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Upstream and downstream sequence elements determine the specificity of the rice tungro bacilliform virus promoter and influence RNA production after transcription initiation

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Abstract

The contribution of sequences upstream and downstream of the transcription start site to the strength and specificity of the promoter of rice tungro bacilliform virus was analysed in transgenic rice plants. The promoter is strongly stimulated by downstream sequences which include an intron and is active in all vascular and epidermal cells. Expression in the vascular tissue requires a promoter element located between −100 and −164 to which protein(s) from rice nuclear extracts bind. Elimination of this region leads to specificity for the epidermis. Due to the presence of a polyadenylation signal in the intron, short-stop RNA is produced from the promoter in addition to full-length primary transcript and its spliced derivatives. The ratio between short-stop RNA and full-length or spliced RNA is determined by upstream promoter sequences, suggesting the assembly of RNA polymerase complexes with different processivity on this promoter.

Introduction

In the analyses of plant promoters, most emphasis has been on sequences upstream of the transcription start site (Guilfoyle, 1997). However, in recent years, important regulatory sequences downstream of the transcription start site have been detected in an increasing number of plant promoters both in translated and untranslated regions (Bovy *et al.*, 1995; Caspar and Quail, 1993; Curie *et al.*, 1993; Curie and McCormick, 1997; De Almeida *et al.*, 1989; Dean *et al.*, 1989; Douglas *et al.*, 1991; Fu *et al.*, 1995; Hohn *et al.*, 1996; Kyozuka *et al.*, 1994; Larkin *et al.*, 1993; Lois *et al.*, 1989; Pierce *et al.*, 1987; Sieburth and Meyerowitz, 1997; Wong *et al.*, 1992). Particularly in monocots, enhancement of gene expression by downstream sequences is often linked to the presence of an intron (Callis *et al.*, 1987; Donath *et al.*, 1995; Fütterer *et al.*; 1994; Li *et al.*, 1995; Luehrsen and Walbot, 1991; Maas *et al.*, 1991; Mascarenhas *et al.*, 1990; McElroy *et al.*, 1990; Oard *et al.*, 1989; Rathus *et al.*, 1993; Sinibaldi and Mettler, 1992; Tanaka *et al.*, 1990; Vasil *et al.*, 1989; reviewed in Simpson and Filipowicz, 1996). In dicots, enhancement by introns is less pronounced (Genschick *et al.*, 1994; Norris *et al.*, 1993; Tanaka *et al.*, 1990), but a recent study with transgenic *Arabidopsis* plants revealed that expression was stimulated by introns of the PAT1 gene (Rose and Last, 1997). It appears that the splicing process itself often contributes to the enhancement. However, since not all intron-promoter combinations function in the same way and since in some cases position effects are observed (Callis *et al.*, 1987; Snowden *et al.*, 1996; Vasil *et al.*, 1989), it is likely that other

features of these sequences are also important (e.g. intron-based enhancers; Gidekel *et al.*, 1996). For the maize *shrunken 1* gene, contribution of the first exon to enhanced expression has been proven in a transient expression system (Clancy *et al.*, 1994; Maas *et al.*, 1991), but again the underlying mechanism is unclear. For the cauliflower mosaic virus (CaMV) 35S promoter it has been reported that expression can be enhanced by including CaMV sequences downstream of the transcription start site; however, the mechanism of this enhancement has not been studied further (Hohn *et al.*, 1996; Pierce *et al.*, 1987).

For many applications in monocot transformation, downstream exons and introns are incorporated into gene expression cassettes to achieve high expression levels. The CaMV 35S promoter is strongly enhanced by insertion of monocot introns in some plant species. The promoters of the rice *actin1* gene and the maize *ubiquitin1* gene are the two other most widely used constitutive promoters for monocot transformation (Christensen *et al.*, 1992; McElroy *et al.*, 1990). In these promoter cassettes, the first exon and intron are routinely present and without these sequences, the activity of the promoters is negligible.

Rice tungro bacilliform virus (RTBV) belongs to the badnaviruses which are one group of the plant pararetroviruses; RTBV is thus related to the caulimovirus CaMV (Hull, 1996). As part of our study of the molecular biology of RTBV we became interested in the promoter that controls the synthesis of the genome-length, pregenomic RNA from the circular DNA template. In addition to the 35S promoter of CaMV, the corresponding promoters of figwort mosaic virus (Sanger *et al.*, 1990), commelina yellow mottle virus (CoYMV; Medberry and Olszewski, 1993), cassava vein mosaic virus (Verdaguer *et al.*, 1996) and sugarcane bacilliform virus (Tzafrir *et al.*, 1998) have been analysed and have always turned out to be strong in those tissues where they were active. The RTBV promoter has been studied in transformed rice plants and in transfected protoplasts. The transcription initiation site has been mapped to genome positions 7404 and 7405 (Bao and Hill, 1993; Yin and Beachy, 1995). In transgenic rice plants, phloem-specific activity was found first for a DNA fragment spanning positions 6671 to 7390 of the RTBV genome (Bhattacharyya-Pakrasi *et al.*, 1993) (numbering according to Qu *et al.*, 1991) and later for fragments covering positions 6671 to 7449 (Matsumura and Tabayashi, 1995; Yin and Beachy, 1995). In protoplasts, the activity of the RTBV promoter depended not only on upstream elements but also on stimulatory elements in the transcribed region (up to position 7500) (Chen *et al.*, 1994, 1996), which were absent from the constructs tested so far in transgenic plants (Yin and Beachy, 1995). The first 250 nucleotides downstream of the transcription start site contain not only a DNA-dependent, position-independent enhancer element, but also a position-dependent element (Chen *et al.*, 1996). In addition, a splice donor site to generate an mRNA for the open reading frame (ORF) IV (Fütterer *et al.*, 1994), the transcription terminator for the generation of the terminally redundant RTBV pregenomic RNA (Rothnie, 1996), and elements involved in translation of the complex RTBV mRNA (Chen *et al.*, 1994; Fütterer *et al.*, 1996) are present within this region.

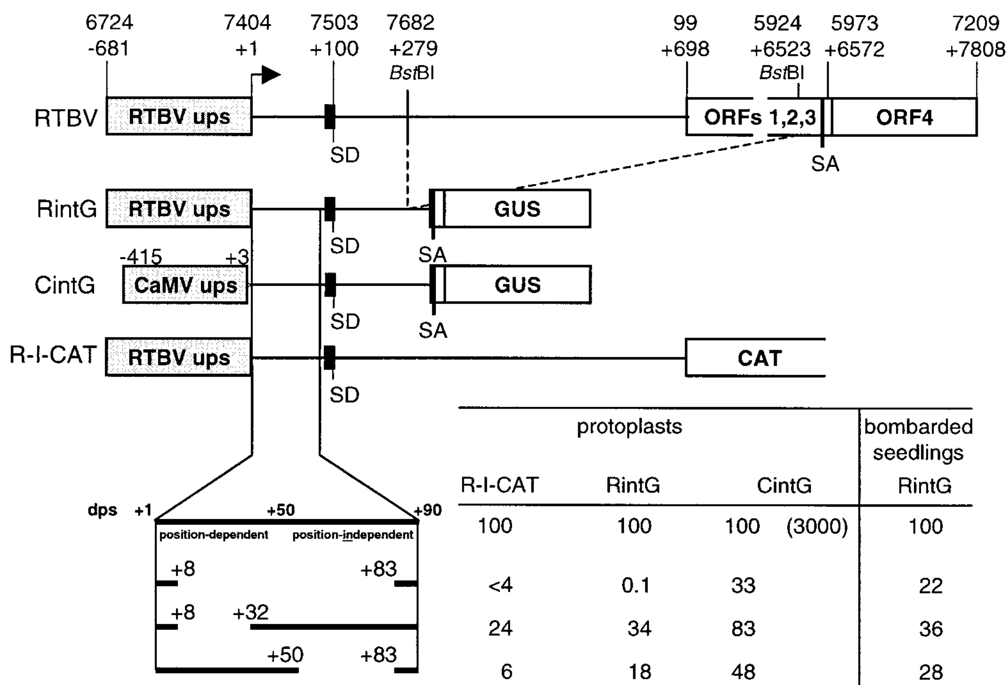
In this report, we evaluate whether downstream promoter sequences (dps) contribute to RTBV promoter activity or influence the tissue specificity *in planta*. Reporter gene constructs which express β-glucuronidase (GUS) under the control of RTBV promoter fragments containing different deletions in the regions upstream and downstream of the transcription start site were analysed. The expression characteristic of the RTBV promoter was compared to the CaMV 35S promoter for which various levels of strength and specificity in rice have been previously reported (Battraw and Hall, 1993; Turner *et al.*, 1996).

Our results show that the RTBV promoter that includes downstream sequences is active in a wider range of rice cell types than one where these sequences are lacking, and is comparable in strength to the CaMV 35S promoter, which is also enhanced by the RTBV downstream sequences. The analysis of RNAs produced under the control of the viral sequences revealed a complex picture, indicating that processes after transcription initiation are affected by deletions upstream and downstream of the transcription start site. The data also suggest that RNA polymerase II complexes with different activities may exist in plant cells.

Materials and methods

Construction of plasmids

Plasmid CintG (Figure 1) was constructed by exchange of the chloramphenicol acetyltransferase (CAT) ORF from plasmid pC4CΔBB7253 (Fütterer *et al.*, 1994) with a GUS ORF (as *Hin*dIII-*Pst*I fragment). Insertion of a CaMV 35S promoter-*aph4*-CaMV 35S terminator cassette as a blunted *Kpn*I



protoplasts				bombarded seedlings
R-I-CAT	RintG	CintG		RintG
100	100	100	(3000)	100
<4	0.1	33		22
24	34	83		36
6	18	48		28

Figure 1. The structure of the RTBV transcription unit is indicated schematically. The locations of features important for the work described in this manuscript are indicated with their position in the genome (following the numbering of Hay *et al.* 1991) and relative to the transcription start site at position 7404 (+1). A line represents the leader sequence of the pregenomic RNA and a small black box the short ORF A. The other short ORFs in the leader are not shown. The splice donor (SD) and splice acceptor (SA) and the *Bst*BI sites used to create the shortened version of the intron are indicated. RTBV ups: upstream promoter sequences of RTBV. In the lower part, the constructs with a CAT ORF fused to the first ORF downstream of the leader sequence (R-I-CAT) and with a GUS ORF fused to RTBV ORF IV downstream of a truncated version of the intron (RintG) are shown schematically and different deletion variants of these constructs with their respective expression levels are listed. Features of the downstream promoter sequences (dps) as characterized by Chen *et al.* (1996) are shown. The leader variants were also tested under the control of CaMV 35S upstream promoter sequences (CintG; the sequence coordinates relative to the CaMV transcription start site are given); the value in brackets in the CintG column represents the expression level relative to RintG, the other expression values are presented in relation to the value for the undeleted dps, which was set to 100. The data represent the average of 4 to 10 independent transfection experiments. Variation between different experiments was ± 20% of the indicated value.

fragment from plasmid pSBH1 (Wünn *et al.*, 1996) into the *Nae*I site of pCintG created pHCintG. The resulting plasmid contains the gene for *aph4* and for GUS in the same orientation (see Figure 2). The junction between the *Hin*dIII site and the GUS ORF is AAGCTTctaCCATGG (*Hin*dIII and *Nco*I restriction sites in capital letters, GUS start codon underlined) creating an optimized translation context for the GUS start codon. The GUS ORF together with parts of the intron was transferred from pHCintG to RTBV promoter constructs containing various deletions in the regions upstream and downstream of the transcription start site (Chen *et al.*, 1994, 1996) as a *Bst*BI (partial) to *Pst*I fragment inserted into the respective sites of the RTBV promoter constructs. The hygromycin resistance gene was inserted as a *Kpn*I fragment (as above) into the *Kpn*I site of the RTBV promoter-GUS plasmids downstream of the polyadenylation signal in the same transcription orientation as the GUS gene (Figure 2).

The plasmid for the antisense probe contains a *Mun*I-*Bam*HI fragment from HRintG inserted into the *Eco*RI and *Bam*HI site of pGem1 (Promega). The probe covers the first 140 nt of the GUS ORF, the intron and the first exon plus 220 nt of the promoter.

RTBV sequences are derived from the clone described by Hay *et al.* (1991).

Transient expression assays

Assays were performed with protoplasts derived from a rice Oc suspension culture which were transfected with circular plasmid DNA by PEG-mediated transformation as described previously (Fütterer *et al.*, 1994). GUS expression was normalized to a cotransfected CAT expression construct. For bombardment of

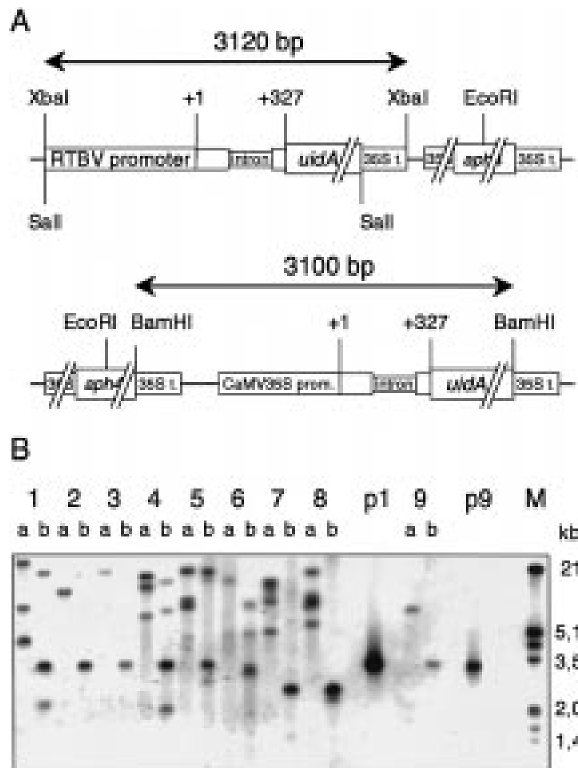


Figure 2. Southern analysis of transgenic rice plants. A. The structure of the transgenes is shown schematically (not to scale). The restriction sites used to either excise the promoter-GUS region (*Xba*I, *Sal*I or *Bam*HI, respectively) or to cut once in the unit (*Eco*RI) are indicated and the size of the expected fragment (for the full-length promoter) is given. 35S t.: CaMV polyadenylation signal. B. Southern analysis. The probe was specific for the GUS coding region. Lanes: a, digestion with the single cutting enzyme *Eco*RI; b, digestion to release a complete GUS gene (1–4, p1: *Xba*I; 5–8: *Sal*I, 9, p9: *Bam*HI). One exemplary transgenic line for each construct is shown: 1, HRintG-680, 2, HRintGΔ8-83; 3, HRintGΔ8-32; 4, HRintGΔ50-83; 5, HRintG-280; 6, HRintG-218; 7, HRintG-101; 8, HRintG-54; p1, plasmid HRintG-680; 9, HCintG; p9, plasmid HCintG; M, size marker.

seedlings, surface-sterilized seeds of rice (cv. Taipei 309) were germinated and grown for 4 days in the dark and bombarded with gold microparticles conjugated to the respective DNA with a Biolistic PDS-100 system (BioRad). GUS expressing cells were visualized after vacuum infiltration with GUS staining solution (0.1 M sodium phosphate buffer pH 7.0, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Biosynth, Switzerland), 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide) (Mendel *et al.*, 1989), incubation overnight at 37 °C, rinsing in sodium phosphate buffer, fixation in 5% formaldehyde, 5% acetic acid and 20% ethanol, and removal of chlorophyll by overnight incubation in 70% ethanol. Blue cells were counted under a light microscope.

Plant transformation

Transgenic plants were obtained by particle bombardment of immature rice embryos (cv. Taipei 309) as described by Burkhardt *et al.* (1997).

Southern analysis

DNA isolation and Southern analysis was performed as described (Burkhardt *et al.*, 1997). Genomic DNA was digested with *Eco*RI, which cuts in the *aph4* gene and only once in the transgene, or with *Sal*I, *Xba*I or *Bam*HI, which all release an (almost) complete copy of the GUS coding region fused to the respective promoter. Different enzymes had to be used for the different constructs since the restriction sites at the 5′ end of the promoters vary (Figure 2). DNA blots were probed with a digoxygenin-labelled 320 bp fragment derived from the GUS region (Burckhardt *et al.*, 1997).

Histological GUS assay

Explants were either stained whole or first sliced manually with a razor blade and then the slices were stained in filter-sterilized GUS staining solution (as above, but with 1 g/l X-Gluc and 1 g/l Triton X-100) at room temperature for 45 min to 16 h. The staining was stopped and chlorophyll was removed by incubating the slices for 30 min in 96% ethanol. Whole explants were photographed with a stereo-microscope, slices were inspected and photographed in 80% glycerol under an inverted light microscope.

Fluorometric GUS assay

Fluorometric GUS assays were performed as described by Jefferson (1987). Ca. 200 mg of callus material or different parts of rice seedlings of variable age were ground in 300 μl extraction buffer. For each extract, the respective parts of three plants were analysed. Extracts were shock-frozen in liquid nitrogen and stored at −80 °C until further processing. Protein concentration was determined by Bradford staining. GUS enzyme activity was determined as the production in nmol per minute per microgram of protein of 4-methylumbelliferone (MU) from 4-methylumbelliferyl-β-D-glucuronide.

RNA analysis

Total RNA was isolated from six-day old seedlings by the method of Chomczynski and Sacchi (1987) and 3 to 5 μg subjected to an RNase A/T1 mapping as described (Fütterer *et al.*, 1994). The antisense RNA was generated by *in vitro* transcription of the *Mun*I to *Bam*HI fragment described above by T7 RNA polymerase in the presence of [α-^{32}P]UTP (30 TBq/mmol). RNA quantification were performed in two or three independent analyses with one or two independent transgenic lines per construct (construct pHRintG-54 was analysed only once). The amount of the different RNA species was measured by determination of the radioactivity in the different bands, correction for the length and U content of the particular protected region, and normalization with respect to the first exon of the rice *actin1* mRNA.

Gel retardation assays

Protein extracts from suspension cultures or from rice seedlings were prepared as described (Chen *et al.*, 1996). Preparation of labelled probes and of competitor fragments, reaction with the protein extracts and analysis by gel electrophoresis were performed as described (Chen *et al.*, 1996). As probe, a synthetic double-stranded DNA oligonucleotide covering the RTBV genome from position 7250 to 7307 was used.

Results

RTBV promoter-GUS constructs and analysis of transient expression

To test the influence of upstream or downstream promoter sequences (ups and dps, respectively) on RTBV promoter activity in transgenic rice plants, we constructed a number of reporter genes containing different RTBV ups and dps regions, a shortened version of the RTBV intron, and a GUS ORF fused to RTBV ORF IV (RintG, Figure 1). These constructs were first tested in transfected rice protoplasts and compared to previously described constructs with a chloramphenicol acetyltransferase (CAT) ORF fused to RTBV ORF I (R-I-CAT, Figure 1; Chen *et al.*, 1994, 1996). For both types of constructs efficient expression was heavily dependent on downstream promoter sequences. Upstream sequences beyond −54 were not required (not shown) whereas a deletion between +8 and +83 drastically reduced expression. Partial deletions in the dps also reduced expression, although less dramatically (RintG and R-I-CAT, Figure 1).

Replacement of the RTBV ups by the CaMV 35S ups resulted in a 30-fold enhancement of expression. In this case, the influence of the RTBV dps was much less significant (CintG, Figure 1). Constructs containing the RTBV promoter with different deletions in the dps region were also bombarded into 4-day old rice seedlings and promoter activities were quantified as number of blue-staining cells. Similar results as in the protoplast assay were obtained except that the complete removal of the dps (deletion +8 to +83) resulted in a much less severe reduction of activity (Figure 1) compared with the results in protoplasts.

Generation of transgenic plants

The different RTBV promoter/GUS cassettes or equivalent constructs with the CaMV 35S ups were each combined in one plasmid with a CaMV 35S promoter-driven *aph4* gene; the latter gene confers hygromycin resistance (Waldron *et al.*, 1985). Transgenic rice plants (cv. Taipei 309) were obtained after gene transfer by particle bombardment to calli derived from the scutellum of immature embryos. In total, 233 transgenic plants were regenerated, 84 (36%) of which expressed GUS in the T_0 generation. Of these, 41 independent lines were identified by Southern analysis. Between one and six transgene copies had been integrated in the plant genomes, as estimated from Southern analysis (a representative blot is shown in Figure 2). GUS activity was analysed in the T_0 plants. As is usually the case in transformation experiments, GUS activities of independent transformants containing the same expression cassette sometimes varied widely. However, RNA analysis revealed that all plants with a comparatively low GUS activity produced additional, unexpected RNAs (not shown). Since such RNAs are probably indicative of either rearrangement of the transgene or post-transcriptional gene silencing, the corresponding plants were excluded from further analysis and will be described elsewhere. After elimination of these plants, GUS expression levels of a given construct were quite uniform between independent lines. For each construct two to four lines were chosen for a more detailed analysis in the T_1 generation.

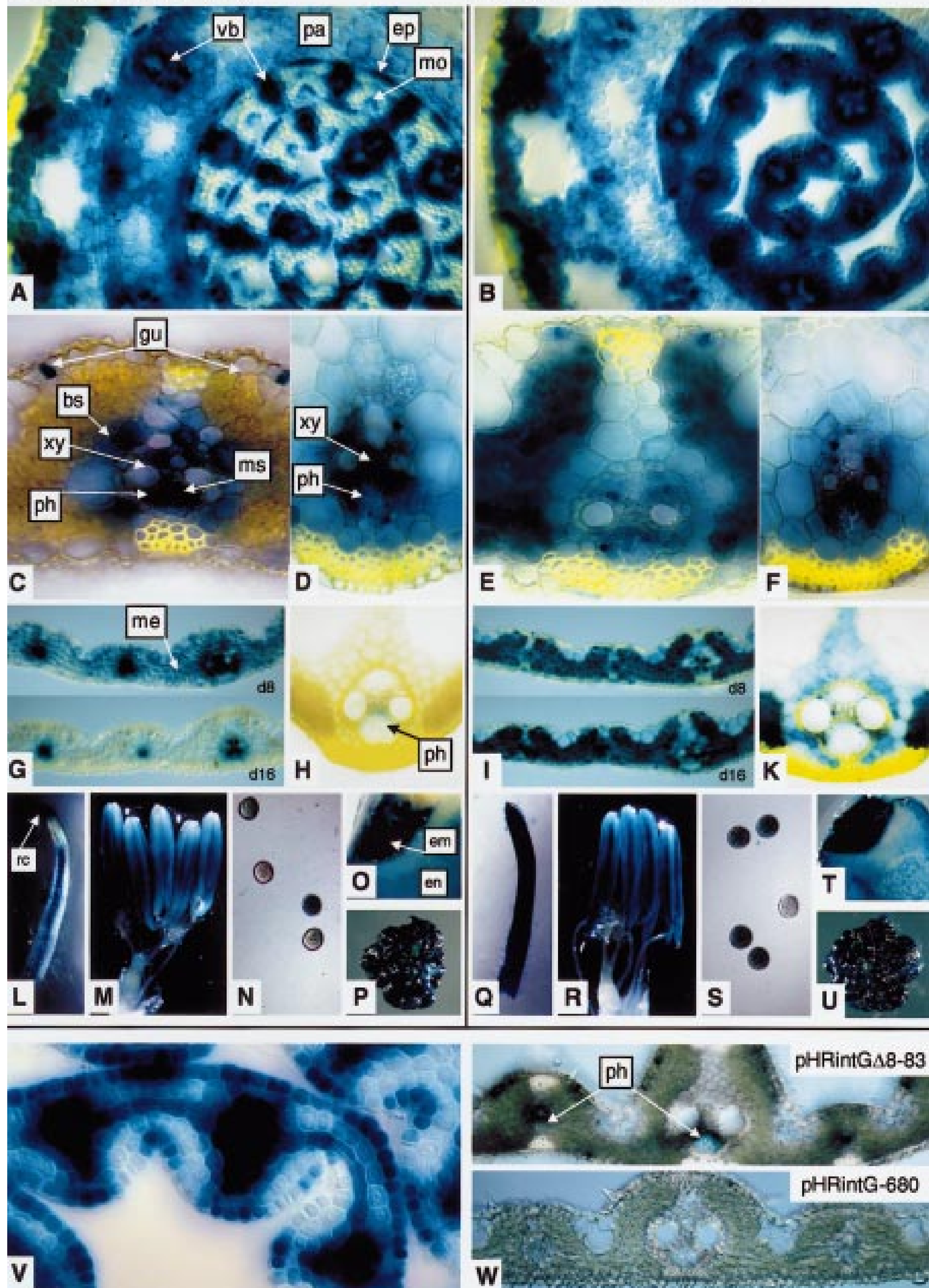

RTBV promoter + RTBV dps
CaMV 35S promoter + RTBV dps
vb
pa
ep
mo
A
B
gu
bs
xy
ms
ph
C
xy
ph
D
E
F
me
d8
d16
G
ph
H
d8
d16
I
K
rc
L
M
N
em
en
O
P
Q
R
S
T
U
V
ph
pHRintGΔ8-83
pHRintG-680
W

Figure 3. Histological GUS staining showing the activities of the RTBV (A, C, D, G, H, L–P, V, W) and the CaMV 35S (B, E, F, I, K, Q–U) promoter, combined with the RTBV downstream promoter sequences, in transgenic rice plants. A, B. Cross sections of 6-day old seedlings at the base; 160×. C, E. Cross sections of the blade of leaf 2 of 6-day old seedlings, showing large vascular bundles; 400×. D, F. Cross sections of the sheath of leaf 2 of 4-day old seedlings, showing large vascular bundles; 400×. G, I. Cross sections of the blade of leaf 3 of 8- or 16-day old seedlings; 160×. H, K. Cross sections of the midrib of a leaf blade of 60-day old plants, showing large vascular bundles; 400×. L, Q. Tips of primary roots of 4-day old seedlings; 160×. M, R. Anthers and pistils of flowering plants. N, S. Pollen of flowering plants. O, T. Cross sections of mature T2 seeds. P, U. Primarily transformed callus after 30 min in GUS staining solution. V. Cross section of 6-day old seedling at the base of a pHRintG-218 transformed plant; 400×. W. Cross sections of the flag leaf of flowering plants: top: pHRintG∆8-83; bottom: pHRintG-680. vb, vascular bundles; pa, sheath parenchym; ep, epidermis cells; mo, motor cells; gu, guard cells; pbs, parenchymal bundle sheath; mbs, mestome bundle sheath; xy, xylem cells; ph, phloem cells; me, mesophyll cells; rc, root cap; em, embryo; en, endosperm.

Tissue-specific activity of the full-length RTBV promoter in transgenic rice

So far, RTBV promoter analyses focused on upstream sequences and promoter activity has been found mainly in phloem tissue (Bhattacharyya-Pakrasi *et al.*, 1993; Yin and Beachy, 1995; Yin *et al.*, 1997b). In the presence of the dps, the full-length RTBV promoter shows strong activity in all cells of the vascular bundle, including xylem parenchyma, mestome and parenchymal bundle sheath cells, and in epidermal cells, including differentiated cells like guard cells and motor cells (Figure 3A, C, D, V). Activity is also clearly detectable in parenchyma cells of the leaf sheath (Figure 3A, D) and, albeit more weakly, in the mesophyll cells of the leaf blade (Figure 3G). Promoter activity is strongest in young tissues and drops gradually with the age of a leaf, persisting longest in the vascular bundles (Figure 3G, H). In the root, most GUS activity can be detected in the vascular bundle and in the root cap as judged from staining of whole roots from young seedlings (Figure 3L). In the spikelet, low GUS activity is detected in the anthers (Figure 3M) and in pollen (Figure 3N). In the seed, promoter activity is detectable in the endosperm and the embryo (Figure 3O). The promoter is also highly active in undifferentiated callus tissue (Figure 3P).

The activity of the RTBV ups was compared to that of the corresponding CaMV ups (Figure 3). The activity of the CaMV 35S promoter in rice plants has been described either as predominantly vascular (Terado and Shimamoto, 1990) or as more or less ubiquitous and constitutive (Battraw and Hall, 1990). In the presence of the RTBV dps, the CaMV promoter was active in almost all rice tissues and cell types. If rapidity and intensity of the histological GUS staining is taken as a measure of promoter activity, the CaMV 35S ups confers lower activity than the RTBV ups in the vascular bundles but higher in the mesophyll, resulting in similar overall GUS activity in extracts from transgenic plants (Table 1). In most cells, activities of RTBV and CaMV ups are similar but not identical (Figure 3D, F, M, R; N, S; O, T; P, U). The activity of the CaMV ups seems to be more stable in the course of plant development and can also be detected in most older leaves (Figure 3I, K), whereas the activity of the RTBV promoter is reduced to phloem cells or is no longer detectable (Figure 3G, H). In roots, the RTBV promoter is mainly active in the vascular tissue and the root cap (Figure 3L), whereas the CaMV 35S ups confers similar activity in all the tissues (Figure 3Q). The differential activities of the two promoters in the different tissues show that the specificity of GUS staining is not due to experimental artefacts like preferential uptake of staining solution or specific inhibition of GUS enzyme activity in certain cells. The staining of single cells also confirms that the staining patterns reflect expression patterns and are not influenced significantly by diffusion of enzymes or products under the staining conditions used (see e.g. Figure 3V).

Deletions in the RTBV promoter upstream of the transcription start site

Deletion of DNA sequences upstream of −218 did not result in major changes of apparent gene expression or specificity (Figure 4B). However, as shown below, GUS expression does not necessarily reflect the complete transcriptional activity of the different promoters.

Further deletion up to position −101 or −54 strongly reduced expression in the vascular bundle whereas expression in the epidermis remained unchanged (Figure 4C, D). GUS expression was found exclusively in epidermal cells in four out of eleven lines (36%) in constructs deleted to position −101 and in five out of eight lines (63%) in constructs deleted to position −54. The remainder of the lines showed some GUS activity in and around the vascular bundle or in the parenchymal cells of the leaf sheaths, but did not have the clearly defined expression pattern of the ‘full-length’ promoter (Figure 4E). It is likely that

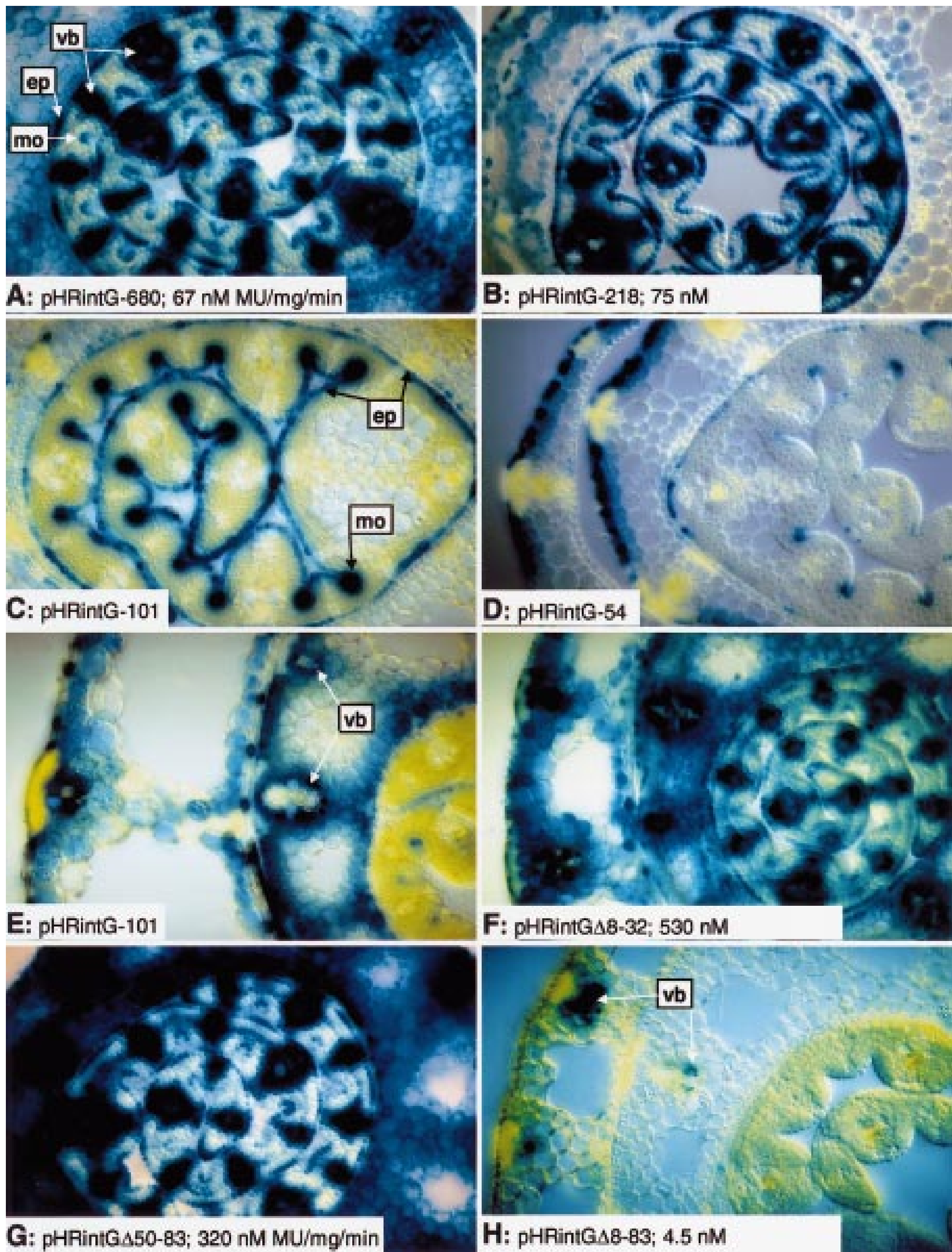

vb
ep
mo
A: pHRintG-680; 67 nM MU/mg/min
B: pHRintG-218; 75 nM
ep
mo
C: pHRintG-101
D: pHRintG-54
vb
E: pHRintG-101
F: pHRintGΔ8-32; 530 nM
G: pHRintGΔ50-83; 320 nM MU/mg/min
vb
H: pHRintGΔ8-83; 4.5 nM

Figure 4. Histological GUS staining showing the activity of the RTBV promoter, either 'full-length' (pHRintG-680) or with different deletions in up- or downstream sequences in transgenic rice plants. Fluorometrically determined GUS activities measured in parallel with the same tissue are given. A. RTBV 'full-length' promoter. B. RTBV promoter with deletion of sequences upstream of −218. C. RTBV promoter with deletion upstream of −101. D. RTBV promoter with deletion upstream of −54. E. RTBV promoter with deletion upstream of −101. Unusual expression type. F. RTBV promoter with deletion in dps (Δ8-32). G. RTBV promoter with deletion in dps (Δ50-83). H. RTBV promoter with deletion in dps (Δ8-83). vb, vascular bundles; ep, epidermis cells, mo, motor cells.

Table 1. Quantitative correlation between GUS expression and RNA products.

Construct	Promoter	GUS[a] (nM MU mg^{-1} min^{-1})	Total RNA[a,b]	Unspliced[c] (%)	Spliced[c] (%)	Short stop[c] (%)	Spliced RNA[d]	GUS per spliced RNA[c]	Number of assays × lines
pHCintG	CaMV	158	136	3	89	8	428	0.37	2×2
pHRintG-680	RTBV	260	**100**	3	28	69	**100**	2.6	3×2
pHRintG-280	RTBV	180	34	6	29	65	35	4.6	3×1
pHRintG-218	RTBV	155	60	1	60	39	129	1.20	2×2
pHRintG-101	RTBV	37	9	2	59	39	19	1.95	2×2
pHRintG-54	RTBV	10	15	2	88	10	46	0.21	1×1
pHRintGΔ50-83	RTBV	218	174	2	73	25	454	0.48	3×2
pHRintGΔ8-32	RTBV	275	72	3	87	10	224	1.2	3×2
pHRintGΔ8-83	RTBV	3	160	1	1	98	2	[e]	3×1

[a]Absolute values for GUS expression or RNA production for indendent lines deviated by about ± 30% from the indicated average.
[b]Total RNA is the sum of detected unspliced, spliced and short-stop RNA and is given in arbitrary units relative to that of the full-length RTBV promoter construct (pHRintG-680).
[c]The proportions of the different RNAs and the GUS/mRNA correlation factor varied by ±10% of the indicated average.
[d]Amount of spliced RNA relative to pHRintG-680.
[e]Not calculated because the measured values for spliced RNA and GUS are too low to be determined with accuracy.

gene expression in the vascular bundles in these lines is due to a weak regulator element that is present in the remaining promoter fragment.

A protein binding site between −101 and −164

The deletion analysis of upstream promoter elements suggests that a sequence element that confers efficient gene expression in vascular tissue is located between −218 and −101. In a previous analysis by Yin and Beachy (1995), such an element was postulated to reside between −164 and +45. Protein binding studies suggested that sequence elements from −53 to −39 and −3 to +8 are major determinants of tissue specificity and promoter strength (Yin and Beachy, 1995; Yin *et al.*, 1997a). Later a protein binding element important for vascular expression was also localized in the −164 to −101 region (Yin *et al.*, 1997b). We have independently tested protein extracts from the nuclei of rice seedlings or cell suspension cells for protein binding activity to the −163 to −100 region and also found strong band shifts with both extracts (Figure 5 and data not shown). The exact pattern varied between different extracts but protein binding always led to the appearance of two shifted major bands, indicative of binding of two different (forms of) proteins or of formation of a multiprotein complex. The binding was sequence-specific. Only a DNA fragment covering the promoter region from −1 to −100 competed slightly for both binding complexes whereas other oligonucleotides from outside of the binding region did not influence protein binding efficiency (Figure 5B). In the previous work (Yin *et al.*, 1997b) a GATA motif was suggested to be responsible for protein binding. Our competition experiments with smaller portions from the binding region showed also that oligonucleotides containing the GATA motif (−125 to −150) would compete in the assay, albeit more weakly than the longer −125 to −163 fragment, but an oligonucleotide covering −140 to −163, lacking the GATA sequence, competed as well (Figure 5). This suggests that the GATA sequence may not be involved directly in protein binding but rather the region between −140 and −150 or a motif present in both subfragments. Preliminary evidence indicates that an AGAAG motif, which occurs twice in this region and also twice further upstream in the promoter, is important for binding (not shown). Flanking sequences may also be required,

Figure 5. Gel shift assays with RTBV promoter fragment −101 to −163. A. Position of DNA fragments used for competition is shown schematically. The sequence of the binding region F1 is given with the coordinates (relative to the transcription start site) of subfragments used to determine the binding site. A GATA motif as defined in by Yin *et al.* (1997b) is underlined, two AGAAG motives are double-underlined. B. Protein from nuclei of rice seedlings was bound to a synthetic, labelled, double-stranded oligonucleotide (probe) representing the RTBV promoter region from −100 to −163. Specificity of binding was ascertained by competition with 50- to 1000-fold molar excess of other RTBV DNA fragments. Experiments in the two parts were performed with independently isolated protein extracts.

since a fragment from −137 to −153 did not compete for binding in these assays.

Deletions downstream of the transcription start site

The expression pattern of the RTBV promoter in transgenic rice plants described here differs from the previously reported one (Bhattacharyya-Pakrasi *et al.*, 1993; Yin and Beachy, 1995) which was obtained with constructs lacking at least large parts of the downstream promoter regions. To investigate whether and how the downstream region is responsible for the observed differences, we analysed three deletion mutants in transgenic rice plants.

In protoplasts, the dps had been dissected in two regions: one between +50 and +83 containing a position-independent enhancer element, and an element that is inactivated by a deletion between +8 and +32 containing a position-dependent element (Chen *et al.*, 1996). A complete deletion of the +8 to +83

Figure 6. Analysis of RNA from transgenic rice plants by RNase A/T_1 protection. A. The 'full-length' RTBV promoter (pHRintG-680) and constructs with different deletions in the dps are shown schematically. Position and extent of the anti-sense probe created by T7 RNA polymerase is indicated at the top. Expected RNAs with the sizes of the protected fragments are shown below each construct. B. Results of a RNase protection assay with RNAs from different transgenic plants transformed with the different deletion constructs. The positions of molecular weight markers are given with the fragment size in nucleotides. The relevant protected fragments are labelled at the left side: a, unspliced RNA; b, short-stop RNA; c, fragments derived from the 5′ end; d, spliced RNA, 3′ exon-derived.

Figure 7. Organization of elements influencing expression from the RTBV promoter. The locations of regions containing different elements with apparent activity in transcription control that can be deduced from the presented work are indicated below a schematic presentation of the transcription unit; stippled lines: supposed activities. Sequence elements defined by others (Yin and Beachy, 1995; Lin *et al.*, 1997a, 1997b) are indicated above. Positions are given relative to the transcription start site. GAGA-like elements are indicated by ∗; SD, splice donor; SA, splice acceptor; lppc+, regions stimulating assembly of low processivity polymerase complexes.

region led to a strong reduction of GUS activity in transgenic plants (pHRintGΔ8-83; Figure 4H); expression was very low in the blade of the youngest leaf. GUS activity was detected in the vascular tissue of the leaf sheath and the guard cells. Protein extracts from these plants contain only about 2 to 7% of the GUS activity of typical plants transformed with the full-length promoter construct (Table 1, Figure 4). However, in older tissue, such as the blade of the flag leaf at flowering stage, clear phloem-specific expression was detected with pHRintGΔ8-83, whereas with the 'full-length' promoter construct (pHRintG-680), no expression was detected (Figure 3W). The expression pattern obtained with the +8 to +83 promoter deletion construct resembles that described by Yin and Beachy (1995) for a promoter fragment ending at position +46. This suggests that the dps does, in fact, contribute to expression strength *in planta* and allows visualization of promoter activity in cells outside the phloem. However, in contrast to the effects described for protoplasts (Chen *et al.*, 1996), removal of either one of the two subregions of the dps did not result in a drastic decrease of GUS activity in transgenic plants. In some staining experiments, an increase of GUS activity without change of the cell specificity was observed (Δ8-32, Figure 4F; Δ50-83, Figure 4G). This is reflected in the faster staining in histochemical GUS assays and in the increase of GUS activity in protein extracts (compared to the full-length promoter; Figure 4). These latter findings indicate that the region downstream of the transcription start site may also contain elements that can negatively influence expression from the reporter constructs and that the effects of this region depend on the assay system.

Upstream and downstream sequences influence RNA types and levels in transgenic plants

Sequences downstream of the transcription start site can influence expression at many different levels, particularly in a complex situation like that of the RTBV pregenomic RNA, where signals for transcription initiation, transcription termination, splicing, translation and possibly other processes are located within this region (Rothnie *et al.*, 1994). Based on our earlier work in protoplasts (Fütterer *et al.*, 1994), we expected both spliced and unspliced RNA to be generated from the transformed expression constructs. While this work was in progress, it became clear that an RNA product resulting from transcription termination and/or polyadenylation at the promoter proximal polyadenylation signal in the intron might also be generated (Fütterer *et al.*, 1997). This was initially unexpected since for CaMV it had been shown that a polyadenylation signal in this proximity to the promoter is almost inactive (Sanfaçon and Hohn, 1990). The RNA generated by the use of the promoter-proximal polyadenylation signal has been termed short-stop RNA and has counterparts in some animal retroviruses such as human immunodeficiency virus type 1 (HIV-1) (Scott and Imperiale, 1997).

Spliced, unspliced, and short-stop RNA could be detected by RNase protection assays in the total RNA fraction from all GUS-expressing transgenic plant lines (Figure 6).

RNAs produced from the CaMV 35S promoter differ from those from the RTBV promoter by the addition of a few nucleotides at the very 5′ end. It is possible that this difference causes some alterations in RNA stability. Since deletions in the dps alter the respective transcripts and possibly the stability of these transcripts, expression levels for dps deletion constructs may be influenced by post-transcriptional processes. In contrast, RNAs produced from ups variants of the RTBV promoter are identical and their levels thus truly reflect the transcriptional activity.

Splicing in transgenic plants proved to be more efficient than expected from our previous work with transfected protoplasts (Fütterer *et al.*, 1994). In all transgenic plants only very small amounts of unspliced RNA was detected (Figure 6, Table 1). Spliced RNA appears as two protected fragments: one of 150 nucleotides in length, representing the second exon which is identical for all constructs, and one of 100 nucleotides or less, representing the first exon, which varies for the different dps deletion constructs (Figure 6). For the full-length RTBV or CaMV promoters, upstream deletion variants of the RTBV promoter, and for the downstream deletion variant Δ8-32, both exons were present in equimolar amounts. For the dps deletion Δ8-83, the first exon is too short to be detectable and for dps deletion Δ50-83, the protected fragment representing the RNA 5′ end can be derived from all the RTBV promoter-derived RNAs since its size is determined by the deletion endpoint and not by RNA processing.

For different constructs, the amount of spliced RNA represented a variable proportion of the total transgene-derived RNA. In the lines containing the full-length RTBV promoter or the −280 upstream deletion variant, 30% of the total RNA was spliced RNA and around 70% was short-stop RNA (Table 1).

When more of the upstream promoter sequence was deleted, the proportion of spliced RNA increased to 60% for the −218 and −101 and to 88% for the −54 deletion mutants. Similar high proportions of spliced RNA are produced from the CaMV 35S promoter and are observed for the two partial deletion variants of the dps. The Δ8-83 variant produced almost exclusively short-stop RNA (Table 1).

Since all the upstream deletion variants of the RTBV promoter produce identical transcripts and the CaMV 35S promoter at least a very similar one, these latter findings show that the generation of short-stop RNA is linked to transcriptional features and not only caused by sequences in the transcript acting in a post-transcriptional processing step. Sequence elements between −280 and −218 and between −101 and −54 influence the ratio between spliced and short-stop RNA; the CaMV 35S promoter seems to lack features that lead to the synthesis of short-stop RNA.

Based on total RNA levels, the CaMV 35S promoter is slightly stronger than the full-length RTBV promoter; similarly the Δ8-32 and Δ50-83 variants may be stronger than the full-length promoter. The upstream deletion to −280 reduces RNA levels about three-fold (Table 1), suggesting the presence of an enhancer element in this region.

A comparison of the levels of spliced RNA and GUS activity (determined in the same batch of rice seedlings) suggests that GUS expression does not necessarily reflect the transcriptional activity of a given promoter but that other, probably post-transcriptional, processes can influence the amount of gene products. In general, less GUS per RNA is produced in plants with relatively high mRNA levels, for example plants containing GUS transgenes with either the CaMV promoter or the RTBV promoter dps deletion variants Δ8-32 and Δ50-83 (Table 1). In order to correlate GUS activity and RNA levels, the data from plants transformed with the −54 and −101 deletion variants must be treated separately since GUS is expressed in these plants only in a subset of cells and thus total RNA levels translate into different RNA concentrations per cell. For these two deletion variants, RNA usage seems to be also significantly more efficient in lines with a lower RNA level.

Discussion

The dps expands the tissue specificity of the RTBV promoter

In previous studies, RTBV promoter activity was reported mainly in the phloem of transgenic plants (Bhattacharyya-Pakrasi *et al.*, 1993; Matsumara and Tabayashi, 1995; Yin and Beachy, 1995; Yin *et al.*, 1997a, 1997b). In these studies the RTBV promoter used contained at most 45 bp downstream of the transcription start site. In a rice protoplast system, RTBV promoter activity depended on at least two sequence elements located downstream of the transcription start site (Chen *et al.*, 1994, 1996). In the present analysis, 680 bp of upstream promoter sequences were supplemented with 278 bp of downstream promoter sequences (dps), containing these two elements and a shortened version of the RTBV intron. The RTBV promoter including the dps showed activity in a wider range of cells than previously observed in the absence of the dps. We found that expression was not restricted exclusively to phloem cells, but was also detected in other cells of the vascular bundles, in the epidermis, in the parenchyma cells of the leaf sheath and, albeit weakly, in leaf mesophyll cells. Promoter activity was detected mainly in young tissues and was lost gradually with increasing age. The overall effect of the dps may be a strong general enhancement of expression, which is reflected in higher GUS levels in plant extracts and a much more rapid staining of individual cells than obtained with the Δ8-83 dps variant. This is in line with our expectations from the analyses performed in transfected rice protoplasts (Chen *et al.*, 1994, 1996). The dps may also contain additional specificity elements for expression in the epidermis and the sheath parenchyma. The dps effects were apparent in our study with the Δ8-83 variant, which showed a very similar expression pattern (restricted to the vascular tissue) as a previously described construct including only the first 45 transcribed nucleotides (Yin and Beachy, 1995). Expression from the Δ8-83 construct, albeit low, persisted in plants much longer than that from the other constructs (Figure 3W) suggesting that also tissue- or development-specific, negative regulators may be involved in expression control or that the relatively high expression levels with the full-length promoters lead to consistent silencing of the transgenes later in development.

Partial deletions of the dps did not lead to a change in tissue specificity or expression strength, suggest-

ing that the elements involved are either redundant or present in the undeleted region between +32 and +50.

Recently a rice transcriptional activator interacting with RTBV promoter elements located between nucleotides −53 and −39 was reported (RF2a); antibodies raised against RF2a showed the presence of this activator protein in most cell types in the vascular bundles, in parenchyma and in epidermal cells (Yin *et al.* 1997a). This localization correlates exactly with the expression pattern of the RTBV promoter supplemented with the dps as described here.

Upstream specificity elements for expression in the vascular bundle

Promoter sequences upstream of −218 were dispensable for the reported activity. However, a deletion to −101 abolished expression in the vascular bundle in many transgenic lines, while strong expression in the epidermis remained. This effect was even stronger with a promoter deleted to −54. Combining these data with the promoter analysis by Yin and Beachy (1995) suggests that no essential promoter elements are located upstream of −164 and that an element very important for expression in the vascular tissue is located between −163 and −101. Previously, the phloem specificity element was tentatively localized to two regions closer to the transcription start site (BoxII from −32 to −66 and ASL Box from −66 to −103) because binding of nuclear proteins (including the bZIP protein RF2a) could be detected at these regions (Yin and Beachy, 1995; Yin *et al.*, 1997a). Only recently, a protein binding site in a GATA-like motif has been identified at a position around −140 (Yin *et al.*, 1997b). Individual mutations of these motifs led to strong reduction of protein binding and slight reduction of phloem-specific expression. A combination of any two of these mutations led to strong reduction, and a combination of all three mutations led to a complete abolishment of phloem-specific expression (Yin *et al.*, 1997b). We also found protein binding to a DNA fragment from −163 to −101 with extracts prepared from rice nuclei. A binding assay resulted in two differently shifted DNA fragments, suggesting the binding of two proteins. Our competition experiments suggest that binding is determined by sequences outside the GATA sequence (Figure 5) either between −140 and −150 or, more likely, by two redundant motifs. The sequence AGAAG, which is also indicated by an ongoing more detailed mutagenesis analysis (X. He and T. Hohn, unpublished observations), may represent such a motif. The published data about protein binding to this promoter region (Yin *et al.*, 1997b) are compatible with AGAAG as the actual binding site since this sequence was altered in the attempt to inactivate the GATA motif. The work of Yin *et al.* (1997b) suggests that the three motifs (GATA, ASL and BoxII) equally contribute to phloem-specific expression. Our data show that for a promoter including the dps, deletion of the −101 to −163 region has a more drastic effect for vascular tissue-specific expression than additional deletion of the ASL box and part of BoxII. The −101 to −163 region may itself act as a specificity element or as a quantitative element modulating specificity element(s) within −101 and +8 or even further downstream. Interestingly, we found that the protein binding to the −101 to −164 region is weakly competed by the region from −100 to −1.

Promoter strength and potential usefulness

The promoter constructs described here might be useful for expression of transgenes in rice. The overall strength of the 'full-length' RTBV promoter is comparable to a CaMV 35S promoter, which itself seems to be enhanced in the context of the RTBV dps and the intron. Soluble extracts from transgenic plants contained an average GUS activity of about 200 nM MU per minute per mg protein for both promoters. For some deletion variants of the RTBV promoter up to 530 nM MU per minute per mg protein were obtained in some experiments. This compares favourably to values obtained with other promoter-GUS constructs in transgenic rice and analysed with comparable protocols (Battraw and Hall, 1990; Cornejo *et al.*, 1993; Terada and Shimamoto 1990; Verdaguer *et al.*, 1996; Xu *et al.*, 1993, 1995; Yin and Beachy, 1995).

It is likely that the presence of an intron contributes to the strength of expression. Both the RTBV and the CaMV promoter constructs are much more active in a seedling bombardment assay when the intron is included (our unpublished observation), and for the CaMV promoter a direct comparison of an intron-containing expression construct and a cDNA derivative showed a difference of up to 50-fold in transfected protoplasts (Fütterer *et al.*, 1994). Similar effects have been described for a number of other promoter-reporter cassettes, particularly in monocots (see Introduction). In our hands, the magnitude of this effect is very variable (our unpublished observations). The value of the RTBV promoter as a tool for gene expression in rice may reside in its preferential activity in

the vascular bundle and the epidermis in young leaves. It also may be useful to express gene products that can interfere with RTBV infection, which can cause severe yield losses (Hibino, 1996).

Correlation between GUS protein and mRNA levels

Simultaneous determination of GUS mRNA and enzyme levels revealed that not only differences in overall transcriptional activity are responsible for the different GUS levels. More GUS protein was synthesized per mRNA molecule when levels of RNA were low, such as with the full-length RTBV promoter, upstream promoter deletions, or the Δ8-83 deletion. In plants that produced higher RNA levels, for example from the promoter downstream deletions 8–32 and 50–83 or the CaMV 35S promoter, apparently 2–3-fold less GUS protein was produced per available mRNA. This might be due to differences in mRNA transport or translation, which could be caused by the sequence differences due to deletions in the dps region or due to a few additional 5′ nucleotides from the CaMV 35S transcription initiation site. It also appears possible that a post-transcriptional process might become saturated. In some experiments (e.g. Figure 3), the plant lines showing this inefficient RNA usage exhibited a higher GUS activity than measured in the particular experiments (summarized in Table 1) where also the RNA levels were determined. We cannot exclude the possibility that growth conditions or differences between individual seedlings influence mRNA maturation or usage. Discrepancies between RNA and protein production have been observed previously with a number of different transgenic plants displaying features of post-transcriptional gene silencing (discussed in Depicker and Van Montague, 1997).

Short-stop RNA and transcription processivity

A striking feature of most of our plant lines is the production of short-stop RNA. This RNA might be generated by 3′ end processing of full-length pre-mRNA in competition with splicing or by co-transcriptional 3′ end processing at the promoter proximal polyadenylation signal. In the first case, the ratio between spliced RNA and short-stop RNA would depend on post-transcriptional competition of the respective signals for processing factors; the ratio would thus depend on the RNA sequence and not on the promoter. However, our results show that the ratio is influenced drastically by the untranscribed promoter sequence: while short-stop RNA constitutes about 70% of the steady-state RNA from the full-length RTBV promoter and from the −280 deletion derivative, this value drops to 10–40% with variants with larger upstream deletions and to less than 10% with the CaMV promoter. The best explanation for these findings is that the termination or 3′ end processing activity is a property of the transcription complex which associates with the respective initiator regions and that these complexes are different for the different promoters.

Our data obtained with the full-length RTBV promoter are compatible with the association of two different polymerase complexes: one with a high processivity that generates long primary transcripts that mature mainly by splicing, and one with a low processivity that generates only short transcripts. Gene-specific control of transcription processivity is a feature of many genes in pro- and eukaryotes (Gu and Reines, 1995; Krumm *et al.*, 1995; Rasmussen and Lis, 1995; Reeder and Hawley, 1996; Wang *et al.*, 1997). For instance, the promoter of HIV-1 produces short-stop RNA (Scott and Imperiale, 1997), requires activation to produce long instead of short transcripts (Jones, 1997; Jones and Peterlin, 1994; Keen *et al.*, 1997) and may associate with polymerase complexes of different processivity (Lu *et al.*, 1993). The short transcripts initially produced from such genes are normally unstable and difficult to detect. In RTBV, the presence of a proper polyadenylation signal may enhance pausing of low-processivity complexes and also stabilize the ensuing short transcripts. In the better studied cases of HIV-1 and *Drosophila* hsp genes, GAGA or complementary TCTC sequences and interacting factors are crucial to recruite low-processivity complexes (Jones and Peterlin, 1994; Shopland *et al.*, 1995; Wilkins and Lis, 1997). GAGA-binding proteins have been identified in plants (Frustaci *et al.*, 1995). The RTBV dps contains four copies of this sequence motif (Figure 7). In the dps deletion variant and in the −54 mutant compared to the −101 mutant, the number of these motifs is reduced (Figure 7) and in the respective plants, the proportion of short-stop RNA drops and the amount of spliced RNA increases. The increase could be due to increased binding of high-processivity complexes either because of lack of competition by the low-processivity complex for binding to the initiator region or because of the absence of a negative element. The RTBV promoter region upstream of −280 influences production of short-stop RNA and spliced RNA equally. In contrast, the region between −280 and −218 contains an element that specifically affects short-stop RNA production since its deletion

leads to reduced short-stop RNA levels. Concomitantly, an increase in spliced RNA (compared to the −280 deletion) is observed similarly as for the dps mutations.

Differences among assay systems

In this and previous studies a number of sequence regions with effect on RTBV promoter activity have been identified (Figure 7). Many have been defined only roughly and probably more exist. Some of those described here are not apparent if GUS expression is taken as the only indication of transcriptional activity. Their presence is only revealed when RNA levels are analysed directly. The activity of the elements may also vary in different assy systems. The Δ8-32 and Δ50-83 mutants express GUS as well as the full-length promoter in plants while they express less in protoplasts. The dps region from +8 to +83 was of much lower importance in the seedling bombardment assay than either in protoplasts or transgenic plants. The CaMV 35S promoter is about 30 times more active than the RTBV promoter in protoplasts but both have a similar activity in plants. The reasons for these differences remain obscure. All this probably reflects the complexity of the transcription unit and the many possible levels of regulation.

For instance, DNA topology can have dramatic effects on promoter activity or specificity (Frisch *et al.*, 1995). One function of the GAGA factor, or of GAGA-dependent assembly of low-processivity polymerase complexes, is the alteration of nucleosome association with promoter regions (Shopland *et al.*, 1995; Wal *et al.*, 1995; Wilkins and Lis, 1997). Such processes might well be different on linear and circular templates and nucleosome association with plasmid DNA in transient expression experiments may be very different from that of low-copy-number integrated genes.

Analysis of a plant viral promoter outside its viral context has been most extensively performed with the CaMV 35S promoter (reviewed by Lam, 1994). Several crucial elements in the CaMV 35S promoter are dispensible for virus viability (Turner *et al.*, 1996), suggesting that the control within the virus context can be different again from what can be concluded from all isolated promoter studies. Nevertheless, such studies remain of great value for our general knowledge about transcription control in plants and for the generation of efficiently expressed transgenes.

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