were self-fertilized, and T_1 seeds were collected. The T_1 seeds were germinated on Murashige and Skoog medium (29) with kanamycin (100 mg/liter) selection, and Kan^r seedlings were grown in a greenhouse for observation of phenotypic changes.

Protein Overlay

2 μg of each protein sample was subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The transferred proteins were denatured in 20 mM Hepes-KOH, pH 7.9, 10% glycerol, 60 mM KCl, 6 mM $MgCl_2$, 0.6 mM EDTA, 1 mM dithiothreitol, and 6 M guanidine hydrochloride for 45 min. The proteins were then renatured by incubation in the same buffer containing decreasing amounts of guanidine hydrochloride (3 M, 1.5 M, 0.75 M, 300 mM, and 100 mM) for 20 min each followed by two washes without guanidine hydrochloride. The membrane was blocked in TBST buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween 20) containing 5% nonfat milk powder for 1 h before adding 20 μg of GUS-TBP fusion protein to the 3-ml reaction buffer and incubated at 4 °C overnight. After intensive washing with TBST, the membrane was blocked with TBST containing 5% milk for 30 min prior to addition of goat anti-GST antibody (Amersham Biosciences). After 2 h of incubation at room temperature, the membrane was intensively washed with TBST and

[2] I. Ordiz and R. N. Beachy, unpublished data.

blocked again with TBST containing 5% milk for another 30 min before adding the horseradish peroxidase-conjugated anti-goat secondary antibody. The membrane was washed with TBST after 2 h of incubation. The GST was detected by applying Super Signal Substrates for horseradish peroxidase (Pierce) and exposure to x-ray film. The membrane was then stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM mercaptoethanol, 2% SDS) at 70 °C for 1 h. The membrane-bound proteins of mutants of RF2a were detected through the same Western immunoblot reaction procedure as described below.

Western Immunoblot Reactions

Protein samples from transgenic tobacco leaf tissues were extracted in buffer (50 mM Na_3PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine) and quantified using the DC protein assay kit (Bio-Rad). 40 μg of each protein samples were subjected to SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was stained with Ponceau S (Sigma) to monitor protein loading prior to immunodetection. The primary antibody used in the immunodetection was raised in rabbits against full-length RF2a; the secondary antibody was horseradish peroxidase-conjugated to goat anti-rabbit antibody (Southern Biotechnology Associates).

In Vitro Transcription Assay

Whole cell extracts of rice cv. IR72 suspension cultures were used to transcribe a template comprising nucleotides −164 to +45 of the RTBV promoter ligated to the *uidA* gene as described (5, 25). The recombinant protein RF2a, mutants of RF2a, and rice TBP2 were added to the reaction mixture 10 min prior to the addition of the whole cell extract. Transcription products were analyzed by primer extension with a primer located in the GUS coding region (2).

RESULTS

RF2a Mutants with Deletions of Functional Domains Bind to the DNA Target Sequence—In previous studies, we showed that the bZIP protein RF2a enhances transcription *in vivo* and *in vitro* (5, 6). Based on the enrichment of amino acids in specific regions of RF2a, we proposed that proline-rich, acidic, and glutamine-rich regions might play specific roles in the function of RF2a (5). To analyze the function of each region, mutants of RF2a were created by removing one or more of the putative domain(s) (Fig. 1*A*). The coding sequence for each mutant was cloned into the bacterial expression vector pET28a, in which a His_6 tag was placed at the N terminus of the fusion protein. The derived plasmids were named pET-RF2a-ΔP, pET-RF2a-ΔQ, pET-RF2a-ΔPΔA, and pET-RF2a-ΔPΔQ. pET-RF2a and pET-RF2a-3Δ were described by Petruccelli *et al.* (6). Proteins from each plasmid are designated RF2a, RF2a-ΔP, RF2a-ΔQ, RF2a-ΔPΔA, RF2a-ΔPΔQ, and RF2a-3Δ, respectively (Fig. 1*B*).

It has been reported that in some cases, protein domains other than the bZIP domain are required for establishing stable protein-DNA complexes (30, 31). Box IIm1 is a mutant of Box II *cis*-element with increased binding affinity to RF2a (compared with Box II; 5). RF2a and RF2a-3Δ are capable of binding Box IIm1 DNA sequence as either homodimers or heterodimers (6). To confirm that each of the mutant proteins bind to the DNA target, gel mobility shift assays were carried out with purified recombinant proteins (Fig. 1*B*). The data presented in Fig. 1*C* demonstrate that proteins ΔP, ΔQ, ΔPΔA, ΔPΔQ, and 3Δ of RF2a bind to Box IIm1.

Contribution of Sequence Domains to the Function of RF2a—Previous studies demonstrated that phloem-specific expression of the RTBV promoter was retained on a DNA fragment comprising nucleotides −164 to +45 (the "E" fragment) (2, 4). Tissue specificity of the promoter is governed primarily by Box II, whereas GATA and ASL elements showed strong effects on the level of promoter expression (3, 4). To facilitate the analysis of the functions of RF2a and its mutants, a chimeric promoter was developed with a single copy of Box II fused to a minimal CaMV 35S promoter comprising nucleotides −48 to +8. The chimera was ligated to the *uidA* coding sequence to create the reporter pBII-48Ca:GUS. The activity of this chimeric promoter is about 16% of the activity of RTBV promoter activity and 6.5% of the activity of the enhanced CaMV 35S promoter in BY-2 protoplasts (not shown).

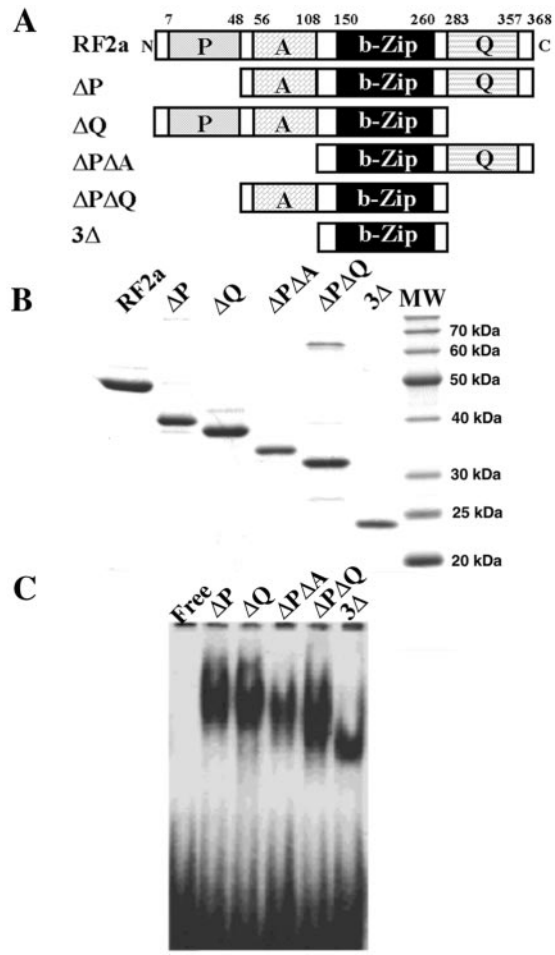

FIG. 1. **Electrophoretic mobility shift assay of protein-DNA complexes formed between mutants of RF2a and Box II *cis*-element of the RTBV promoter.** *A*, schematic diagram of mutants of RF2a. *A*, acidic domain; *P*, proline-rich domain; *Q*, glutamine-rich domain. *B*, gel mobility shift assay using purified mutant proteins of RF2a as labeled. A control lane without protein (free) is included in the assay. Box IIm1 DNA was labeled with ^{32}P, and radioactivity was detected by autoradiography.

To analyze the function of domains of RF2a, effectors were created by inserting coding sequences of mutants of RF2a downstream of the enhanced CaMV 35S promoter in pMON999 vector (a gift from Monsanto Co., St. Louis, MO). The resultant constructs, p35S:RF2a, p35S:RF2a-ΔP, p35S:RF2a-ΔQ, p35S:RF2a-ΔPΔA, p35S:RF2a-ΔPΔQ, and p35S:RF2a-3Δ, were co-transfected into BY-2 protoplasts with pBII-48Ca:GUS (Fig. 2*A*). Plasmid pCat-GFP, in which the GFP gene was driven by CaMV 35S promoter, was co-introduced to serve as an internal control.

As shown in Fig. 2*B*, the transactivation function of RF2a was not decreased by removing either the proline-rich (RF2a-ΔP) or glutamine-rich (RF2a-ΔQ) domains or both of the domains (RF2a-ΔPΔQ). In fact, the activation function of each of these mutants was greater than that of full-length RF2a; RF2a-ΔP was significantly different from RF2a at the $P_{0.05}$ level, whereas RF2a-ΔQ and RF2a-ΔPΔQ were significantly different from RF2a at the $P_{0.01}$ level (Student's *t* test). Also, the difference between the activity of RF2a-ΔP and RF2a-ΔQ was significant at the $P_{0.01}$ level, and there was no difference between RF2a-ΔQ and RF2a-ΔPΔQ. The data suggest that the proline-rich and glutamine-rich domains do not contribute in a positive way to the activation function of RF2a; on the contrary,

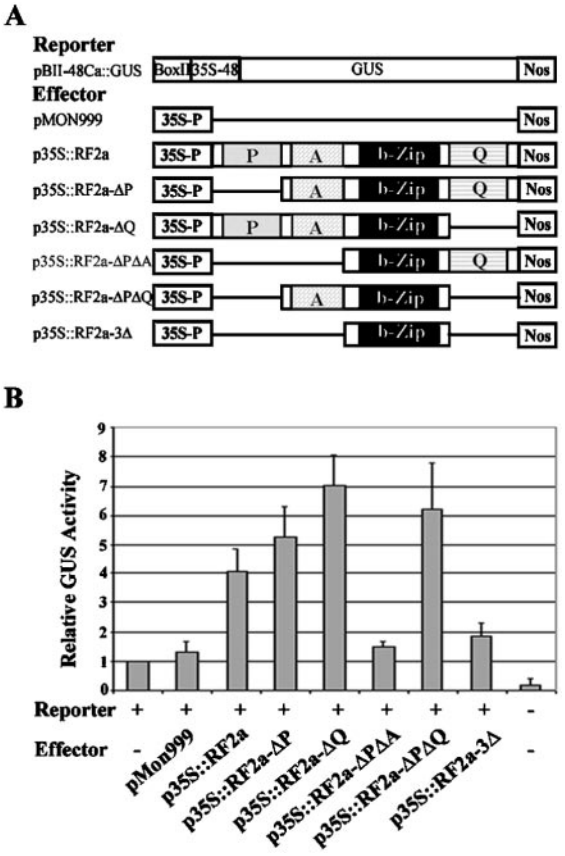

FIG. 2. **Effects of RF2a and mutants of RF2a on gene expression in BY-2 protoplasts.** *A*, reporter and effector gene constructs. The GUS reporter gene contains Box II with nucleotides −48 to +8 of the CaMV 35S as promoter and nopaline synthase 3′ terminator sequence (pBII-48Ca:GUS). Effectors include RF2a and mutants ligated with the CaMV 35S promoter and nopaline synthase terminator. *B*, relative GUS activities in BY-2 protoplasts that were co-transfected with reporter gene and effector gene constructs as indicated. The results are the averages with S.D. of three independent experiments, three samples experiment, after normalization with GFP.

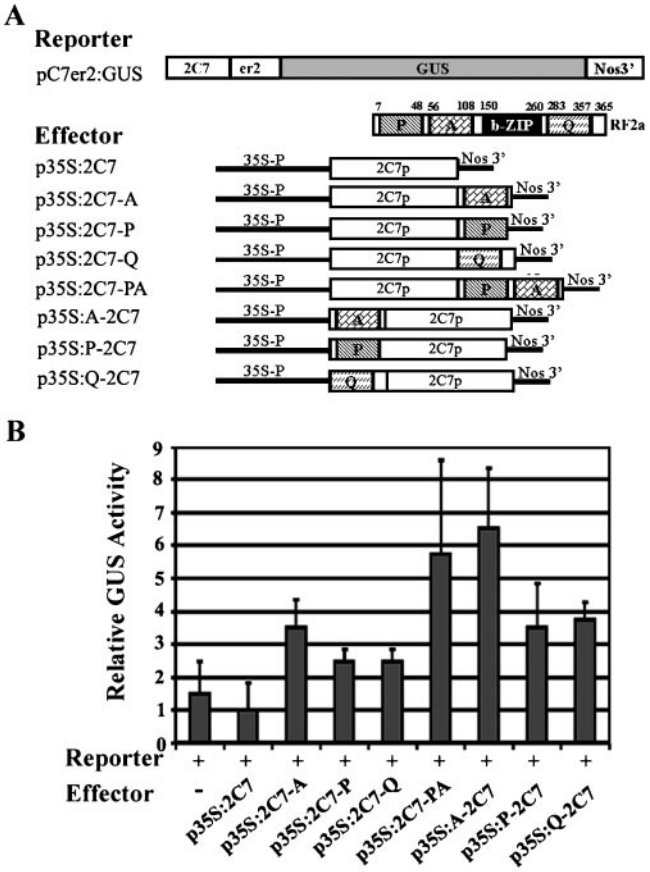

FIG. 3. **Effects of RF2a domains on gene expression when in fusion with 2C7 DNA-binding domain.** *A*, diagram of reporter and effector constructs used for transient assays of fusion proteins of RF2a function domains with 2C7 synthetic zinc finger DBD. *B*, relative GUS activities in BY-2 protoplasts that were co-transfected with reporter gene and effector gene constructs as indicated. The results are the averages with S.D. of three independent experiments, three samples/experiment, after normalization with GFP.

these domains may reduce the activity of RF2a. In contrast, the activity dropped to near basal level when the acidic domain was removed (RF2a-ΔPΔA and RF2a-3Δ) (Fig. 2*B*). These results suggest that the acidic domain is responsible for the activation of gene expression by RF2a.

Functions of RF2a Domains in Fusion Proteins with 2C7 DBD—To determine whether domains of RF2a can serve as independent modules to regulate transcription, putative functional domains were fused with the synthetic 2C7 protein, a synthetic zinc finger DBD that specifically binds to the 2C7 DNA-binding site (26, 32) (Fig. 3*A*); domains were placed either at the N terminus or the C terminus of the DBD. The reporter construct pC7er2:GUS carried the *uidA* coding sequence located downstream of a chimeric promoter comprising 6x2C7-binding sites ligated with the minimal promoter of erbB-2 (26). p35S:2C7 encodes the 2C7 protein without an activation domain and served as a control (Fig. 3*A*) (26).

As shown in Fig. 3*B*, when domains of RF2a were placed C-terminal of the 2C7 protein, 2C7-A and 2C7-PA showed significant activation function. When the domains were fused individually at the N terminus of 2C7, the acidic domain (A-2C7) gave stronger activation than the P (P-2C7) or Q (Q-2C7) domains did. The function of the acidic domain in the fusion proteins is consistent with its function in RF2a, although the position of this domain in the fusion proteins affects its activity. The proline- and glutamine-rich domains had no effect on gene expression when they were placed at the C terminus of the 2C7 DBD; however, these two domains showed mild activation function when they were fused at the N terminus of the 2C7 DBD. Neither of these two domains showed repression function as they did in the context of RF2a.

Impact of Mutants of RF2a on Plant Development—We previously demonstrated that transgenic rice (5) and tobacco (6) plants that overexpressed RF2a were normal in appearance and reproduction. To determine whether mutants of RF2a from which one or more domains were removed had a positive or negative effect on plant development, we produced transgenic plants that overexpress mutants of RF2a. Fifteen or more independent transgenic tobacco lines were developed with each construct through *Agrobacterium*-mediated transformation. After PCR analysis, transgenic lines with each mutant were observed for phenotypic changes. T_1 generation plants with 35S:RF2a, 35S:RF2a-ΔP, 35S:RF2a-ΔQ, and 35S:RF2a-ΔPΔQ did not exhibit abnormal phenotypes (Fig. 4*A*). However, 11 of 15 independent transgenic lines with 35S:RF2a-ΔPΔA exhibited mild to severe stunting with curved leaves and substantial delay in flowering times (Fig. 4, *A* and *B*). The internodal elongation of transgenic plants was strongly repressed by RF2a-ΔPΔA (Fig. 4*C*, *panel 1*). The phenotype caused by 35S:RF2a-ΔPΔA was similar to but less severe than the phenotype caused by 35S:RF2a-3Δ (6). Cross-sections of the stem of transgenic plants with either RF2a-ΔPΔA or RF2a-3Δ showed that the xylem of stunted plants was not uniformly lignified and

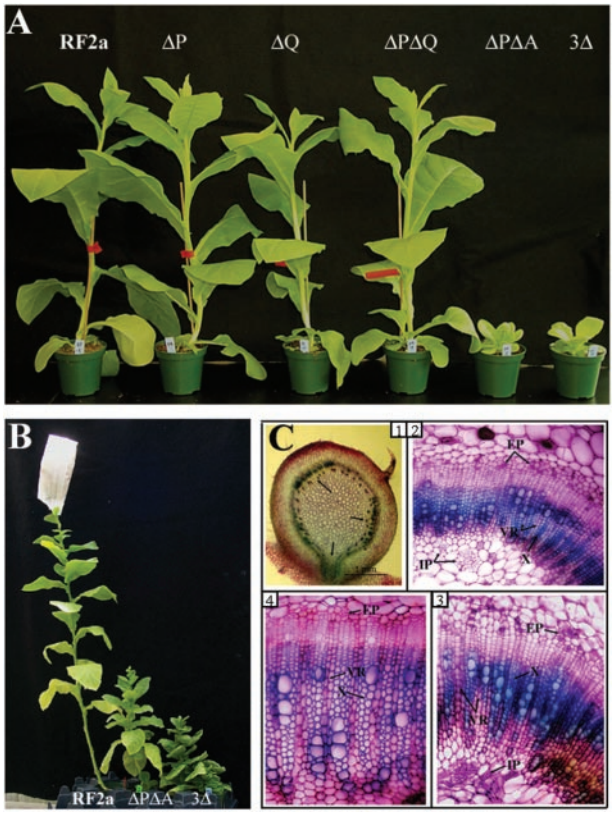

FIG. 4. **Impact of RF2a and mutants on development of transgenic tobacco plants.** *A*, two-month-old transgenic tobacco plants with 35S:RF2a and mutants of RF2a driven by the 35S promoter were grown in the greenhouse. Only transgenic plants with mutants lacking the acidic domain (RF2a-ΔPΔA and RF2a-3Δ) showed severe stunting phenotype. *B*, transgenic plants at 105 days. Leaves of plants with RF2a-ΔPΔA and RF2a-3Δ were curved downward, and flowering time was significantly delayed. *C*, *panel 1*, transversal section of the stem of transgenic plants with RF2a-DPDA in low magnification. The regions in response to leaf formation position in the vascular cylinder are indicated with *arrows*. The section indicates the strong repression of RF2a-ΔPΔA on internodal elongation. *Panels 2–4*, transverse sections of the lower part of stems of two-month old tobacco plants stained with toluidine blue O. *Panel 2*, transgenic plant with RF2a-ΔPΔA; *panel 3*, transgenic plant with RF2a-3Δ; *panel 4*, nontransgenic plant. *EP*, external phloem; *IP*, internal phloem; *VR*, vascular ray; *X*, xylem.

that phloem development was altered (Fig. 4*C*).

To confirm that the phenotype was related to transgene expression, leaf protein samples of transgenic plants with RF2a-ΔPΔA were analyzed via a Western blot assay using antibody against RF2a. Fig. 5 shows that there is a direct correlation between the abnormal phenotype and the accumulation of RF2a-ΔPΔA.

RF2a Interacts with TBP via the Glutamine-rich Domain—Zhu *et al.* (25) reported that RF2a physically interacts with rice TBP, and the results of current studies raised the question of whether this interaction affects RF2a function. To address this issue, we first conducted experiments to identify the regions of RF2a that interact with TBP. Gel mobility shift assays of mutants of RF2a were carried out with and without the addition of purified TBP (Fig. 6*A*). ^{32}P-Labeled Box IIm1 DNA element was used as probe in the gel shift assays. As shown in Fig. 6*B*, RF2a-ΔQ and RF2a-ΔPΔA form DNA-protein complexes with Box IIm1. When TBP was added to the reactions, only the RF2a-ΔPΔA-DNA complex exhibited a band with slower mobility (supershift) compared with the sample without TBP. When a higher amount of TBP was added to the reaction, both the RF2a-ΔPΔA-DNA complex and the supershift band was enhanced. In samples with RF2a-ΔQ, no supershift of

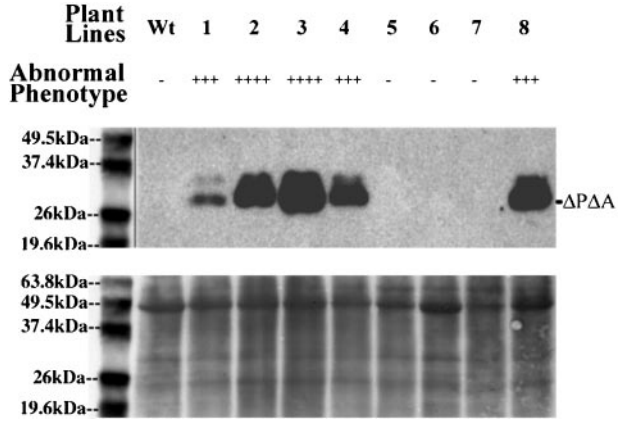

FIG. 5. **Correlation between severity of abnormal phenotypes and accumulation of RF2a-ΔPΔA.** Severity of the abnormal phenotype of transgenic tobacco plants was marked with +++ for stunting and ++++ for severe stunted phenotype, whereas − indicates that no abnormal phenotype. *Upper panel*, 40-μg protein samples were separated in 10% SDS-PAGE and detected with antibody against full-length RF2a after blotting on the membrane. The band that contains RF2a-ΔPΔA is marked on the *right*. *Lower panel*, the membrane used in the immunoblot was stained with Ponceau S (Sigma) prior to the antibody reaction.

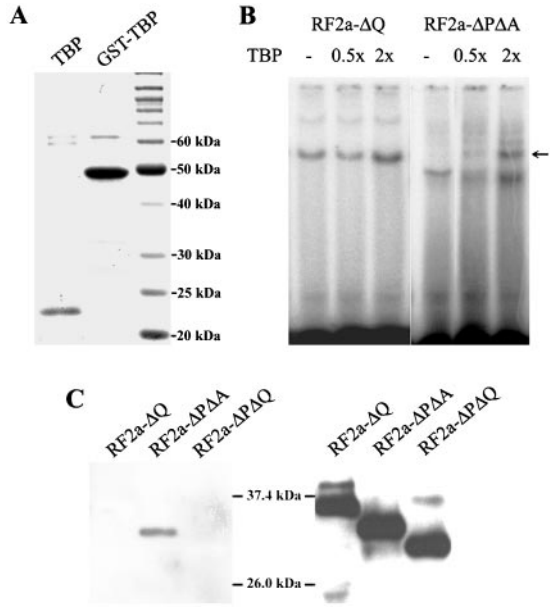

FIG. 6. **Physical interactions between RF2a mutants and rice TBP.** *A*, SDS-PAGE of purified TBP and GST-TBP. TBP was released from glutathione-cross-linked agarose beads bound GST-TBP by thrombin digestion; GST-TBP fusion protein was eluted from agarose beads with 10 mM reduced glutathione. *B*, electrophoretic mobility shift assay of RF2a mutants in interaction with TBP. 100 ng of purified ΔQ and ΔPΔA of RF2a were incubated with 0.5- or 2-fold molar ratio of TBP as labeled at room temperature for 30 min prior to electrophoretic mobility shift assay with ^{32}P-labeled Box IIm1 as probe. The supershift bands in the lane with RF2a-ΔPΔA and TBP are indicated with a *solid arrow*. *C*, protein overlay assay. Purified proteins of mutants of RF2a were separated by SDS-PAGE and electroblotted to nitrocellulose membrane. After reaction with GST-TBP, the GST-TBP fusion protein was detected using anti-GST antibody (*left panel*). The membrane was stripped after reaction with GST-TBP. The remaining membrane bound proteins were detected with anti-RF2a antibody (*right panel*).

DNA-protein complexes was observed. This experiment shows that the proline-rich and acidic domains did not directly interact with TBP, whereas the glutamine-rich domain did.

To confirm the data from gel supershift assays, protein overlay assays were carried out. Mutants of RF2a were separated by SDS-PAGE and blotted to nitrocellulose membrane, and the

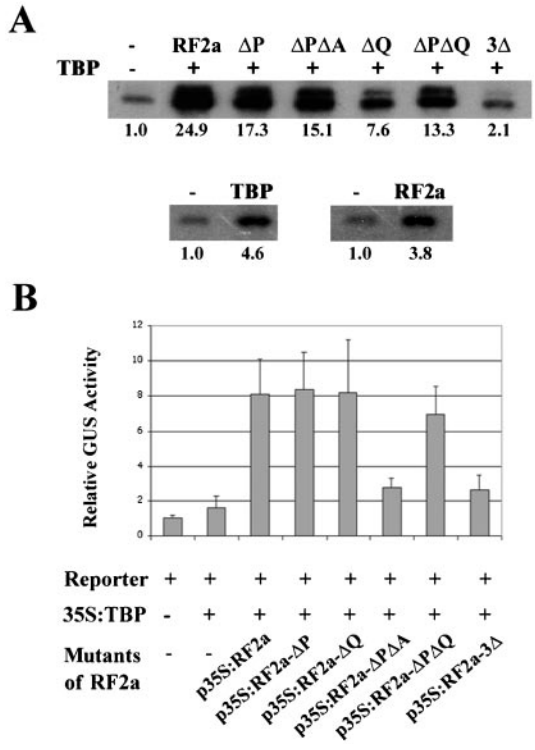

FIG. 7. **Functional interactions between RF2a and mutants and TBP *in vitro* and *in vivo*.** *A*, *in vitro* transcription assays in a rice whole cell extract system. The GUS gene driven by the E promoter was added as template. For each reaction, purified TBP and one of the purified RF2a and mutants of RF2a proteins were added as indicated. The amount of gene-specific transcript in each reaction was compared with reactions that were not enriched by recombinant proteins (control = 1). *B*, relative GUS activity of protoplast samples that were co-transfected with the GUS reporter gene pBII-48Ca:GUS, gene constructs that encode RF2a or mutants of RF2a with (+) or without (−) 35S:TBP. The results are the averages with S.D. of three independent experiments, three samples/experiment, after normalization with GFP.

bound proteins were renatured and incubated with GST-TBP fusion protein. The GST-TBP on the membrane was detected using anti-GST antibody. In this assay, mutants of RF2a that contain the glutamine-rich domain bound TBP, whereas other mutants did not (Fig. 6*C*).

Activity of Mutants of RF2a with TBP in Vitro and in Vivo—As previously reported, RF2a and TBP stimulated transcription by 4- and 5.5-fold, respectively, in an *in vitro* transcription assay (25, 33). When both RF2a and TBP were added to the same reactions, transcription was increased by about 17-fold (25). To determine the contribution of the interaction between mutants of RF2a and TBP on transcription from the E promoter of RTBV, purified proteins were used in the *in vitro* transcription assay system as previously described (25, 33). As shown in Fig. 7*A*, reactions supplied with RF2a, RF2a-ΔP, and RF2a-ΔPΔA enhanced transcription from the E promoter in the presence of TBP. The reactions supplied with RF2a-ΔQ showed only mild activation in presence of TBP (Fig. 7*A*), a result that is similar to the sum of effects that RF2a and TBP achieved in separated reactions (25). This result suggests that the N terminus of RF2a did not contribute to the synergistic activation between RF2a and TBP. RF2a-ΔPΔQ showed stronger activity in this assay than did RF2a-ΔQ but not as strong as RF2a and RF2a-ΔP. This result is somewhat inconsistent with the studies showing that RF2a interacts with TBP through the glutamine-rich domain (Fig. 6). However, the proline-rich and glutamine-rich domains each exhibited a modest degree of repression of the activity of RF2a *in vivo* (Fig. 2). This result may indicate that RF2a-ΔPΔQ alone has stronger activation function than does RF2a alone (25) in this *in vitro* system but lacks synergistic effect with TBP (Fig. 7*A*). Data from these assays further support the conclusion that RF2a interacts with TBP through the glutamine-rich domain, and the acidic domain is important for the activation function of RF2a even though it does not directly interact with TBP.

To further evaluate the effect of the interaction of TBP and RF2a on gene expression, protoplasts were co-transfected with the effectors used in Fig. 2 plus p35S:TBP using pBII-48Ca:GUS as the reporter gene. In the plasmid p35S:TBP, rice TBP2 was under the control of the enhanced CaMV ^{35}S promoter. RF2a-ΔQ and RF2a-ΔPΔQ provided strong activation in the absence of additional TBP (Fig. 2*B*), and co-transfection with TBP did not enhance the activities of RF2a-ΔQ, RF2a-ΔPΔQ, and RF2a-3Δ (compare Fig. 7*B* with Fig. 2*B*). In contrast, the activities of RF2a, RF2a-ΔP, and RF2a-ΔPΔA were enhanced when TBP was co-expressed (compare Fig. 7*B* and Fig. 2*B*). The data agree with the *in vitro* assays that TBP enhanced the function of RF2a and mutants that contain the glutamine-rich domain, although the level of enhancement in these transient assays was not as great as in the *in vitro* transcription assays.

DISCUSSION

RF2a binds to Box II, a *cis*-element adjacent to the TATA box, to regulate expression of the RTBV promoter (5, 6). Results of *in vitro* transcription studies indicated that interactions of RF2a with TBP contribute to the function of RF2a (25). In the present study, we defined the functional domains of RF2a using *in vivo* assays and examined the mechanisms by which these domains contribute to its activity.

The proline-rich and glutamine-rich domains of RF2a do not directly contribute to the activation function of RF2a, although the acidic (A) domain is essential for its activity. The A domain is a strong activation domain when fused with a heterologous DNA-binding domain. The P and Q domains showed weak activation function only when they were placed at the N terminus of the 2C7 DBD; this activity is different from functions in the context of RF2a. Nevertheless, RF2a is a strong activator of the expression of the RTBV promoter (6).

The bZIP domain of RF2a shares high similarity with a small family of bZIP proteins that include members from *Arabidopsis*, tobacco, tomato, and other plants (34–36). This group of proteins has a lysine residue at the −10 position relative to the first leucine residue of the leucine zipper domain (5). The amino acid sequence signature of the DNA-binding regions of the proteins of this class is N*XXX*SA*XX*SK (37). The identification of this subgroup of bZIP proteins may imply that members are essential for plant growth and development. Based upon the finding that the RTBV promoter is expressed in vascular tissues in rice (2, 4), tobacco (6), and *Arabidopsis*,[3] we suggest that this group of bZIP proteins is involved in regulating gene expression in these tissues. Yin *et al.* (5) used an antisense approach to demonstrate that RF2a is important for development of the vascular system in rice seedlings. Ectopic expression of RF2a-ΔPΔA and RF2a-3Δ in transgenic tobacco (Fig. 4) and rice[3] caused severe stunting and abnormal development. These phenotypes apparently result from dominant negative effects of RF2a-ΔPΔA and RF2a-3Δ on the expression of genes that require function of RF2a-like homologs in tobacco plants, such as RSG (34, 38). Fukazawa *et al.* (34) determined that RSG regulates GA biosynthesis through the GA3 gene.

Interestingly, the proline-rich and glutamine-rich domains of RF2a did not directly contribute to the activation function of RF2a in BY-2 protoplasts even though these two domains showed limited activation functions in fusions with 2C7 DBD.

[3] S. Dai and R. N. Beachy, unpublished data.

Like the proline-rich domain in p45 NF-E (39), the proline-rich domain of RF2a may be dispensable for the activity of RF2a. In contrast, the C-terminal region of RF2a, including the glutamine-rich domain, may repress the function of the acidic domain (Fig. 3). Previous studies demonstrated that glutamine-rich domains can act as repression domains (23, 24). It will be important to further map and characterize the potential repression domain at the C terminus of RF2a.

The binding of TBP is important for formation of the transcription preinitiation complex (7, 19). Therefore, interactions between gene-specific transcription factors and TBP or TAFs and recruitment of TFIID are important mechanisms of gene regulation. Because the acidic domain is a primary contributor to the activation of RF2a *in vivo*, we expected that this domain would interact with TBP. Surprisingly, as in the case of AtHSF1, which interacts with AtTBP1 and AtTBP2 through its N-terminal sequence rather than the acidic C terminus (40), the glutamine-rich domain was responsible for interaction of RF2a with TBP (Figs. 6 and 7). Our studies indicate that the acidic domain activates transcription through mechanisms other than direct binding to TBP. For example, the acidic domain may interact with other components of the transcriptional machinery upon binding of RF2a to the *cis*-element. Alternatively, the acidic domain may recruit TFIID through interaction with certain TAFs (18).

The roles of the glutamine-rich domain and TBP interaction were somewhat different *in vitro* and *in vivo* (Fig. 7). In the cell-free *in vitro* assay, RF2a and TBP enhanced transcription in a synergistic manner when the glutamine-rich domain was included but not when this domain was removed. In transient *in vivo* assays, the addition of TBP in the transient assay obviated the repression effect of the glutamine-rich domain but did not further stimulate gene expression. The apparent differences in the two systems may be the result of differences in relative concentrations of the proteins in *in vitro* and *in vivo* systems and/or the presence of other related proteins that modify the function of RF2a and interactions with TBP.

Recently, it was reported that the acidic domain of Gal4 interacts with TBP in biochemical assays (41). However, the Gal4 transactivator and TBP did not bind cooperatively to target promoters (42). This may indicate that recruitment of TFIID is not the primary activation mechanism of the Gal4 protein (42). The situation may be different with regard to the role of interactions between RF2a and TBP. However, the outcome is essentially the same, *i.e.* the interaction between TBP and the glutamine-rich domain did not fully reconstruct the activity of RF2a in the absence of the acidic domain. Of course, this does not necessarily mean that there is not a biological consequence of the interaction. The interactions of the glutamine-rich domain with TBP may play an important role in regulation of transcription that we were not able to detect in the current work.

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REFERENCES

1. Bhattacharyya-Pakrasi, M., Peng, J., Elmer, J. S., Laco, G., Shen, P., Kaniewska, M. B., Kononowicz, H., Wen, F., Hodges, T. K., and Beachy, R. N. (1993) *Plant J.* **4,** 71–79
2. Yin, Y., and Beachy, R. N. (1995) *Plant J.* **7,** 969–980
3. He, X., Hohn, T., and Futterer, J. (2000) *J. Biol. Chem.* **275,** 11799–11808
4. Yin, Y., Chen, L., and Beachy, R. (1997) *Plant J.* **12,** 1179–1188
5. Yin, Y., Zhu, Q., Dai, S., Lamb, C., and Beachy, R. N. (1997) *EMBO J.* **16,** 5247–5259
6. Petruccelli, S., Dai, S., Carcamo, R., Yin, Y., Chen, S., and Beachy, R. N. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7635–7640
7. Roeder, R. G. (1991) *Trends Biochem. Sci* **16,** 402–408
8. Yankulov, K., Blau, J., Purton, T., Roberts, S., and Bentley, D. L. (1994) *Cell* **77,** 749–759
9. Holmes, M. C., and Tjian, R. (2000) *Science* **288,** 867–870
10. Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002) *Cell* **108,** 475–487
11. Martens, J. A., and Winston, F. (2002) *Genes Dev.* **16,** 2231–2236
12. Silverman, N., Agapite, J., and Guarente, L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11665–11668
13. Hall, D. B., and Struhl, K. (2002) *J. Biol. Chem.* **277,** 46043–46050
14. Meshi, T., and Iwabuchi, M. (1995) *Plant Cell Physiol.* **36,** 1405–1420
15. Giniger, E., Varnum, S. M., and Ptashne, M. (1985) *Cell* **40,** 767–774
16. Mitchell, P. J., and Tjian, R. (1989) *Science* **245,** 371–378
17. Truant, R., Xiao, H., Ingles, C. J., and Greenblatt, J. (1993) *J. Biol. Chem.* **268,** 2284–2287
18. Uesugi, M., Nyanguile, O., Lu, H., Levine, A. J., and Verdine, G. L. (1997) *Science* **277,** 1310–1313
19. Pugh, B. F. (2000) *Gene* (*Amst.*) **255,** 1–14
20. Klemm, R. D., Goodrich, J. A., Zhou, S., and Tjian, R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5788–5792
21. Hayashi, F., Ishima, R., Liu, D., Tong, K. I., Kim, S., Reinberg, D., Bagby, S., and Ikura, M. (1998) *Biochemistry* **37,** 7941–7951
22. Stargell, L. A., and Struhl, K. (1995) *Science* **269,** 75–78
23. Madden, S. L., Cook, D. M., Morris, J. F., Gashler, A., Sukhatme, V. P., and Rauscher, F. J., III. (1991) *Science* **253,** 1550–1553
24. Licht, J. D., Hanna-Rose, W., Reddy, J. C., English, M. A., Ro, M., Grossel, M., Shaknovich, R., and Hansen, U. (1994) *Mol. Cell Biol.* **14,** 4057–4066
25. Zhu, Q., Ordiz, M. I., Dabi, T., Beachy, R. N., and Lamb, C. (2002) *Plant Cell* **14,** 795–803
26. Ordiz, M. I., Barbas, C. F., III, and Beachy, R. N. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13290–13295
27. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) *EMBO J.* **6,** 3901–3907
28. Horsch, R. B., Fry, J., Hoffmann, N., Neidermeyer, J., Rogers, S. G., and Fraley, R. T. (1988) in *Plant Molecular Biology Manual* (Gelvin, S. B., and Schilperoort, R. A., eds) pp. A5 and 1–9, Kluwer, Dordrecht, The Netherlands
29. Murashige, T., and Skoog, F. (1962) *Physiol. Plantarum* **15,** 473–497
30. Haas, N. B., Cantwell, C. A., Johnson, P. F., and Burch, J. B. (1995) *Mol. Cell Biol.* **15,** 1923–1932
31. Katagiri, F., Seipel, K., and Chua, N. H. (1992) *Mol. Cell Biol.* **12,** 4809–4816
32. Liu, Q., Segal, D. J., Ghiara, J. B., and Barbas, C. F., III. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5525–5530
33. Zhu, Q., Chappell, J., Hedrick, S. A., and Lamb, C. (1995) *Plant J.* **7,** 1021–1030
34. Fukazawa, J., Sakai, T., Ishida, S., Yamaguchi, I., Kamiya, Y., and Takahashi, Y. (2000) *Plant Cell* **12,** 901–915
35. Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T., and Parcy, F. (2002) *Trends Plant Sci.* **7,** 106–111
36. Ringli, C., and Keller, B. (1998) *Plant Mol. Biol.* **37,** 977–988
37. Fujii, Y., Shimizu, T., Toda, T., Yanagida, M., and Hakoshima, T. (2000) *Nat. Struct. Biol.* **7,** 889–893
38. Olive, M., Williams, S. C., Dezan, C., Johnson, P. F., and Vinson, C. (1996) *J. Biol. Chem.* **271,** 2040–2047
39. Bean, T. L., and Ney, P. A. (1997) *Nucleic Acids Res.* **25,** 2509–2515
40. Reindl, A., and Schoffl, F. (1998) *FEBS Lett.* **436,** 318–322
41. Xie, Y., Denison, C., Yang, S. H., Fancy, D. A., and Kodadek, T. (2000) *J. Biol. Chem.* **275,** 31914–31920
42. Xie, Y., Sun, L., and Kodadek, T. (2000) *J. Biol. Chem.* **275,** 40797–40803
43. Watanabe, Y., Meshi, T., and Okada, Y. (1987) *FEBS Lett.* **219,** 65–69