The Mungbean Yellow Mosaic Begomovirus Transcriptional Activator Protein Transactivates the Viral Promoter-Driven Transgene and Causes Toxicity in Transgenic Tobacco Plants

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The *Begomovirus* transcriptional activator protein (TrAP/AC2/C2) is a multifunctional protein which activates the viral late gene promoters, suppresses gene silencing, and determines pathogenicity. To study TrAP-mediated transactivation of a stably integrated gene, we generated transgenic tobacco plants with a *Mungbean yellow mosaic virus* (MYMV) AV1 late gene promoter-driven reporter gene and supertransformed them with the MYMV *TrAP* gene driven by a strong 35S promoter. We obtained a single supertransformed plant with an intact 35S-*TrAP* gene that activated the reporter gene 2.5-fold. However, 10 of the 11 supertransformed plants did not have the *TrAP* region of the T-DNA, suggesting the likely toxicity of TrAP in plants. Upon transformation of wild-type tobacco plants with the *TrAP* gene, six of the seven transgenic plants obtained had truncated T-DNAs which lacked *TrAP*. One plant, which had the intact *TrAP* gene, did not express TrAP. The apparent toxic effect of the *TrAP* transgene was abolished by mutations in its nuclear-localization signal or zinc-finger domain and by deletion of its activation domain. Therefore, all three domains of TrAP, which are required for transactivation and suppression of gene silencing, also are needed for its toxic effect.

Additional keywords: geminiviruses, negative selection.

Geminiviruses encapsidate small, circular, single-stranded DNA genomes of 2.5 to 3 kb. Based on their genome organization, species of insect that transmits the virus, and host range, geminiviruses are classified into four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus* (Fauquet and Stanley 2005). In begomoviruses, expression of the late genes coding for coat protein (AV1) and nuclear shuttle protein (BV1), is dependent on the viral transcriptional activator protein (TrAP/AC2/C2/AL2) encoded by the early gene *TrAP* (Haley et al. 1992; Shivaprasad et al. 2005; Sunter and Bisaro 1991, 1992). TrAP also is involved in the suppression of gene silencing (Bisaro 2006; Trinks et al. 2005; Vanitharani et al. 2005; Voinnet et al. 1999; Wang et al. 2005) and acts as a pathoge-

nicity determinant (Hong et al. 1996, 1997). TrAP has three functional domains: a basic domain with the bipartite nuclear localization signal (NLS) at the N-terminus, a nonclassical zinc-finger domain in the middle, and an acidic activation domain at the C-terminus (Hartitz et al. 1999; Trinks et al. 2005). Because of high conservation of the functional domains, TrAP mutation in one begomovirus can be functionally complemented by TrAP of a second begomovirus (Saunders and Stanley 1995; Sunter et al. 1994). TrAP of monopartite begomoviruses share a high level of sequence similarity with that of bipartite begomoviruses (Noris et al. 1996; van Wezel et al. 2001) and are comparable in domain organization (Dong et al. 2003; van Wezel et al. 2001, 2002, 2003), transactivation, suppression of gene silencing, and pathogenicity determination (Dong et al. 2003; Dry et al. 2000; Krake et al. 1998; Rigden et al. 1994; van Wezel et al. 2001, 2002, 2003). Even though a conserved *cis*-element in the late begomoviral promoters (Arguello-Astorga et al. 1994) was shown to be needed for the activation by TrAP in *Pepper huasteco virus* (PHV) (Ruiz-Medrano et al. 1999), no consensus TrAP-responsive sequence has been identified to date (Shivaprasad et al. 2005; Sunter and Bisaro 2003). Sunter and Bisaro (2003) showed that the regulation of the AV1 promoter is more complex and involves a bipartite sequence. Because TrAP binds to double-stranded DNA in a sequence-nonspecific manner, the interaction between TrAP and late begomoviral promoters also may be indirect, as in the case of E1a protein of adenovirus (Hartitz et al. 1999; Noris et al. 1996).

Mungbean yellow mosaic virus (MYMV) is a bipartite begomovirus (Karthikeyan et al. 2004) and its TrAP has all three conserved domains (Trinks et al. 2005). TrAP-mediated transactivation of the MYMV AV1 and BV1 promoters as well as the BC1 promoter was demonstrated by transient expression studies in *Nicotiana plumbaginifolia* protoplasts (Shivaprasad et al. 2005; Trinks et al. 2005). In addition, RNA profiling in *Arabidopsis* protoplasts has revealed that transient expression of MYMV TrAP and its homolog from *African cassava mosaic virus* (ACMV) activates transcription of the same set of host genes (Trinks et al. 2005). Earlier, using tobacco and cassava protoplasts, Frey and associates (2001) showed transactivation of the ACMV promoters by TrAP. Furthermore, transactivation of *Tomato golden mosaic virus* (TGMV) late gene promoters by TrAP was demonstrated in protoplasts of *N. tabacum* and *N. benthamiana* (Sunter and Bisaro 1991, 1992, 2003). Integrated copies of begomovirus late gene promoters in transgenic

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plants were shown to be transactivated by transient expression of TrAP, using agroinoculation of leaf discs of transgenic plants (Haley et al. 1992) and agroinoculation of the transgenic plants with the infectious virus clones (Hong et al. 1996; Sunter and Bisaro 1997) or by transient expression of TrAP from a PVX vector (Hong et al. 1997). Sunter and Bisaro (1997) showed that TrAP is required for the expression of the AV1 promoter in both mesophyll and phloem tissues. In the mesophyll cells, TrAP is involved in the activation of the AV1 promoter whereas, in the phloem tissue, TrAP causes its derepression. Transactivation of an integrated copy of the PHV AV1 promoter in transgenic plants also was demonstrated by particle bombardment with a TrAP-expressing plasmid (Ruiz-Medrano et al. 1999). Transactivation of begomovirus promoter has not been demonstrated to date with an integrated copy of a *TrAP* gene. Interestingly, TrAP has been proposed to be toxic (Hao et al. 2003; Hartitz et al. 1999; Hong et al. 1997; Sunter et al. 2001). However, no clear experimental evidence has been reported thus far for this toxicity.

Legume-infecting Old World begomoviruses are evolutionarily distinct from the remaining viruses in this genus (Qazi et al. 2007). Thus, it is of interest to study the function of TrAP in this group of begomoviruses. In this article, we report for the first time that an integrated copy of MYMV *TrAP* transactivates the MYMV AV1 promoter in a transgenic *N. tabacum*

plant. We also report that almost all of the transgenic tobacco plants obtained by transformation with the 35S promoter-driven *TrAP* gene had truncated T-DNAs lacking either all or part of *TrAP*. These results suggest that constitutive expression of TrAP is toxic to plants. Mutations in the TrAP NLS and zinc-finger domains and deletion of the activation domain, which are required for transactivation and suppression of silencing, abolished the toxic effect of TrAP.

RESULTS

Transactivation of MYMV AV1 promoter by TrAP in protoplasts and transgenic plants.

The AV1 promoter, defined as the 420-bp fragment upstream of the AV1 open reading frame, was fused to a bean catalase intron-containing β -glucuronidase (*GUS*) gene and introduced into the binary plasmid pPZP211 with the 35S promoter-driven neomycin phosphotransferase II gene (*NPTII*) as selection marker (pPZP-AV1) (Fig. 1A). First, transactivation of the AV1 promoter by TrAP was evaluated by transient expression in *N. plumbaginifolia* protoplasts. For this purpose, the protoplasts were transfected with either pPZP-AV1 alone or together with p35S-*TrAP*, a plasmid expressing *TrAP* under 35S promoter. A plasmid constitutively expressing the *CAT* gene (p35S-*CAT*) was cotransfected to serve as an internal control and to

Fig. 1. T-DNA regions of the binary vectors used for transfection of *Nicotiana plumbaginifolia* protoplasts. **A**, The plasmid pPZP-AV1 harbors AV1 promoter (420 bp) fused to int- β -glucuronidase (*GUS*) in the binary vector pPZP211. It has the 35S promoter-driven *NPTII* gene as a plant transformation marker. **B**, The plasmid pPZP Δ 35S-AV1 harbors AV1-*GUS* fusion in pPZP211 with a promoter-less *NPTII* gene. RB: T-DNA border-right, int: bean catalase intron, NOS 3': nopaline synthase polyadenylation signal, p35S: *Cauliflower mosaic virus* (CaMV) 35S promoter, *NPTII*: neomycin phosphotransferase II gene, 35S 3': CaMV 35S polyadenylation signal, LB: T-DNA border-left.

Fig. 2. Transactivation of AV1 promoter by transcriptional activator protein (TrAP) in transgenic tobacco. **A**, Plasmid pGA-AV1 harbors AV1 promoter (420 bp) fused to int- β -glucuronidase (*GUS*) in pGA472, a binary vector with pNOS-driven *NPTII* as plant transformation marker. **B**, Plasmid pCAM-*TrAP* harbors *TrAP* gene of *Mungbean yellow mosaic virus* (MYMV)-Vig under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter and hygromycin phosphotransferase gene (*HPH*) as plant transformation marker. RB: T-DNA border-right, pNOS: nopaline synthase gene promoter, *NPTII*: neomycin phosphotransferase II gene, NOS 3': nopaline synthase polyadenylation signal, int: bean catalase intron, p35S: CaMV 35S promoter, *TrAP*: MYMV-Vig *TrAP* gene, 35S 3': CaMV 35S polyadenylation signal, LB: T-DNA border-left. Probes used are marked in dotted lines. The junction fragment sizes and internal T-DNA fragment (*TrAP*) size are marked below the arrows. **C**, Southern blot analysis of tobacco plants transformed with pGA-AV1. Total DNA from 11 transformed plants that regenerated and rooted under kanamycin selection was digested with *HindIII* and probed with *NPTII* coding sequence. Undigested DNA from one of the transgenic plants (U) and DNA from nontransgenic control tobacco plant digested with *HindIII* (C) were used as controls. λ /*HindIII* was used as marker. Junction fragments of >2.4 kb from transgenic plants are expected to hybridize. **D** and **E**, Southern blot analysis of AV1-*GUS* plants supertransformed with pCAM-*TrAP*. Total DNA from six plants of line T1 (1.1 to 1.6), three plants of line T3 (3.1 to 3.3), three plants of line T4 (4.1 to 4.3), and three plants of line T10 (10.1 to 10.3) supertransformed with pCAM-*TrAP* was digested with *EcoRI* and probed with *HPH* coding sequence. Junction fragments of >2.3 kb from transgenic plants are expected to hybridize. **F**, Southern blot analysis of T1.1 plant with *TrAP* probe. DNA from T1.1 was digested with *EcoRI* and probed with *TrAP* coding sequence. The binary plasmid pCAM-*TrAP* digested with *EcoRI* (B) was used as a positive control. A 1-kb ladder (Invitrogen, Carlsbad, CA, U.S.A.) was used as marker (M). An internal *EcoRI* T-DNA fragment of 0.4 kb is expected to hybridize from transgenic plants and the binary plasmid control (B). **G**, Northern blot analysis of T1.1 plant. Total RNA samples from the untransformed control (C), parent (T1), and a plant supertransformed with *TrAP* (T1.1) were electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *TrAP* coding sequence. The 18S rRNA portion of the ethidium-bromide-stained gel is placed at the bottom to show equal loading of RNA in all the lanes. Sizes of an RNA standard are marked on the left.

normalize GUS expression levels. The AV1-*GUS* fusion in pPZP-AV1 showed a low basal GUS activity and was activated fourfold by TrAP (Table 1). A basal level of GUS expression also was observed in tobacco plants stably transformed with pPZP-AV1 (data not shown). It is likely that the enhancer element of the 35S promoter of the *NPTII* gene in pPZP-AV1 contributed to the moderate basal activity of AV1-*GUS*, a problem previously reported by Yoo and associates (2005).

To check this possibility, the 35S promoter of the *NPTII* gene in pPZP-AV1 was deleted (Fig. 1B). As expected, the basal (TrAP-independent) AV1-*GUS* activity was reduced (11-fold) in *N. plumbaginifolia* protoplasts upon deletion of the 35S promoter of *NPTII* gene. Nevertheless, the AV1-*GUS* gene in pPZPA35S-AV1 was activated 4.3-fold in the presence of TrAP (Table 1). To obtain tighter control of AV1-*GUS* expression, the AV1-*GUS* fusion was subcloned in the pGA472 binary vector with the *NPTII* gene under the control of *NOS* promoter (pGA-AV1) (Fig. 2A). Tobacco plants transformed with pGA-AV1 were selected on kanamycin and integration of the T-DNA was confirmed by Southern blotting (Fig. 2C). Of the 11 plants transformed with pGA-AV1, 10 showed very weak or no TrAP-independent GUS expression and 1 plant (T10) had moderate GUS activity (data not shown). We found that plants T1, T3, T4, and T8 had single T-DNA insertions, whereas other plants had two or more insertions.

To study transactivation of the integrated AV1-*GUS*, T1, T3, T4, and T10 plants were supertransformed with *Agrobacterium* spp. harboring the binary plasmid pCAM-TrAP (Fig. 2B), which contains a hygromycin phosphotransferase gene (*HPH*) as plant transformation marker and 35S-*TrAP*. Six supertransformed plants were obtained for T1 and three each for the plants T3, T4, and T10. All 15 supertransformed plants had one to two copies of the *HPH* gene (Fig. 2D and E). Unexpectedly, polymerase chain reaction (PCR) analysis showed that only one of them (T1.1) had the *TrAP* gene (data not shown). To check the presence of intact *TrAP* in the T1.1 plant, its DNA was digested with *EcoRI* and probed with the *TrAP*-coding sequence. Because there are two *EcoRI* sites flanking the *TrAP* gene in pCAM-TrAP (Fig. 2B), the presence of intact *TrAP* coding sequence will result in the hybridization of an internal T-DNA fragment of 0.4 kb. Any truncation in *TrAP* prior to T-DNA integration will result in the formation of a direct junction between *TrAP* and plant DNA. Consequently, *EcoRI* digestion will release a *TrAP*-hybridizing junction frag-

ment larger or smaller than 0.4 kb. Hybridization of *TrAP* to a 0.4-kb fragment in the plant T1.1 (Fig. 2F) confirmed that an intact *TrAP* is integrated. In a Northern hybridization analysis with *TrAP*, the plant T1.1 was found to accumulate the expected approximately 0.7-kb *TrAP* transcript (Fig. 2G). Furthermore, sequencing of a fragment obtained by reverse-transcription PCR confirmed the presence of the wild-type *TrAP* sequence (data not shown). Fluorometric GUS assay revealed a 2.5-fold increase in GUS-specific activity (data not shown), confirming its transactivation function. None of the other supertransformed plants showed an increase in GUS staining (data not shown).

Expression of TrAP causes toxic effect in transgenic plants.

To further evaluate why a large number of *TrAP*-transformed plants show deletion of *TrAP* in the integrated T-DNA, *N. tabacum* plants were transformed with a binary vector containing the MYMV 35S-*TrAP* gene flanked by a 35S-*BAR* gene and 35S-*GUS* gene (pCAM-TrAP-GUS) (Fig. 3A). In this configuration of the T-DNA, phosphinothricin (PPT)-resistant and GUS-positive plants are expected to have the intact T-DNA, including the MYMV *TrAP*. Southern blot analysis (Fig. 3B) of 10 PPT-resistant plants (W1 to W10) with the *BAR* probe showed that all of them had one or more copies of the *BAR* gene (Table 2). Junction fragment analysis revealed that W5/W6/W8 and W9/W10 were independent plants that regenerated from the same transformation event. Southern blot analysis with the *TrAP* probe (Fig. 3C) showed that only one of the transformed plants (W2) had the intact *TrAP* gene and only two of them (W1 and W2) had the intact *GUS* gene (data summarized in Table 2). W2 also had a second insert with a truncated version of *TrAP*.

The plant W2 was subjected to Northern hybridization analysis with the *TrAP* probe to check whether it was recovered only because *TrAP* was not expressed. The RNA from the known *TrAP*-expressing plant T1.1 (Fig. 2G) hybridized to *TrAP*, but no hybridization was seen for the plant W2 (Fig. 3D). Although W1, which had the *GUS* gene (Table 2), showed GUS activity in histochemical staining (data not shown), W2 had the *GUS* gene but did not show GUS activity (data not shown). Digestion of DNA of the plant W2 with methylation-sensitive enzymes *AluI* and *MspI* and Southern hybridization analysis with 35S promoter probe showed incomplete digestion (data not shown), suggesting methylation of the 35S promoter and consequent silencing of *TrAP*. In summary, upon transformation with *TrAP*, only transgenic plants with truncated, missing, or silenced *TrAP* were recovered. These observations provide a strong evidence for the toxicity of *TrAP* in plants.

The three functional domains of TrAP are associated with toxicity.

Mutations in the three essential domains of *TrAP* had been constructed and analyzed earlier (Trinks et al. 2005). In Δ NLS, three basic amino acids (KKR) within the N-terminal basic domain were replaced by alanine residues destroying the NLS. In Δ ZF, the first two cysteine residues of the central zinc-finger motif were replaced by alanine residues and, in Δ AD, amino acids 105 to 135 within the C-terminal acidic activation domain (Hartitz et al. 1999) were deleted. These mutant *TrAP* genes were subcloned in the binary plasmid pCAMBIA1300 to yield pCAM- Δ ZF (Fig. 4A), pCAM- Δ NLS (Fig. 5A), and pCAM- Δ AD (Fig. 6A).

In all, 10 transgenic plants (Z1 to Z10) were obtained by transformation with pCAM- Δ ZF, 13 (N1 to N13) with pCAM-NLS, and 12 (A1 to A12) with pCAM- Δ AD. Southern blot analysis showed that all of these had one to several integrated copies of the T-DNA with the *HPH* gene (Figs. 4B, 5B, and 6B)

Table 1. Transactivation of AV1- β -glucuronidase (*GUS*) fusion by 35S-transcriptional activator protein (*TrAP*) gene in *Nicotiana plumbaginifolia* protoplasts^a

AV1- <i>GUS</i>		Δ 35S AV1- <i>GUS</i>	
- <i>TrAP</i>	+ <i>TrAP</i>	- <i>TrAP</i>	+ <i>TrAP</i>
76 $\pm$ 31	302 $\pm$ 132	7 $\pm$ 3	30 $\pm$ 7

^a GUS activity of AV1 promoter fusion in pPZP-AV1 (AV1-*GUS*) and pPZPA35S-AV1 (Δ 35S-AV1-*GUS*) is shown in the absence and presence of the 35S-*TrAP*. GUS activity is shown as mean percentage value normalized using CAT levels. GUS values are represented as mean $\pm$ standard deviation of three replicate assays.

Table 2. Summary of Southern blot analysis of tobacco plants transformed with pCAM-transcriptional activator protein- β -glucuronidase

Gene	Plant number							Total plants (%)
	W1	W2	W3	W4	W5	W7	W10	
<i>BAR</i>	+	+	+	+	+	+	+	100
<i>GUS</i>	+	+	-	-	-	-	-	28.5
<i>TrAP</i>	-	+	-	-	-	-	-	14

and most of them with the intact *TrAP* mutant (Figs. 4C, 5C, and 6C) in obvious contrast to the wild-type *TrAP* experiments (Fig. 3C). Exceptions were Z8 and N2, where the mutated *TrAP* was deleted and N6, A6, and A7, where it was truncated.

Northern analysis was carried out in a selection of these transgenic plants (with one to three copies of the T-DNA) (Fig. 7A and B). Except for plant N7 (Δ NLS), all of the analyzed plants accumulated the *TrAP* transcript, suggesting that the mutated *TrAP* genes were transcribed well in these transgenic

plants. Obviously, the expression of the mutated *TrAP* gene did not cause any toxic effect in the transgenic plants.

DISCUSSION

We used tobacco as a model system to study the transactivation of the integrated AV1-*GUS* gene by TrAP in a transgenic plant. pPZP-AV1-transformed tobacco plants unexpectedly showed a moderate amount of TrAP-independent GUS activ-

Fig. 3. Analysis of tobacco plants transformed with wild-type transcriptional activator protein (*TrAP*) gene. **A**, pCAM-Trap- β -glucuronidase (*GUS*) harbors wild-type *TrAP* in pCAMBIA3301. It has the *BAR* gene as plant transformation marker and *GUS* gene as reporter gene. **B**, Southern blot analysis of tobacco plants transformed with pCAM-Trap-GUS. Total DNA from 10 transformed plants that regenerated and rooted under phosphinothricin selection (W1 to W10) was digested with *Eco*RI and probed with *BAR* coding sequence. Undigested DNA from one of the transgenic plants (U) and DNA from nontransgenic control tobacco plant digested with *Eco*RI (C) were used as controls. λ /*Hind*III was used as marker. Junction fragments of >1.84 kb from transgenic plants are expected to hybridize. Results showed that plants W5/W6/W8 and W9/W10 were independent plants that regenerated from the same transformation event. The total number of independently transformed plants is seven. **C**, DNA from plants W1 to W10 was digested with *Eco*RI and probed with *TrAP* coding sequence. The binary plasmid pCAM-Trap-GUS digested with *Eco*RI (P) was used as a positive control. λ /*Hind*III was used as marker. An internal T-DNA fragment of 0.4 kb from transgenic plants and the binary plasmid control (B) is expected to hybridize. **D**, Northern blot analysis of pCAM-Trap-GUS-transformed plant W2. Total RNA samples from nontransgenic control plant (C) and W2 were electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *TrAP* coding sequence. RNA from a transgenic tobacco plant which transcribed the *TrAP* gene was loaded as positive control (P). The 18S rRNA portion of the ethidium-bromide-stained gel is placed at the bottom to show the equal loading of RNA in all the lanes. Sizes of an RNA standard are marked on the left.

ity. This basal activity, which was due to the enhancer element of the 35S promoter of the *NPTII* gene, was abolished when the 35S promoter was replaced by the *NOS* promoter. This finding agrees with the earlier report of Yoo and associates (2005) and highlighted the importance of avoiding the presence of the 35S promoter in the same T-DNA while evaluating the properties of new promoters in transgenic plants. In the present study, we demonstrated that an integrated copy of the *TrAP* gene transactivates the AV1 promoter in a transgenic plant. The degree of activation of the AV1 promoter in the transgenic plant T1.1 is relatively low (2.5-fold) but similar to those reported in protoplast systems (Frey et al. 2001; Haley et al. 1992; Sunter and Bisaro 1992; Sunter et al. 1994).

TrAP of ACMV (Hong et al. 1996, 1997), TYLCV-C (van Wezel et al. 2001, 2002), TGMV, and BCTV (Sunter et al.

2001) are reported to function as pathogenicity determinants, perhaps connected to their function as silencing suppressors. *TrAP* of TGMV causes enhanced susceptibility to the virus in transgenic plants by inactivating SNF1 kinase and redirecting plant metabolism (Hao et al. 2003). The toxicity of MYMV *TrAP*, interfering with the recovery of *TrAP*-expressing transgenic tobacco plants, may be a reflection of the pathogenicity factor of *TrAP*. All transformants obtained with a plasmid containing the *TrAP* gene in addition to a selection marker had integrated T-DNAs in which the *TrAP* gene was either deleted or truncated or, if present in an intact form, was not expressed. Based on these results, it is clear that *TrAP* is inherently toxic to plants.

As reported earlier (Trinks et al. 2005), mutations in ZF, NLS, and AD of *TrAP* abolished the activity of *TrAP* as a transcrip-

Fig. 4. Analysis of tobacco plants transformed with transcriptional activator protein (*TrAP*) gene containing mutation in zinc finger (ZF). **A**, pCAM-ΔZF has *TrAP* gene with a mutation in the ZF motif cloned in pCAMBIA1300. Plasmids pCAM-ΔZF, pCAM-ΔNLS, and pCAM-ΔAD have hygromycin phosphotransferase gene (*HPH*) as the plant transformation marker. RB: T-DNA border-right, p35S: *Cauliflower mosaic virus* (CaMV) 35S promoter, 35S 3': CaMV 35S polyadenylation signal, LB: T-DNA border-left. Probe used is marked in dotted lines. The junction fragment sizes and internal T-DNA (*TrAP*) size are marked below the arrows. **B**, Southern blot analysis of tobacco plants transformed with pCAM-ΔZF. Total DNA from 10 transformants that regenerated and rooted under hygromycin selection (Z1 to Z10) was digested with *EcoRI* and probed with *HPH* coding sequence. Undigested total DNA from one of the transgenic plants (U) and total DNA from the nontransgenic control tobacco plant digested with *EcoRI* (C) were used as controls. λ /HindIII was used as marker. Junction fragments of >2.1 kb from transgenic plants are expected to hybridize. **C**, Southern blot analysis of tobacco plants transformed with pCAM-ΔZF using *TrAP* probe. DNA from 10 Southern-positive plants (Z1 to Z10) was digested with *EcoRI* and probed with *TrAP* coding sequence. The binary plasmid ΔpCAM-ΔZF digested with *EcoRI* (B) was used as a positive control. An internal T-DNA fragment of 0.4 kb from transgenic plants and the binary plasmid control (B) is expected to hybridize.

tional activator and a silencing suppressor. The same mutations also relieved its toxicity, suggesting that the three activities (gene activation, silencing suppression, and toxicity) are linked.

Constitutive expression of HcPro of *Turnip mosaic virus*, p19 of *Tomato bushy stunt virus*, and many other silencing suppressors caused developmental defects resembling the pathogenicity of the virus (Chapman et al. 2004; Dunoyer et al. 2004; Kasschau et al. 2003). The pathogenicity is attributed to the interference of silencing suppressors in the miRNA pathway (Kasschau et al. 2003; Silhavy and Burgyan 2004). Similarly, interference with the miRNA-controlled pathways could be the mechanism through which TrAP controls pathogenicity and symptom development. Additionally, transcriptome analysis (Trinks et al. 2005) showed that MYMV TrAP induced expression of a large number of host genes and upregu-

lation of one or many of these host genes also could have resulted in toxicity.

Also, TrAP of other begomoviruses (TGMV and ACMV) have been proposed to have toxic effects and difficulties have been reported in generating transgenic plants with them (Hao et al. 2003; Hartitz et al. 1999; Hong et al. 1997; Sunter et al. 2001). Contrary to these results, Chellappan and associates (2005) reported that *Arabidopsis* plants transformed with TrAP of *East African cassava mosaic virus* (EACMV) did not have any toxic effect and had a normal phenotype. However, in this case, it was not analyzed whether the TrAP gene remained intact and active.

This study raises the interesting question of how plants cope with the extreme toxicity of TrAP during MYMV infections. It is likely that TrAP may be accumulating at low, subtoxic levels

Fig. 5. Analysis of tobacco plants transformed with transcriptional activator protein (TrAP) gene containing mutation in nuclear localization signal. **A**, pCAM-ΔNLS has TrAP gene with a mutation in the nuclear localization signal in pCAMBIA1300. **B**, Southern blot analysis of tobacco plants transformed with pCAM-ΔNLS. Total DNA from 13 transformed plants that regenerated and rooted under hygromycin selection (N1 to N13) was digested with *EcoRI* and probed with hygromycin phosphotransferase gene coding sequence. Undigested DNA from one of the transgenic plants (U) and DNA from the nontransgenic control tobacco plant digested with *EcoRI* (C) were used as controls. λ /HindIII was used as marker. Junction fragments of >2.1 kb from transgenic plants are expected to hybridize. **C**, Southern blot analysis of tobacco plants transformed with pCAM-ΔNLS. DNA from 13 hygromycin-resistant Southern-positive plants (N1 to N13) was digested with *EcoRI* and probed with TrAP coding sequence. An internal T-DNA fragment of 0.4 kb from transgenic plants and the binary plasmid control (B) is expected to hybridize.

Fig. 6. Analysis of tobacco plants transformed with transcriptional activator protein (*TrAP*) gene with deletion of the activation domain. **A**, pCAM-ΔAD has *TrAP* gene with a deletion of the activation domain in pCambia1300. **B**, Southern blot analysis of tobacco plants transformed with pCAM-ΔAD. DNA from 12 transformed plants (A1 to A12) that regenerated and rooted under hygromycin selection was digested with *EcoRI* and probed with hygromycin phosphotransferase gene coding sequence. Junction fragments of >2.1 kb from transgenic plants are expected to hybridize. **C**, Southern blot analysis of tobacco plants transformed with pCAM-ΔAD. DNA from 12 Southern-positive plants (A1 to A12) was digested with *EcoRI* and probed with *TrAP* coding sequence. An internal T-DNA fragment of 0.3 kb is expected to hybridize from transgenic plants and the binary plasmid control (B).

in MYMV-infected plants. Alternatively, TrAP in MYMV-infected plants may be sequestered by multiple copies of AV1 and BV1 promoters and, thus, may be diverted from its toxic effect. This could explain how we managed to obtain a TrAP-expressing transgenic plant T1.1 which harbored both AV1 promoter and *TrAP* gene. Additionally, expression of TrAP in rapidly dividing cells during the callus phase of growth in tobacco transformation could be toxic, as against whole plants infected with the virus. The toxic nature of MYMV TrAP can find applications as a negative selection marker in gene targeting (Terada et al. 2004) and for the elimination of transgenic events with “long T-DNA transfers” in which binary vector backbone is transferred to the plants (Hanson et al. 1999).

MATERIALS AND METHODS

Plasmid constructs.

The binary plasmid pPZP-AV1 was constructed as follows: A 2.1-kb *SalI/EcoRI* fragment containing the second exon of

the *GUS* gene and the *NOS* terminator was subcloned from pIG221 (Ohta et al. 1990) into the corresponding sites of pUC18 (pUC-GUS). A 223-bp fragment containing the first exon of *GUS* and the bean catalase intron was PCR amplified (cycling conditions: 30 cycles of 95°C for 40s, 54°C for 40s, and 72°C for 1 min and a final extension of 72°C for 5 min) from pIG221 with oligonucleotides 5'-agagcatgcacccctacagg 3' (with a *SphI* site, underlined) and 5'-cgagtcgacgtttctgtaac 3' (with a *SalI* site, underlined). After verifying the sequence, this PCR product was digested with *SphI* and *SalI* and cloned upstream of the second exon of *GUS* gene in pUC-GUS to yield pUC-GUS-int. The 434-bp AV1 promoter fragment between ATG start codon of AC1 (at position 2,599) and the AV1 ATG (at position 307) was PCR amplified (cycling conditions: 30 cycles of 95°C for 40s, 54°C for 40s, and 72°C for 1 min and a final extension of 72°C for 5 min) from MYMV DNA A (GenBank accession number AJ132575) with oligonucleotides 5'-gtctaagcgtattttggcg 3' (with a *HindIII* site, underlined) and 5'-ttggcatgcttcctgtatc 3' (with a *SphI* site, underlined). After veri-

Fig. 7. Northern blot analysis of tobacco plants transformed with transcriptional activator protein (*TrAP*) mutants. **A**, Total RNA from the nontransgenic plant (C) and five plants (Z1, Z10, Z4, Z5, and Z7) transformed with pCAM- Δ ZF was electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *TrAP* coding sequence. A tobacco plant which expresses *TrAP* gene was loaded as a positive control (P). **B**, Total RNA from four plants each of pCAM- Δ NLS-transformation and pCAM- Δ AD-transformation was electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *TrAP* coding sequence. The 18S rRNA portion of the ethidium-bromide-stained gel is placed at the bottom to show equal loading of RNA in all the lanes. Sizes of an RNA standard (Ambion, Austin, TX, U.S.A.) are marked on the left.

fication of the sequence, this PCR product was digested with *Hind*III and *Sph*I and cloned upstream of the first exon of *GUS* in pUC-*GUS*-int to create pUC-AV1-*GUS*. A 2.7-kb AV1-*GUS* cassette, as an *Eco*RI/*Hind*III fragment, was subcloned from pUC-AV1-*GUS* into the corresponding sites of pBSIIKS⁺ to yield pBS-AV1-*GUS*. The 2.7-kb AV1-*GUS* cassette, as an *Eco*RI/*Kpn*I fragment, was cloned into the corresponding sites of pPZP211 (Hajdukiewicz et al. 1994) to yield pPZP-AV1. pPZP Δ 35S-AV1 was constructed by digestion of pPZP-AV1 with *Bgl*II and *Eco*RI, end filled, and self-ligated to remove the 1,049-bp fragment containing the 35S promoter. pGA-AV1 was constructed by subcloning the 2.7-kb AV1-*GUS* cassette as a *Sma*I/*Kpn*I fragment into the *Kpn*I site and an end-filled *Xho*I site of pGA472 (An et al. 1985). pCAM-*TrAP* was constructed as follows: a 477-bp *Scal*/*Dra*I fragment from MYMV DNA A (coordinates 1,194 to 1,671) containing the *TrAP* coding sequence was subcloned into the *Sma*I site of pJIC35S cassette (Hellens et al. 2000) to yield p35S-*TrAP*. A 1.15-kb *Eco*RV fragment with 35S-*TrAP* cassette was subcloned into the *Sma*I site of pCAMBIA1300 to yield pCAM-*TrAP*. pCAM-*TrAP*-*GUS* was constructed by subcloning the 1.15-kb 35S-*TrAP* cassette as an *Eco*RV fragment from p35S-*TrAP* into the *Sma*I site of pCAMBIA3301. *TrAP* constructs with mutations in NLS, ZF, and AD have been described previously (Trinks et al. 2005). The binary plasmids were mobilized from *Escherichia coli* into *Agrobacterium tumefaciens* LBA4404 by triparental mating and mobilizations were confirmed by Southern blot analysis.

Tobacco transformation and transfection of protoplasts.

Tobacco (*N. tabacum* L. cv. Wisconsin 38) leaf discs were transformed using *Agrobacterium* spp. as described earlier (Sunilkumar et al. 1999). Transgenic shoots were rooted in BGS medium (Murashige-Skoog salts, 0.001% folic acid, myoinositol at 100 mg/liter, thiamine at 0.4 mg/liter, 0.057 μ M indole-3-acetic acid, 0.14 μ M kinetin, 3% [wt/vol] sucrose, 0.9% [wt/vol] agar, pH 5.7) supplemented with cefotaxime at 250 mg/liter and kanamycin at 50 mg/liter (for pPZP-AV1 and pGA-AV1), hygromycin at 50 mg/liter (for pCAM-*TrAP*, pCAM- Δ ZF, pCAM- Δ NLS, and pCAM- Δ AD) or PPT at 5 mg/liter (for pCAM-*TrAP*-*GUS*). Transient expression in *N. plumbaginifolia* leaf protoplasts was performed as described by Trinks and associates (2005). GUS histochemical staining

and GUS fluorometric assay were performed as described by Sunilkumar and associates (1999).

Southern and Northern blot analysis.

Total DNA was extracted as described by Rogers and Bendich (1994). Plant DNA was estimated using a Hoefer DNA fluorometer (DyNA Quant 200) using Hoechst dye 33258. DNA samples (10 μ g) from control and transgenic tobacco plants were digested with appropriate enzymes, electrophoresed in 0.8 or 1% agarose gels in 1 $\times$ Tris-borate-EDTA, and subjected to Southern blot analysis. Total RNA extraction and Northern hybridization analysis was carried out as described by Pawlowski and associates (1994).

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LITERATURE CITED

- An, G., Watson, B. D., Stachel, S., Gordon, M. P., and Nester, E. W. 1985. New cloning vehicles for transformation of higher plants. EMBO (Eur. Mol. Biol. Organ.) J. 4:277-284.
- Arguello-Astorga, G. R., Guevara-Gonzalez, R. G., Herrera-Estrella, L. R., and Rivera-Bustamante, R. F. 1994. Geminivirus replication origins have a group-specific organization of iterative elements: A model for replication. Virology 203:90-100.
- Bisaro, D. M. 2006. Silencing suppression by geminivirus proteins. Virology 344:158-168.
- Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. V., and Carrington, J. C. 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev. 18:1179-1186.
- Chellappan, P., Vanitharani, R., and Fauquet, C. M. 2005. MicroRNA-binding viral protein interferes with *Arabidopsis* development. Proc. Natl. Acad. Sci. U.S.A. 102:10381-10386.
- Dong, X., van Wezel, R., Stanley, J., and Hong, Y. 2003. Functional characterization of the nuclear localization signal for a suppressor of post-transcriptional gene silencing. J. Virol. 77:7026-7033.
- Dry, I., Krake, L., Mullineaux, P., and Rezaian, A. 2000. Regulation of to-

- mato leaf curl viral gene expression in host tissues. *Mol. Plant-Microbe Interact.* 13:529-537.
- Dunoyer, P., Lecellier, C. H., Parizotto, E. A., Himber, C., and Voinnet, O. 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* 16:1235-1250.
- Fauquet, C. M., and Stanley, J. 2005. Revising the way we conceive and name viruses below the species level: A review of geminivirus taxonomy calls for new standardized isolate descriptors. *Arch. Virol.* 150:2151-2179.
- Frey, P. M., Schärer-Hernandez, N. G., Futterer, J., Potrykus, I., and Puonti-Kaerlas, J. 2001. Simultaneous analysis of the bidirectional *African cassava mosaic virus* promoter activity using two different luciferase genes. *Virus Genes* 22:231-242.
- Hajdukiewicz, P., Svab, Z., and Maliga, P. 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* 25:989-994.
- Haley, A., Zhan, X., Richardson, K., Head, K., and Morris, B. 1992. Regulation of the activities of *African cassava mosaic virus* promoters by the AC1, AC2, and AC3 gene products. *Virology* 188:905-909.
- Hanson, B., Engler, D., Moy, Y., Newman, B., Ralston, E., and Gutterson, N. 1999. A simple method to enrich an *Agrobacterium*-transformed population for plants containing only T-DNA sequences. *Plant J.* 19:727-734.
- Hao, L., Wang, H., Sunter, G., and Bisaro, D. M. 2003. Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *Plant Cell* 15:1034-1048.
- Hartitz, M. D., Sunter, G., and Bisaro, D. M. 1999. The *Tomato golden mosaic virus* transactivator (TrAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain. *Virology* 263:1-14.
- Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S., and Mullineaux, P. M. 2000. pGreen: A versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* 42:819-832.
- Hong, Y., Saunders, K., Hartley, M. R., and Stanley, J. 1996. Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants. *Virology* 220:119-127.
- Hong, Y., Saunders, K., and Stanley, J. 1997. Transactivation of dianthin transgene expression by *African cassava mosaic virus* AC2. *Virology* 228:383-387.
- Karthikeyan, A. S., Vanitharani, R., Balaji, V., Anuradha, S., Thillaichidambaram, P., Shivaprasad, P. V., Parameswari, C., Balamani, V., Saminathan, M., and Veluthambi, K. 2004. Analysis of an isolate of *Mungbean yellow mosaic virus* (MYMV) with a highly variable DNA B component. *Arch. Virol.* 149:1643-1652.
- Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A., and Carrington, J. C. 2003. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Dev. Cell* 4:205-217.
- Krake, L. R., Rezaian, M. A., and Dry, I. B. 1998. Expression of the tomato leaf curl geminivirus C4 gene produces virus-like symptoms in transgenic plants. *Mol. Plant-Microbe Interact.* 11:413-417.
- Noris, E., Jupin, I., Accotto, G.P., and Gronenborn, B. 1996. DNA-binding activity of the C2 protein of tomato yellow leaf curl geminivirus. *Virology* 217:607-612.
- Ohta, S., Mita, S., Hattori, T., and Nakamura, K. 1990. Construction and expression in tobacco of a β -glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol.* 31:805-813.
- Pawlowski, K., Kunze, R., DeVries, S., and Bisseling, T. 1994. Isolation of total, poly(A) and polysomal RNA from plant tissues. Pages D5/1-D5/13 in: *Plant Molecular Biology Manual*. S. B. Gelvin and R. A. Schilperoort, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Qazi, J., Ilyas, M., Mansoor, S., and Bridson, R. W. 2007. Legume yellow mosaic viruses: Genetically isolated begomoviruses. *Mol. Plant Pathol.* 8:343-348.
- Rigden, J. E., Krake, L. R., Rezaian, M. A., and Dry, I. B. 1994. ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity. *Virology* 204:847-850.
- Rogers, S. O., and Bendich, A. J. 1994. Extraction of total cellular DNA from plants, algae and fungi. Pages D1:1-8 in: *Plant Molecular Biology Manual*. S. B. Gelvin and R. A. Schilperoort, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Ruiz-Medrano, R., Guevara-Gonzalez, R. G., Arguello-Astorga, G. R., Monsalve-Fonnegra, Z., Herrera-Estrella, L. R., and Rivera-Bustamante, R. F. 1999. Identification of a sequence element involved in AC2-mediated transactivation of the *Pepper huasteco virus* coat protein gene. *Virology* 253:162-169.
- Saunders, K., and Stanley, J. 1995. Complementation of *African cassava mosaic virus* AC2 gene function in a mixed bipartite geminivirus infection. *J. Gen. Virol.* 76:2287-2292.
- Shivaprasad, P. V., Akbergenov, R., Trinks, D., Rajeswaran, R., Veluthambi, K., Hohn, T., and Pooggin, M. M. 2005. Promoters, transcripts, and regulatory proteins of mungbean yellow mosaic geminivirus. *J. Virol.* 79:8149-8163.
- Silhavy, D., and Burgyn, J. 2004. Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends Plant Sci.* 9:76-83.
- Sunilkumar, G., Vijayachandra, K., and Veluthambi, K. 1999. Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. *Plant Sci.* 141:51-58.
- Sunter, G., and Bisaro, D. M. 1991. Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression. *Virology* 180:416-419.
- Sunter, G., and Bisaro, D. M. 1992. Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. *Plant Cell* 4:1321-1331.
- Sunter, G., and Bisaro, D. M. 1997. Regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): Evidence for activation and de-repression mechanisms. *Virology* 232:269-280.
- Sunter, G., and Bisaro, D. M. 2003. Identification of a minimal sequence required for activation of the *Tomato golden mosaic virus* coat protein promoter in protoplasts. *Virology* 305:452-462.
- Sunter, G., Stenger, D. C., and Bisaro, D. M. 1994. Heterologous complementation by geminivirus AL2 and AL3 genes. *Virology* 203:203-210.
- Sunter, G., Sunter, J. L., and Bisaro, D. M. 2001. Plants expressing *Tomato golden mosaic virus* AL2 or Beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. *Virology* 285:59-70.
- Terada, R., Asao, H., and Iida, S. 2004. A large-scale *Agrobacterium*-mediated transformation procedure with a strong positive-negative selection for gene targeting in rice (*Oryza sativa* L.). *Plant Cell Rep.* 22:653-659.
- Trinks, D., Rajeswaran, R., Shivaprasad, P. V., Akbergenov, R., Oakeley, E. J., Veluthambi, K., Hohn, T., and Pooggin, M. M. 2005. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J. Virol.* 79:2517-2527.
- Vanitharani, R., Chellappan, P., and Fauquet, C. M. 2005. Geminiviruses and RNA silencing. *Trends Plant Sci.* 10:144-151.
- van Wezel, R., Dong, X., Liu, H., Tien, P., Stanley, J., and Hong, Y. 2002. Mutation of three cysteine residues in *Tomato yellow leaf curl virus-China* C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. *Mol. Plant-Microbe Interact.* 15:203-208.
- van Wezel, R., Liu, H., Tien, P., Stanley, J., and Hong, Y. 2001. Gene C2 of the monopartite geminivirus *Tomato yellow leaf curl virus-China* encodes a pathogenicity determinant that is localized in the nucleus. *Mol. Plant-Microbe Interact.* 14:1125-1128.
- van Wezel, R., Liu, H., Wu, Z., Stanley, J., and Hong, Y. 2003. Contribution of the zinc finger to zinc and DNA binding by a suppressor of post-transcriptional gene silencing. *J. Virol.* 77:696-700.
- Voinnet, O., Pinto, Y. M., and Baulcombe, D. C. 1999. Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. U.S.A.* 96:14147-14152.
- Wang, H., Buckley, K. J., Yang, X., Buchmann, R. C., and Bisaro, D. M. 2005. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. *J. Virol.* 79:7410-7418.
- Yoo, S. Y., Bomblies, K., Yoo, S. K., Yang, J. W., Choi, M. S., Lee, J. S., Weigel, D., and Ahn, J. H. 2005. The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. *Planta* 221:523-530.