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Final Technical Report

Grant No.: DE-FG03 91 ER 20039

"Regulating Expression of Cell and Tissue Specific Genes by Modifying *Trans* Factors"

P.I.: Roger N. Beachy, Ph.D.

Co-P.I.: Peter Ghazal, Ph.D.
The Scripps Research Institute
La Jolla, CA

A. Summary of Research Project, as proposed.

Regulated expression of genes that results in the cell and tissue-specific accumulation of gene products is largely, but not solely, controlled at the level of transcription. We have studied the expression of genes that encode the β -conglycinin seed storage proteins and the promoter from the rice tungro bacilliform badnavirus (RTBV), whose expression in rice is limited to phloem-associated cells. We previously identified several sequences located 5' of the TATA element that are important in expression of this promoter. Very recently we isolated and characterized a transactivator protein, RF2a, that binds to one of the important *cis* sequence elements, and characterized its role in binding DNA and its activity in *in vitro* transcription reaction assays using a cell-free extract derived from rice cell cultures (in collaboration with Q. Zhu and C. Lamb, The Salk Institute). RF2a, a member of the bZIP class of transcriptional regulators, is found in many cell and tissue types in rice plants, and is involved in the tissue-specific expression of the RTBV promoter. We propose to develop dominant negative mutants of RF2a that reduced expression of the RTBV promoter *in vitro* and *in vivo*, but have no effects on the expression of other genes that require RF2a for expression. Mutants of RF2a will be developed by deletion analyses and site-directed mutations: the effects of the mutations on binding of the target sequence (designated the Box II sequence in the RTBV promoter) will be evaluated by the affinity constant of binding (using BIAcore analyses), electrophoretic mobility shift assays (EMSA), *in vitro* transcription assays and transient *in vivo* assays. Because it is likely that this bZIP factor requires dimerization for binding, we propose a series of experiments to develop a single protein mutant of RF2a that does not require dimerization to bind target DNA sequences. The results of these studies will make it possible in the future to develop strategies to control the expression of one or several genes in a given gene family that require a common transcription factor; dominant negative mutants may be useful to redirect plant metabolism to alter the production of proteins, starches or other products. A similar approach may be useful to restrict the replication of DNA containing viruses, including the pararetroviruses and geminiviruses.

B. Summary of Research Progress

Introduction

As indicated in the Summary of Research, the goal of this project was to determine the role of the transcription factor RF2a in regulating expression of the promoter from RTBV (rice tungro bacilliform badnavirus), a plant pararetrovirus. The long-term goal was to characterize the proteins that bind the promoter, and to develop strategies to control, at will, the expression of the

promoter. Success would make it possible to control replication of the virus, and would be the first example of control of replication of a plant pararetrovirus by regulating transcription of the promoter. A second goal was to use the knowledge of regulation of the promoter to develop a new system for control of gene expression in plants using unique combinations of DNA sequences, elements of the RTBV promoter, and well-characterized transcriptional transactivator protein(s). Here we describe the progress made on several of the stated goals of the proposed research: it is important to note that the proposal was written for 3 years of research, and the project was supported for 18 months.

We previously demonstrated that the RTBV promoter fragment comprising nucleotides -628 to +45 (Bhattacharyya *et al.*, 1993) is expressed only in phloem tissues of transgenic rice plants: it was previously shown that RTBV infection is largely limited to vascular tissues, in particular to the phloem. We used site-directed mutagenesis and sequence deletions to introduce mutations in the RTBV promoter and determined the effects of the mutations on expression of the promoter in transgenic rice plants. From these studies we determined that tissue-specific expression was retained on a promoter fragment comprising nucleotides -164 to +45 (Yin *et al.*, 1997a). Within this region, nucleotides -66 to -32, which contains 'Box II' (n.t.-53 to -39), had a great impact on expression of the promoter. Sequences similar to the AS-1 element found in other plant promoters (including the well-characterized CaMV 35S promoter) and the GATA sequence element (Lam and Chua, 1989) are also located on the -164 to +45 fragment of the promoter. Based on the studies of Yin *et al.*, (1997a) it was concluded that expression of the RTBV promoter resulted from the combined interactions between *cis* elements and multiple protein factors and the effects of these interactions on transcription.

Because other research groups have spent considerable effort to characterize the GATA and AS1-like sequence elements in relation to their roles in regulating gene expression, we focused our research on the isolation and characterization of proteins that bound the Box II sequence. Using a yeast-based, one-hybrid system we isolated, and subsequently characterized, a protein that bound to nucleotides in Box II. The protein, was named RF2a (Rice Factor, Box II, Factor a), was characterized as a bZIP protein. The protein is somewhat unique in that it contains three putative sequence domains that were predicted to play a role in regulating gene expression, namely, a Proline-rich domain (P-domain), a Glutamine-rich domain (Q-domain) and an Acidic domain (A-domain) (see Fig. 1). RF2a is, as expected, primarily found in nuclei, and is present in all or most leaf cells in rice plants, as well as in rice tissue culture suspension cells; by contrast, it is in very low abundance, or is absent, from root tissues.

In a collaboration with Q. Zhu and C. Lamb (Salk Institute), who developed an *in vitro* transcription system from whole cell extracts of rice cell suspension cultures, we found that the RTBV promoter was transcribed appropriately *in vitro*. Using an antibody against RF2a, endogenous RF2a was depleted from the extract; this substantially reduced the activity of the extract for the RTBV promoter, but not other promoters. Furthermore when RF2a, was purified from *E. coli* and added to the depleted *in vitro* transcription system, expression of the RTBV promoter was restored (Yin *et al.*, 1997b). We concluded that the *in vitro* transcription system might be useful to determine the role of the putative regulatory domains (P, Q, and A) on the activity of RF2a. Furthermore, by expressing RF2a and mutants of RF2a in transgenic plants we would be in the position to compare and contrast the results of *in vitro* and *in vivo* (*in planta*) assays related to the function of RF2a, and draw certain conclusions of the function of the putative regulatory domains on the protein. The results of these studies are described here and reflect our studies to January 31, 1999.

I. Determining the role of RF2a in growth and development of rice. This research goal was extended to include tobacco plants as well as rice plants in order to gain a better understanding of the role of RF2a in plant growth and development, and in regulating expression of the RTBV promoter. The work in tobacco was conducted by Dr. Sylvana Petrucci, and the work in rice was conducted by Yanhai Yin (2 months; previous graduate student) and Shunhong Dai (10 months; current graduate student): support of Dr. Petrucci was described above. Tissue culture was carried out by Ms. Rosa Carcamo, who receives partial support from this grant.

Effects of expression of antisense and sense gene sequences of RF2a in transgenic rice plants. The cloned cDNA encoding RF2a was ligated with the maize ubiquitin gene promoter (*Ubi*) in the sense and antisense orientation and the genes were expressed in transgenic rice plants. Transgenic RO plants that contained the *Ubi::rf2a* gene and accumulated high levels of RF2a showed leaf discoloration but were otherwise normal in appearance and development. By contrast, 11 of 34 transgenic plants that contained the antisense gene showed marked suppression of RF2a levels in leaf tissues, and leaves with reduced levels of RF2a exhibited abnormal growth. Most such plants were dwarfed and their leaves were twisted, and development of vascular tissues was abnormal: in some leaves the size of the vascular bundles was reduced. In contrast, root development appeared to be normal (Yin *et al.*, 1997b). The abnormal development of plants that expressed the antisense gene sequences were reduced as the plants reached maturity, and the plants set seed in a normal manner. We concluded from these studies that RF2a was probably important for development of vascular tissues in young leaf tissues, but not in root tissues. To further define the developmental role of RF2a, both sense and antisense RF2a sequences were put into constructs, driven by different tissue-specific promoters, namely the Cab3 promoter, the Pal2 promoter, the SH promoter, and the RTBV promoter (Fig. 2). Rice transformation of each of these constructs is in progress.

II. Characterizing the role of RF2a sequence domains. As diagrammed in Fig. 1, the sequence of RF2a predicts the presence of several sequence domains (described by Yin *et al.*, 1997b). We proposed to identify, or characterize, the function of the various domains by several *in vitro* assays, in protoplast based assays, and in transgenic plants. It has not been possible to complete each of the proposed studies given the brevity of the award, but we have made substantial progress on several assays.

A. Determining the minimal length of RF2a for binding to Box II sequences. The wild type Box II sequence, and a mutant referred to as Box IIml are given below. Box IIml has substantially higher binding constant for RF2a than wild type Box II sequence (see Fig. 4 and Table I in Yin *et al.*, 1997b; attached) and was used for some of the studies.

Box II.	-53 CCAGTGTGCCCCTGG
Box IIml	-38 CCAGTGTGGCGCTGG

Mutants of RF2a that lacked one or several domains (*i.e.*, P, A, Q domains) were constructed and expressed in *E. coli* as fusions with 6 histidine residues to aid in purification of the proteins: the constructs are shown in Fig. 1 with designations for each mutant.

Each of the proteins was tested in electrophoretic mobility shift assays (EMSA) using either Box II or Box IIml. Since RF2a is a bZIP protein, it binds to target DNA as a homodimer with a characteristic mobility in these assays (for example, see Fig. 3, Yin *et al.*, 1997b). The results of these studies were not unexpected, and demonstrated that: (1) the bZIP domain of RF2a, apart

from other domains, was sufficient to bind Box II. Mutants that lack either the basic domain or the leu-rich sequence are incapable of binding target DNA sequence. (2) RF2a from which a single (P, A, or Q) domain, or multiple (P + A, P + Q, etc.) domains were deleted bound target DNA. Furthermore, changes in the size of the binding protein caused by the deletions, resulted in greater mobility in EMSA than did full-length RF2a, presumably due to reduced sizes of the DNA:protein complexes (Fig. 3).

In the proposal we described experiments to develop N-terminal and C-terminal deletions, in 25 or 50 amino acid increments, from RF2a, and to determine the effect of these mutations on DNA binding, and on biological function of RF2a. This has not yet been done, and based upon the results of studies described here which show that DNA binding is due to the bZIP sequences and not to sequences in the P, A, or Q domains, it seems less important to complete these studies in preference to other studies.

B, Determining the binding affinity of RF2a and truncated mutants for Box II DNA. We determined the DNA binding constants for purified RF2a and Box II and Box IIml by standard techniques of DNA:protein EMSA, using increasing amounts of labeled probe in the assays. Using the Scatchard equation, RF2a bound Box II with $K_d=3.2$ nM, and to Box IIml with $K_d=1.0$ nM.

We also used a nuclear extract and located the band that contained RF2a (confirmed by a supershift in band mobility when the reaction products were reacted with anti-RF2a antibody prior to EMSA) following EMSA using either Box II or Box IIml as probe. The band, referred to as RF2, had a lower K_d with Box II ($K_d=0.6$ nM) than with Box IIml ($K_d=0.9$ nM; Fig. 3, panel B, and Table I; Yin *et al.*, 1997b). The RF2 DNA-protein complex had a more rapid mobility in these assays than did complexes containing RF2a:DNA, which suggests that RF2 contains, in addition to RF2a, a second protein. In a subsequent section, we describe the results of experiments to identify a candidate protein that we propose is the second protein, RF2b, that forms a heterodimer with RF2a to form the RF2 complex.

C. Activity of RF2a and mutants in *in vitro* transcription assays. (The experiments described here were conducted by Dr. Isabel Ordiz, in collaboration with Q. Zhu and C. Lamb at the Salk Institute. Supplies and other expenses were paid by this grant.) We recently reported that RF2a purified from *E. coli* is active as a transcriptional activator in *in vitro* transcription reactions derived from whole cell extracts from rice suspension cultures (Yin *et al.*, 1997b). In these studies it was shown that RF2a is essential for accurate initiation of the RTBV promoter, but had no apparent effect on expression of the PAL promoter.

Experiments were conducted to determine if there are direct interactions between RF2a and components of RNA Pol II or general transcription factors that influence the activity of RF2a in the transcription reactions. Q. Zhu isolated cloned cDNAs encoding the TATA-binding protein (TBP) and transcription factor IIB (TFIIB from rice based upon homologies to other TBP and TFIIB protein sequences (Zhu *et al.*, manuscript in preparation). *In vitro* transcription reactions using the RTBV promoter were supplemented with various combinations of RF2a, TBP, and TFIIB; the results of a typical reaction is shown in Fig. 4. RF2a, TBP and TFIIB each stimulated transcription 3 to 6 fold in these reactions; however, there was greater activity when TBP and RF2a were both added to the reaction (16x increase). These results suggested that there may be interaction between RF2a and TBP, but not between RF2a and TFIIB in these assays. To determine which functional domains of RF2a contributed to the increase in transcription, we constructed mutants of RF2a from which the P-domain, and/or A domain were deleted, while retaining the Q-domain (see Fig. 1). When these proteins were added to similar reactions there was little difference compared with reactions containing wild type RF2a. In contrast, mutants of

RF2a that lacked the Q domain resulted in significantly less product in such reactions; however, the amount of transcription in reactions containing mutant RF2a- Δ PAQ was consistently greater than in reactions containing RF2a- Δ Q or RF2a- Δ P Δ A Δ Q (Fig. 5). These results indicated that in these reactions the Q domain plays a significant role in initiating transcription from the RTBV promoter. In addition, the A domain, may play a minor role in activating transcription in absence of the Q domain.

To determine if there is significant interaction between TBP and RF2a we conducted EMSA reactions using Box IIml as labeled probe (Fig. 6). These reactions have proven to be somewhat difficult and the results between experiments are somewhat inconsistent; perhaps because of the difficulty in renaturing the proteins used for the reactions. Reactions contained purified RF2a or mutants that were deleted in P, A, or Q domains (described in Fig. 1), with or without TBP. In these reactions RF2a bound the probe as previously reported (Fig. 3A). In some but not all experiments, addition of TBP to reactions containing RF2a resulted in a supershift of the protein-DNA complex, (Fig. 6) consistent with the hypothesis that TBP interacts with RF2a. Results of experiments with mutants of RF2a are clear and are currently being repeated.

D. Activity of RF2a and mutants in a protoplast-based transient assay . (These studies were conducted by S. Dai and S. Petrucci). We spent considerable effort attempting to establish a viable transient assay system in which to quantitate the effect of RF2a on expression of a reporter gene construct comprising the short RTBV promoter (n.t. -164 to +45; with wild type Box II) with the *uidA* reporter gene and the nos 3' end. Protoplasts were prepared from suspension cell cultures of rice (Taipei 309) and from the tobacco culture BY-2, and the reporter plasmid and a plasmid comprising the *Ubi* promoter with RF2a cDNA were introduced by electroporation: a luciferase reporter gene was included as an internal standard. The rice protoplasts, which contain RF2a (Yin et al., 1997b), proved to be technically more difficult to maintain in a receptive condition than BY-2 cells, and were dropped from the study. In BY-2 protoplasts there was basal level of expression from the *uidA* (GUS) reporter plasmid in absence of RF2a; cells that received the reporter gene plus genes encoding RF2a driven by the 35S promoter, or any of the mutants that lacked P, Q, or A domains, produced less GUS than cells that received only the reporter . We consider the possibility that RF2a is either not active in this assay in absence of a partner' for forming a heterodimer, or that other co-factors are missing. Alternatively, the experiments failed because reporter and effector genes were introduced on two plasmids. We are currently constructing single plasmids that contain both the reporter and the effector genes and will repeat the experiments described above. We are also developing rice cell lines in which similar studies will be conducted.

III. Identification of other gene(s) whose expression requires RF2a. No progress was made on this goal, and it will be eliminated from the project.

IV. To determine the structure of RF2a with Box II DNA. This remains one of the long-term goals of the research project, but until this time has not been pursued.

V. Developing dominant negative mutants of RF2a that reduce expression of the RTBV promoter. We originally proposed to construct regulatory molecules based upon constructing molecules that contain two bZIP domains, and to undertake a mutagenesis strategy to increase the specificity and affinity of the dimerized protein for the RTBV promoter. We have not yet initiated this part of the work due to the short time between award of the last grant, and re-application. Instead, we spent considerable time and effort to determine if it is possible to control, in a positive or negative manner, expression of the RTBV promoter in transgenic

tobacco and rice plants. This work has been done over the past 18 months by Dr. Petruccelli (tobacco) and S. Dai (rice).

A. Expression of the RTBV promoter in tobacco plants and the role of the P, A, and O domains on gene expression. For these studies transgenic tobacco (*Nicotiana tabacum*) plants were produced that contained: (1) the reporter gene comprising the short, or long, RTBV promoter plus *uidA* coding sequences and nos 3' end; or (2) the reporter gene, plus a second gene, driven by the CaMV 35S promoter, that encodes RF2a or mutants of RF2a. The two genes were placed on the same intermediate plasmid, and introduced into tobacco leaf discs by *Agrobacterium tumefaciens*, and 10 to 15 transgenic plants were produced from each transformation. Fig. 7 diagrams each of the plasmid constructs that were introduced: below are summarized the results of analysis of these plants to date. This is an ongoing study that has been implemented by the return of Dr. Petruccelli to Argentina where she will establish a research lab: funds are requested to make it possible for Dr. Petruccelli to return to the lab for 6 months each year to continue this aspect of the research project.

Transgenic plants are being analyzed by several methods: (1) the level of GUS activity is quantitated in leaf extracts using standard protocols (Jefferson *et al.*, 1986); (2) leaf tissues are stained histochemically to determine the distribution of GUS; (3) the amount of RF2a is analyzed by ELISA using leaf extracts. For such assays, Dr. Petruccelli developed a new, polyclonal antibody raised in rabbits against the RF2a holoprotein. The limitation of the ELISA reaction is that each mutant protein lacks some of the antigenic determinants that are present on the RF2a, making comparative quantitation of each mutant protein less accurate compared with wild type RF2a; and (4) each plant is visualized for abnormal phenotypes that might reflect impacts of the gene(s) on plant growth and development.

Fig. 8 summarizes the results of GUS assays on each of the R₀ plant lines tested to date. In this figure, plants that contain only the Short (S) and Long (L) RTBV promoter-GUS gene are presented, as well as plants that contain the gene plus the second gene that produced wild type RF2a. As expected, we observed a relatively wide range of activities of GUS in the transgenic plants, as is common with most promoter assays in transgenic plants. We will continue these studies to the R₁ generation to confirm the levels of gene expression, particularly with respect to gene segregation. Second, there was a greater level of GUS activity in plants that contain RF2a than in plants that do not; this was the case when the GUS gene was driven by the Long or Short version of the promoter (compares with S+RF2a; and L with L+RF2a). These results clearly demonstrate that expression of the S and L mutants of the RTBV promoter are increased by production of RF2a: this is one of a few examples in which expression of a single transcription factor affects the level of expression of the cognate promoter in a transgenic organism.

Leaf tissues were taken from many of the transgenic plants and stained with X-gluc reagent to identify tissues in which the S and L promoters were expressed in the presence of RF2a, or mutants thereof. These experiments are in progress, and a summary of conclusions to date is presented here. First, the S and L variants of the promoter are expressed only in vascular tissues in tobacco plants (Fig. 9A, C), similar to transgenic rice plants (Bhattacharyya-Pakrasi *et al.*, 1993; Yin and Beachy, 1995; Yin *et al.*, 1997a). Secondly, transgenic plants that co-express the GUS gene and full length RF2a show expression of GUS throughout the leaf, including the epidermal cells, and leaf mesophyll cells (Fig. 9B, D). Since the 35S promoter is expressed in most plant tissues one would anticipate GUS activity in cells that contain both the reporter gene and RF2a. The results suggest, but do not conclusively prove, that the regulatory activity is effected by homodimeric RF2a: it is possible, though somewhat unlikely, that there exists in

tobacco tissues, a 'partner' that interacts with RF2a to confer the activity. This is one of very few examples in which expression of a single transcription co-factor alters the pattern of expression of the promoter.

During these studies it was observed that a large percentage of transgenic plants that 'contain the reporter gene plus the gene encoding the bZIP domain, but not other RF2a mutants, exhibit an abnormal growth phenotype (we have not yet produced transgenic plants that contain only the bZIP domain). The abnormal growth is characterized by a downward curling of leaves that involves the midvein, but not the petiole; similarly, in older leaves the secondary and tertiary veins are misshapen, and cause a downward curling in various parts of the aging leaf (Fig. 9E). This is a very recent observation, and is not yet fully described. Nevertheless, we have drawn several conclusions from the studies to date. First, observed that development of vascular tissue was abnormal, and resulted in overproduction of certain cell types: we propose (based upon limited experimental data!) that this may cause the leaf to be distorted. Second, abnormal growth is more severe in some plants than in others, and becomes more severe as the plant ages. These observations suggest the possibility that the bZIP domain of RF2a, when produced in absence of the A, P, and Q domains, has a negative impact on expression of genes in tobacco plants that are involved in development of vascular tissues. This impact might be caused by interaction of the bZIP domain with other bZIP proteins and altering their activity, or by its binding to transcriptional promoters of genes that are involved in vascular development, and altering their pattern of expression. It is interesting to recall that the distortion of leaf development is not apparent in plants that contain other mutants of RF2a. These studies are ongoing.

B. Expression of mutants of RF2a in transgenic rice plants. As summarized above (see Introduction of Progress Report) we found that expression in transgenic rice plants of a gene encoding (-) sense sequences of the RF2a gene resulted in altered development of the young leaves (Yin *et al.*, 1997b), and expression of bZIP domain of RF2a in transgenic tobacco plants caused abnormal growth. To determine if the bZIP domain has an effect on rice plants and can be used as a dominant negative mutant to control RTBV promoter, gene constructs with the bZIP coding sequence of RF2a under the control of different tissue-specific promoters were built (Fig. 10). These genes are currently being used to retransform transgenic rice lines which contain the short RTBV promoter (-164 to +45, referred to as the E fragment, Yin and Beachy, 1995) ligated with the uidA gene sequence.

New Research: Isolation of RF2b, a Putative Partner for RF2a. As indicated above, it appears that there are two nuclear proteins that bind to the Box II sequence element. The bZIP transcription factor RF2a was isolated based upon its binding to Box II using the yeast one-hybrid system. We used the yeast two-hybrid system to clone the putative partner of RF2a. Because bZIP proteins normally form a homodimer or heterodimer through the bZIP domain, this domain of RF2a was used as the bait to screen a yeast cDNA expression library. In total, 1.7 x 10⁶ colonies were screened. After carrying out a His⁻ selection on the colonies, we isolated 39 positive clones; a *lacZ* assay eliminated two-thirds of the false positive clones, after which 12 candidates remained. Lastly, we carried out a mating assay and found that 11 of the 12 candidates restored *HIS3* gene expression. Among these clones, one was identified (based on sequence analysis) as RF2a; the others belong to four different cDNA species. All candidate cDNA species were isolated at least twice. Two of the four candidate cDNAs contain a bZIP domain with high similarity to the bZIP domain of RF2a, and were designated RF2b and TF15. The bZIP domain of RF2b shares 72% nucleotide identity with RF2a and 83% identity at the amino acid (a.a.) sequence level (see Fig. 11 for comparison of bZIP sequences of RF2a and RF2b, and other similar proteins); the bZIP domain of TF15 has a low (36%) relatedness to RF2a at the a.a. level. Based on these data we focused on the analysis of RF2b.

RF2b is a 1640 bp cDNA which has a long ORF, encoding a protein of 329 a.a. Southern blot assays showed that it is a single copy gene in rice (as is RF2a), and Northern blots showed a single band of about 1.7 kb. In addition to the bZIP domain, RF2b has two putative functional domains, namely an acidic domain at the N-terminus and a proline-rich domain at the C-terminus. To determine if RF2b has the capacity to bind to cis-element Box II. EMSA was carried out using RF2b expressed and isolated from *E.coli*. The results demonstrated that RF2b has the ability to bind to Box II and Box IIml (described above). In addition, we found that RF2b forms a heterodimer with RF2a in EMSA conditions, indicating that RF2b may be a partner of RF2a. We will propose to investigate the functional relationship between RF2a and RF2b in terms of activities in expression of the RTBV promoter.

Publications that cite support during current grant period:

- Voloudakis, A.E., Yin, Y., and Beachy, R.N. 1998. "Recombinant protein expression in plants." Gene Expression Systems: Using Nature for the Art of Expression. Eds. J. Fernandez, and J.P. Hoeffler. San Diego: Academic Press. (in press)
- Yin, Y., Chen, L., and Beachy, R.N. 1997 a. Promoter elements required for phloem-specific gene expression from the RTBV promoter in rice. *Plant J.* 12:1179-1188.
- Yin, Y., Zhu, Q., Dai, S., Lamb, C., and Beachy, R.N. 1997b. RF2a, a bZIP transcriptional activator of the phloem-specific rice tungro bacilliform virus promoter, functions in vascular development. *EMBO J.* 16:5247-5259.

Rice tungro bacilliform virus (RTBV) promoter is activated by expression of RF2a, a transcriptional activator.

Silvana Petruccelli **, Shunhong Dai, Rosa Carcamo, Yanhai Yin, Shouyi Chen ***, Roger N. Beachy*.

Division of Plant Biology, BCC206, Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

*Current address: The Donald Danforth Plant Science Center, 7425 Forsyth Blvd.Box 1098, Clayton, MO 63105, U.S.A. e-mail: RnBeachy@danforthcenter.org

**Current address: Centro de Investigación y Desarrollo en Criotecnología de Alimentos, 47 y 116, 1900 La Plata, Argentina.

***Current address: Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China.

Abstract

We studied the role of the rice transcription factor RF2a to regulate expression of the promoter from Rice Tungro Bacilliform Virus (RTBV) in transgenic plants. The RTBV promoter expressed in phloem cells in transgenic rice plants and RF2a, a b-ZIP protein, is known to binds a *cis* DNA sequence element in the promoter. Here, we demonstrate that the full length RTBV promoter comprising nucleotides -731 to +45 relative to the transcription, and a fragment, named 'E', comprising nucleotides -164 to +45 are phloem specific in tobacco. When a gene encoding RF2a was constitutively co-expressed with a reporter gene (uid A) under control of the RTBV promoter, there was a 5-20 fold increase in GUS activity. Besides the expression of the RTBV promoter was altered from phloem specificity to constitutive. The increase in activity was positively correlated with the amount of RF2a in transgenic plants. RF2a does not produce any morphological changes. We also show that ectopic expression of the b-ZIP domain of RF2a reduces expression of the RTBV promoter. Furthermore, constitutive expression of the b-ZIP domain alters leaf development in tobacco plants.

Fig. 1 Schematic representation of RF2a (w.t.) with the bZIP domain and three potential activation domains: proline (P), acidic (A), and glutamine (Q). Mutants from which single or multiple domains were deleted (Δ) are indicated.

Fig.2 Diagram of constructs with either sense (+) or antisense (-) orientation of *rf2a* driven by different tissue specific promoters. Ubi, maize ubiquitin; Sh, synthase-1 promoter; RTBV, RTBV promoter; Cab3, Arabidopsis Cab3 promoter; Pal2, bean pal2 gene.

Fig. 3 Influence of deleting domains of RF2a on binding to BoxII1. w.t. RF2a and mutants (see Fig. 1) purified from *E. coli* were used in EMSAs with BoxII1 as ³²P labeled probe. The reaction products were separated on a 5% polyacrilamide gel. All proteins, except those from which the basic or leucine zipper were deleted, bound to the probe.

Fig. 4 Functional interaction between rice TBP and RF2a transcription factor. RTBV promoter:GUS construct was used as template for In vitro transcription reactions. 100 ng each of TBP, TFIIB, and RF2a were added 10 min. before the addition of rice whole cell extracts. Products of the reaction were separated on 8% polyacrilamide denaturing gel. Figures at the bottom refer to quantitation of the product, relative to the left-most lane.

Fig.5 Functional interactions between rice TBP and RF2a transcription factor. 100 ng each of TBP and RF2a or different mutants of RF2a were added 10 min. before the addition of rice whole cell extracts to the in vitro transcription reaction.

TBP	+	-	+
Rf2a	-	+	+

Fig.6 Physical Interactions between rice TBP and RF2a transcription factor. EMSA. Box1m1 of RTBV promoter was used as 32P labeled probe. Adding TBP with RF2a resulted in decrease in mobility of the complex.

Fig.7 Diagram of constructs used for Agrobacterium mediated transformation. E = RTBV promoter fragment, -164 to 45; RTBV = full-length (L) promoter fragment, -628 to +45; 35S = 35S promoter from CaMV; P, proline-rich domain of rf2a; A, acidic domain of rf2a; Q, glutamine-rich domain of rf2a; bZIP, bZIP domain of rf2a; uidA = GUS encoding gene.

Fig.8 GUS activity in extracts prepared from transgenic tobacco plants that harbor the reporter gene p-RTBV:GUS (E, short promoter; R, long promoter) with or without a gene encoding full length (F) RF2a (P35S:rf2a).

Fig.12 EMSA of RF2a, bZIP domain of RF2a (3Δ), and RF2b with Box1m1 probe

Fig.9 GUS staining of transgenic tobacco plants. A) Cross section of leaf main vein of plants transformed only with the reporter gene. B) Cross section of leaf main vein of plants transformed with reporter gene plus RF2a. C) Whole leaf of plant as in A. D) Partial leaf of plant, as in B. E) Misshapen leaf of plant transformed with the reporter gene, plus the bZIP domain of RF2a.

Fig.10 Diagram of gene constructs with the bZIP coding sequence of rf2a driven by different tissue specific promoters (as above)

RF2b	DFFRAKRI LANRQSAARSKERKARYITELERKVQTLQTEATTLSAQLTLFQRDITGLSAXNAEL
B-ZIP	DFFRAKRI LANRQSAARSKERKARYITELERKVQTLQTEATTLSAQLSLFQRDITGLSAXNAEL
RF2a	DFFRAKRI LANRQSAARSKERKARYITELERKVQTLQTEATTLSAQLALLQRDITGLTENSEL
PoaP21	DFFRAKRI LANRQSAARSKERKARYITELERKVQTLQTEATTLSAQLTLLQRDITGLTENSEL
VSP-1	DFFRAKRI LANRQSAARSKERKARYITELERKVQTLQTEATTLSAQLTLLQRDITGLTENSEL
GSF1	ELKREKRRQSHRESAARRSRLRQAETTELATQVENLAENSTSLRSEIORLTESSEKLRLENSAL
O2	EEVRKRRKESHRESAARRSRYRKAANLKELEDQVAQLKANSCLLRRIAAALNQYMDANVDNRVL
TGA1a	VEKVLRLRLAQRHRAAKSRLRKKATVQQLENSRLKLIQLNQELESRAKQGMCVGGVDASQLST
TF15	VCHADQEKSLKRRSGQRSEVRKLYTIADLERIVDSLQNGADLAVRVASLFLQLRNALSHENKQL
Consensus	-----BB--BN--AA-B-R-BB-----LE-----L-----L-----L-----L-----

Fig.11 Alignment of amino acid sequences in the bZIP domains of RF2b and TF15 with RF2a and other known bZIP proteins