VirusDis. (January–June 2015) 26(1-2):55–61
DOI 10.1007/s13337-015-0251-2

ORIGINAL ARTICLE

Transgenic tobacco plants expressing siRNA targeted against the *Mungbean yellow mosaic virus* transcriptional activator protein gene efficiently block the viral DNA accumulation

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Received: 16 August 2014 / Accepted: 18 March 2015 / Published online: 18 April 2015
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Abstract *Mungbean yellow mosaic virus* (MYMV) is a bipartite begomovirus that infects many pulse crops such as blackgram, mungbean, mothbean, Frenchbean, and soybean. We tested the efficacy of the transgenically expressed intron-spliced hairpin RNA gene of the transcriptional activator protein (hp*TrAP*) in reducing MYMV DNA accumulation. Tobacco plants transformed with the MYMV hp*TrAP* gene accumulated 21–22 nt siRNA. Leaf discs of the transgenic plants, agroinoculated with the partial dimers of MYMV, displayed pronounced reduction in MYMV DNA accumulation. Thus, silencing of the *TrAP* gene, a suppressor of gene silencing, emerged as an effective strategy to control MYMV.

Keywords Geminivirus · Hairpin RNA · RNA silencing · Silencing suppressor · siRNA · *TrAP*

Introduction

Mungbean yellow mosaic virus (MYMV) is a bipartite begomovirus species (family *Geminiviridae*) that causes severe losses in economically important pulse crops such as blackgram, mungbean, Frenchbean, mothbean, and soybean. Geminiviruses constitute a large group of plant viruses that possess unique geminate virion morphology and have a genome of one or two small circular single-stranded (ss) DNA molecules. They replicate in the nucleus through a double-stranded DNA intermediate using the rolling-circle replication mechanism [37].

The family *Geminiviridae* has seven genera: *Begomovirus, Mastrevirus, Curtovirus, Becurtovirus, Eragrovirus, Topocuvirus,* and *Turncurtovirus* classified on the basis of their genome structure, host range and insect vector [9, 13]. Members of the genus *Begomovirus* are either monopartite (one component of DNA A, ~2.9 kb) or bipartite (two components ~2.7 kb each of DNA A and DNA B). They infect dicotyledonous plants and are transmitted by whitefly [12]. MYMV is a bipartite begomovirus. DNA A of MYMV has six overlapping open reading frames (ORFs). Rep/AC1 (replication-associated protein), TrAP/AC2 (transcriptional activator protein), REn/AC3 (replication enhancer protein), and AC4 (silencing suppressor) are encoded on the complementary-sense strand. AV1 (coat protein), and AV2 (pre-coat protein) are on the virion-sense strand of DNA A. MYMV DNA B encodes two ORFs; BV1 (nuclear shuttle protein) in the virion-sense strand and BC1 (movement protein) in the complementary-sense strand [17, 20, 22]. DNA A is responsible for replication and encapsidation, whereas DNA B controls the virus movement in the host plant.

A number of strategies have been developed to engineer geminivirus resistance in transgenic plants. Expression of truncated/mutated/defective viral coat protein [19], movement protein [48], nuclear shuttle protein [15], and replication-associated protein [2, 24, 35] proved to be effective in developing geminivirus resistance. Antisense RNA-based approaches conferred resistance against many geminiviruses [3, 6, 8, 14, 52]. RNA silencing based on siRNA [46], and miRNA [29, 49] production in transgenic plants has gained interest following its efficiency in engineering resistance against plant viruses. Resistance against *Tomato yellow leaf curl virus* (TYLCV) [10], *Bean golden mosaic virus* (BGMV) [7], *African cassava*

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mosaic virus (ACMV) [44], and *Maize streak virus* (MSV) [25] were achieved by expressing the intron-spliced hairpin RNA genes of replication-associated protein. Coat protein [53], and *AC4* [28, 32, 40] hpRNA genes yielded resistance against geminiviruses. Intron-spliced hairpin RNA genes, comprising inverted repeats of the common region which harbour the bidirectional promoter, conferred a high degree of resistance in MYMV [27], and ACMV [45].

TrAP, a 15 kDa protein also known as AC2/AL2 in bipartite begomoviruses and as C2/L2 in monopartite begomoviruses, is a multifunctional protein. TrAP is a transcriptional factor which acts on the promoters of *AV1* and *BV1* [30, 34, 41, 43]. TrAP activates transcription of cellular genes that negatively regulate the silencing pathways [43]. TrAP inhibits transcriptional gene silencing by interacting with and inactivating adenosine kinase (ADK), resulting in the reduction of DNA methylation [50, 51].

Since TrAP acts as a suppressor of silencing besides functioning as a transcriptional activator, an RNA silencing strategy based on *TrAP* can be very effective in controlling geminiviruses. In the study reported here, we raised transgenic tobacco plants with the intron-spliced *TrAP* hairpin RNA gene (hp*TrAP*). The transgenic plants accumulated the *TrAP* siRNA. Agroinoculation of leaf discs of the transgenic tobacco plants with MYMV-[IN-Vig] partial dimers showed a pronounced reduction in MYMV DNA accumulation.

Materials and methods

Construction of pGA-hp*TrAP*

The P35S-hp*TrAP*-35S3′ cassette in pRT100 [42], comprising a 443-bp fragment of MYMV-*TrAP* gene (co-ordinates 1186–1629, accession number AJ132575.1) in sense and antisense orientations with an intervening 120-bp synthetic intron [11] was received from Thomas Hohn, University of Basel. The 1.15-kb hp*TrAP* cassette was excised as a *Sac*I fragment and subcloned into the *Sac*I site of pOK12. The 1.15-kb hp*TrAP* cassette was taken as a *Hin*dIII/*Bgl*II fragment and cloned in the corresponding sites of pGA643 [1] to yield pGA-hp*TrAP*. pGA-hp*TrAP* was mobilized into the *Agrobacterium tumefaciens* strain EHA105 by triparental mating. The presence of the complete binary plasmid in *Agrobacterium* was confirmed by Southern blot analysis.

Tobacco transformation

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin38) leaf discs were transformed using *A. tumefaciens* EHA105 (pGA-hp*TrAP*) as described by Sunilkumar et al. [38]. Transgenic shoots were selected on the shoot-induction medium (Murashige and Skoog [MS] salts [21], B5 vitamins, 0.5 μM NAA, 4 μM BAP, 3 % sucrose [*w/v*], 0.8 % agar [*w/v*], pH 5.7) containing 100 mg/l kanamycin and 250 mg/l cefotaxime. The shoots were kept for root induction on the plant establishment medium (MS salts, 1 mg/l folic acid, 100 mg/l myoinsitol, 0.4 mg/l thiamine, 0.057 μM indole-3-acetic acid, 0.14 μM kinetin, 3 % sucrose [*w/v*], 0.9 % agar [*w/v*], pH 5.7). The primary transformants were axenically maintained in a plant tissue culture room at 25 °C with a light intensity of 100 μE m^{-2} s^{-1} and with a light–dark cycle of 16 h/8 h. Plants were maintained by subculturing shoots on the plant establishment medium at 6-week intervals.

Southern and northern blot analysis

Plant DNA was extracted as described by Rogers and Bendich [33]. DNA concentration was estimated using the Hoechst dye 33258 in the DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, San Francisco, USA). Southern blot analysis of transgenic plants was done as described earlier [31, 36]. DNA samples (10 μg) from control and transgenic plants were digested with appropriate restriction enzymes and electrophoresed in a 0.8 % [*w/v*] agarose gel in 1x Tris–borate-EDTA (TBE) buffer. After ethidium bromide staining, DNA was alkali denatured and transferred to the Zeta-probe membrane (Bio-Rad Laboratories, Hercules, USA) and hybridized to [α-^{32}P]dCTP-labeled probes prepared using the Megaprime DNA labeling system (GE Healthcare UK Ltd., Little Chalfont, UK).

Viral DNA titre determination of agroinoculated leaf discs was done by electrophoresis of undigested plant DNA (5 μg) in a 0.8 % agarose [*w/v*] gel in 1x TNE (40 mM Tris–acetate, pH 7.5, 20 mM sodium acetate, 2 mM EDTA) buffer. Total RNA was extracted using the Tri Reagent (Sigma-Aldrich, St. Louis, USA) and northern blot analysis was carried out as described by Pawlowski et al. [26].

siRNA analysis

Total RNA was extracted and the small RNAs were fractionated using the RNA clean up protocol of the Midi RNeasy kit (Qiagen GmBH, Hilden, Germany). Small RNA fraction (20 μg) was loaded onto a 15 % polyacrylamide [*w/v*] gel with 8 M urea and was electrophoresed at 300 V. Small RNAs were blotted onto the positively charged nylon membrane (Roche Diagnostics, Indianapolis, USA) using the Trans blot-SD semidry transfer apparatus (Bio-Rad, Hercules, USA) in 1 × TBE at 7 V for 45 min. The membrane was crosslinked twice in a UV cross linker (Hoefer Scientific Instruments, San Francisco, USA). A 0.4-kb *TrAP* fragment was labeled with

[α-^{32}P]dCTP using the Megaprime DNA labeling system (GE Healthcare UK Ltd., Little Chalfont, UK) and hybridization was performed at 37 °C for 16 h. Post-hybridization washes were done four times (20 min each) with 2 × SSC/0.2 % SDS at 50 °C.

Agroinoculation

Tobacco leaf disc agroinoculation assay was done to study MYMV replication. Leaf discs (8 mm diameter) were cut from 6-week-old, axenically grown tobacco plants and were agroinoculated with the *A. tumefaciens* strain Ach5 harbouring the partial dimers of both DNA A and B of MYMV-[IN-Vig] [16, 35]. After 2-day co-cultivation, the leaf discs were transferred onto tobacco shoot induction medium supplemented with 250 mg/l cefotaxime. Total DNA of leaf discs was extracted 10 days post-agroinoculation, 5 µg DNA was electrophoresed in a 0.8 % agarose [*w/v*] gel in 1x TNE buffer and Southern blot analysis was done with the MYMV DNA A probe.

Results

Generation of *TrAP*-hpRNA transgenic tobacco plants

The binary plasmid pGA-hp*TrAP* harbours a *TrAP*-hpRNA cassette comprising the CaMV 35S promoter, MYMV-*TrAP* inverted repeat, a 120-bp synthetic intron and the 35S polyadenylation signal. The *neomycin phosphotransferase* II (*npt*II) gene under the transcriptional control of the *nopaline synthase* (*nos*) promoter served as the plant selectable marker (Fig. 1a). Tobacco transformation was performed with *A. tumefaciens* EHA105 (pGA-hp*TrAP*) and nine transgenic plants were established on the selection medium containing 100 mg/l kanamycin.

Southern blot analysis to study the T-DNA integration

Plant DNA was digested with *Hin*dIII and the blot was probed with the *npt*II gene. Junction fragments longer than 2.6 kb are expected to hybridize (Fig. 1a). All nine transgenic plants displayed one or more junction fragments (Fig. 1b). Plants *TrAP*-2, and -3 displayed junction fragments of the same size of 7.4 kb. Similarly, the plants *TrAP*-5, -7, and -9 displayed junction fragments of 5.2 kb. The results suggested that *TrAP*-2, and -3 may represent two plants which regenerated from one transformed callus. Similarly, plants *TrAP*-5, -7, and -9 may represent a second set of plants derived from one transformation event.

To further confirm the nature of integration events, junction fragment analysis was repeated by digesting the plant DNA with a second enzyme *Bgl*II. Junction fragments longer than 3.7 kb are expected to hybridize to the *npt*II probe (Fig. 1a). All nine transgenic plants displayed junction fragments longer than 3.7 kb (Fig. 1c). Plants *TrAP*-2, and -3 in one set and *TrAP*-5, -7, and -9 in a second set showed junction fragments of same sizes. On the basis of the two Southern blot analyses, plants *TrAP*-1, -2, -4, -5, -6, and -8 were inferred as independent transformation events and were taken up for MYMV agroinoculation studies.

It is important to check whether the transgenic plants have the complete hp*TrAP* gene since a high frequency of T-DNA truncations was reported in T-DNAs harbouring hpRNA genes [39]. Internal T-DNA fragment analysis was done to check whether the transgenic plants harboured the complete hp*TrAP* gene. A 1.1-kb fragment is expected to hybridize to the *TrAP* probe when the plant DNA is digested with *Hin*dIII + *Bgl*II (Fig. 1a). Eight out of nine transgenic plants displayed hybridization of the 1.1-kb fragment, which indicated the presence of the complete *TrAP* gene (Fig. 2). Plant *TrAP*-6 displayed a junction fragment instead of the 1.1-kb internal T-DNA fragment which indicated the presence of a truncated hp*TrAP* gene. *TrAP*-1, -2, -4, -5, and -8 are the five independent transgenic plants which harboured the complete hp*TrAP* gene. All hp*TrAP* transgenic plants, which were maintained axenically in tissue culture conditions for 10 cycles (6-week subculture period for each cycle), were phenotypically comparable to axenically-grown control plants.

siRNA analysis

Northern blot analysis was done to check siRNA accumulation in *TrAP*-1, -2, -4, -5, -6, and -8 plants which harboured the hp*TrAP* gene. The blot was probed with the *TrAP* coding sequence. The transgenic plants with the complete hp*TrAP* gene *TrAP*-1, -2, -4, -5, and -8 accumulated high levels of siRNA, whereas the plant hp*TrAP*-6, with a truncated hp*TrAP* gene, accumulated a lower level of siRNA (Fig. 3a).

MYMV replication assay

Although tobacco is not a natural host for MYMV, tobacco leaf discs agroinoculated with the partial dimers of MYMV accumulated the viral DNA [35]. Leaf discs of control and hp*TrAP*-transgenic plants were agroinoculated with the *A. tumefaciens* strain Ach5 which harboured the partial dimers of DNA A and B of MYMV. Ten days post-agroinoculation, total DNA extracted from leaf discs was

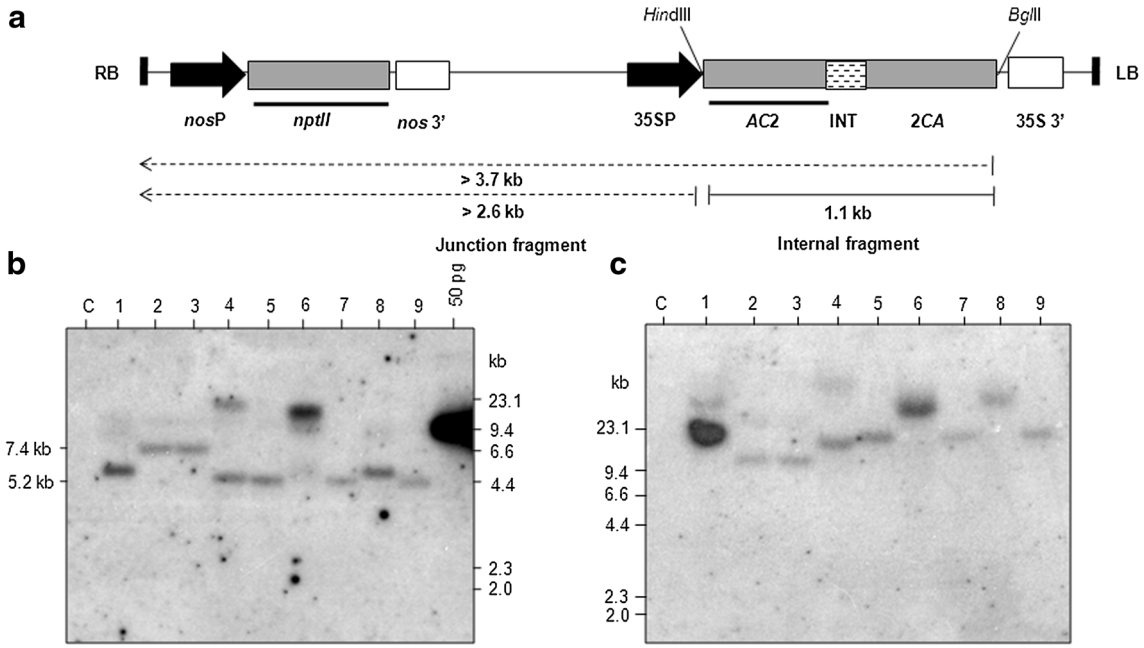

Fig. 1 Analysis of tobacco plants transformed with the binary vector pGA-hp*TrAP*. **a** T-DNA of pGA-hp*TrAP*. *RB* T-DNA border-right, *P35S Cauliflower mosaic virus* (CaMV) 35S promoter, *npt*II neomycin phosphotransferase II gene, *35S 3′* CaMV 35S polyadenylation signal, *nos 3′* nopaline synthase polyadenylation signal, *LB* T-DNA border-left. Junction fragment sizes (>2.6-kb and >3.7-kb) are marked with dashed lines with arrows. Probes are marked with the *bold lines*. **b** The *npt*II probe-based Southern blot analysis of tobacco plant DNA digested with *Hin*dIII. Binary plasmid (*50 pg*) digested with *Hin*dIII was used as the positive control. **c** The *npt*II probe-based Southern blot analysis of tobacco plant DNA digested with *Bgl*II. In **b** and **c**, total DNA (10 μg) from nine transformants hp*TrAP*-1 to hp*TrAP*-9 (*1–9*) was digested with *Hin*dIII or *Bgl*II and hybridization was carried out with the [α-^{32}P]dCTP-labeled *npt*II coding sequence. Total DNA from the control tobacco plant (*C*) digested with *Hin*dIII was used as the negative control

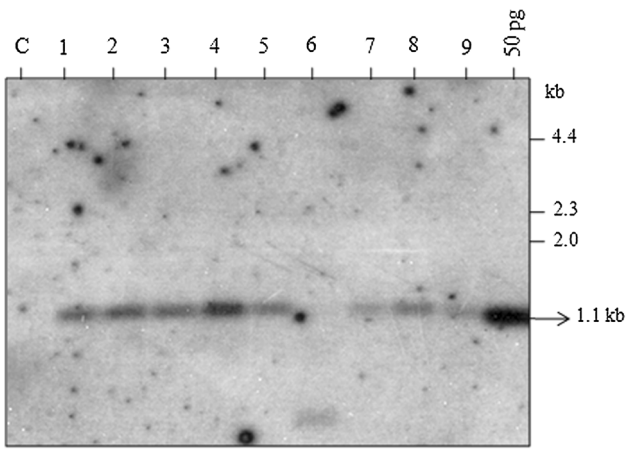

Fig. 2 Internal T-DNA fragment analysis of tobacco plants transformed with the binary plasmid pGA-hp*TrAP*. Total DNA from nine transgenic plants hp*TrAP*-1 to hp*TrAP*-9 (*1–9*) was double-digested with *Hin*dIII and *Bgl*II and the blot was probed with the *TrAP* gene. DNA from the control tobacco plant digested with *Hin*dIII and *Bgl*II (*C*) was used as the negative control. Binary plasmid (*50 pg*) digested with *Hin*dIII and *Bgl*II was used as the positive control. An internal T-DNA fragment of 1.1 kb from transgenic plants and the binary vector is expected to hybridize

subjected to Southern blot analysis with the MYMV DNA A probe. An intense band of 1.8 kb representing both the ss viral DNA, and the super-coiled (sc) double-stranded DNA (dsDNA) forms of the virus, was seen in the leaf discs upon agroinoculation of non-transformed, control tobacco plant (CI). However, the leaf discs of the hp*TrAP* transgenic plants did not display hybridization at the 1.8-kb position (Fig. 3b) suggesting very little or no accumulation of MYMV DNA in agroinoculated leaf discs. The agroinoculation experiment was repeated once and the same results were obtained.

Discussion

Yellow mosaic disease caused by MYMV and *Mungbean yellow mosaic India virus* (MYMIV) is a serious constraint in the cultivation of many pulse crops in India [47]. Gene silencing has been very successfully used to develop resistance against many geminiviruses [44]. Pooggin et al. [27] designed a hpRNA gene of the MYMV common region comprising the bidirectional promoter and showed

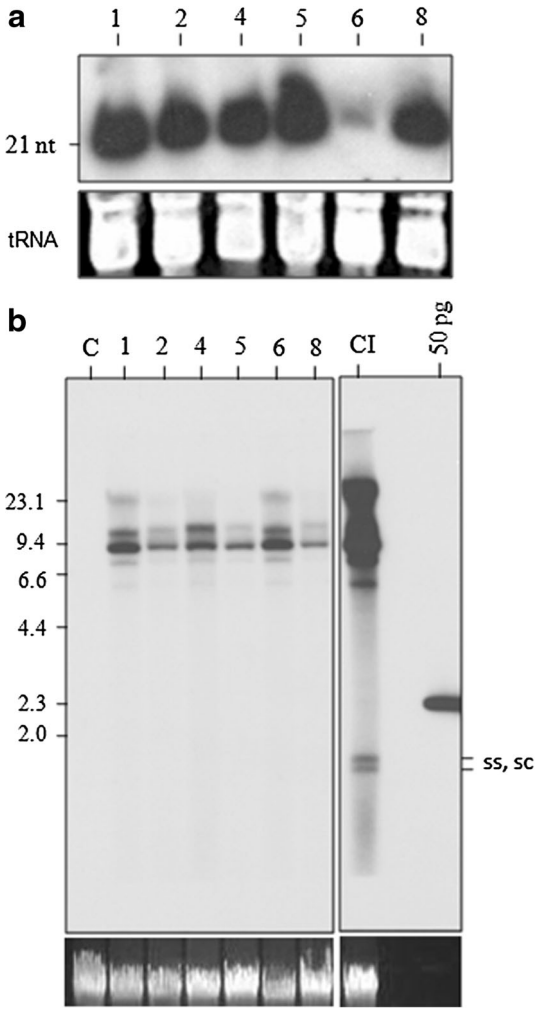

Fig. 3 **a** Northern blot analysis of siRNA in tobacco plants transformed with the binary vector pGA-hp*TrAP*. Low molecular weight RNA (20 μg) samples from six hp*TrAP* transgenic plants (*1, 2, 4, 5, 6,* and *8*) were analysed by hybridization with the [α-^{32}P]dCTP-labeled MYMV-*TrAP*. The tRNA portion of the ethidium bromide-stained gel (*tRNA*) is placed at the bottom to show equal loading of RNA in all lanes. **b** Southern blot analysis of MYMV-[IN:Vig] DNA A in agroinoculated leaf discs of control plants and transgenic tobacco plants which harboured the MYMV hp*TrAP* gene. Leaf discs of six hp*TrAP* transgenic plants (*1, 2, 4, 5, 6,* and *8*) and an untransformed control tobacco plant (*C*) were agroinoculated with MYMV DNA A and DNA B partial dimers. The blot with the total DNA from non-agroinoculated leaf discs of the control plant (*C*), agroinoculated leaf discs of the control plant (*CI*) and six hp*TrAP* transgenic plants (*1, 2, 4, 5, 6,*and *8*) was hybridized to the full-length DNA A (2.7 kb) probe. The positions of viral single-stranded DNA (*ss*), super-coiled, double-stranded DNA (*sc*) are indicated. *Agrobacterium* binary plasmid (*50 pg*) with the partial dimers of MYMV is marked. High molecular weight bands between the 9-kb and 23-kb positions of the gel represent the binary vector. The 23-kb portion of the ethidium bromide-stained gel is placed in the bottom panel to show equal loading of plant DNA in all lanes

that direct delivery of the hpRNA gene into the MYMV-infected blackgram plants brought about recovery from viral infection.

Agrobacterium-mediated blackgram transformation has yielded very limited success owing to difficulty in achieving regeneration. Therefore, *N. tabacum* was used as a model system in this study to evaluate whether the transgenically expressed hp*TrAP* gene of MYMV is effective in controlling MYMV infection. Six independent transgenic plants were identified by junction fragment analysis with the *npt*II probe (Fig. 1a). Five of the six transgenic plants (*TrAP*-1, -2, -4, -5, and -8) harboured the complete hp*TrAP* gene, whereas *TrAP*-6 harboured a truncated hp*TrAP* gene (Fig. 2). We previously reported a high frequency of hpRNA gene deletions in transgenic plants when both the selectable marker gene and the hpRNA gene were placed under the transcriptional control of the CaMV 35S promoter [39]. In this work, cloning the selectable marker gene (*npt*II) under the transcriptional control of the *nos* promoter and the hp*TrAP* gene downstream of the CaMV 35S promoter yielded a much higher number of transgenic plants with the complete hp*TrAP* gene.

Five transgenic plants *TrAP*-1, -2, -4, -5, and -8 with the complete hp*TrAP* gene accumulated high levels of siRNA (Fig. 3a), whereas the plant *TrAP*-6 with the truncated hp*TrAP* gene accumulated much lower levels of siRNA. Agroinoculation-based MYMV infection of transgenic tobacco leaf discs showed profound reduction of MYMV DNA accumulation in all six hp*TrAP* transgenic plants (Fig. 3b). Very interestingly, MYMV DNA accumulation was effectively reduced even in the plant *TrAP*-6 in which siRNA accumulation was much lower.

TrAP is a multifunctional protein. It transactivates the rightward promoters in both DNA A and DNA B [30, 41], suppresses RNA silencing [43, 51], regulates basal cell metabolism by interacting with SNF1 kinase and influences the methyl cycle [50]. Silencing of *TrAP* transgenic tobacco plants deprives the virus of all these key functions. As a consequence MYMV DNA accumulation is reduced in the leaf discs of all the hp*TrAP* transgenic plants. Recently, Krenz et al. [18] reported that *Abutilon mosaic virus TrAP* regulated viral DNA replication by acting as a replication brake, which in turn is counteracted by *AC3* (*REn*). In such a situation, it can be argued that *TrAP* silencing should have caused an increase in MYMV DNA accumulation. In view of the observation of Krenz et al. [18] that the effect of *TrAP* as a replication brake is counteracted by *AC3* (*REn*) (which is expressed from MYMV DNA A) and because *TrAP* plays a critical role as a transcriptional activator and suppressor of silencing, it is explainable that *TrAP* silencing causes a reduction in MYMV DNA accumulation.

Co-delivery of synthetic siRNA targeting the *Rep/AC*1 of ACMV in BY2 protoplasts reduced the viral mRNA and DNA levels [46]. Geminivirus resistance has been shown previously in transgenic plants expressing the hpRNA of

the *Rep* genes of TGMV [5], ACMV [46, 52], CLCV [4], ToLCV [28], and BGMV [7]. Rep, TrAP, REn, and AC4 are translated from the same transcript. Therefore, it is difficult to infer from the *Rep* hpRNA studies whether lowering viral DNA accumulation is achieved through the reduction of Rep, TrAP, REn or AC4 proteins.

Viruses deploy one or more viral suppressors of silencing to counter RNA silencing which is manifested as a defence mechanism by plants. Attempts have been made to target the viral suppressors to engineer resistance against RNA viruses [23] and DNA viruses [28, 40]. Hairpin RNA-mediated silencing of *Plum pox potyvirus* (PPV) *P1* and *HC-Pro* genes conferred resistance against PPV [23]. Artificial microRNA strategy has been successfully employed to develop resistance against *Tomato leaf curl New Delhi virus* (ToLCNDV) in the transgenic tomato plants by targeting the *AV2* gene, which encodes a silencing suppressor [49].

Transgenic tobacco plants expressing the hpRNA gene of MYMV-*AC4,* a suppressor of silencing, effectively blocked the MYMV DNA accumulation [40]. Similarly, the present work clearly shows that silencing of *TrAP,* a viral suppressor of silencing in MYMV [43], is very effective in reducing MYMV DNA accumulation. A transgenic strategy that targets both the viral suppressors of silencing (AC4 and TrAP) can be very effective because that would mitigate the ability of MYMV to suppress RNA silencing.

Acknowledgments We thank Prof. Thomas Hohn, University of Basel for providing the P35S-hp*TrAP*-35S3′ cassette and Dr. K. Dharmalingam, School of Biotechnology, Madurai Kamaraj University for his permission to use the radioisotope facility. This work was funded by the Department of Biotechnology (Project Number: BT/PR9823/AGR/36/10/2007), Ministry of Science and Technology, Government of India. The University Grants Commission (UGC), Govt. of India is thanked for the Research Fellowship to GS under the UGC-UPE project to Madurai Kamaraj University.

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