Essential role of the Box II cis element and cognate host factors in regulating the promoter of *Rice tungro bacilliform virus*

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*Rice tungro bacilliform virus* (RTBV) is a double-stranded DNA virus with a single, tissue-specific promoter that is expressed primarily in phloem tissues. Rice transcription factors RF2a and RF2b bind to Box II, a cis element adjacent to the TATA box, and control gene expression from the promoter. Mutations were made in the promoter to delete or mutate Box II and the mutated promoters were fused to a reporter gene; the chimeric genes were expressed in transient BY-2 protoplast assays and in transgenic *Arabidopsis* plants. The results of these studies showed that Box II is essential to the activity of the RTBV promoter. A chimeric β-glucuronidase (GUS) reporter gene containing the Box II sequence and a minimal promoter derived from the *Cauliflower mosaic virus* 35S promoter were co-transfected into protoplasts with gene constructs that encoded RF2a or RF2b. The reporter gene produced threefold higher GUS activity when co-transfected with RF2a, and 11-fold higher activity when co-transfected with RF2b, than in the absence of added transcription factors. Moreover, chimeric reporter genes were activated by approximately threefold following induction of expression of the RF2a gene in transgenic *Arabidopsis* plants. The work presented here and earlier findings show that Box II and its interactions with cognate rice transcription factors, including RF2a and RF2b, are essential to the activity of the RTBV promoter and are probably involved in expression of the RTBV genome during virus replication.

INTRODUCTION

Rice tungro disease (RTD) is a significant threat to rice production in South-East Asia (Hull, 1996). RTD is caused by two plant viruses, namely *Rice tungro spherical virus* (RTSV) and *Rice tungro bacilliform virus* (RTBV) (Hibino *et al.*, 1978; Hull, 1996). Whilst RTSV is required for disease transmission, RTBV is the primary causative agent of RTD (Hull, 1996). RTBV is a plant pararetrovirus with a genome of circular, double-stranded DNA (Qu *et al.*, 1991) and belongs to the genus 'Rice tungro bacilliform-like viruses' of the family *Caulimoviridae* (Mayo & Pringle, 1998). The 8 kbp RTBV genome consists of four open reading frames (ORFs) and a single transcriptional promoter that is located within the intergenic region between ORF IV and ORF I (Bhattacharyya-Pakrasi *et al.*, 1993; Qu *et al.*, 1991). The RTBV promoter is expressed predominantly in phloem tissues in transgenic rice and tobacco (Bhattacharyya-Pakrasi *et al.*, 1993; Petruccelli *et al.*, 2001; Yin & Beachy, 1995). Promoter activity in the epidermis and other cell types has also been observed in transgenic rice when sequences downstream of the transcription start site were included in the analysis (Klöti *et al.*, 1999). Tissue specificity of the promoter correlates somewhat with the tissue-specific accumulation of RTBV particles in infected plants (Bhattacharyya-Pakrasi *et al.*, 1993; Cruz & Koganezawa, 1991; Saito *et al.*, 1986; Yin & Beachy, 1995).

The E fragment of the RTBV promoter, as described in earlier studies (Yin & Beachy, 1995), includes nt −164 to +45 relative to the transcription start site; this fragment (hereafter referred to as the ‘E promoter’) retains significant activity and tissue specificity of expression in transgenic rice and tobacco plants (Bhattacharyya-Pakrasi *et al.*, 1993; Petruccelli *et al.*, 2001; Yin & Beachy, 1995). In addition to the TATA box (nt −31 to −25), the E promoter contains four cis elements identified through footprint analysis and electrophoretic mobility-shift assays (Yin & Beachy, 1995; Yin *et al.*, 1997a). The cis elements include a GATA motif (nt −143 to −135), an AS1-like (ASL) box (nt −98 to −79), Box II (nt −53 to −39) and Box I (nt −2 to +8). The roles of each motif in regulating the activity of the promoter have been partially characterized (Yin & Beachy, 1995; Yin *et al.*, 1997a). Similarly, four cis elements within the E promoter, namely the vascular bundle expression (VBE) element (nt −169 to −100), the activator element (AE) (nt −70 to
−35), a TATA box (−35 to +1) and the downstream promoter sequence (DPS), which includes dps-a (nt +1 to +35), dps-b (nt +20 to +55) and dps1 (nt +50 to +90), were subsequently defined (He et al., 2000, 2001, 2002). The GATA motif, Box II and Box I are coincident with the VBE, AE and DPS elements, respectively. Box II is a unique cis element located immediately 5′ of the TATA box; sequences similar to those in Box II have been identified among other vascular tissue-specific promoters that are expressed in plants (Yin et al., 1997a).

The genes that encode rice basic leucine zipper (bZIP) transcription factors RF2a and RF2b are expressed predominantly in vascular tissues, the tissues in which RTBV replication occurs and where the RTBV promoter is expressed (Bhattacharyya-Pakrasi et al., 1993; Cruz & Koganezawa, 1991; Dai et al., 2004; Saito et al., 1986; Yin & Beachy, 1995). RF2a and RF2b interact with Box II (Dai et al., 2004; Yin et al., 1997b) and activate transcription from the E promoter (Dai et al., 2003, 2004; Petruccelli et al., 2001; Yin et al., 1997b). In transgenic plants that overexpress RF2a or RF2b constitutively, the expression pattern of the RTBV promoter was altered from vascular tissue-specific to constitutive (Dai et al., 2004; Petruccelli et al., 2001). Down-regulating the expression level of RF2a and RF2b in transgenic rice plants by expressing antisense genes of RF2a and RF2b causes phenotypes that in part resemble RTD symptoms (Dai et al., 2004; Yin et al., 1997b). These data suggest that RF2a and RF2b regulate expression of the RTBV promoter during infection. The purpose of the present study was to characterize further the role of Box II and its interaction with cognate rice transcription factors such as RF2a and RF2b in regulating the expression level of RF2a and RF2b in transgenic rice plants by expressing antisense genes of RF2a and RF2b constitutively, the expression pattern of the RTBV promoter was altered from vascular tissue-specific to constitutive (Dai et al., 2004; Petruccelli et al., 2001). Down-regulating the expression level of RF2a and RF2b in transgenic rice plants by expressing antisense genes of RF2a and RF2b causes phenotypes that in part resemble RTD symptoms (Dai et al., 2004; Yin et al., 1997b). These data suggest that RF2a and RF2b regulate expression of the RTBV promoter during infection. The purpose of the present study was to characterize further the role of Box II and its interaction with cognate rice transcription factors such as RF2a and RF2b in regulating expression of the RTBV promoter and to gain a better understanding of replication of RTBV.

In this report, data from transient tobacco BY-2 protoplast assays and transgenic Arabidopsis plants indicated that Box II, or the Box II portion of AE, is essential for the activity of the RTBV promoter. Furthermore, we demonstrated that chimeric promoters comprising Box II fused to minimal promoters derived from Cauliflower mosaic virus (CaMV) 35S promoter could be activated by RF2a and RF2b. We also showed that chimeric promoters could be activated by induction of RF2a using the edscyne receptor-based, chemical-inducible gene-expression system. The work published here and our earlier findings show that Box II and its interactions with cognate rice transcription factors such as RF2a and RF2b are essential for expression of the RTBV promoter and potentially for virus replication.

**METHODS**

**Plasmid construction**

**Plasmids for tobacco BY-2 protoplast transfection.** The promoters used in this study included the E promoter (nt −164 to +45) and deletions of the promoter that were generated via PCR amplification from the chimeric gene pE::GUS (Yin & Beachy, 1995). For mutagenesis, we used the β-glucuronidase (GUS) 3′ primer with complementary sequence located in the uid A (GUS gene)-encoding sequence and one of three 5′ primers (RTBV-100 5′, RTBV-68 5′ or RTBV-32 5′) with a HindIII site and sequences up to designated positions at −100, −68 and −32 of the E fragment (see primer list below) (Yin & Beachy, 1995; Yin et al., 1997a). Promoters with mutations in the Box II cis element, namely Box II1 and Box II2 (described previously by Yin et al., 1997a; see Fig. 1 for sequence information), or deletion of Box II were produced by fusion PCR with pE::GUS as template. PCR products that

![Fig. 1. Box II is essential for the activity of the E promoter in BY-2 protoplasts. (a) Diagram of 5′ deletions of the E promoter, point mutations of Box II and Box II deletions in the E promoter region. Each promoter fragment was ligated to the uidA reporter gene and the nopaline synthase (Nos) 3′ terminator sequence. Asterisks indicate the positions of point mutations. (b) Sequence information for Box II and the Box II1 (Box1) and Box II2 (Box2) mutants and their relative positions in the E promoter (Yin & Beachy, 1995). (c) Relative GUS activity of gene constructs following transfection of BY-2 protoplasts. GUS activity from each construct was compared with that of p−32:GUS, which was assigned a value of 1. Each column represents the mean ± SD of three independent experiments, with three repeats per experiment. All results were normalized against the internal-control plasmid p35S::GFP.](image-url)
produced the deletion or other mutations of the E promoter were inserted into pE:GUS via HindIII/NcoI restriction sites to replace the E promoter, resulting in p−100:GUS, p−68:GUS, p−32:GUS, pE(Bllm1):GUS, pE(Bllm2):GUS and pE(ABII):GUS, respectively.

To construct plasmids with the uid A-coding sequence driven by CaMV 35S minimal promoters, PCR products were amplified from p35S:GUS (Petruccelli et al., 2001) by using specific primer sets, namely GUS 3′ primer (Yin & Beachy, 1995) and primers CaMV-35S 5′ and CaMV-48 5′, which contained a HindIII site and truncated mutants of the CaMV 35S promoter to −35 or −48, respectively. PCR products were inserted into pE:GUS (Yin & Beachy, 1995) to replace the E promoter via HindIII/NcoI restriction sites and resulted in pSm−38:GUS and pSm−48:GUS. Similarly, to construct plasmids with a GUS gene driven by chimeric promoters that contained Box II or AE sequences and the 5′ region of the E promoter, resulting in pSm−2−38:GUS, pSm−2−48:GUS and AE3m−38:GUS, pAE3m−38:GUS and pAESm−38:GUS, pAESm−48:GUS and pAESm−38:GUS, respectively.


To construct plasmids with a gene encoding RF2a that can be induced by methoxyfenozide, the coding sequence of RF2a was released from p35S:RF2a (Petruccelli et al., 2001) by using EcoRI (blunt)/BamHI (blunt). The resulting fragment was inserted into pG35Sm:Luc (Padidam et al., 2003) to replace the luciferase gene. This gene is controlled by a chimeric promoter comprising five repeats of the Gal4 DNA-binding site and a 35S minimal promoter through ligation at the NcoI (blunt)/XhoI (blunt) site; this resulted in plasmid pG35Sm:RF2a. 5G35Sm:RF2a was released from pG35Sm:RF2a by using SalI/BamHI and cloned into pSPL301 (Invitrogen) through the same set of restriction enzymes to yield intermediate vector pSPL-5G35Sm:RF2a. DNA fragments encoding E:GUS, Sm−38:GUS, BIISm−38:GUS and AESm−32:GUS were released from pE:GUS, pSm−38:GUS, pBIISm−38:GUS and pAESm−32:GUS, respectively, through BamHI and HindIII (blunt) restriction sites. These DNA fragments were inserted into the intermediate vector pSPL-5G35Sm:RF2a through BamHI and Ndel (blunt) and resulted in plasmids pSPL-E:GUS/5G35Sm:RF2a, pSPL-Sm−38:GUS/5G35Sm:RF2a, pSPL-BIISm−38:GUS/5G35Sm:RF2a and pSPL-AESm−32:GUS/5G35Sm:RF2a, respectively.

The pCambia1300-based binary plasmid pS-5Grbm: E/U6/GUS: VGE, which carries the 5Grbm: E5 gene and the chimeric receptor VGE (V, VP16; G, Gal4 DNA-binding site; E, ligand-binding domain of the edcsyne receptor) driven by the promoter of Cassava vein mosaic virus (CaMV) (Padidam et al., 2003) was used to construct the plasmids for inducible expression of RF2a and the GUS reporter gene. DNA fragments encoding E:GUS/5G35Sm:RF2a, Sm−38:GUS/5G35Sm:RF2a, BIISm−38:GUS/5G35Sm:RF2a and AESm−32:GUS/5G35Sm:RF2a were released from pSPL-E:GUS/5G35Sm:RF2a, pSPL-Sm−38:GUS/5G35Sm:RF2a, pSPL-BIISm−38:GUS/5G35Sm:RF2a and pSPL-AESm−32:GUS/5G35Sm:RF2a by digestion of the plasmid DNAs with HindIII (blunt). These DNA fragments were inserted into pC-5Grbm:E5/CaMV:VGE through the SalI (blunt) site to replace 5Grbm:E5 and yielded plasmids pC-E:GUS/2aVGE, pC-Sm−38:GUS/2aVGE, pC-BIISm−38:GUS/2aVGE and pC-AESm−38:GUS/2aVGE (see Fig. 4a).

Primers. The primers used for the above experiments were: GUS 3′, GATTTCACGGGGTGGGGATTCTA; RTBV−100 5′, ATCAAGCTT-GATCTAATCTTACGAT; RTBV−68 5′, ATCAAGCTTAAAGGACGGACAGGAT; CaMV−35 5′, TGATCACAAGCTTTCTCTCATATAGGAAGT; CaMV−48 5′, TGATCACAAGCTTTGGCAAGAAGTCGCTCTCTCTC; CaMV−58 5′, TGATCACAAGCTTTGGCAAGAAGTCGCTCTCTC; CaMV−68 5′, TGATCACAAGCTTTGGCAAGAAGTCGCTCTCTC; CaMV−78 5′, CAAGGCTGTAAGTGGTATAAGGACGGACAGGAT.

Transfection of tobacco BY-2 protoplasts. Protoplast transfection was conducted as previously described (Dai et al., 2003). Approximately 106 protoplasts were transfected by electroporation with 20 μg effector DNA, 15 μg herring-sperm DNA, 2.5 μg reporter gene DNA and 15 μg pCat-GFP DNA (Dai et al., 2003). In samples with reporter gene alone, the total amount of DNA was adjusted by adding 20 μg herring-sperm carrier DNA.

Arabidopsis transformation. pCE:GUS, pC−100:GUS, pC−68:GUS, pC−32:GUS, pCE(Bllm1):GUS, pCE(Bllm2):GUS, pC-Sm−38:GUS/2aVGE, pC-BIISm−38:GUS/2aVGE and pC-AESm−38:GUS/2aVGE were introduced into Agrobacterium tumefaciens GV3101 and used to transform Arabidopsis thaliana Col-0 via an Agrobacterium-mediated flower-dip method (Clough & Bent, 1998). T1 generation transgenic plants were selected from seeds collected from the primary transformants on MS medium (Murashige & Skoog, 1962) plus 50 mg hygromycin B 1 L−1 and 100 mg carbenicillin 1 L−1. Arabidopsis plants were maintained in a growth room at 22 °C with a 16 h light/8 h dark cycle.

Induction of Arabidopsis gene expression. Transgenic plants harbouring pC-Sm−38:GUS/2aVGE, pC-BIISm−38:GUS/2aVGE and pC-AESm−38:GUS/2aVGE were selected and transplanted into soil. Intrepid 2F (Dow AgroSciences) was diluted to a working concentration of 62.5 μg methoxyfenozide and applied through a single soil drench to the plants 2 weeks after transplanting. Two samples were collected from each plant, one prior to the induction of the expression of RF2a and another at 1 week after the induction. Each treatment was repeated three times with nine plants pooled as one repeat.

Western blot analysis. Leaf samples from each transgenic Arabidopsis line, which carried either pE-C:GUS/2aVGE or pE-E:GUS, were collected, one prior to the application of methoxyfenozide and one week after the application of inducer. Forty micrograms of protein isolated from each leaf sample was resolved by SDS-PAGE (12 % gel). Immoblot analysis was carried out as described previously (Dai et al., 2003) using antibody against RF2a. The membrane was stained with Ponceau S (Sigma) prior to immunoblot analysis.

Quantitative analysis of GUS activity and green fluorescent protein (GFP). Protein samples from protoplasts and plants were prepared by using protein-extraction buffer (Jefferson et al., 1987) and quantified by using a DC Protein Assay kit (Bio-Rad). GUS activity was measured as described previously using 4-methylumbelliferyl-β-D-glucuronide as substrate (Jefferson et al., 1987). GFP was
measured with a Gemini fluorescence spectrophotometer (Molecular Devices). The excitation and emission wavelengths were set at 460 and 510 nm, respectively. Similar extracts from non-transgenic plants were used as the ‘blank’ in these assays.

RESULTS AND DISCUSSION

Box II DNA cis element is essential for RTBV promoter activity in BY-2 cells

Previous reports have indicated that the RTBV promoter is expressed predominantly in phloem tissues in transgenic tobacco, rice and Arabidopsis plants; promoter activity was also observed in epidermal cells and other cell types in transgenic rice when a large fragment of DNA sequence of the virus downstream of the transcription start site was included (Klöti et al., 1999). The E fragment of the RTBV promoter, comprising nt −164 to +45 relative to the start site of transcription, retains vascular tissue-specific expression similar to a DNA fragment that included sequences from −731 to +45 (Bhattacharyya-Pakrasi et al., 1993; Yin & Beachy, 1995; Yin et al., 1997a). The E promoter is also active in Orychophragmus violaceus (dicot) and tobacco BY-2 protoplasts (Chen et al., 1994; Dai et al., 2003, 2004). The activity of the E promoter in BY-2 protoplasts was approximately 40% of the activity of the enhanced CaMV 35S promoter, a strong constitutive promoter (data not shown). Transient assays were conducted in BY-2 protoplasts to determine the role of the Box II cis element in activity of the E promoter.

To establish the function of Box II in the E promoter, we truncated the promoter from the 5’ end to remove the GATA motif, the ASL box and Box II. These 5’-end deletion mutants were fused to the uidA coding sequence in constructs p−100:GUS, p−68:GUS and p−32:GUS (Fig. 1a). These constructs and the E:GUS reporter gene (Dai et al., 2004) were introduced into BY-2 protoplasts via electroporation in transient assays. The results shown in Fig. 1(c) indicated that the Box II sequence was essential for promoter activity in this assay. The promoter activity remained unchanged in p−68:GUS compared with the E:GUS construct, whereas promoter activity dropped to basal level when Box II was removed (p−32:GUS). This result is in agreement with results from rice protoplasts reported by He et al. (2000).

Mutations were made to either alter or delete the Box II element in the E promoter. Mutated promoters were ligated to the uidA coding sequence to create pE(BIIm1):GUS, pE(BIIm2):GUS and pE(ΔBII):GUS [see Fig. 1(b) for sequence information]. Plasmids were transfected into BY-2 cells by electroporation. Transient assays were conducted in BY-2 protoplasts to determine the role of the Box II cis element in activity of the E promoter. As the promoter is active in BY-2 cells, we concluded that endogenous transcription factor(s) in BY-2 cells interact with Box II, but not mutants of Box II, to maintain expression of the reporter gene (Fig. 1c). A transcription factor (repression of shoot growth or RSG) with high sequence identity to RF2a has been identified from BY-2 cells (Fukazawa et al., 2000).

It was noted that, although RF2a and RF2b showed higher binding affinities to Box II than to native Box II in in vitro assays (Dai et al., 2004; Yin et al., 1997b), the mutation did not enhance promoter activity in the protoplast assays. He et al. (2001) reported that the RTBV promoter is subject to the regulation of DNA methylation. A possible explanation of the results obtained in protoplast transient assays is that, by mutating the CCCC sequence in Box II to GCGC in Box IIIm1, we coincidently introduced methylation site(s) that may potentially be methylated in vivo. Another possible explanation is that efficient transcription relies on dynamic interactions between transcription factors to the promoter rather than tight binding.

Box II is essential for expression of the E promoter in transgenic Arabidopsis plants

The E:GUS gene is expressed in vascular tissues in transgenic Arabidopsis, rice and tobacco plants (Petruccelli et al., 2001; Yin & Beachy, 1995). Arabidopsis was chosen to characterize further the role of Box II in expression of the E promoter. Gene constructs that included deletions and mutations of the E promoter were introduced by Agrobacterium-mediated transformation as described in Methods. More than 30 independent transgenic lines were developed for most constructs and GUS activity was analysed in plants of the T1 generation of each line (Fig. 2). As shown in Fig. 2, the data from transgenic plants agreed, in general, with the data from the transient assays (Fig. 1) and showed that Box II is essential for expression of the E promoter in transgenic plants. Promoter activity dropped significantly, but was not abolished, when Box II was removed (compare pCE−68:GUS with pCE−32:GUS) or mutated [compare pCE:GUS with pCE(BIIm1):GUS and pCE(BIIm2):GUS].

RF2a and RF2b activate transcription from chimeric promoters that contain Box II

As described above, Box II is essential for the activity of the E promoter. Rice bZIP host factors RF2a and RF2b interact with the promoter through this cis element and activate transcription from the E promoter (Dai et al., 2004; Yin et al., 1997b). We wished to determine whether RF2a and RF2b could activate chimeric promoters that contain Box II. He et al. (2000) reported that the space between Box II and TATA is important; we made similar observations in other studies (Z. Zhang, S. Dai & R. N. Beachy, unpublished data). To maintain the same relative position and orientation between Box II and TATA in the chimeric promoter, Box II was fused to the 5’ end of the core promoter sequence from...
the CaMV 35S promoter at either nt −38 or nt −48, generating reporter constructs pBIISm−38 : GUS and pBIISm−48 : GUS (Fig. 3a). These reporter genes were co-transfected independently into BY-2 cells with pCs : RF2a or pCs : RF2b (Dai et al., 2004). As shown in Fig. 3(b), RF2a and RF2b activated transcription from both chimeric promoters, whereas RF2a had no effect on control plasmids that lacked Box II [pE(ABII) : GUS] and the minimal promoter of CaMV (pSm−48 : GUS) (Fig. 3c). Similarly, the vector-control plasmid (pCs) showed no effect on expression of the reporter genes (Fig. 3b). As in the case of a reporter gene driven by the E promoter (Dai et al., 2004), RF2b showed stronger activity than RF2a in activating transcription from the chimeric promoters.

To evaluate further the role of interactions between Box II and the transcription factors in regulating gene expression, additional studies were carried out in transgenic Arabidopsis plants. For these studies, genes were constructed in which expression of RF2a was under the control of an inducible promoter that contained five repeats of the Gal4 DNA-binding site and the 35S minimal promoter (5G35Sm). This promoter is silent unless the VGE receptor protein activates its expression (Fig. 4b). The VGE chimeric receptor was placed under the control of the CsVMV promoter; in the presence of methoxyfenozide, VGE binds the Gal4 DNA-binding site to induce expression of RF2a, which in turn binds to Box II and regulates expression of the target gene. Genes encoding RF2a and VGE were cloned into binary vectors together with the reporter gene BIISm−38 : GUS to yield pC-BIISm−38 : GUS/2aVGE (Fig. 4a). In this

![Activities of promoters with mutations or 5′ deletions in Box II of the E promoter in transgenic Arabidopsis plants.](http://vir.sgmjournals.org)

**Fig. 2.** Activity of promoters with mutations or 5′ deletions in Box II of the E promoter in transgenic Arabidopsis plants. The GUS activity (per unit soluble protein) from leaf samples of 3-week-old Arabidopsis plants from each independent transgenic line was presented as one point in each column. The total number of transgenic lines assayed for each construct (n) is shown. RU, Relative units.

![Regulation of RTBV promoter activity](http://vir.sgmjournals.org)

**Fig. 3.** Transcription factors RF2a and RF2b activate transcription from Box II containing chimeric promoters with the Box II cis element in BY-2 protoplasts. (a) Diagram of reporter- and effector-gene constructs. Chimeric promoters with Box II fused to the minimal promoter of CaMV 35S promoter sequences are referred to as BIISm−38 and BIISm−48. BoxII, Box II of the E promoter of RTBV; Sm−38, −38 to +8 of CaMV 35S promoter; Sm−48, −48 to +8 of CaMV 35S promoter; CsVMV, CaMV promoter; Nos, nopaline synthase (Nos) 3′ terminator sequence. pE(ABII) : GUS is a deletion mutant of the E promoter in which the Box II cis element was removed. (b) Relative GUS activity of reporter genes in BY-2 protoplasts co-transfected with RF2a or RF2b. The data presented were compared with the pBIISm−38 : GUS gene co-transfected with the pCs plasmid (assigned a value of 1). Each bar represents the mean±SD of three independent co-transfection experiments with three repeats of each experiment. All results were normalized against the internal control p35S : GFP prior to further analysis. (c) RF2a has no effect on reporter genes without the Box II cis element and the transcription factors in regulating gene expression, additional studies were carried out in transgenic Arabidopsis plants. For these studies, genes were constructed in which expression of RF2a was under the control of an inducible promoter that contained five repeats of the Gal4 DNA-binding site and the 35S minimal promoter (5G35Sm). This promoter is silent unless the VGE receptor protein activates its expression (Fig. 4b). The VGE chimeric receptor was placed under the control of the CsVMV promoter; in the presence of methoxyfenozide, VGE binds the Gal4 DNA-binding site to induce expression of RF2a, which in turn binds to Box II and regulates expression of the target gene. Genes encoding RF2a and VGE were cloned into binary vectors together with the reporter gene BIISm−38 : GUS to yield pC-BIISm−38 : GUS/2aVGE (Fig. 4a). In this
More than 40 independent transgenic Arabidopsis were constructed with Box II. The reporter gene AESm was also taken into consideration when chimeric promoters TATA. The relative orientation between Box II and TATA promoter of CaMV to nt of the TATA box as reported by He potential effects that might be caused by the relative position were developed for each construct. Expression of RF2a and negative-control constructs included pC-E : GUS, AESm (2000), was fused to the CaMV 35S minimal promoter (Fig. 4a). Positive- and negative-control constructs included pC-E : GUS, pC-E : GUS/2aVGE and pC-Sm – 38 : GUS/2aVGE (Fig. 4a). More than 40 independent transgenic Arabidopsis lines were developed for each construct. Expression of RF2a following induction was monitored by Western blot analysis. As an example, induction of the expression of RF2a in transgenic lines with pC-E : GUS/2aVGE is shown in Fig. 4(b).

Results of studies of representative lines of each construct are shown in Fig. 4(c). As shown in the left panel, induction of RF2a enhanced expression of the E : GUS gene as anticipated based on results obtained when RF2a was expressed constitutively in transgenic tobacco plants (Petruccelli et al., 2001). GUS activity in plants that harboured E : GUS only were not affected by the application of methoxyfenozide. As shown in the right panel of Fig. 4(c), induction of RF2a activated expression from BIISm – 38 and AESm – 32 chimeric promoters (pC-BIISm – 38 : GUS/2aVGE and pC-AESm – 32 : GUS/2aVGE), whilst induction of RF2a did not affect the CaMV 35S minimal promoter without Box II (pC-Sm – 38 : GUS/2aVGE). Note that absolute GUS activity in transgenic plants in which the E : GUS gene was activated by RF2a was much higher than in transgenic plants in which the E : GUS gene was activated by RF2a (the expression of E : GUS was approximately 12-fold higher than that in uninduced transgenic plants with BIISm – 38 : GUS/2aVGE (right panel).
Conclusions

Understanding transcriptional regulation of the RTBV promoter is important for studies of RTBV replication and RTD. Data from transient assays and stable transgenic plants have shown that the activity and tissue specificity of the RTBV promoter require Box II, a cis element proximal to the 5’ end of the TATA box (Petruccelli et al., 2001; Yin & Beachy, 1995). Multiple nuclear protein-binding complexes are formed on the RTBV promoter, including complexes formed on the ASL sequence (nt −98 to −79) and on Box II (nt −53 to −39), both of which are within the E promoter (Yin & Beachy, 1995; Yin et al., 1997a). He et al. (2000) identified a single AE (nt −70 to −40) within the region nt −100 to −32 of the E promoter. Rice transcription factors RF2a and RF2b interacted with the promoter via Box II and functioned as strong activators to stimulate the expression of the E promoter in cell types in which the promoter is not normally expressed.

It is important to clarify the role of AE and Box II in contributing to the activity of the RTBV promoter. The results presented in this paper indicate that Box II, which is included in AE, plays an essential role in the E promoter and is a prerequisite for correct functioning of the promoter (Figs 1 and 2). The reported differences in the activity of Box II and AE may be a result of using different nuclear proteins and DNA complexes as the starting material for footprint analyses (He et al., 2000; Yin & Beachy, 1995; Yin et al., 1997a). Studies of the P97 promoter of human papillomavirus type 16 identified several functional cis elements that were adjacent to or overlapped the YY1-binding site by different groups (Dong & Pfister, 1999; O’Connor et al., 1996). We suggest the possibility that AE is a combination of two or more elements, including Box II. Our studies confirm that Box II and its interaction with rice transcription factors such as RF2a and RF2b are essential for promoter activity. Furthermore, we suggest that this interaction is essential for expression of the RTBV promoter during virus replication and may contribute to the severity of RTD.

ACKNOWLEDGEMENTS

We thank Drs Y. Yin and S. Petruccelli for their input in the project. We thank Dr M. Padidam (RheoGene Company) for providing the inducible gene-expression system for this study. We thank Dr Y. Liu for critical reading of the manuscript. This research is supported by DOE grant DE-FG02-99ER20355 to R. N. B. and a grant from the Rohm & Haas Company.

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