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ORIGINAL PAPER

The *TrAP/REn* monodirectional promoter of Mungbean yellow mosaic geminivirus (MYMV) displays root-specific expression in transgenic tobacco

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Abstract The *TrAP/REn* monodirectional promoter of *Mungbean yellow mosaic virus* (MYMV) comprises many root-specific motifs. The *TrAP/REn* promoter fused to the *β*-glucuronidase (*gus*) reporter gene was used to transform tobacco. Histochemical staining of various parts of seven transgenic plants showed a preferential root-specific expression pattern. Tobacco transformation with the *TrAP* gene under the transcriptional control of the *TrAP/REn* promoter yielded nine transgenic plants, of which six harboured the complete *TrAP* gene in the integrated T-DNAs. Transcript analysis indicated root tissue-specific expression of the *TrAP* gene. *TrAP* transgenic plants were phenotypically normal. Expression of the *TrAP* gene under its own promoter obviated the toxicity which was observed when the *TrAP* gene was expressed under the CaMV 35S promoter.

Keywords *AC2* · *Cis*-regulatory elements · Geminivirus · Root-specific promoter · *TrAP*

Abbreviations

Rep	Replication-associated protein
TrAP	Transcriptional activator protein
REn	Replication enhancer protein
CP	Coat protein
MP	Movement protein
NSP	Nuclear shuttle protein
IGR	Intergenic region

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Introduction

Plant viruses serve as a good repository for regulatory elements which can be used to drive transgene expression in plants. Promoters of *Cauliflower mosaic virus* (CaMV) (Odell et al. 1985), *Cestrum yellow leaf curling virus* (CmYLCV) (Stavolone et al. 2003), *Figwort mosaic virus* (FMV) (Sanger et al. 1990) and *Cassava vein mosaic virus* (CVMV) (Verdaguer et al. 1996) display strong, constitutive expression in transgenic plants. Generation of transgenic plants with promoter-reporter gene fusions have enabled the characterization of vascular tissue-specific expression of cinnamoyl CoA reductase (*LICCR*) and cinnamoyl alcohol dehydrogenase (*LICAD*) (Prashant et al. 2011), tuber-specific expression of granule-bound starch synthase I (GBSSI) (Bansal et al. 2011) and fruit-specific expression of *2A12* (Wang et al. 2011). The *uid*A and *gfp* genes are the two widely used reporter genes (Mußmann et al. 2011; Ramadan et al. 2011).

Geminiviruses are plant DNA viruses characterized by one or two small, circular, single-stranded DNA genomes. Based on the genome organization, host-range and vector transmission, geminiviruses are classified into the genera, *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*. Geminiviruses replicate in infected plant cell nuclei by producing double-stranded replicative forms, which serve as the template for transcription (Preiss and Jeske 2003). Geminiviruses rely heavily on the host transcription machinery for viral gene expression (Hanley-Bowdoin et al. 1999). Thus, geminiviral promoter elements are well suited for transgene expression in plants. *Mungbean yellow mosaic virus* (MYMV) is a whitefly-transmitted, bipartite begomovirus which infects mungbean (Balaji et al. 2004; Morinaga et al. 1993) and blackgram (Karthikeyan et al. 2004). DNA A encodes the replication-associated protein

(Rep) and replication enhancer protein (REn) which are required for replication. DNA A also encodes the coat protein (CP) and transcriptional activator protein (TrAP) which are needed for encapsidation and transactivation, respectively. DNA B encodes the nuclear shuttle protein (NSP) and movement protein (MP) which are required for viral movement.

The intergenic region (IGR) of DNA A and DNA B of begomoviruses share a common region (CR) of about 160–200 bp. Chromatin modification of the geminivirus genome into minichromosomes modulates gene expression. The nucleosome-free gaps are likely to be accessible for host replication and transcription factors. Fine mapping of the replicative form of *Abutilon mosaic virus* (AbMV) revealed that the minichromosomes comprised 11–13 nucleosomes (Pilartz and Jeske 2003). One gap in the IGR and a second gap upstream of the translation initiation codon of TrAP were identified on AbMV DNA A, which suggested the presence of promoter elements in these regions. The *cis*-acting elements in the CR regulate the leftward transcription of the *Rep* gene and rightward transcription of the *CP* gene. TATA box and G-box consensus elements were identified as essential elements for the transcriptional activation of the *Rep* gene (Eagle and Hanley-Bowdoin 1997). Rep also functions as a negative feedback regulator of its own transcription by binding to a direct repeat iteron sequence between the TATA box and the transcription start site (Behjatnia et al. 1998; Eagle et al. 1994).

The potential promoter activity of the *cis*-elements of the *Rep* gene was demonstrated in tobacco protoplasts by transient expression of the *African cassava mosaic virus* (ACMV) IGR regulatory element fused to the *gus* reporter gene (Haley et al. 1992; Hong and Stanley 1995; Zhan et al. 1991). The firefly luciferase gene fused to the ACMV IGR regulatory element expressed well in tobacco and cassava protoplasts (Frey et al. 2001). Similar transient expression studies of the *Tomato golden mosaic virus* (TGMV) element in *Nicotiana tabacum* protoplasts (Sunter et al. 1993) and the MYMV element in *N. plumbaginifolia* protoplasts (Shivaprasad et al. 2005) confirmed the host-dependent transcriptional activity of the bidirectional promoter.

Agroinfiltration of *N. benthamiana* and many legume leaves and agroinoculation of roots with *Mungbean yellow mosaic India virus* (MYMIV) *Rep* promoter-*gus* fusions showed a preferential GUS expression in the vascular and mesophyll cells of leaf and no expression in roots (Usharani et al. 2006). Transgenic expression from the ACMV *Rep* regulatory elements in *N. tabacum* (Haley et al. 1992) and *Tomato mottle Taino virus* (ToMoTV) elements in tobacco and potato (Ramos et al. 2004) showed a vascular tissue-specific expression pattern. However, the *Rep* regulatory elements of the monopartite begomovirus *Tomato leaf curl virus* (TLCV) exhibited constitutive expression in transgenic *N. tabacum* (Dry et al. 2000).

The expression of the virion-sense (CP) promoter is weak but is strongly upregulated by transcriptional activators. Several groups demonstrated the TrAP-mediated transactivation of the CP promoter in protoplasts (Dry et al. 2000; Frey et al. 2001; Hong et al. 1996; Shivaprasad et al. 2005; Sunter et al. 1990; Sunter and Bisaro 1991; 1992) and in transgenic plants (Rajeswaran et al. 2007). A conserved late element (CLE) in the IGR was found to be essential for the TrAP-mediated transactivation in *Pepper huasteco virus* (PHV) (Ruiz-Medrano et al. 1999). However, CLE was found to be dispensable in TGMV (Sunter and Bisaro 2003) and *Bean golden mosaic virus* (BGMV) which lacked the element (Hung and Petty 2001). The CLE motif acted as an enhancer element in PHV (Cazzonelli et al. 2005) and *Beet curly top virus* (BCTV) (Hur et al. 2008).

The rightward promoter of TGMV did not drive GUS expression in the phloem and mesophyll cells of transgenic *N. benthamiana* (Sunter and Bisaro 1997). However, the CP promoter of TLCV was active in phloem and mesophyll in transgenic plants even in the absence of TrAP (Dry et al. 2000). The transgenically expressed GUS reporter gene under the truncated CP promoters of TGMV (Sunter and Bisaro 1997) and *Cabbage leaf curl virus* (CaLCuV) (Lacatus and Sunter 2008) exhibited TrAP-independent activity in the phloem cells of *N. benthamiana*. The full-length CP promoter possessed a repressor element that repressed the TrAP-independent activity in the phloem cells. TrAP-mediated transactivation was achieved by activation of the CP promoter in the mesophyll cells and by derepression in the phloem cells (Lacatus and Sunter 2008; Sunter and Bisaro 1997, 2003). Phloem-specific expression of the virion-sense promoter of *Wheat dwarf virus* (WDV) was shown in various transgenic dicotyledonous plants (Dinant et al. 2004).

In addition to the strong bidirectional promoter of ACMV, a region upstream of the *TrAP* ORF was shown to possess moderate promoter activity in *N. clevelandii* protoplasts (Zhan et al. 1991). Detailed transcriptome mapping of MYMV (Shivaprasad et al. 2005) and TGMV (Shung et al. 2006) confirmed the presence of a separate monodirectional promoter which transcribed the *TrAP*/*REn* sequences. The current study focuses on the expression pattern of the monodirectional *TrAP*/*REn* promoter of MYMV in transgenic *N. tabacum* plants. Although this promoter transcribes both *TrAP* and *REn* open reading frames, it is referred as the *TrAP* promoter in the rest of the paper. We report a preferential root-specific expression pattern of the *TrAP* promoter for the first time. We also report that the toxicity manifested by constitutive expression of *TrAP* in tobacco under the 35S

promoter (Rajeswaran et al. 2007) can be alleviated by the regulated, tissue-specific expression of *TrAP* under its native promoter.

Materials and methods

Plasmid constructs

The binary plasmid pPZP-P*TrAP-gus* was constructed as follows: A 359-bp sequence of the *TrAP* promoter (coordinates 1974 to 1615, accession number AJ132575.1) was amplified and cloned into the *Eco*RV site of pBSIIKS$^+$ (Stratagene, West Cedar, USA) (pBS-P*TrAP*). The *Pst*I and *Nco*I restriction sites were introduced in the forward and reverse primers, respectively. The *TrAP* promoter fragment from pBS-P*TrAP* was subcloned as a *Hin*cII/*Nco*I fragment into the corresponding sites of pRT100 (Topfer et al. 1987) (pRT-P*TrAP*-35S 3′). A 2.1-kb *Nco*I/*Sac*I intron-*gus* gene fragment was subcloned into the corresponding sites of pRT-P*TrAP*-35S 3′ (pRT-P*TrAP-gus*-35S 3′). Independently, a 2.1-kb P*nos-npt*II-*ocs* 3′ fragment was cloned as a *Stu*I/*Nru*I fragment in the *Eco*RI site of pPZP200 (Hajdukiewicz et al. 1994), in which process the *Eco*RI site was recreated (pPZP200-M) (Courtesy: Pradeep Burma, UDSC, New Delhi). The 2.8-kb P*TrAP-gus* cassette from pRT-P*TrAP-gus*-35S 3′was cloned as a *Hin*dIII fragment into the corresponding site of pPZP200-M (pPZP-P*TrAP-gus*) and the binary plasmid was mobilized by triparental mating into the *Agrobacterium tumefaciens vir* helper strain C58C1 (pGV2260).

The binary plasmid pGA-P*TrAP-TrAP* was constructed as follows: A 278-bp fragment with the CaMV 35S polyA signal was cloned as an *Eco*RI/*Xho*I fragment in pBSIIKS$^+$ (pBS-35S 3′). An 847-bp fragment comprising the MYMV-*TrAP* promoter and the coding sequence (coordinates 1982 to 1135, accession number AJ132575.1) was amplified and cloned in pGEM-T (Promega, Madison, USA) (pGEM-P*TrAP-TrAP*). The *Sac*I and *Bam*HI recognition sites were introduced in the forward and reverse primers, respectively. An 850-bp *Sac*I/*Bam*HI fragment was excised from pGEM-P*TrAP-TrAP* and subcloned in the corresponding sites of pBS-35S 3′ (pBS-P*TrAP-TrAP*-35S 3′). The 1.1-kb P*TrAP-TrAP*-35S 3′ cassette was cloned as a *Sac*I/*Xho*I fragment in the corresponding sites of pGA472-M (Sunitha et al. 2012) (pGA-P*TrAP-TrAP*) and the binary vector was mobilized by triparental mating into the *A. tumefaciens* strain LBA4404.

Tobacco transformation

Axenic tobacco (*N. tabacum* L. cv. Wisconsin 38) plants were grown in a tissue culture room under 16 h light (100 μE m^{-2} s^{-1})/8 h dark cycles at 25 ± 1°C. Tobacco leaf discs were transformed using *A. tumefaciens* as described by Sunilkumar et al. (1999). Transgenic shoots were selected on Murashige and Skoog (MS) medium which contained 100 mg l^{-1} kanamycin + 250 mg l^{-1} cefotaxime and were kept for root induction on the BGS medium (MS salts, 0.001 mg l^{-1} folic acid, 100 mg l^{-1} myoinositol, 0.4 mg l^{-1} thiamine, 0.057 μM indole-3-acetic acid, 0.14 μM kinetin, 3% [w/v] sucrose, 0.9% [w/v] agar, pH 5.7) supplemented with 250 mg l^{-1} cefotaxime and 100 mg l^{-1} kanamycin. GUS histochemical staining and GUS fluorometric assay were done as described by Sunilkumar et al. (1999).

Southern blot and RT-PCR analyses

Total plant DNA was extracted as described by Rogers and Bendich (1994). Plant DNA concentration was estimated using the Hoechst dye 33258 in the DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, San Fransisco, USA). T-DNA integration was analysed by Southern blotting. Total DNA (10 μg) was digested with appropriate restriction enzymes and electrophoresed in a 0.8% agarose gel in 1× Tris–borate-EDTA buffer. Viral titre in agroinfected blackgram leaves and roots was determined by DNA blot analysis of undigested plant DNA following agarose gel electrophoresis in 1× TNE (40 mM Tris–acetate, pH 7.5, 20 mM sodium acetate, 2 mM EDTA) buffer (Karthikeyan et al. 2004). DNA was transferred to the Zeta-probe nylon membrane (Biorad, Hercules, USA) and hybridized to [α-^{32}P]dCTP-labelled probes. RT-PCR analysis using the *TrAP* primers and tobacco actin primers was done as described earlier (Sunitha et al. 2011).

Results

Sequence analysis of the *TrAP* promoter

The 359-bp *TrAP* promoter sequence was submitted to the PLACE database (Higo et al. 1999) to identify the putative *cis*-elements. The transcription start site (TSS) nucleotide which was identified by Shivaprasad et al. (2005) was numbered as +1 (Fig. 1). A TATA box (TATAA) at the −27 position and multiple CAAT boxes at −51, −249 and −277 positions were identified (Fig. 1) upstream of the TSS. The stress-regulated motifs ABRELATERD1 (ACGTG) and MYCCONSENSUSAT (CANNTG) were identified at −266 and −231 positions, respectively. At positions −176 and −296, light-regulated motifs ASF1MOTIFCAMV (TGACG) and GATA box were identified. Cytokinin-responsive motifs CPBCSPOR (TATTAG) (at −317 position) and ARR1AT (NGATT) (at positions −122 and −306)

Fig. 1 Nucleotide sequence of the 357-bp *TrAP* promoter and 407-bp *TrAP* coding sequence of MYMV. The transcription start site (TSS +1) and the translation start site (ATG) are marked with *arrows* and the translation termination codon (TAG) is marked in a *bold line*. Important *cis*-elements identified by the software tool PLACE for root-specific expression have been marked in *boxes*. Other regulatory motifs identified are marked in *thin lines*

were also found. Interestingly, several root-specific motifs ROOTMOTIFPOX1 (ATATT), SORLIP1AT (GCCAC), OSE2ROOTNODULE (CTCTT) and RHERPATEXPA7 (KCACGW) were identified at −84, −234, −32 and −268 positions, respectively (Fig. 1). The presence of four root-specific motifs in the *TrAP* promoter prompted us to analyse the pattern of the promoter activity in transgenic tobacco plants.

Southern blot analysis of tobacco plants transformed with P*TrAP-gus*, histochemical and fluorometric GUS assay

The *TrAP* promoter sequence fused with the *gus* reporter gene was placed in pPZP200-M to yield pPZP-P*TrAP-gus*, which harboured the neomycin phosphotransferaseII (*npt*II) gene as the plant selectable marker (Fig. 2a). Care

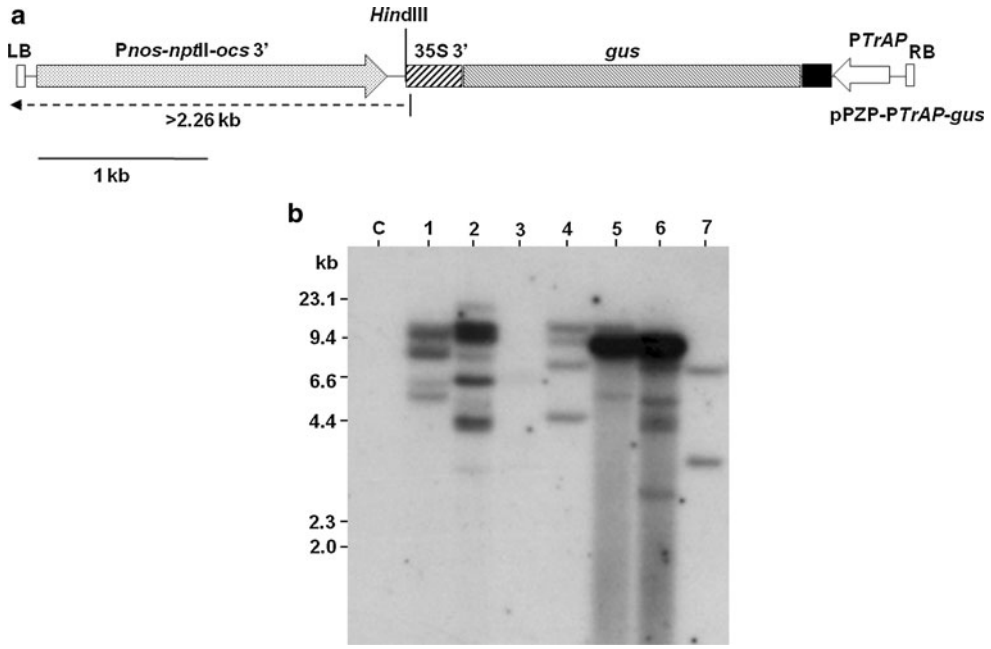

Fig. 2 Southern blot analysis of tobacco plants transformed with pPZP-P*TrAP-gus*. **a** The T-DNA of pPZP-P*TrAP-gus*. The MYMV *TrAP* promoter was fused to an intron-containing *β*-glucuronidase (*gus*) gene in the binary vector pPZP200-M. *PTrAP* MYMV *TrAP*/*REn* promoter; *RB* T-DNA border-right; *Pnos* nopaline synthase promoter; *nptII* neomycin phosphotransferaseII gene; *ocs3′* octopine synthase polyadenylation signal; *35S 3′* CaMV 35S polyadenylation signal; *LB* T-DNA border-left. The probe used for Southern blotting was the *npt*II gene. The junction fragment size (>2.26 kb) has been marked in a *dashed arrow*. **b** The *npt*II probe-based Southern blot analysis of tobacco plants transformed with pPZP-P*TrAP-gus*. Total DNA (10 μg) from seven transgenic plants TG-1 to TG-7 (*1–7*) was digested with *Hin*dIII and the blot was probed with the *npt*II coding sequence. Total DNA from an untransformed, control tobacco plant digested with *Hin*dIII (*C*) was used as the negative control

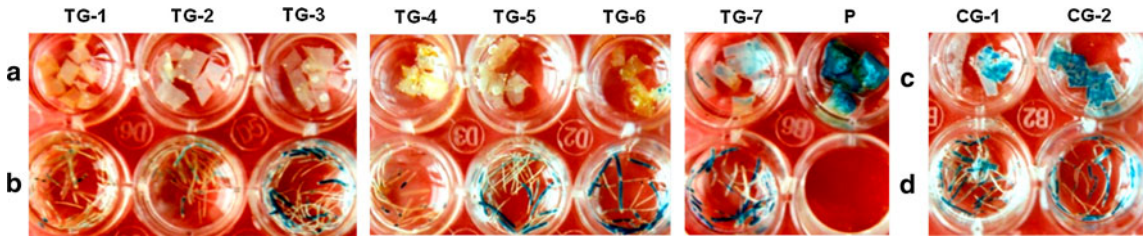

Fig. 3 GUS histochemical analysis of P*TrAP-gus* transgenic plants. *a*, *b* GUS staining pattern of P*TrAP-gus* transformed plants TG-1 to TG-7 in leaf and root, respectively. *c*, *d* GUS staining pattern of P35S-*gus* transformed plants CG-1 and CG-2 in leaf and root, respectively. A GUS-positive transgenic tobacco callus was used as a positive control (*P*)

was taken to avoid the presence of the CaMV 35S promoter in the T-DNA. Tobacco transformation with pPZP-P*TrAP-gus* yielded seven kanamycin resistant shoots which formed roots on a medium containing 100 mg l^{-1} kanamycin. T-DNA integration of kanamycin-resistant plants was studied by Southern blotting with the *npt*II probe. Digestion of DNA with *Hin*dIII and hybridization with the *npt*II probe will yield junction fragments longer than 2.26 kb (Fig. 2a). TG-1 displayed hybridization to four integrated T-DNA copies (9.4, 7.2, 5.8 and 5.0 kb), TG-2 to six copies (10.2, 10.0, 7.4, 6.0, 4.2 and 3.3 kb), TG-4 to four copies (10.2, 9.4, 7.2 and 4.4 kb), TG-5 to three copies (10.2, 9.4 and 5.0 kb), TG-6 to five copies (9.4, 7.2, 5.0, 4.6 and 2.9 kb), and TG-7 to two copies (7.2 and 3.3 kb) (Fig. 2b). One junction fragment of 6.6 kb hybridized weakly in TG-3.

Seven P*TrAP-gus* (TG-1 to TG-7) and two P35S-*gus* (CG-1 and CG-2) transgenic tobacco plants were used to study the GUS expression pattern in leaf and root tissues by histochemical staining (Fig. 3). Five of the seven P*TrAP-gus* plants (TG-1 to TG-5) did not show GUS staining in the leaf segments (Fig. 3a). Plants TG-6 and TG-7 exhibited very mild GUS expression in the leaf tissue (Fig. 3a). Leaf segments of CG-1 and CG-2 plants transformed with P35S-*gus* showed intense GUS staining (Fig. 3c). Interestingly, all seven transgenic P*TrAP-gus* plants (TG-1 to TG-7) exhibited GUS staining in roots (Fig. 3b) similar to the intensity found in P35S-*gus* transformed plant roots (Fig. 3d).

GUS histochemical staining pattern was analysed in other parts of the transgenic plants. The P35S-*gus* transgenic plant CG-1 exhibited intense staining in petals (Fig. 4a), stigma (Fig. 4b), sepals (Fig. 4c), fruit wall, immature seeds and placenta (Fig. 4d), anther (Fig. 4e) and stem (Fig. 4f). In five of the seven P*TrAP-gus* transgenic plants (TG-1 to TG-5) no GUS staining was observed in petals, sepals, immature seeds and stamen (Fig. 4a, c–e, respectively). Very weak GUS staining in the stem (Fig. 4f) was observed in five of the seven P*TrAP-gus* transgenic plants (TG-2, TG-3, TG-5, TG-6 and TG-7). Weak GUS staining was observed in the stigma of only

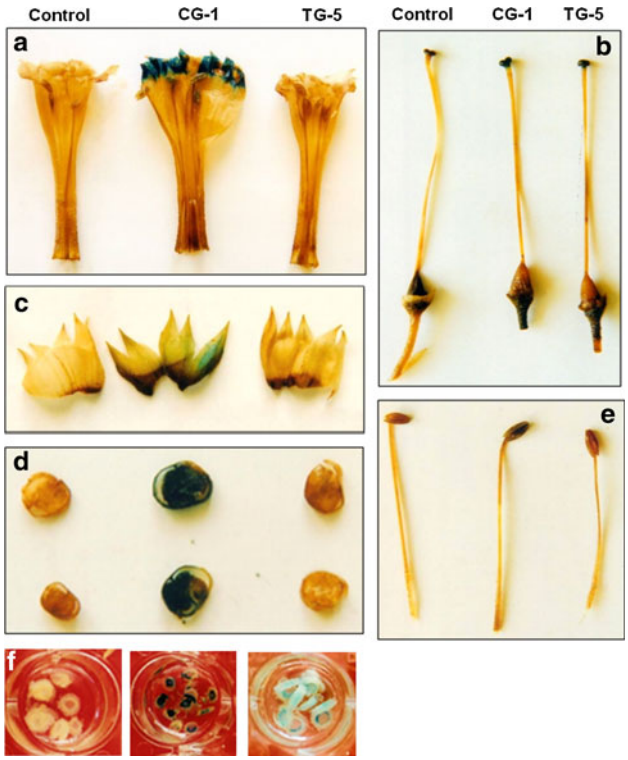

Fig. 4 GUS histochemical analysis of different parts of P*TrAP-gus* (TG-5) and P35S-*gus* (CG-1) transgenic plants. **a** Petal, **b** pistil, **c** sepal, **d** ovary (a cross section), **e** stamen, **f** stem (a transverse section). An untransformed tobacco plant served as negative control (*Control*)

TG-5 (Fig. 4b). Plants TG-6 and TG-7 exhibited mild GUS staining in all analysed parts (data not shown).

GUS fluorometric assay was done in leaf and root protein extracts of P*TrAP-gus* and P35S-*gus* plants to quantitate GUS activity. A high level of GUS specific activity was found in the leaves and roots of P35S-*gus* transformed plants (Table 1). In the P*TrAP-gus* transgenic plants, GUS specific activity was high in roots but was very low in leaves. The ratio of GUS specific activities in roots versus leaves was in the range of 15–110 in P*TrAP-gus* plants in comparison to the range of 6.3–7.0 in P35S-*gus* transgenic plants. Fluorometric GUS analysis confirmed a strong and

Table 1 Fluorometric assay of β-glucuronidase (GUS) activity in leaf and root tissues of P*TrAP-gus* and P35S-*gus* transgenic tobacco plants

	Plant no.	GUS specific activity in leaves	GUS specific activity in roots	Ratio of GUS specific activity roots/leaves
Control		21[a]	312[a]	
P*TrAP-gus*	1	57	2386	42
	2	65	4054	62
	3	119	9455	79
	4	69	3333	48
	5	83	3478	41
	6	186	20540	110
	7	217	3243	15
P35S-*gus*	1	1982	12567	6.3
	2	1069	7500	7.0

The specific activity of GUS is expressed as pmoles of 4-methylumbelliferone released/mg protein/min

[a] Fluorometric readings in control plants reflect background fluorescence in plant extracts

preferential expression of the *TrAP* promoter in roots. The fluorometric GUS assays were done in duplicates which yielded similar values.

Southern blot analysis of MYMV infected blackgram plants

Since the P*TrAP-gus* fusion gene preferentially expressed in roots, the ability of MYMV to infect and replicate in blackgram roots was evaluated. MYMV DNA accumulation in top crop (TC), young fully expanded leaf (FL), mature leaf (ML) and roots (R) was evaluated in blackgram plants agroinoculated with MYMV DNA A and DNA B partial dimers. DNA blot analysis with the MYMV DNA A probe revealed that the top crop (comprising the shoot bud and young leaves), young fully expanded leaves and mature leaves accumulated high levels of MYMV DNA A (Fig. 5). Interestingly, blackgram roots accumulated detectable levels of DNA A, although the levels were much lower in comparison to the levels found in the leaves. Thus, MYMV infected and replicated in blackgram plants.

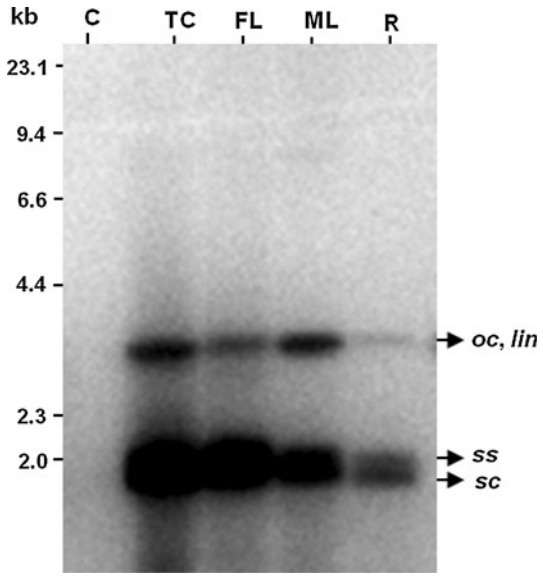

Fig. 5 Southern blot analysis of leaves and roots of MYMV-infected blackgram plants. A blot with total DNA from top crop (*TC*), young fully expanded leaf (*FL*), mature leaf (*ML*) and roots (*R*) from MYMV-infected blackgram plants and leaves of uninfected blackgram plant (*C*) was hybridized to the full-length DNA A probe. The positions of open circular dsDNA (*oc*), linear dsDNA (*lin*), single-stranded DNA (*ss*) and super-coiled dsDNA (*sc*) of MYMV are marked

Southern blot analysis of tobacco plants transformed with the MYMV *TrAP* gene under its native promoter

The expression of the MYMV *TrAP* gene under the transcriptional control of the CaMV 35S promoter proved to be toxic in transgenic plants (Rajeswaran et al. 2007). We studied whether the toxicity of *TrAP* can be alleviated by expressing it under its native promoter. MYMV *TrAP* promoter plus its coding sequence along with the 35S polyA signal was cloned in the binary vector pGA472-M (Sunitha et al. 2012) to yield pGA-P*TrAP-TrAP*. There is no 35S promoter in the T-DNA. The binary vector harboured the *npt*II gene as the plant selectable marker (Fig. 6a). Nine transgenic plants which formed roots on a selection medium with kanamycin were obtained upon transformation with pGA-P*TrAP-TrAP*. Digestion of DNA with *Hin*dIII and hybridization with the *npt*II probe will yield junction fragments longer than 2.8 kb (Fig. 6a). Plants TT-3 (6.2 kb), TT-5 (3.4 kb), TT-7 (7.0 kb) and TT-8 (18.0 kb) displayed hybridization to single junction fragments. Plants TT-1 (8.2 and 2.9 kb), TT-2 (8.2 and 5.6 kb), TT-4 (7.4 and 2.8 kb), TT-6 (4.8 and 4.6 kb) and TT-9 (7.6 and 6.8 kb) had two integrated T-DNA copies (Fig. 6b). The presence of the complete *TrAP* gene in the integrated T-DNA was analysed using the *TrAP* probe. Upon digestion of the plant DNA with *Eco*RI and hybridization with the *TrAP* probe, six of the nine plants (TT-2, TT-3, TT-4, TT-6, TT-7 and TT-9) exhibited

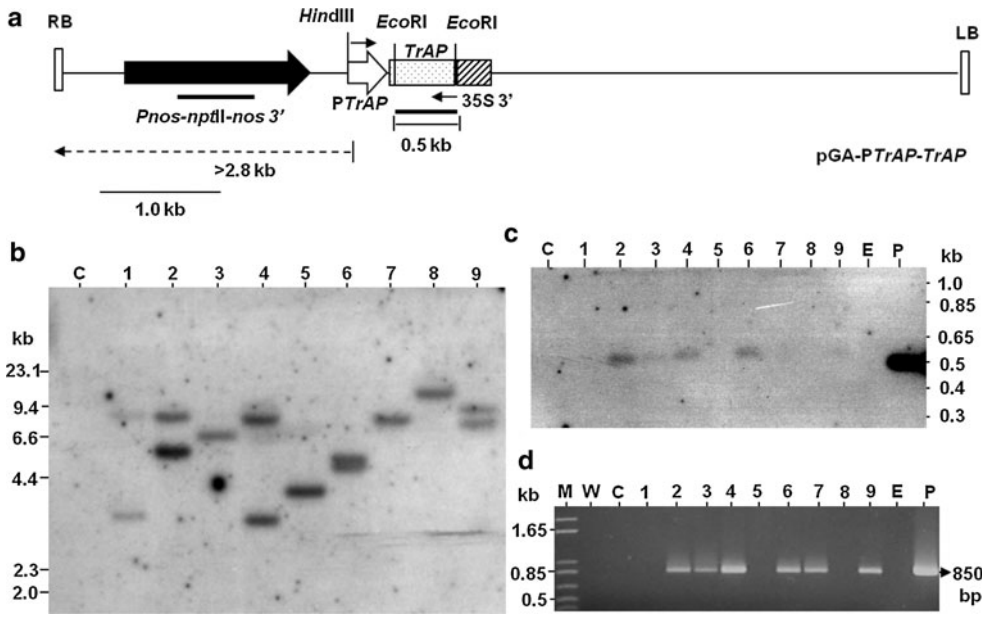

Fig. 6 Southern blot analysis of tobacco plants transformed with pGA-P*TrAP-TrAP*. **a** The T-DNA of pGA-P*TrAP-TrAP*. The MYMV *TrAP* promoter and *TrAP* coding region were cloned in the binary vector pGA472-M. *RB* T-DNA border-right; *Pnos* nopaline synthase promoter; *nptII* neomycin phosphotransferaseII gene; *nos 3′* nopaline synthase polyadenylation signal; *35S 3′* CaMV 35S polyadenylation signal; *PTrAP* MYMV *TrAP*/*REn* promoter; *LB* T-DNA border-left. Probes used for hybridization have been marked in *bold lines*. The junction fragment size (>2.8 kb) and internal T-DNA fragment size (0.5 kb) have been marked in *dashed arrow* and *line*, respectively. The primers used for PCR analysis have been marked in *filled arrows*. **b** The *npt*II probe-based Southern blot analysis of tobacco plants transformed with pGA-P*TrAP-TrAP*. Total DNA (10 µg) from nine transformants TT-1 to TT-9 (*1–9*) was digested with *Hin*dIII and the blot was probed with the *npt*II coding sequence. Total DNA from the untransformed, control tobacco plant digested with *Hin*dIII (*C*) was used as the negative control. **c** The *TrAP* probe-based Southern blot analysis of tobacco plants transformed with pGA-P*TrAP-TrAP*. DNA from the transgenic plants TT-1 to TT-9 (*1–9*) was digested with *Eco*RI and the blot was probed with the *TrAP* coding sequence. The binary plasmid pGA-P*TrAP-TrAP* (50 pg) digested with *Eco*RI was used as the positive control (*P*). An internal T-DNA fragment of 0.5 kb was expected to hybridize in transgenic plants and the binary plasmid control (*P*). *E* empty lane. **d** PCR analysis of tobacco plants transformed with pGA-P*TrAP-TrAP*. DNA (100 ng) from the untransformed, control tobacco plant (*C*) and from nine transgenic plants TT-1 to TT-9 (*1–9*) was used as the PCR template. Binary plasmid DNA (50 pg) was used as the positive control (*P*). *W* water control, *E* empty lane, *M* 1 kb^+ marker

hybridization to the expected internal T-DNA fragment of 0.5 kb (Fig. 6c). PCR with P*TrAP-TrAP*-specific primers (Fig. 6a) amplified an 850-bp fragment in all these six transgenic plants (TT-2, TT-3, TT-4, TT-6, TT-7 and TT-9) (Fig. 6d). Thus, six of the nine transgenic plants harboured the complete *TrAP* gene.

Transcript and phenotypic analyses of P*TrAP-TrAP* transgenic tobacco plants

RT-PCR was done using the *TrAP*-specific primers in the six plants which harboured the complete *TrAP* gene. RNA was extracted from leaf and root tissues and analysed for *TrAP* transcript accumulation. No amplification was observed in control and transgenic leaf tissues (Fig. 7a). Interestingly, the expected 400-bp fragment was amplified in the roots of four of the six transgenic plants (TT-4, TT-6, TT-7 and TT-9) (Fig. 7a). No amplification was observed in the control reaction in which the reverse transcription step was omitted (data not shown), thus ruling out DNA contamination. RT-PCR with tobacco actin primers amplified a 467-bp fragment in leaf and root tissues of control and all six transgenic plants (Fig. 7a).

Silencing suppressors are known to cause phenotypic abnormalities upon expression in transgenic plants. TrAP of MYMV was reported to suppress RNA silencing (Trinks et al. 2005). The transgenic P*TrAP-TrAP* plants were established in the greenhouse. All plants were phenotypically normal (Fig. 7b). Thus, the expression of P*TrAP-TrAP* did not cause any phenotypic abnormality.

Discussion

TrAP/*REn* transcripts are made from monodirectional promoters in ACMV (Zhan et al. 1991), MYMV (Shivaprasad et al. 2005) and TGMV (Shung et al. 2006). The constitutively expressed *TrAP* promoter was further activated by TrAP in *N. plumbaginifolia* protoplasts (Shivaprasad et al. 2005). In contrast, the ACMV *TrAP* promoter displayed only constitutive expression (Haley et al. 1992). The autoregulation of *Rep* enhanced the *TrAP* and *REn* expression in TGMV (Shung

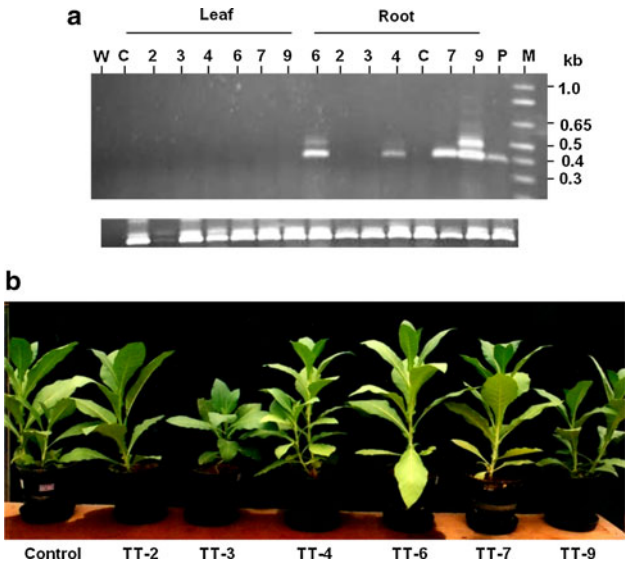

Fig. 7 Expression and phenotypic analyses of P*TrAP-TrAP* transgenic plants. **a** RT-PCR of the P*TrAP-TrAP* transgenic plants with the *TrAP*-specific primers. Total RNA extracted from the leaf and root tissues of untransformed, control tobacco plant (*C*) and from six transgenic plants TT-2, TT-3, TT-4, TT-6, TT-7 and TT-9 harbouring the *TrAP* gene (*2*, *3*, *4*, *6*, *7* and *9*) was used as the RT-PCR template. A Southern blot-positive transgenic plant expressing a *TrAP* mutant gene (Rajeswaran et al. 2007) was used as the positive control (*P*). The *bottom panel* presents an equal loading control with samples amplified with the tobacco actin gene-specific primers. *W* water control. **b** Phenotype of P*TrAP-TrAP* transgenic plants. Six transgenic tobacco plants TT-2, TT-3, TT-4, TT-6, TT-7 and TT-9 which harboured the *TrAP* gene and untransformed, control tobacco plant were established in the greenhouse. Six-week-old plants were photographed

and Sunter 2007). A 9-bp conserved site in the TGMV *TrAP* promoter, which binds to host nuclear proteins, is essential for *TrAP*/*REn* expression and viral replication (Tu and Sunter 2007). Interestingly, the leftward transcription of the bidirectional promoter inhibited the *TrAP* promoter activity, since the regulatory sequence was within the *Rep* coding region.

Cis-elements in the MYMV *TrAP* promoter suggest root-specific expression

The *TrAP* promoter sequence harboured four motifs associated with root-specific expression (Fig. 1). ROOTMOTIFPOX1 was reported in the root-specific *rol* D promoter from *Agrobacterium rhizogenes* (Elmayan and Tepfer 1995). SORLIP1AT upregulated many root-specific genes (Jiao et al. 2005). The motifs OSE2ROOTNODULE2 and RHERPATEXPA7 controlled expression in root nodule (Fehlberg et al. 2005) and root hair (Kim et al. 2006), respectively. Interestingly, the cytokinin-dependent elements CPBCSPOR and ARR1AT also were attributed to root-specific expression (Fusada et al. 2005; Ross et al. 2004).

Root-specific expression of the MYMV *TrAP* promoter in transgenic tobacco

P*TrAP-gus* displayed a preferential root-specific expression pattern of GUS in five out of seven transgenic plants (Fig. 3b). No GUS staining was observed in leaf (Fig. 3a), petals, sepals, immature seeds and stamen (Fig. 4) in those transgenic plants. The mild GUS staining observed in stem sections of five P*TrAP-gus* plants (TG-2, TG-3, TG-5, TG-6 and TG-7) could be attributed to the presence of the bean *GRP 1.8* gene stem elements 1 and 2 (SE1, SE2) (Keller and Baumgartner 1991) with 75 and 71% nucleotide sequence similarity, respectively, in the MYMV *TrAP* promoter (data not shown). The weak GUS staining exhibited by the plants TG-6 and TG-7 in all analysed parts of the plants may be due to the impact of flanking plant DNA sequences at the sites of T-DNA integration. Integration of multiple T-DNA copies did not have an adverse effect on GUS expression in roots. TG-3, a plant in which a single T-DNA copy was integrated and the plant TG-6, in which five copies of the T-DNA were integrated had comparable levels of GUS activity (Fig. 2b; Table 1). As a majority of the transgenic plants (TG-1 to TG-5) expressed P*TrAP-gus* only in roots, it is inferred that the *TrAP* promoter displays preferential root-specific expression. The ability of MYMV to invade and replicate in the roots (Fig. 5) lends significance to the expression of the *TrAP* promoter in roots. It is important to note that Shivaprasad et al. (2005) reported accumulation of the *TrAP*/*REn* transcript in the MYMV-infected blackgram leaves. Two explanations may be given for the preferential root-specific expression of the P*TrAP-gus* gene in transgenic tobacco: (1) The promoter sequence we studied in this report might lack a leaf-specific regulatory sequence element, which may be present in the complete MYMV DNA A. (2) A protein encoded in another part of the viral genome may alter the spatial expression pattern of the *TrAP* promoter during MYMV infection in blackgram. The transgenic tobacco plants which expressed the C2-*gus* or C3-*gus* translational fusions of TLCV showed GUS activity in vascular tissues of leaf, stem and root (Dry et al. 2000). However, it is not very clear from this report whether the C2 and C3 proteins were translated from the *Rep* transcript initiated from the bidirectional promoter or from the *C2*/*C3* transcript initiated from the monodirectional *TrAP* promoter. The root versus leaf GUS specific activity ratio was in the range of 15–110 in P*TrAP-gus* plants (Table 1) in comparison to the range of 6.3–7.0 displayed by P35S-*gus* transgenic plants. These observations substantiate the preferential root-specific expression pattern of the P*TrAP-gus* plants. Thus, the MYMV *TrAP* promoter is useful to achieve root-specific expression of transgenes.

Expression of MYMV *TrAP* under its own promoter alleviated its toxicity

Begomovirus *TrAP* acts as a transcriptional activator of late genes (Haley et al. 1992; Shivaprasad et al. 2005; Sunter and Bisaro 1991, 1992), as a suppressor of gene silencing (Bisaro 2006; Trinks et al. 2005) and as a pathogenicity determinant (Hong et al. 1996, 1997). Constitutive expression of MYMV-*TrAP* resulted in truncated T-DNA integrations which were attributed to TrAP-mediated toxicity (Rajeswaran et al. 2007). This lead to the intriguing question on how MYMV infection in blackgram circumvented the TrAP-mediated toxicity. Shung et al. (2006) demonstrated that the TGMV *TrAP* expression is tightly regulated. Of the three complementary-sense transcripts AL62, AL1629 and AL1935, AL1629 is the only transcript that encodes TrAP. Rajeswaran et al. (2007) proposed that TrAP may accumulate at low, subtoxic levels in MYMV infected plants. Six of the nine (67%) transgenic plants with the P*TrAP-TrAP* gene (TT-2, TT-3, TT-4, TT-6, TT-7 and TT-9) harboured the complete *TrAP* portion of the T-DNA (Fig. 6). This should be compared with the previous report (Rajeswaran et al. 2007) in which six out of seven (86%) transgenic plants which were raised with the P35S-*TrAP* gene were found to harbour truncated T-DNAs without the complete *TrAP* gene. Transgenic expression of the ACMV *TrAP* gene under the 35S promoter caused severe and moderate phenotypic abnormalities in *N. tabacum* and *N. benthamiana*, respectively (Siddiqui et al. 2008). No phenotypic abnormality was observed in any of the MYMV P*TrAP-TrAP* transgenic tobacco plants (Fig. 7b) although four transgenic plants accumulated the *TrAP* transcript in the roots (Fig. 7a). Thus, expression of MYMV-*TrAP*, under its own promoter was not toxic to tobacco plants.

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