Virus Genes
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Antibegomoviral activity of the agrobacterial virulence protein VirE2

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Abstract Mungbean yellow mosaic geminivirus (MYMV) causes severe yellow mosaic disease in blackgram, mungbean, Frenchbean, pigeonpea, soybean and mothbean. We attempted to induce resistance against this virus using the transcriptional activator protein gene deleted in the C-terminal activation domain (*TrAP*-ΔAD) and *Agrobacterium tumefaciens vir*E2. MYMV is known to replicate in agroinoculated tobacco leaf discs. Three transgenic tobacco plants which harboured a truncated MYMV transcriptional activator protein gene and two tobacco plants transformed with the octopine type *A. tumefaciens vir*E2 gene were agroinoculated with an *A. tumefaciens* strain which harboured the partial dimers of both DNA A and DNA B of MYMV. The level of viral DNA accumulation in leaf discs of transgenic plants correlated inversely to the level of the MYMV *TrAP*-ΔAD transcript. Two VirE2-transgenic plants, which complemented tumorigenesis of a *vir*E2 mutant *A. tumefaciens* strain, effectively reduced MYMV DNA accumulation in the leaf disc agroinoculation assay.

Keywords AC2 · *Agrobacterium vir*E2 · Agroinoculation · Geminivirus · MYMV · TrAP

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Introduction

Geminiviruses are encapsidated in twinned icosahedral particles comprising small, circular, ssDNA genomes [1, 2]. Their genomes are either monopartite or bipartite. Based on the genome-organization, host-range and vector transmission, geminiviruses are classified into four genera namely *Mastrevius*, *Curtovirus*, *Topocuvirus* and *Begomovirus* [3]. *Mungbean yellow mosaic virus* (MYMV) is a whitefly-transmitted bipartite begomovirus that causes yellow mosaic disease in mungbean [4] and blackgram [5]. MYMV and *Mungbean yellow mosaic India virus* (MYMIV) infect many important pulse crops, blackgram, mungbean, Frenchbean, pigeonpea, mothbean and soybean and cause an annual yield loss of about US $ 300 million [6].

Pathogen-derived resistance (PDR), which involves unmodified viral genes, has been reported for many geminiviruses. Genes of the coat protein (CP) and the replication-associated protein have been successfully deployed to generate geminivirus resistance [7–9].

Mutants of many geminiviral genes and non-viral genes have been tested for *trans*-dominant negative inhibition of geminivirus replication and movement. Truncated Rep (T-Rep) of *Tomato yellow leaf curl virus* (TYLCV) interfered with the virion-sense strand synthesis and repressed its own transcription and thereby acted as a *trans*-dominant negative mutant in *Nicotiana benthamiana* [10]. Expression of the NTP-binding domain mutant and the DNA-nicking domain mutant of *Bean golden mosaic virus*-Guatemala (BGMV-GA) Rep in the tobacco cell suspension culture inhibited the replication of BGMV-DR, BGMV-BZ and *Bean dwarf mosaic virus* (BDMV) [11]. Expression of the oligomerization domain of the *Tomato leaf curl New Delhi virus* (ToLCNDV) Rep in

N. benthamiana interfered with its DNA accumulation by binding to the origin of replication and by the formation of dysfunctional oligomers [12]. A similar mechanism was reported by Lucioli et al. [13] when the *Tomato yellow leaf curl Sardina virus* (TYLCSV) *Rep* gene was evaluated in *N. benthamiana* for *trans*-dominant inhibition of viral replication.

Resistance mediated by *trans*-dominant negative mutation was also achieved by the expression of the mutant movement proteins (MPs) of *Tomato golden mosaic virus* (TGMV) in *N. benthamiana* [14] and *Cabbage leaf curl virus* (CabLCV) in *N. tabacum* [15]. Delayed infection was observed in transgenic tomato plants which expressed the mutants of BDMV MP and nuclear shuttle protein (NSP) genes [16].

The effectiveness of a non-viral protein in conferring geminiviral resistance by *trans*-dominant negative inhibition was evaluated by Padidam et al. [17]. The gene 5 protein (g5p) from the *Escherichia coli* phage M13 displayed sequence non-specific binding to ssDNA. The *Tomato leaf curl virus* (ToLCV) genome was engineered to express g5p as a fusion protein. M13-g5p was fused to the N-terminal 66 amino acid-portion of CP with an intervening linker of six glycine residues. The chimaeric ToLCV did not spread efficiently in *N. benthamiana* plants and the inoculated plants developed only very mild symptoms. The efficient ssDNA binding property of g5p in the chimaeric ToLCV was proposed to interfere with the interaction of NSP to the viral DNA and inhibit the viral DNA movement in *N. benthamiana*.

We tested the effectiveness of *Agrobacterium* VirE2, a nuclear targeted ssDNA binding protein, in contributing geminivirus resistance. *Agrobacterium tumefaciens* Ti plasmid-encoded VirE2 is a 60 kDa ssDNA-binding protein [18–20]. VirE2 binding of ssDNA is co-operative, sequence non-specific [21] and protects the T-strand from host nucleases [22].

Transgenic plants which expressed VirE2 complemented the virulence of a *vir*E2 mutant strain of *A. tumefaciens* [23, 24]. This finding proved that T-complex formation occurred in the plant cell. VirE2 comprised two nuclear localization signals [23, 25]. VirE2 interaction with VIP1 (VirE2 interacting protein 1), a phosphoprotein with a conventional NLS, facilitated the interaction with karyopherin α and transported the VirE2 protein into the nucleus [26]. Bimolecular fluorescence complementation studies co-localized VirE2 to the nucleus upon interaction with the importin α isoform IMPa-4 [27]. Phosphorylation of VIP1 was proposed to be the deciding factor for nuclear trafficking of VirE2 [28].

We report in this article the effect of transgenic expression of a mutant of the MYMV *TrAP* and the wild-type *vir*E2 on the accumulation of MYMV. Three transgenic *TrAP*-ΔAD-tobacco plants which accumulated varying levels of the *TrAP*-ΔAD transcript and two VirE2-transgenic tobacco plants which complemented a *vir*E2 mutation in *Agrobacterium* were evaluated for inhibition of MYMV DNA replication using the leaf disc agroinoculation assay [29]. The plants which accumulated higher levels of the *TrAP*-ΔAD and *vir*E2 transcripts displayed a pronounced reduction in MYMV DNA accumulation. We report for the first time that geminivirus DNA accumulation is reduced in transgenic plants which expressed a TrAP mutant and the wild-type *Agrobacterium* VirE2.

Materials and methods

Viral clones

Accession numbers of DNA A and DNA B (KA22) of *Mungbean yellow mosaic virus* (MYMV-[IN:Vig]) in EMBL/Genbank are AJ132575 and AJ132574, respectively. Agroinoculation of partial dimers of MYMV-[IN:Vig] DNA A and DNA B caused the yellow mosaic disease in blackgram (*Vigna mungo*) [5] and mungbean [30].

Plasmid constructs

The binary plasmid pCAM-*TrAP*-ΔAD which harboured the MYMV *TrAP* gene with the C-terminal activation domain deletion (*TrAP*-ΔAD) has been described previously [31]. pCAM-*TrAP*-ΔAD was introduced into the *A. tumefaciens* strain LBA4404 by triparental mating [32]. The binary vector pCAM-*vir*E2, which harboured the octopine type *A. tumefaciens vir*E2 gene, was constructed as follows: A 1.6-kb *vir*E2 gene with the CaMV 35S promoter and an intron sequence was cloned as a *Bam*HI/*Pst*I fragment in the corresponding sites of pCAMBIA1380. pCAM-*vir*E2 was introduced into the *A. tumefaciens vir* helper strain GV3101 which harboured the disarmed vector pPM6000 [33].

Tobacco transformation

Tobacco (*N. tabacum* L. cv. Wisconsin 38) leaf discs were transformed using *A. tumefaciens* as described by Sunilkumar et al. [34]. Transgenic shoots were selected on a selection medium [35] which contained 50 mg/l hygromycin + 250 mg/l cefotaxime and were kept for root induction on the BGS medium (Murashige and Skoog [MS] salts [35], 0.0001% [*w*/*v*] folic acid, 100 mg/l myoinositol, 0.4 mg/l 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 cefotaxime, 150 mg/l timentin and 50 mg/l hygromycin.

*Agrobacterium vir*E2-complementation assay

Tobacco leaf discs transformed with the wild-type *A. tumefaciens* strain A348 (with the Ti plasmid pTiA6) formed tumours on a hormone-free MS medium. The *A. tumefaciens* strain pTi358 with a Tn*3*-HoHo1 insertion mutation in *vir*E2 did not induce tumours in tobacco leaf discs [36]. However, a plant expressing *vir*E2 complemented the *vir*E2 mutation in pTi358 and caused tumorigenesis. *Agrobacterium vir*E2 complementation assay was performed by the co-cultivation of leaf discs of 6-week-old, axenic, *vir*E2-transgenic plants with the *A. tumefaciens* strain pTi358 for 2 days. The leaf discs were then placed on the hormone-free MS medium with 250 mg/l cefotaxime. Appearance of tumours was taken as an index of functional complementation by the plant-expressed VirE2.

Tobacco leaf disc agroinoculation with MYMV partial dimers

Leaf discs (8 mm diameter) were cut from 6-week-old tobacco plants and agroinoculated with the *A. tumefaciens* strain Ach5 which harboured the partial dimers of both DNA A and DNA B of MYMV-[IN:Vig] [37]. After 2 days of co-cultivation, the leaf discs were placed for 10 days on the tobacco shoot-induction medium (MS salts, B5 vitamins, 0.5 μM NAA, 4 μM BAP, 3% [*w*/*v*] sucrose, 0.8% [*w*/*v*] agar, pH 5.7) supplemented with 250 mg/l cefotaxime.

Southern blot analysis

Total plant DNA was extracted as described by Rogers and Bendich [38]. DNA concentration was estimated using the Hoechst dye 33258 in the DyNA Quant 200 flourometer (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 determination of agroinoculated leaf discs was performed by electrophoresis of undigested total plant DNA in a 0.8% agarose gel in 1× TNE (40 mM Tris–acetate, pH 7.5, 20 mM sodium acetate, 2 mM EDTA) buffer [39]. DNA was transferred to the Zeta probe nylon membrane and hybridized with [α-^{32}P]dCTP-labelled probes.

RT-PCR analysis

Total RNA was extracted using the Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA) and estimated in a spectrophotometer. Total RNA (50 μg) was treated with 1 U of DNase in 50 μl of 40 mM Tris–Cl (pH 7.5) at 37°C for 30 min. DNA-free RNA was estimated and 100 ng of the RNA template was used for RT-PCR analysis using the *vir*E2-specific primers (FP: 5′-GGCAGCGAGCACGGAAATC-3′ and RP: 5′-CTGGCTGCTGTCCCATATTTAC-3′). Tobacco actin primers (FP: 5′-GCTATTCAGGCTGTCCTTTCCTTGTATG-3′ and RP: 5′-CCGATATCAACATCACACTTCATAATG-3′) were used to amplify the endogenous actin gene.

Results

Agrobacterium-mediated transformation of blackgram (*Vigna mungo*) [40] and generation of transgenic blackgram plants with the *npt*II and *gus* genes have been reported [41]. However, due to high degree of sensitivity to variations in tissue-culture conditions, blackgram transformation has not become a routine method. Alternatively, we found that tobacco (cv. Wisconsin 38) leaf discs, agroinoculated with the partial dimers of MYMV DNA A and DNA B, replicated and accumulated MYMV DNA A [29]. We raised transgenic tobacco plants with the candidate genes and used the tobacco leaf disc agroinoculation assay to evaluate the effect of transgenes on MYMV DNA accumulation.

Effect of expression of MYMV *TrAP*-ΔAD in transgenic tobacco on MYMV DNA accumulation

Three transgenic tobacco plants A2, A3 and A10 which we raised in our earlier study accumulated low, high and moderate levels of the *TrAP*-ΔAD transcript, respectively [32]. They were chosen to study whether the expression of the mutant *TrAP* can alter the MYMV DNA accumulation. Leaf discs of an untransformed tobacco plant (C) and the three *TrAP*-ΔAD transgenic plants (A2, A3 and A10) were agroinoculated with the *A. tumefaciens* strain Ach5 which harboured the partial dimers of both DNA A and DNA B of MYMV [37]. Total DNA was extracted from the leaf discs, and Southern blot analysis was done with the 2.7-kb MYMV DNA A probe. Agroinoculated leaf discs from an untransformed tobacco plant (C) accumulated high levels of the viral DNA corresponding to single-stranded (*ss*) DNA/supercoiled, double-stranded (*sc*) DNA forms (Fig. 1). Densitometry analysis of the autoradiogram (Fig. 1) was done using the AlphaEase™ software (Version 5.5, Alpha Innotech Corporation, San Leandro, CA). The integrated density value (IDV) at the *ss/sc* position in lane C was 50480. The plant A2 with a low level of the *TrAP*-ΔAD transcript caused 50% reduction (IDV-24624) in the accumulation of MYMV DNA A. The plant A10 with a moderate *TrAP*-ΔAD transcript level caused 75% reduction (IDV-11376) in viral DNA accumulation. In the leaf discs of the plant A3, which

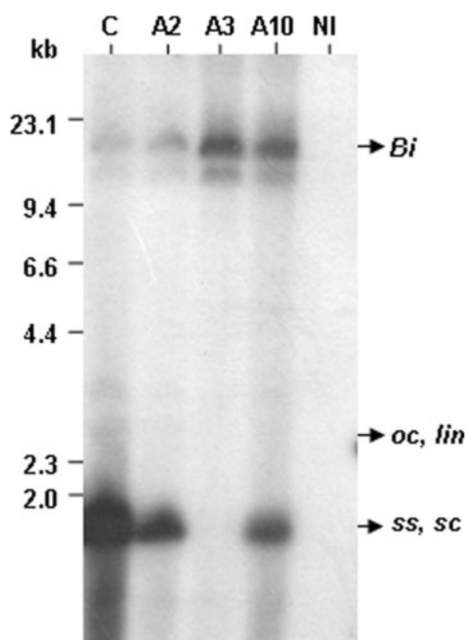

Fig. 1 Southern blot analysis of MYMV-[IN:Vig] DNA A in agroinoculated leaf discs of control plants and transgenic tobacco plants which harboured the MYMV *TrAP*-ΔAD gene. Leaf discs of three *TrAP*-ΔAD transgenic plants (*A2*, *A3* and *A10*) [32] and an untransformed tobacco plant (*C*) were agroinoculated with MYMV. A blot with the total DNA from non-agroinoculated leaf discs (*NI*) of the control plant, agroinoculated leaf discs of the control plant (*C*) and three *TrAP*-ΔAD transgenic plants (*A2*, *A3* and *A10*) was hybridized to the full-length DNA A (2.7 kb) probe. Open circular (*oc*), linear (*lin*), single-stranded (*ss*) and super-coiled (*sc*) forms of MYMV-[IN:Vig] [39] and *Agrobacterium* binary vector (*Bi*) with the partial dimers of MYMV are marked

accumulated the highest level of the *TrAP*-ΔAD transcript, MYMV DNA accumulation was completely blocked. The leaf disc agroinoculation experiments were repeated thrice and similar results were obtained.

Transformation of tobacco with the *A. tumefaciens* *vir*E2 gene and functional evaluation of VirE2

The octopine type *A. tumefaciens* *vir*E2 gene with an intron, cloned under the control of the CaMV 35S promoter, was used to construct the binary vector pCAM-*vir*E2, which harboured the hygromycin phosphotransferase (*hph*) gene as the plant selectable marker (Fig. 2). Tobacco transformation with pCAM-*vir*E2 yielded six hygromycin-resistant shoots which formed roots on a medium containing 50 mg/l hygromycin. All six *vir*E2-transgenic plants were evaluated for the functional competence of VirE2 by testing tumorigenesis of leaf discs infected with the *vir*E2 mutant *A. tumefaciens* strain pTi358 [36]. In a preliminary screening, three of the six hygromycin-resistant plants (E2-1, E2-5 and E2-7) complemented the *vir*E2 mutation efficiently and formed tumours, whereas one plant weakly complemented the *vir*E2 mutation (E2-4) (data not shown). *vir*E2 complementation analysis of two transgenic plants (E2-1 and E2-7) is shown in Fig. 3. Tumour formation on hormone-free MS medium was used as an index of tumorigenesis. Leaf discs of untransformed tobacco plants infected with the wild-type *A. tumefaciens* strain A348 harbouring pTiA6 formed tumours (C-pTiA6). The leaf discs of the untransformed tobacco plant and the *vir*E2 transgenic plant E2-1 did not form tumours on the hormone-free medium without infection with *Agrobacterium* (Fig. 3, C and E2-1). Leaf discs of control tobacco plants infected with the *A. tumefaciens* *vir*E2 mutant strain (C-pTi358) did not form tumours. Two *vir*E2 transgenic plants formed tumours upon infection with the *A. tumefaciens* *vir*E2 mutant (E2-1-pTi358 and E2-7-pTi358). Thus, VirE2 expression in transgenic plants efficiently complemented the *vir*E2 mutation in *A. tumefaciens*. The VirE2 complementation experiment with E2-1 and E2-7 was repeated once and similar results were attained.

Southern blot analysis of E2-1 and E2-7 plants was done with the *hph* and *vir*E2 probes. The transgenic plant DNA, digested with *Eco*RI and analysed with the *hph* probe, was expected to display hybridization of junction fragments longer than 2.2 kb (Fig. 2). The plant E2-1 displayed hybridization to one intense band of 3.5 kb and one weak band of 12.0 kb corresponding to junction fragments (Fig. 4a). The plant E2-7 displayed one junction fragment

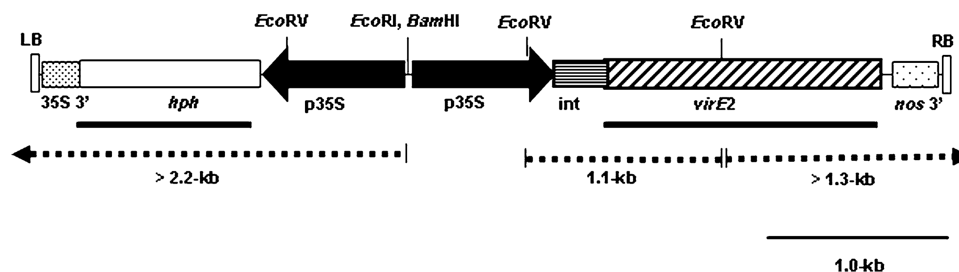

Fig. 2 The T-DNA of the binary vector pCAM-*vir*E2. *RB*: T-DNA border-right, *p35S*: *Cauliflower mosaic virus* (CaMV) 35S promoter, *35S 3′*: CaMV 35S polyadenylation signal, *hph*: hygromycin phosphotransferase gene, *nos* 3′: polyadenylation signal of the nopaline synthase gene, *LB*: T-DNA border-left. Probes used (*hph* and *vir*E2) have been marked in *bold lines*. The junction fragment sizes (>2.2 kb and >1.3 kb) and an internal T-DNA fragment of 1.1-kb released by *Eco*RV and scale (1.0 kb) have been marked

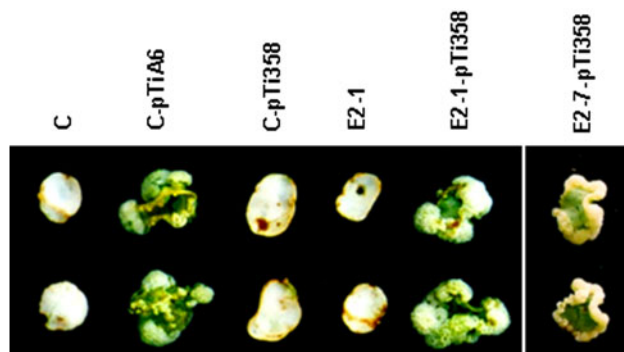

Fig. 3 Functional complementation of the *A. tumefaciens vir*E2 mutation in tobacco plants transformed with pCAM-*vir*E2. The *A. tumefaciens* strain harbouring pTi358 with *vir*E2 mutation [36] was used to infect the leaf discs of the untransformed control plant (*C-pTi358*) and two *vir*E2 transgenic tobacco plants E2-1 (*E2-1-pTi358*) and E2-7 (*E2-7-pTi358*). *C*: leaf discs of uninfected, untransformed control tobacco plant, *E2-1*: uninfected leaf discs of the transgenic plant E2-1 (uninfected leaf discs of E2-7 are not shown). Untransformed control tobacco leaf discs infected with the wild-type *A. tumefaciens* strain A348 [57] which harboured pTiA6 (*C-pTiA6*) served as the positive control and those infected with the *vir*E2 mutant *A. tumefaciens* strain pTi358 (*C-pTi358*) served as the negative control

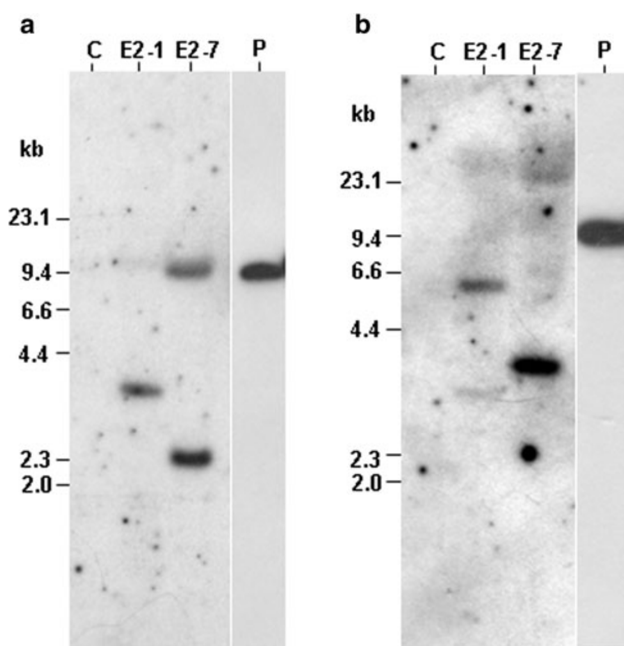

Fig. 4 Southern blot analysis of the *vir*E2-transgenic plants E2-1 and E2-7 with the *hph* probe. **a** Analysis of *Eco*RI-digested plant DNA. **b** Analysis of *Bam*HI-digested plant DNA. **a** and **b** DNA from non-transgenic control plant (*C*) and two *vir*E2-transgenic plants (*E2-1*, *E2-7*). *A. tumefaciens* (pCAM-*vir*E2) DNA samples (1 ng) digested with *Eco*RI (**a**) or *Bam*HI (**b**) were used as positive controls (*P*). Shorter exposures of the lane '*P*' from the same gels were placed in **a** and **b**

of 2.3-kb which indicated true T-DNA integration. However, a second fragment hybridized at the 11.0-kb position (Fig. 4a) which corresponded in size to the *Eco*RI-digested binary vector fragment (P) which invoked the possibility of *Agrobacterium* contamination. To distinguish between T-DNA integration and *Agrobacterium* persistence in plant E2-7, the *hph* Southern blot analysis was repeated by digesting the plant DNA with a second enzyme, *Bam*HI. It is very unlikely that the junction fragment size and the binary vector size will remain the same when digestion was performed with two different restriction enzymes. The *hph* probe was expected to display hybridization of junction fragments longer than 2.2 kb upon *Bam*HI digestion of plant DNA (Fig. 2). Both plants (E2-1 and E2-7) displayed two junction fragments each (Fig. 4b). One intense band of 6.0 kb and a weak band of 3.0 kb were seen for E2-1. Similarly, one intense band of 3.5 kb and a weak band of 23.0 kb were seen for E2-7. The two junction fragments of the plant E2-7 did not match with the binary vector fragment generated by *Bam*HI digestion. Hence, the possibility of *Agrobacterium* contamination in the plant E2-7 was ruled out. Both E2-1 and E2-7 harboured two copies of the *hph* gene. The weak bands of 12.0 kb (Fig. 4a) and 3.0 kb (Fig. 4b) of the plant E2-1, which hybridized to the *hph* probe, may represent a truncated T-DNA.

The presence of the *vir*E2 transgene in the transgenic plants was evaluated by Southern blot analysis with the *vir*E2 probe. Upon digestion of the transgenic plant DNA with *Eco*RV, the *vir*E2 probe was expected to hybridize to an internal T-DNA fragment of 1.1 kb and to a junction fragment longer than 1.3 kb (Fig. 2). As expected, the plants E2-1 and E2-7 displayed hybridization to an internal T-DNA fragment of 1.1 kb (Fig. 5a). Besides, E2-1 hybridized to one junction fragment and E2-7 displayed hybridization of two junction fragments. The results confirmed the presence of integrated copies of *vir*E2 in both E2-1 and E2-7.

PCR with *hph*- and *vir*E2-specific primers amplified 1.0-kb and 817-bp fragments in the plants E2-1 and E2-7, respectively (Fig. S1a and b). PCR was done with *vir*G primers to check *Agrobacterium* persistence in the transgenic plants. *A. tumefaciens* DNA was used as a positive control (P) in which a 627-bp fragment was amplified. The plants E2-1 and E2-7 amplified the *hph* and *vir*E2 fragments, but failed to amplify the *vir*G fragment (Fig. S1c). Thus, the absence of *Agrobacterium* contamination in both E2-1 and E2-7 was confirmed.

Expression of the *vir*E2 gene in the *vir*E2 transgenic plants E2-1 and E2-7

Reverse transcriptase-PCR (RT-PCR) was done in the plants E2-1 and E2-7 using the *vir*E2-specific primers. An 817-bp fragment was amplified in both E2-1 and E2-7 plants (Fig. 5b). No amplification was observed in the control reaction in which the reverse transcriptase step was

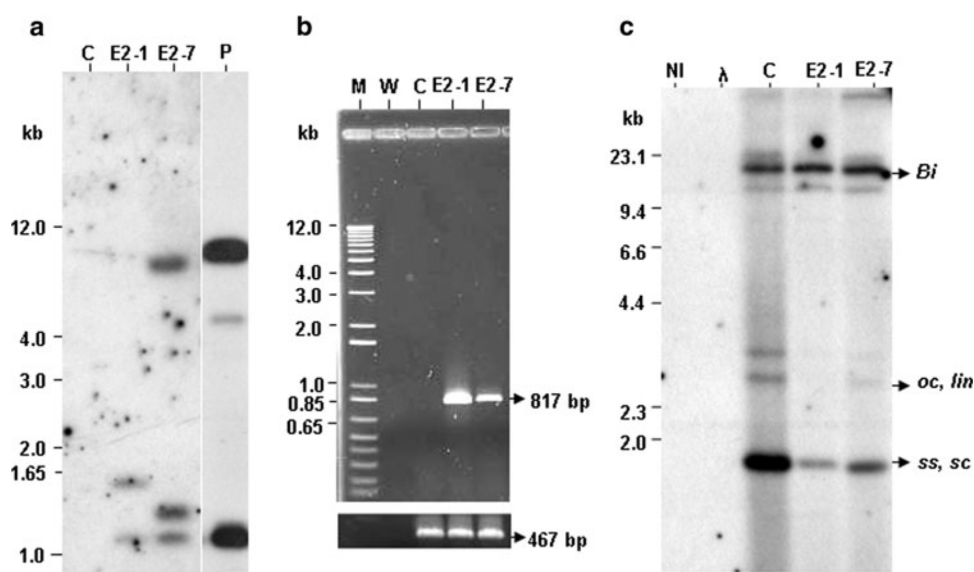

Fig. 5 Study of the *vir*E2 gene integration in the *vir*E2-transgenic plants, *vir*E2 expression analysis and leaf disc agroinoculation assay to study the effect of *vir*E2 on MYMV DNA accumulation. **a** Southern blot analysis of the *vir*E2-transgenic tobacco plants. DNA from two *vir*E2-transgenic plants (*E2-1* and *E2-7*) was digested with *Eco*RV and the blot was probed with the *vir*E2 gene. DNA from the untransformed control tobacco plant digested with *Eco*RV (*C*) was used as the negative control. *A. tumefaciens* (pCAM-*vir*E2) DNA (1 ng) digested with *Eco*RV was used as the positive control (*P*). Two fragments of 1.1 and 10.0 kb were expected to hybridize. A shorter exposure of the lane *P* from the same gel was shown in the figure. **b** RT-PCR analysis of the *vir*E2-transgenic plants with the *vir*E2-specific primers. Total RNA from the untransformed control tobacco plant (*C*) and from two transgenic plants (*E2-1* and *E2-7*) was used as the RT-PCR template. The bottom panel presents an equal loading control with samples amplified with the actin gene-specific primers. **c** Southern blot analysis of DNA (5 μg) from leaf discs of *vir*E2-transgenic tobacco plants agroinoculated with MYMV. The blot was hybridized to the full-length DNA A probe. *NI*: DNA from non-agroinoculated leaf discs of an untransformed control plant, *C*: DNA from leaf discs of an untransformed plant agroinoculated with MYMV. Leaf discs from two *vir*E2-transgenic plants (*E2-1* and *E2-7*) were agroinoculated. The positions of open circular (*oc*), linear (*lin*), single-stranded (*ss*) and super-coiled (*sc*) forms of MYMV [39] and *Agrobacterium* binary vector (*Bi*) with the partial dimers of MYMV are marked

omitted (data not shown), thus ruling out DNA contamination. The intensity of the of the *vir*E2 transcript signal was much higher in the plant E2-1 than in E2-7. RT-PCR with tobacco actin primers amplified a 467-bp fragment with equal intensity in control and in both transgenic plants, which confirmed equal loading of RNA in all three plants (Fig. 5b).

Leaf disc agroinoculation assay to determine the effect of VirE2 on the accumulation of MYMV DNA

Leaf discs from an untransformed control plant and two *vir*E2-expressing transgenic plants E2-1 and E2-7 were agroinoculated with the wild-type *A. tumefaciens* strain Ach5 which harboured the partial dimers of both DNA A and DNA B of MYMV. Viral titre of agroinoculated leaf discs was determined by Southern blot analysis using the MYMV DNA A probe. Leaf discs of the untransformed tobacco plant accumulated MYMV DNA corresponding to single-stranded DNA (*ss*)/double-stranded super-coiled DNA (*sc*), and linear DNA (*lin*)/open circular DNA (*oc*) (Fig. 5c). The band corresponding to the *ss*/*sc* forms was more intense in the untransformed plant with an IDV of 26174. The plant E2-1 with a higher *vir*E2 transcript level showed a marked reduction (70%) in the viral DNA level with an IDV of 8424. The plant E2-7, which accumulated a comparatively lower level of the *vir*E2 transcript, displayed an intermediate level (50%) of viral titre (IDV-11808). Thus, the transgenic plants which expressed the functional VirE2 reduced the accumulation of MYMV DNA. A good correlation was seen between the *vir*E2 transcript level (Fig. 5b) and the extent of inhibition of MYMV DNA accumulation (Fig. 5c).

Discussion

Several examples of *trans*-dominant inhibition of replication of animal viruses [42] and plant geminiviruses [10–16] have been reported. We used the tobacco leaf disc agroinoculation assay as a heterologous system to study *trans*-dominant inhibition of MYMV accumulation by transgenic expression of MYMV *TrAP*-ΔAD and *Agrobacterium* *vir*E2. Though tobacco is not a natural host of MYMV,

tobacco leaf disc replication assay was found to be an efficient method to evaluate the effectiveness of transgenically expressed MYMV-derived genes in inhibiting the viral DNA accumulation. T-Rep of MYMV, with a mutation in the ATPase domain, caused a reduction in MYMV DNA accumulation [29].

Begomovirus TrAP has three functional domains namely a basic bipartite NLS at the N-terminus, a Zn-finger domain in the middle, and an acidic activation domain in the C-terminus [31, 43]. MYMV TrAP transactivated the CP and NSP gene expression [44], suppressed gene silencing [31] and caused toxicity when expressed in transgenic plants [32]. Deletion of the 31-amino acid C-terminal activation domain of MYMV TrAP abolished all the three functions [31, 32, 44] whereas the corresponding TGMV mutant retained the silencing suppression property [45]. Thus, *TrAP*–ΔAD emerged as a good candidate gene for causing *trans*-dominant inhibition of MYMV accumulation. The ability of TrAP to interfere with the host defence mechanism prompted Vanderschuren et al. [9] to propose *TrAP* as a good candidate gene to engineer geminiviral resistance.

Three *TrAP*-ΔAD transgenic tobacco plants from our previous report [32] which accumulated varying levels of the *TrAP*-ΔAD transcript were evaluated for *trans*-dominant inhibition of MYMV replication. Agroinoculated leaf discs of all three transgenic plants displayed reduced levels of viral DNA accumulation in comparison to those of the untransformed control plant (Fig. 1). The plant A3 with the highest level of the *TrAP* transcript displayed almost 100% inhibition of viral DNA accumulation. The plant A10 with a moderate level of the *TrAP* transcript displayed 75% inhibition. The plant A2 with a low level of the *TrAP* transcript inhibited MYMV replication by about 50%. Higher level of *TrAP*-ΔAD transcript caused a greater level of reduction in MYMV accumulation. The activation domain-deleted TrAP mutant protein may bind to the late gene promoters of MYMV but may not activate their expression. This may result in the *trans*-dominant inhibition of the viral TrAP and cause inhibition of virus accumulation. Because the TrAP function is highly conserved in begomoviruses [46, 47], it is viewed as a good candidate gene for *trans*-dominant inhibition. Hence, *TrAP*-ΔAD is likely to confer broad-spectrum geminivirus resistance.

VirE2, a ssDNA binding protein of *A. tumefaciens*, was chosen as a non-viral candidate gene for developing MYMV resistance. The non-sequence specific, co-operative ssDNA binding property and the presence of NLS [48] made VirE2 an attractive candidate to interfere with geminivirus replication. Two *vir*E2-transgenic plants, which efficiently complemented the *vir*E2 mutation in *Agrobacterium* (Fig. 3), harboured integrated *vir*E2 copies (Fig. 5a) and were devoid of *Agrobacterium* contamination (Fig. S1).

RT-PCR analysis indicated that the plant E2-1 accumulated a higher level of the *vir*E2 transcript than the plant E2-7 (Fig. 5b). Agroinoculation of the leaf discs of both *vir*E2-transgenic plants with the partial dimers of MYMV DNA A and DNA B showed a pronounced reduction in viral DNA accumulation when compared to agroinfected leaf discs of the untransformed control plant (Fig. 5c). The plant E2-1 with a higher *vir*E2 transcript level inhibited MYMV replication by 70%, whereas the plant E2-7 with a moderate *vir*E2 transcript level inhibited MYMV replication by 50%. The inhibition observed in these two plants could be protein-mediated since these plants accumulated the functional VirE2 protein.

Geminiviruses possess ssDNA genomes which replicate in the nuclei through dsDNA intermediates [49]. The form of geminiviral DNA (ssDNA or dsDNA) involved in intracellular movement and cell-to-cell spread is not clearly known [50]. The "relay-race model" of viral movement was proposed in BDMV in which the NSP-bound dsDNA/ssDNA was shuttled from the nucleus to the cytoplasm, from where it was delivered to MP to cross the plasmodesmata barrier [51, 52]. The "couple-skating model" was proposed in *Squash leaf curl virus* (SqLCV) and *Abutilon mosaic virus* (AbMV) in which the NSP-bound ssDNA of the virus was shuttled between the nucleus and the cytoplasm as a nucleoprotein complex, which then interacted with MP to move to the adjacent cell for further multiplication [53, 54]. In either of the models, blocking of the binding of viral ssDNA to NSP may effectively interfere with virus movement and cause a block in virus spread and multiplication.

The expression of M13-g5p inhibited ssDNA movement of ToLCV by interfering with NSP binding [17]. Similarly, the nuclear localization of VirE2 and its efficient co-operative binding to MYMV ssDNA might interfere with the binding of NSP to ssDNA. NSP binding to ssDNA in the nucleus is a prerequisite for the shuttling of viral DNA to the cytoplasm [52, 54, 55]. VirE2-bound ssDNA is likely to be restricted to the infected cell nucleus and rendered unavailable for cell-to-cell movement. Consequently, subsequent movement and multiplication of the virus in the adjacent cells may be blocked. As expected, the leaf discs of the transgenic plants E2-1 and E2-7, which expressed a functional VirE2, displayed a pronounced reduction in viral DNA accumulation. Blocking of viral DNA accumulation was higher in the plant E2-1 in which *vir*E2 transcript level was higher. VirE2 expression offered an important advantage over the M13-g5p. The g5p protein lacked its own NLS. It either required the NLS of CP to which it was fused or it gained entry into the nucleus through passive diffusion [17]. In contrast, VirE2 has its own NLS which efficiently localized it to the nucleus. The sequence non-specific, co-operative ssDNA binding property of VirE2

could make it an attractive candidate to develop broad-spectrum resistance against many geminiviruses.

Transgenic expression of MYMV T-Rep in tobacco [29] and particle bombardment of blackgram with the MYMV hpCR gene which comprised an inverted repeat of MYMV common region [56] caused a pronounced reduction in MYMV DNA accumulation. We have reported in this article that transgenic tobacco plants which expressed *TrAP*-ΔAD and *vir*E2 very effectively reduced MYMV DNA accumulation in leaf disc agroinoculation assays. Thus, we report for the first time that the *TrAP*-ΔAD mutant and wild-type VirE2 are additional candidate genes which can be used to develop transgenic plants with broad-spectrum geminivirus resistance.

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