RF2a, a bZIP transcriptional activator of the phloem-specific rice tungro bacilliform virus promoter, functions in vascular development

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Rice tungro bacilliform virus (RTBV) replicates only in phloem cells in infected rice plants and its promoter drives strong phloem-specific reporter gene expression in transgenic rice plants. We isolated a cDNA encoding a basic leucine zipper (bZIP) protein, RF2a, which binds to the Box II *cis* element that is important for expression from the promoter. RF2a, which stimulates Box II-dependent transcription in a homologous *in vitro* transcription system, accumulates in nuclei of phloem and certain other cell types in shoots, but is found at only very low levels in roots. Transgenic antisense plants in which RF2a accumulation was suppressed had normal roots but stunted, twisted leaves with small, disorganized vascular bundles, an enlarged sclerenchyma and large air spaces. We propose that the RTBV promoter exploits a host transcription factor that is critical for leaf tissue differentiation and vascular development for its expression.

Keywords: antisense/bZIP/phloem/RF2a/vascular

Introduction

The plant vascular system comprises phloem, which transports organic materials, and xylem, which transports water and soil-derived nutrients. The phloem contains sieve elements and companion cells, and xylem contains tracheary elements and xylem parenchyma cells. Rice tungro bacilliform virus (RTBV) is a plant pararetrovirus, with a double-stranded DNA genome of 8 kb that, together with the RNA virus rice tungro spherical virus, produces the severe condition known as rice tungro disease (Hay *et al.*, 1991; Qu *et al.*, 1991). Electron microscopic studies of virus-infected rice plants indicate that the virus accumulates primarily in the cytoplasm of the phloem companion cells (Saito *et al.*, 1986). Similarly, expression of the RTBV promoter–reporter gene fusions is limited to phloem in transgenic rice plants and hence the properties of the viral promoter can account for the tissue specificity of RTBV replication (Battacharyya-Pakrasi *et al.*, 1993; Yin and Beachy, 1995).

The evolution of an efficient vascular system was

critical for the emergence of land plants. However, little is known about the transcription factors that are involved in cell type-specific expression in phloem or xylem and the differentiation of vascular tissues. Combinatorial interactions between positive and negative *cis* elements appear to be required for the regulated expression of several xylem-specific promoters. For example, promoters from the *pal2* and *4cl* genes encoding phenylalanine ammonia-lyase and 4-coumarate:CoA ligase, which are involved in the synthesis of a lignin component of the xylem cell wall, contain positive elements for xylem expression and a negative element to suppress expression in phloem (Leyva *et al.*, 1992; Hauffe *et al.*, 1993; Hatton *et al.*, 1995). Likewise, the promoter of the *grp1.8* gene, which encodes a xylem-specific cell wall structural protein, contains a *cis* element, *vs-1*, required both for gene expression in xylem and for suppression of promoter activity in parenchyma (Keller and Baumgartner, 1991). A basic leucine zipper (bZIP) transcription factor, VSF-1, has been cloned by virtue of its binding to *vs-1* (Torres-Schumann *et al.*, 1996). However, while VSF-1 stimulates *grp1.8* transcription in transformed protoplasts, there is no information on the function of this transcription factor in xylem-specific gene expression or its role in vascular development.

Several phloem-specific promoters have been reported, including those from the glutamine synthetase *GS3A* gene (Brears *et al.*, 1991), the *Arabidopsis* *AHA3* gene encoding H⁺-ATPase isoform 3 (DeWitt *et al.*, 1991), the *Agrobacterium* *rolC* promoter (Schmulling *et al.*, 1989), the *Arabidopsis* sucrose synthase gene *Asus1* (Martin *et al.*, 1993) and the maize sucrose synthase-1 gene *Sh1* (Werr *et al.*, 1985; Yang and Russell, 1990), as well as the RTBV promoter. In the *GS3A* promoter, sequences to nucleotide (nt) –132 relative to the transcription start site are sufficient for tissue-specific expression, and a 17 bp imperfect palindrome was identified as a putative *cis* element by virtue of the binding of a nuclear protein complex (Brears *et al.*, 1991). However, the functions of this *cis* element and of the cognate *GS3A-F1 trans* factor are not known.

A small DNA fragment from RTBV, comprising nt –164 to +45 relative to the transcription start site, is sufficient for phloem-specific expression of a reporter gene, and within this region three elements, Box II, the ASL Box and the GATA motif, in combination establish tissue specificity (Yin and Beachy, 1995; Yin *et al.*, 1997). Here we report the molecular cloning of RF2a, a bZIP transcription factor which binds to Box II and stimulates Box II-dependent transcription *in vitro*. RF2a is found in the nuclei of phloem cells and certain other cell types in leaves and stems, but is present at only very low levels in roots. Antisense suppression of RF2a protein accumulation in transgenic plants causes the development of stunted

plants with twisted leaves that contain small disorganized vascular bundles, enlarged sclerenchyma and large air spaces. Our studies support the conclusion that the RTBV promoter interacts with a host transcription factor critical for leaf tissue differentiation and vascular development.

Results

Molecular cloning of a rice protein that binds to the RTBV Box II involved in phloem expression

Box II of the RTBV promoter, together with other DNA sequence elements located between nt -164 and +45, confers phloem-specific gene expression in transgenic rice plants (Yin and Beachy, 1995; Yin *et al.*, 1997). Several nuclear factor-Box II complexes, referred to as RNFG2, have been identified (Yin and Beachy, 1995). We used a one-hybrid yeast genetic selection system (Wang and Reed, 1993) to clone factors that bind to Box II. The system contains three components: (i) yeast strain yWAM2 which is his⁻trp⁻leu⁻; (ii) reporter plasmid vector pRS315HIS containing the *LEU2* gene; and (iii) cDNA library vector pPC86 containing the *TRP1* gene (Wang and Reed, 1993). The reporter plasmid was constructed by fusing a tetramer of RTBV Box II sequences to the minimal promoter-*HIS3* gene fusion in pRS315HIS. A rice seedling cDNA library was constructed in the yeast expression vector pPC86 to give translational fusions with the GAL4 activation domain. Yeast that harbors a GAL4-rice fusion protein capable of binding the Box II sites present in the reporter gene will activate transcription of *HIS3* and the candidate clones can be identified by selection of HIS⁺ phenotypes.

In one screening experiment, 2×10^6 transformants were plated on media lacking histidine, and 12 colonies with HIS⁺, TRP⁺ and LEU⁺ phenotypes were isolated. Plasmids were recovered and transformed back into yeast strains with different reporter plasmids. Plasmid YTJ1-4 from one of the colonies, but not the parental vector pPC86, conferred the HIS⁺ phenotype when transformed into cells harboring a reporter plasmid containing a tetramer of Box II, while YTJ1-4 failed to rescue the HIS⁺ phenotype in yeast harboring a reporter plasmid without Box II (data not shown). These results suggested a specific interaction between the protein encoded by YTJ1-4 and Box II. YTJ1-4 contains a rice cDNA insert of 1.2 kb, and RNA blot hybridization analysis showed that the mature transcript of this gene, designated *rf2a*, was ~1.8 kb (see Figure 6). A full-length cDNA clone of 1777 bp subsequently was isolated from the cDNA library and the nucleotide sequence of both strands determined.

rf2a encodes a bZIP DNA-binding protein

Nucleotide sequence analysis of the full-length cDNA revealed an open reading frame (ORF) predicted to encode a protein of 368 amino acids; the encoded protein was designated RF2a (Figure 1A). A typical bZIP DNA-binding domain could be discerned between residues 150 and 260. A glutamine-rich domain (27 out of 75 amino acid residues, 36%) in the carboxy-terminal region, a proline-rich domain (nine out of 42, 21%) and an acidic domain (15 out of 53, 28%) near the amino-terminus may serve as transactivation domains (Figure 1B).

A search of GenBank revealed a high degree of simi-

Fig. 1. Predicted amino acid sequence and domain structure of RF2a. (A) Deduced amino acid sequence of RF2a is shown with the bZIP domain boxed and the three potential activation domains, the proline-rich, acidic and glutamine-rich domains, underlined. These sequence data have been submitted to DDBJ/EMBL/GenBank under accession No. AF005492. (B) Schematic domain structure of RF2a; the percentages of proline residues, acidic amino acids and glutamine residues in each putative domain are indicated.

arity (overall 64%) between RF2a and a putative transcription factor from *Arabidopsis*, encoded by *PosF21*, a gene of unknown function that was cloned by virtue of its high glutamine content (Aeschbacher *et al.*, 1991). High homology was also found between RF2a and VSF-1, a transcription factor that binds to *vs-1*, a *cis* element involved in vascular-specific *grp1.8* expression (Torres-Schumann *et al.*, 1996). Alignment of the bZIP domains of RF2a, PosF21, VSF-1 and other selected bZIP proteins is shown in Figure 2. Like other bZIP proteins, RF2a contains typical heptad leucine repeats and an adjacent basic region, as well as an invariant Asn at position -18 relative to the first Leu residue in the Leu zipper region (Hurst, 1994). Unlike other bZIP proteins that have a conserved Arg residue at position -10, RF2a, PosF21 and VSF-1 have Lys at this position. The presence of Lys at position -10 in these three plant *trans* factors, together with their highly conserved bZIP domains, suggests that RF2a, PosF21 and VSF-1 comprise a discrete class of bZIP DNA-binding proteins (Torres-Schumann *et al.*, 1996).

The full-length RF2a was expressed in *Escherichia coli*, purified, renatured and used for DNA binding assays; and a fragment comprising amino acids 264-368 was purified from *E. coli* and used to produce antibodies. The DNA-binding properties of RF2a were assessed by electrophoretic mobility shift assays (EMSAs) using purified full-length recombinant protein (Figure 3). Previously we found that a group of rice nuclear factors, comprising complexes EF4-6, and referred to as RNFG2, bind to Box II. The sequences CCA/TGG and CCCC are essential for RNFG2 binding (Yin and Beachy, 1995; Yin *et al.*, 1997; Figure 3A). Two mutants of Box II, Box II_{m1}, in which the CCCC sequence was changed to GCGC, and Box II_{m2}, in which the CCA/TGG sequences were changed to GGA/TTC, failed to form RNFG2 complexes in EMSAs (Yin *et al.*, 1997).

Oligonucleotides containing Box II, Box II_{m1} or Box II_{m2} were used in EMSAs to examine RF2a binding

Fig. 2. Alignment of amino acid sequences in the basic/leucine zipper region. The RF2a bZIP domain was aligned with PosF21 (Aeschbacher *et al.*, 1991), VSF-1 (Torres-Schumann *et al.*, 1996) and other bZIP proteins (summarized by Hurst, 1994). The basic region and the leucine zipper region are indicated. The amino acids that are identical to RF2a are shaded. The critical consensus sequences are indicated below, numbered according to Suckow *et al.* (1994).

activity (Figure 3B). RF2a bound to the probe containing Box II, but not to the probe containing Box IIm2 (Figure 3B, lanes 1 and 3). Surprisingly, Box IIm1, in which a CCCC sequence was changed into GCGC, binds RF2a more efficiently than does wild-type Box II (compare lanes 1 and 2). These results suggest that both the CCA/TGG and the CCCC motifs in Box II are important for RF2a binding. Polyclonal antibodies raised against the carboxy-terminus of RF2a bind complexes comprising RF2a plus Box II, resulting in a supershifted band (lane 4) in EMSA, whereas pre-immune serum did not affect the mobility of the complex (lane 5).

We next tested whether or not the RNFG2 complexes that bind to Box II contain RF2a using anti-RF2a antibodies (Figure 3B, lanes 6–11). Surprisingly, none of the RNFG2 in the reactions was supershifted by anti-RF2a antibodies when both poly(dA–dT) and poly(dI–dC) were used as non-specific competitors in binding reactions (data not shown); these results suggested that RF2a is not a component of RNFG2 complexes. Since Box II is GC-rich, a potential protein–DNA complex could be competed by poly(dI–dC) in the binding reactions. In reactions that contained only poly(dA–dT) as a non-specific competitor, an additional complex different from RNFG2, designated RF2 (Figure 3B, lanes 6 and 7), was observed. Moreover, the RF2 complex was supershifted by anti-RF2a antibodies but not by pre-immune serum, indicating that the RF2 complex contains RF2a (Figure 3B, lanes 8–11). It was therefore concluded that RF2a is one of the proteins that bind to Box II in extracts of rice nuclei.

The DNA sequence of Box II resembles several other *cis* elements that are involved in vascular-specific gene expression, including *vs-1* in *grp1.8* (Torres-Schumann *et al.*, 1996), and AC-I and AC-II in *pal2* (Hatton *et al.*, 1995). These elements contain CCA/TGG repeats and/or CCCC sequences as indicated in Figure 3A. EMSAs were carried out with oligonucleotides that contain these sequences and, as shown in Figure 3C, RF2a binds to *vs-1* and AC-I, but not to either AC-II or the ASL Box from the RTBV promoter. However, while RF2a bound to *vs-1*, anti-RF2a antibodies failed to supershift this complex, possibly because the antibodies modify the conformation of RF2a so that the RF2a–antibody complex does not recognize *vs-1*.

Binding constants for RF2a–DNA interactions

The DNA-binding affinities of RF2a, RF2 and RNFG2 complexes with Box II and Box IIm1 were estimated by EMSAs performed with increasing amounts of probe at constant protein concentration (Figure 4). The amount of ³²P in the DNA–protein complexes versus the free probes was quantified by phosphorimager scanning. Scatchard analysis of RF2a, RF2 and RNFG2 (EF4, EF6) binding to Box II or Box IIm1 is shown in Figure 4, and the estimated binding constants are summarized in Table I. Cloned RF2a bound with higher affinity to Box IIm1 than to Box II (K_d 1.0 and 3.2 nM respectively). RF2 had a higher affinity for Box II than did RF2a (K_d of 0.6 versus 3.2 nM). Unlike RF2a, which bound Box IIm1 better than Box II, RF2 bound Box II better than Box IIm1, consistent with the hypothesis that RF2 is different from RF2a. Two components of RNFG2, EF4 and EF6, bind to Box II with different affinities (K_d 0.07 and 1.5 nM respectively), demonstrating that different factors may bind with different affinities to the same site.

RF2a stimulates transcription in a homologous *in vitro* transcription system

The ability of RF2a to activate transcription was assessed by a homologous *in vitro* transcription system (Zhu *et al.*, 1995a). In this system, whole-cell extracts from rice cell suspension cultures were used to transcribe a template comprising nt –164 to +45 of the RTBV promoter ligated to a fragment of the *E. coli uidA* gene. The *in vitro* transcription product (indicated by an arrow) was detected by primer extension assays (Figure 5A). The transcription initiation site in this reaction was the same as the site used by the promoter in transgenic rice plants (Yin and Beachy, 1995), as indicated by a sequencing reaction (Figure 5A). Transcription was sensitive to low concentrations of α -amanitin, confirming that *in vitro* transcription of the RTBV promoter was mediated by RNA polymerase II (Figure 5A).

When RNFG2, RF2 and other Box II-binding factors were depleted from the whole-cell extract by passage through an affinity column containing a tetramer of Box II, the transcription activity of the RTBV promoter was reduced to 5% of that observed with the untreated control extract (Figure 5B). Addition of recombinant RF2a pro-

Fig. 3. Nucleotide sequence specificity of RF2a binding. (A) Core nucleotide sequences of the oligonucleotides used as probes in EMSAs. Box II, Box II m1, Box II m2 and ASL Box from the RTBV promoter are described in Yin and Beachy (1995) and in Yin *et al.* (1997); vs-1 is described in Torres-Schumann *et al.* (1996) and AC-I and AC-II are described in Hatton *et al.* (1995). The proposed recognition sequence of RF2a, e.g. repeat units of CCA/TGG, are indicated by arrows, and the conserved CCCC sequences in some of the elements are italicized. The positions of these elements relative to the transcription start sites in their respective promoters are indicated. The mutated base pairs in Box II m1 and Box II m2 are underlined. (B) Rice nuclear extracts (RN) and recombinant RF2a purified from *E. coli* were used in EMSAs with Box II (II), Box II m1 (m1) or Box II m2 (m2) as ³²P-labeled probes. Poly(dA-dT) was used as a non-specific competitor in binding reactions (200 ng with RF2a, 2 µg with rice nuclear extracts; RN). The absence (-) and presence (+) of anti-RF2a antibodies or pre-immune IgGs (P) are indicated. Complexes designated RNFG2 (EF4, 5 and 6), RF2 and RF2a are described in the text. The complexes supershifted by anti-RF2a antibodies (+Ab) are indicated. The free probe is not shown. (C) Different probes were used in an EMSA with purified RF2a in the presence of 200 ng of poly(dA-dT) as non-specific competitor. Sequences derived from the vs-1, AC-I, AC-II and the ASL Box are described in (A).

duced in *E. coli* restored the transcription activity to ~20% of the control level (Figure 5B, upper panel). Moreover, when the depleted whole-cell extract was combined with a rice seedling nuclear extract from which the Box II-binding factors had also been depleted, transcription was stimulated from 15% of the control level to >60% of the control level by addition of purified recombinant RF2a (Figure 5B, middle panel). In contrast, RF2a failed to stimulate transcription from a rice *pal* promoter that does not contain an RF2a-binding site (Figure 5B, lower panel).

Thus, RF2a is a transcriptional activator, and stimulates transcription in a sequence-dependent manner.

***rf2a* genomic organization and organ-specific expression**

Blot hybridization analysis of genomic DNA isolated from rice seedlings showed that RF2a is encoded by a single-copy gene; all the restriction enzymes tested produced a single fragment which hybridized with the *rf2a* probe under high stringency conditions (Figure 6A). In RNA

Fig. 4. Determination of DNA-binding constants. Scatchard analyses were performed for RF2a binding to Box II (A) and Box II1 (B), nuclear complex RF2 binding to Box II (C) and Box II1 (D), as well as EF4 and EF6 (components of RNFG2) binding to Box II (E and F). Binding reactions were carried out with increasing amounts of probes (0.04–1.28 nM) and constant amounts of RF2a protein (100 ng) or rice nuclear extract (2.0 μ g protein). The bound and unbound probes were quantified by phosphorimager scanning and plotted according to the Scatchard equation (see Materials and methods). The K_{app} s determined from the slopes of the Scatchard plots are summarized in Table I. Inserts show EMSAs of the binding reactions; RF2a, RF2, EF4 and EF6 are indicated.

blot hybridization reactions, *rf2a* mRNA was present at similar levels in samples prepared from roots, leaf sheaths and leaves (Figure 6B). However, parallel immunoblot analysis showed that the RF2a protein accumulated to relatively high levels in sheaths, moderate levels in leaves and very low levels in roots (Figure 6C), suggesting selective post-transcriptional control of RF2a protein levels in different tissues. The organ-specific accumulation of

RF2a is consistent with the expression of the RTBV promoter in these organs, i.e. expression is highest in sheaths, lower in leaves and >200 times lower in roots (Yin and Beachy, 1995). These results suggest that RF2a plays an important role in RTBV transcription *in vivo* and can account, at least in part, for the organ specificity of the promoter.

RF2a has an apparent M_r of 50 kDa (Figure 6C)

Table I. DNA-binding affinities of RF2a, RF2 and RNFG2 (nM)

Factors	Box II	Box IIml
RF2a	3.2	1.0
RF2	0.6	0.9
RNFG2-EF4	0.07	NB
RNFG2-EF6	1.5	NB

NB, no detectable binding.

although the molecular mass of the protein deduced from the ORF of the *rf2a* cDNA is 40 kDa. The 50 kDa band detected in immunoblot reactions was confirmed to be RF2a because products of *in vitro* translation reactions primed with the transcript of the cloned RF2a cDNA as well as protein produced in *E. coli* migrate to this position in SDS-PAGE (data not shown). The lower band (~40 kDa) appears to be a degradation product of the 50 kDa RF2a band in leaf tissues, since both of the bands increased equally in transgenic plants in which RF2a is over-expressed (see Figure 8, lanes 1 and 3).

RF2a is localized to the nucleus of phloem cells and other cell types in sheath tissue

Because RF2a accumulates to relatively high levels in leaf sheath tissues (Figure 6C), we used this tissue for immunolocalization experiments (Figure 7). Frozen sections containing vascular bundles (A, C, E, G and I) and fundamental parenchyma, the parenchyma cells outside of the vascular bundles (B, D, F, H and J), were stained with various reagents and observed with a fluorescence microscope. Sections stained with ethidium bromide indicated the distribution of nuclei in the vascular bundles and fundamental parenchyma (Figure 7A and B). When sections were stained with anti-RF2a and fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit IgG as the secondary antibody, RF2a was observed in phloem cells, epidermal cells (Figure 7C) and fundamental parenchyma cells (Figure 7D), whereas no staining was observed in similar reactions with pre-immune serum (Figure 7E and F). RF2a appeared to be localized to the nuclei of these cell types, and this was confirmed by comparison of sections co-stained with anti-RF2a antibodies and propidium iodide. The latter stains nuclei and gives red fluorescence when examined under conditions that exclude green fluorescence arising from antibody binding to RF2a (Figure 7I and J). RF2a was detected in most cell types in the vascular bundles as well as in fundamental parenchyma (Figure 7G and H), with some yellow fluorescence from mixing of the green fluorescence with red fluorescence from areas strongly stained with propidium iodide. Comparisons between Figure 7G and I and between Figure 7H and J showed that RF2a was only found at sites that co-stained with propidium iodide. RF2a was localized to the nuclei of phloem, xylem parenchyma, fundamental parenchyma and epidermal cells.

Role of RF2a in plant development

The role of RF2a in development was investigated by ectopic expression and antisense suppression of *rf2a* in transgenic rice plants. Plasmids containing the *rf2a* gene in either sense or antisense orientation under the control of the maize ubiquitin *ubi* promoter (*pUbi::rf2a* and

Fig. 5. RF2a stimulates Box II-dependent transcription *in vitro*.

(A) *In vitro* transcription was performed with rice whole-cell extracts using as template a plasmid containing nt -164 to +45 of the RTBV promoter ligated to the *uidA* gene. The specific transcript (indicated by an arrow) was detected by primer extension assays with a primer from the *uidA* coding sequence. α -Amanitin was added to some of the transcription reactions at the concentrations indicated. Products of a sequencing reaction with the same primer and template are shown (lanes G, A, T and C) with the positions relative to the transcription start site indicated. (B) Box II-binding factors RF2 and RNFG2 were depleted from the rice whole-cell extract and/or leaf nuclear extract by DNA chromatography. *In vitro* transcription reactions were performed with the untreated (control) or treated extracts in the absence or presence of 100 ng of purified RF2a. The relative amounts of transcript were quantified and are indicated below each panel. In the bottom panel, the template for the transcription reaction was a rice *pal* promoter-*uidA* gene fusion (Zhu *et al.*, 1995b). The *pal* promoter does not contain a Box II sequence.

pUbi::a2fr respectively) were introduced into rice cells together with a second plasmid carrying the *hph* gene, conferring resistance to hygromycin, as a selectable marker, and gene integration in regenerated transgenic plants was confirmed by PCR (data not shown).

Thirty four transgenic lines containing the antisense construct (*pUbi::a2fr*) were analyzed in detail. Of these, 11 showed a marked suppression of RF2a levels in immunoblots of whole leaf extracts. Figure 8 is a representative immunoblot showing strong suppression of RF2a protein accumulation in antisense lines 270-12, -37, -47 and -48 (lanes 6–9) compared with RF2a levels in CK1, a transgenic line harboring *hph* only (lane 4), and CK2, an untransformed plant (lane 5).

Six-week-old regenerated plantlets were used to examine phenotypic effects arising from RF2a suppression (Figure 9). All antisense lines with marked reduction of RF2a protein accumulation exhibited abnormal phenotypes characterized by dwarfing of the plants (Figure 9B) and

Fig. 6. Genomic organization and expression of *rf2a*. (A) Ten μ g of rice genomic DNA were digested with the indicated enzymes, and probed with a 32 P-labeled *rf2a* fragment. The sizes of DNA fragments are indicated at the right of the blot. (B) Poly(A)⁺ mRNA isolated from 200 μ g of total RNA each of root (R), sheath (S) and leaf (L) were used in blot hybridization analysis. The blot was first hybridized with a *rf2a* probe (top) and the filter exposed for 5 days. A specific 1.8 kb species is indicated by the arrow. The stripped filter was rehybridized with an rRNA probe and the filter was exposed for 10 h. The RNA size markers are indicated to the left of the blot. (C) Forty μ g of proteins from rice leaf (L), sheath (S) and root (R) tissues were used in immunoblots with anti-RF2a (top panel) or pre-immune (bottom panel) antibodies. The RF2a band is indicated by the arrow and the mobilities of protein molecular weight standards are indicated on the left.

twisting of the leaves (Figure 9B–D), whereas the roots appeared relatively normal. Comparison of cross-sections from normal and twisted leaves revealed dramatic changes in leaf morphology of antisense lines showing strong RF2a suppression (Figure 9E–G). The most prominent change was the formation of large air spaces, resembling aerenchyma, along the vascular bundles in twisted leaves (Figure 9F and G, indicated by arrows). In the most severe cases, much of the interior of the leaf was occupied by these air spaces and the number of mesophyll cells was dramatically reduced (Figure 9G). Moreover, compared with leaves from control plants, the size of the vascular bundles was substantially reduced, especially in severely twisted leaves (Figure 9E–G).

The characteristic autofluorescence from the cell walls of different cell types was exploited for further characterization of the effects of RF2a suppression on the organization of vascular bundles in leaf sheaths (Figure 9H and J). The vascular tissue appeared disorganized and the vascular bundles were often smaller and not fully developed (compare Figure 9H and J). In control plants (Figure 9H), at this stage of development, the sclerenchyma cells (the cell walls of which exhibit bright, green autofluorescence) are separated from the vascular bundles and are composed of 2–3 layers of thick-wall cells. In contrast, the antisense transgenic lines with strong RF2a suppression showed an enlargement of sclerenchyma that failed to separate from the vascular bundles (Figure 9J). The morphological changes shown in Figure 9E–J were confirmed by three specimens from at least five plant lines in each case. Such aberrant morphologies were never observed in wild-type plants, or in transgenic lines harboring only the *hph* selectable marker or in those antisense lines exhibiting little or no suppression of RF2a protein levels.

Five of the 23 transgenic lines harboring the *rf2a* gene sequence in the sense orientation (*pUbi::rf2a*) accumulated

higher levels of RF2a than control plants. Lines with enhanced expression of RF2a, e.g. 269-20 and -24 (Figure 8), showed discoloration (yellowing) of leaves (Figure 9A), but these lines exhibited no obvious changes in leaf morphology. In these lines, the vascular bundles and mesophyll tissue appeared relatively normal and similar to those in control lines (Figure 9I). Transgenic line 269-23, which contains the *pUbi::rf2a* (lane 2), accumulated less RF2a than the control plant lines, presumably due to co-suppression (Matzke and Matzke, 1995) of the wild-type gene by the transgene sequences. This line had the aberrant leaf morphology characteristic of the antisense lines in which there was strong suppression of RF2a protein accumulation.

Discussion

RF2a is a member of a new class of plant bZIP DNA-binding proteins

We have cloned a transcription factor, RF2a, which binds to Box II, a *cis* element that is important for phloem-specific expression of the RTBV promoter (Yin and Beachy, 1995; Yin *et al.*, 1997). While RF2a is a typical bZIP protein with several heptad leucine repeats and an adjacent basic domain, RF2a joins VSF-1 and PosF21 in a distinct class of bZIP proteins based on their highly conserved bZIP domains and the presence of Lys rather than Arg at position –10 (Torres-Schumann *et al.*, 1996). Moreover, while many plant bZIP proteins bind to sites that contain the palindromic ACGT core sequence (Foster *et al.*, 1994), the binding sites of RF2a [CCA(N)_nTGG] and VSF-1 [probably TGG(N)₃TGG] do not contain this sequence. Substitution of Arg by Lys at position –10 changes the optimal binding site of the yeast bZIP protein GCN4 from the palindromic ATF/CREB *cis* element to the pseudo-palindromic AP1 site (Suckow *et al.*, 1994), and an Arg→Lys substitution at this position in the

Fig. 7. Immunolocalization of RF2a in rice sheath tissues. Frozen sections of sheath tissues showing either phloem (p) and xylem (x) tissues (left panels) or fundamental parenchyma (fp) (right panels) were stained with: (A and B) ethidium bromide (EtBr); (C and D) anti-RF2a and FITC-coupled goat anti-rabbit IgG; (E and F) pre-immune IgG and FITC-coupled goat anti-rabbit IgG; and (G–J) anti-RF2a, FITC-coupled goat anti-rabbit IgG and propidium iodide. Specimens were observed with an epifluorescence microscope with an FITC filter (A–H) or a rhodamine filter (I and J). Specific tissues and examples of positive signals are indicated by arrows. The bar represents 25 μ m.

bZIP domain of an *opaque-2* mutant in maize abolished sequence-specific DNA binding to *zein* promoters (Aukerman *et al.*, 1991). Hence, the presence of Lys rather

than Arg at –10 of RF2a probably contributes to binding to a non-palindromic sequence.

The CCA/TGG motifs in Box II of the RTBV promoter

Fig. 8. Immunoblot analysis of RF2a accumulation in leaves of transgenic rice plants. Forty μ g of proteins from transgenic leaf tissues were used in immunoblots with anti-RF2a antibodies as described in Materials and methods. Lanes 1–3, plant lines that carry the *rf2a* sense orientation construct; lane 4, CK1, line 269–38, a transgenic line with the *hph* selectable marker only; lane 5, CK2, an untransformed plant; lanes 6–9, plant lines with the *rf2a* antisense gene construct.

are important for binding of RF2a. A mutant of Box II, Box II_{m1}, which contains a tandem repeat of the sequence TGG [TGG(N)₃TGG], binds the homodimer of cloned RF2a more efficiently than wild-type Box II (Figure 3A and B); RF2a also binds to vs-1 and AC-I elements, both of which contain CCA/TGG repeats. Moreover, methylation interference assays indicate that the G residues in the CCA/TGG repeats are involved in binding of RF2a to Box II (data not shown), and we conclude that the CCA/TGG repeats serve as recognition sequences for RF2a.

Antibodies to RF2a supershift a specific DNA-binding complex, RF2, that is produced by the binding of rice nuclear proteins to Box II of the RTBV promoter. Several observations indicate that RF2 is a heterodimer between RF2a and a second *trans* factor. First, recombinant RF2a forms a complex with a different mobility in EMSA compared with that of RF2; second, anti-RF2a antibodies supershift complexes containing RF2a more dramatically than RF2, suggesting that native RF2a in rice nuclear extracts binds to Box II as a heterodimer whereas recombinant RF2a binds as a homodimer. Moreover, RF2a binds to Box II_{m1} with higher affinity than Box II, implying that Box II_{m1} serves as a better binding site for the RF2a homodimer, whereas Box II is a better binding site for native RF2. Heterodimerization of transcription factors can increase their DNA-binding affinity; for example the binding affinity of Fos–Jun dimers is much greater than for Jun homodimers (Hurst, 1994), and heterodimerization of RF2a in rice nuclear extracts probably accounts for the higher affinity of RF2 compared with RF2a for Box II.

RF2a activates transcription from Box II

RF2a has three potential transactivation domains, the acidic, proline-rich and glutamine-rich domains, that might function in the activation of transcription by interacting with different components of the basic transcription machinery (Zawel and Reinberg, 1995; Verrijzer and Tjian, 1996). RF2a functions as a sequence-specific transcription activator in the *in vitro* transcription system derived from rice whole-cell extracts (Figure 5). This homologous

transcription system gives authentic transcription from a rice *pal* promoter and several other rice promoters (Zhu *et al.*, 1995ab). Stimulation of transcription *in vitro* by recombinant RF2a was sequence specific since depletion of Box II-binding factors from the rice extracts did not inhibit transcription from the unrelated *pal* promoter to a significant level and addition of recombinant RF2a did not stimulate transcription from the *pal* promoter.

These *in vitro* studies demonstrated that RF2a functions as an authentic, sequence-specific bZIP transcription factor. However, addition of RF2a to the rice extracts in which Box II-binding factors were depleted only partially restored Box II-dependent transcription, suggesting that RF2a functions in combination with other transcription factors that bind to the RTBV Box II *cis* element. Alternatively, recombinant RF2a may require post-translational modification for full activity equivalent to that observed with native RF2a. For example, pathogen- or elicitor-induced phosphorylation of the soybean G/HBF-1 *trans* factor promotes binding of this bZIP protein to the cognate *cis* elements in the promoters of immediate/early defense genes (Dröge-Laser *et al.*, 1997). RF2a contains several potential phosphorylation sites and is a good substrate for a calcium-dependent protein kinase from *Arabidopsis thaliana in vitro* (Y.Yin, J.Harper and R.N.Beachy, unpublished observations).

Role of RF2a in plant development

Reducing the amount of RF2a in transgenic rice plants severely disrupted shoot development. Transgenic lines in which RF2a levels were substantially reduced from wild-type exhibited a characteristic set of developmental phenotypes, including severe stunting of shoots and leaf twisting, accompanied by aberrant tissue organization, especially in vascular bundles. These phenotypes were not observed in transgenic plants transformed with vector sequences alone, or transgenic plants in which levels of RF2a were equal to or greater than wild-type levels. We concluded that these phenotypes result from the reduction in levels of RF2a due to the expression of the *rf2a* antisense gene construct rather than the transformation procedure *per se* or the presence of the *ubi* promoter.

Since RF2a is encoded by a single-copy gene, the phenotypes of plants transformed with the *ubi::a2fr* antisense construct are likely to result from the marked suppression of RF2a protein accumulation rather than cross-suppression of other classes of bZIP *trans* factors. This conclusion is supported by the observation that the phenotypic effects in *ubi::a2fr* antisense lines is restricted to shoots, while roots appear to be normal; this is consistent with the finding that RF2a is abundant in shoots but not in roots in wild-type plants. Moreover, RF2a accumulates in the nuclei of several different cell types including phloem, xylem parenchyma, fundamental parenchyma and epidermal cells. It is therefore not unexpected that antisense suppression would induce extensive morphological aberrations in the organization of leaf tissues. We propose that RF2a plays a crucial role in vascular development and shoot tissue organization. The enlargement of sclerenchyma tissue and the development of extensive air spaces at the expense of mesophyll and vascular tissue in plants with reduced levels of RF2a might reflect a delay or disruption of a specific tissue

Fig. 9. Phenotypes derived from ectopic overexpression and antisense suppression of RF2a in transgenic plants. **(A)** Transgenic plant 269-24 that overexpresses RF2a (left) and a normal plant line carrying only the *hph* hygromycin resistance gene (right). **(B)** Plant line 270-12 with reduced RF2a protein levels showing dwarfed phenotype and twisted leaves (left, indicated by an arrow) and a normal plant line carrying the *hph* hygromycin resistance gene only (right). **(C and D)** Two twisted leaves from lines 270-47 and 270-12 with reduced levels of RF2a. **(E–G)** Cross-sections of leaves from a control plant **(E)**, from lines 270-47 **(F)** and 270-12 **(G)**, respectively that contains reduced levels of RF2a. The vascular bundles (VB) and the air spaces (AS) are indicated by arrows. **(H–J)** Fluorescence microscopy of leaf sheath vascular bundles from control plant **(H)**, the RF2a-overexpressing line 269-24 **(I)** and RF2a-suppressed plant line 270-12 **(J)**, respectively. A green filter was used to observe the cell wall-derived autofluorescence from sclerenchyma (SC) and vascular bundles (VB).

differentiation process followed by widespread but secondary, compensatory effects. For example, the enlarged sclerenchyma may reflect a defect in a vascular function that normally restricts sclerenchyma development; likewise the appearance of air space cavities adjacent to vascular bundles could be due to a primary dysfunction in vascular development rather than to a direct effect on mesophyll differentiation. It should be pointed out that *vs-1*, to which RF2a binds *in vitro*, functions not only as a positive *cis* element in xylem but also as a negative *cis* element in parenchyma cells (Torres-Schumann *et al.*, 1996). These results support the hypothesis that RF2a has multiple functions in shoot tissue organization.

The specificity and the mechanisms of the phenotypes

that resulted from reducing the amount of RF2a will be investigated further by expression of RF2a antisense constructs under the control of tissue-specific or inducible promoters.

The RTBV promoter drives phloem-specific expression of reporter genes, whereas RF2a is found not only in phloem cells but also in certain other cell types. How does a ubiquitous factor contribute to phloem-specific expression of the RTBV promoter? Heterotypic interactions between multiple transcription factors is a common mechanism that can establish cell type-specific gene expression (e.g. see Olson, 1993). In addition to Box II, several additional *cis* elements in the –164 to +45 region of the RTBV promoter are required to give high level,

phloem-specific gene expression (Yin *et al.*, 1997). It is possible that the tissue specificity of the RTBV promoter involves combinatorial interactions between several *cis* elements and *trans* factors in addition to Box II and RF2a.

While a large number of plant bZIP proteins have been cloned that bind to specific *cis* elements involved in selective gene regulation by developmental cues, including abscisic acid, UV irradiation, wounding and pathogen-induced signals (Foster *et al.*, 1994; Menkens *et al.*, 1995; Dröge-Laser *et al.*, 1997), only the biological function of Opaque2, a regulator of zein expression, has been established (Schmidt *et al.*, 1992). The present demonstration that RF2a, a bZIP transcription factor which interacts with a *cis* element involved in phloem-specific expression of the RTBV promoter, plays a critical role in leaf tissue differentiation now provides the basis for molecular dissection of the regulatory combinations of genes that govern cell type-specific gene expression, cellular specialization and tissue organization in the plant vascular system.

Materials and methods

Plant material

Rice (*Oryza sativa* L.) cultivar TP 309 was used in all experiments.

Genetic selection in yeast

The yeast genetic selection system was kindly provided by Dr R. Reed (Wang and Reed, 1993). The reporter plasmid pRS315HIS-II4 was constructed by insertion of a Box II tetramer into the *Xba*I and *Bam*HI sites of pRS315HIS. Yeast strain yWAM2 (MATa *gal4 gal80 URA3::GAL1-lacZ lys2801^{amber} his-200trp-63 leu2ade2-101^{ochre} CYH2*) transformed with pRS315HIS-II4 was maintained in synthetic dextrose medium (SD) without leucine.

Rice cDNA was generated from poly(A)⁺ mRNA isolated from 2-week-old seedlings and directly cloned into the *Sall*-*Not*I sites of the TRP-I-marked yeast expression vector pPC86 using the Superscript cDNA synthesis system (BRL). The ligation products were electroporated into *E. coli* strain DH10B, and the transformants (8 × 10⁶, with average insert size of 1.5 kb) were amplified. The plasmid library DNA was purified with the Qiagen plasmid kit and used for yeast transformation by the polyethyleneglycol/lithium acetate method (Gietz *et al.*, 1992). Yeast transformed with the rice cDNA library were plated directly onto SD medium without histidine and incubated for 3 days at 30°C. Transformation efficiency was determined by plating an aliquot of the transformation mixture onto SD medium without leucine and tryptophan. The HIS⁺ yeast colonies were transferred to SD medium without leucine and tryptophan. Plasmids were recovered from the HIS⁺LEU⁺TRP⁺ yeast clones, amplified in *E. coli* and transformed back into yeast strains with reporter plasmids with or without Box II to determine the specificity of the HIS⁺ phenotype. Plasmid from yeast clone YTJ1-4 recovered the HIS⁺ phenotype in a Box II-dependent manner and was analyzed further.

Full-length rf2a cDNA clone

A full-length cloned cDNA of RF2a was obtained using the Marathon cDNA Amplification Kit (Clontech). Briefly, rice cDNAs were ligated to an adaptor containing two nested primers AP1 and AP2. The cDNA was first amplified with primer AP1 and a gene-specific primer (t₈) from clone YTJ1-4 by PCRs using *Pfu* polymerase (Stratagene). Aliquots of the first PCR products were amplified further with AP2 and another nested primer (t₁₀) 5' of primer t₈. The specific products were cloned and amplified in plasmid pBluescript SK (Stratagene).

Expression of recombinant RF2a in *E. coli*

The coding region for RF2a (1104 bp) was cloned into expression vector pET-12a (Novagen) with a 6HIS tag sequence from plasmid AK1-6H (Harper *et al.*, 1994) fused at the C-terminus. The plasmid was amplified in *E. coli* DH10B cells and transformed into *E. coli* BL21(DE3)/pLysE. Cells were grown at 28°C to an OD₆₀₀ of 1.0, induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1–3 h. The cell pellets were processed as described by Pognonec *et al.* (1991). RF2a was found

exclusively in inclusion bodies. The inclusion bodies were solubilized in buffer with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 100 mM NaCl plus 8 M urea, purified over Probond nickel resin (InVitrogen) and renatured by dialyzing in buffers with decreasing molar concentrations of urea (4–1–0–0) for 12 h in each buffer.

RF2a antibodies

A polypeptide comprising amino acids 264–368 (carboxy-terminus) of RF2a was expressed with the GST fusion-based and 6HIS-tagged vector AK1-6H (Harper *et al.*, 1994) and purified consecutively by Probond nickel resin (InVitrogen) and glutathione-Sepharose 4B resin (Sigma). Polyclonal antisera were produced by injecting rabbits (Bio-World) with purified GST fusion proteins. Antibodies were purified first by absorption of serum with excess GST proteins produced from pGEX-2T (Pharmacia) and then by a protein affinity column containing the polypeptide comprising residues 264–368 of RF2a coupled to CNBr-Sepharose 4B (Pharmacia). Antibodies from pre-immune sera were purified by adsorption to a protein A column (Sambrook *et al.*, 1989).

Electrophoretic mobility shift assay

EMSAs were carried out as described (Yin and Beachy, 1995) with some modifications (see Figure 3). For supershift EMSA, the purified RF2a or rice nuclear extracts were incubated with 0.7 µg of purified antibodies for 15 min before adding other components to the binding assays.

DNA-binding constants

DNA-binding affinities were estimated essentially as described by Yin and Gaynor (1996). Briefly, a series of binding reactions were performed in which the concentrations of the ³²P-labeled DNA probes were varied and the concentrations of recombinant RF2a or extracted rice leaf nuclear proteins were kept constant. The percentage of unbound DNA and DNA bound to RF2a, RF2 and RNFG2 in each reaction was determined by a phosphorimager. Data were analyzed by the Scatchard equation: B/F = -K_{app}B + K_{app}B_{max}, where B and F represent bound and free probes respectively, K_{app} is the apparent equilibrium constant, and B_{max} is the maximum binding capacity. A plot of B/F versus B yields a line of slope -1/K_{app}.

In vitro transcription assays

In vitro transcription assays were carried out as described by Zhu *et al.* (1995a). To deplete Box II-binding factors, the extracts were adsorbed with a DNA affinity resin prepared as follows: 2.5 µg of Box II tetramer DNA labeled by biotin-7-dATP through Klenow fill-in reactions were mixed with 0.5 ml of washed avidin-agarose (Sigma) and rotated at 4°C overnight. Rice whole-cell extracts (Zhu *et al.*, 1995a) or rice leaf nuclear extracts (Yin and Beachy, 1995) in a volume of 160 µl were mixed with 150 µl of affinity resin containing Box II at room temperature for 30 min. The supernatants were collected after brief centrifugation of the mixture. The depletion of Box II-binding factors was confirmed by EMSA.

Nucleic acid blot hybridization reactions

For DNA blot hybridization reactions, 10 µg of rice genomic DNA was digested with various restriction enzymes that do not cut in the probe, and transferred to nylon membranes (Hybond, Amersham) according to Sambrook *et al.* (1989). For RNA blot hybridizations, mRNA purified by oligo(dT)-cellulose chromatography from 200 µg of total RNA isolated from rice root, sheath and leaf tissues was electrophoresed under denaturing conditions and transferred to membranes. DNA templates spanning RF2a amino acid residues 82–149 for DNA blots or the 1.2 kb insert of the plasmid YTJ1-4 for RNA blots were labeled with ³²P by random DNA priming (Stratagene). The hybridization conditions were: 5 × SSC, 50% (v/v) deionized formamide, 5 × Denhardt's solution, 0.5–1% SDS, 100 mg/ml single strand salmon sperm DNA, 42°C, 16 h. The membranes were washed in 0.1% SDS and 2 × SSC for 2 × 5 min at room temperature, 1 × SSC for 30 min at 68°C, and 0.1 × SSC for 2 × 30 min at 68°C.

Immunoblotting

Plant tissues (5 g) were ground in liquid N₂, followed by extraction in protein loading buffer (63 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). Protein extracts (40 µg per lane) were loaded onto a 7.5% polyacrylamide gel containing SDS and were transferred to nitrocellulose membranes. The membrane was blocked overnight in TBST buffer (10 mM Tris, pH 7.4, 150 mM NaCl and 0.2% Tween 20) with 2% (w/v) non-fat dry milk at 4°C. Purified anti-

RF2a antibodies or IgG from pre-immune serum (2 µg/ml) were added and incubated at room temperature for 2 h, followed by four washes of 10 min each in TBST buffer. Secondary antibodies (goat anti-rabbit IgG) were diluted 1:5000 in blocking buffer and incubated with the nitrocellulose filter for 1 h at room temperature. The blot was washed again as described above before alkaline phosphatase assay or ECL chemiluminescence reactions (Amersham).

Immunolocalization assays

Rice sheath pieces (2–5 mm) from 10-day-old rice seedlings were fixed in Histochoice tissue fixative (Amresco, H108-1Q) with 20% ethanol for 2 h and embedded in TBS tissue-freezing medium (EMS, H-TFM). Frozen sections (15 µm) were made with a Cryocut (Reichert-Jung, Germany) and placed on poly-L-lysine-treated slides. The sections were cycled with hydrophobic PAP PEN (Kiyota) and the embedding medium was removed by washing with blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.4% Triton X-100). The sections were then incubated in blocking buffer with 2% non-fat dry milk at room temperature for 1 h. After overnight incubation with 5 µg/ml affinity-purified antibodies (anti-RF2a or pre-immune serum IgG) in blocking buffer with 2% dry milk, the sections were washed 3×10 min with blocking buffer containing 0.1% Triton. Secondary antibodies and the anti-rabbit IgG-FITC isomer 1 conjugate (Molecular Probes) were diluted 100-fold in blocking buffer with 2% non-fat dry milk and incubated with sections for 1 h. The slides were then washed 3×10 min with blocking buffer containing 0.1% Triton X-100. For ethidium bromide staining, the tissues were treated with 10 µg/ml ethidium bromide for 10 min. For propidium iodide staining, 100 µg/ml RNase A was added with secondary antibodies and 1 µg/ml propidium iodide in blocking buffer was added after a final wash and incubated at room temperature for 10 min. After a brief rinse with blocking buffer, the samples were mounted with Slow-Fade Kit (Molecular Probes) and observed with a Nikon fluorescent microscope with excitation filters of 450–490 nm for FITC and ethidium bromide staining and 510–560 nm for propidium iodide staining. Photographs were taken using Kodak 1600 slide film.

rf2a transgenic rice plants

The *rf2a* coding region was ligated with a maize ubiquitin *ubi* promoter expression cassette (Christensen *et al.*, 1992) in the sense or antisense orientations, and was introduced by particle bombardment into transgenic rice together with the *hph* gene for hygromycin resistance driven by the cauliflower mosaic virus (CaMV) 35S promoter as described (Li *et al.*, 1993; L.Chen, unpublished). The integration of the *rf2a* gene in the sense or antisense orientations in the rice genome was confirmed by PCR analysis. Representative transgenic plantlets or leaf pieces were photographed. Leaf or leaf sheath pieces were fixed, embedded in 4% agarose and sectioned with a Vibratome. Immunoblotting of whole proteins from transgenic plants was carried out as described above.

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