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ORIGINAL ARTICLE

Agroinfection of tobacco by croton yellow vein mosaic virus and designing of a replicon vector for expression of foreign gene in plant

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Abstract Croton yellow vein mosaic virus (CYVMV, genus *Begomovirus* family *Geminiviridae*) is a proliferating begomovirus in the Indian sub-continent. The infectious constructs in binary vector was developed against the CYVMV genome and its associated betasatellite. Agroinoculation of the genomic construct of CYVMV produced leaf curl symptoms alone in three species of tobacco, *Nicotiana tabacum*, *N. benthamiana* and *N. glutinosa*. Co-inoculation of betasatellite enhanced the severity of the disease and reduced the incubation time. Based on the infectious clone, a replicon vector pCro, with only the ability to replicate inside the plant was developed. In pCro vector, CP and V2 ORFs from genome of CYVMV was deleted, which resulted localised replication of the molecule with no visible symptoms. Besides the partial CYVMV genome, pCro also has a cassette containing a double 35S promoter, multiple cloning sites and a NOS terminator to overexpress any foreign protein in plant. Episomal release of the replicon from the binary vector backbone after agroinoculation was detected by PCR. A GFP gene was cloned in pCro vector (pCro-GFP) and agroinoculated to *N. tabacum* resulted in localized expression of GFP at 5 dpi. The CYVMV replicon vector will be a useful tool for studying functional genomics, vaccine expression and gene silencing in plant.

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Introduction

Croton yellow vein mosaic virus (CYVMV) is a distinct virus species of the genus *Begomovirus*, family *Geminiviridae*. CYVMV is transmitted by whitefly, *Bemisia tabaci*, and occurs commonly on *Croton bonplandianum* causing bright yellow vein mosaic disease. Other than *C. Bonplandianum*, natural infection of CYVMV has been identified in several weeds and economic plant species e.g., *Jatropha* sp., *Acalypha* sp., *Cyamopsis* sp., *Crotalaria juncea*, okra, papaya, radish, rapeseed-mustard and tomato [15, 27, 28, 31]. Under the experimental conditions, CYVMV infects as many as 35 plant species of 11 families [27]. CYVMV having broad host range and being efficiently disseminated by *B. tabaci* is one of the most prevalent begomoviruses in the Indian sub-continent. The complete sequence of the CYVMV genome and the associated croton yellow vein mosaic betasatellite (CroYVMB) are available for several isolates. Biolistic delivery of DNA components generated through rolling-circle amplification (RCA) of monomeric DNA derived from the clones of CYVMV genome and CroYVMB produced typical yellow vein mosaic symptoms in *C. bonplandianum* [27]. Hence, both the CYVMV and its associated betasatellite are proved to be the etiological agent. Agroinoculation has been widely used for obtaining infectivity of the cloned DNA of begomoviruses [1, 9, 18, 19]. However, agroinfection and the role of CYVMV genome and CroYVMB in disease development have not been studied.

Studies on molecular biology of plant virus led to realize it as a useful genetic resource. Plant viruses are attractive

candidates for the molecular farming of pharmaceutical protein in plant system. The idea of plant virus as vector for the production of foreign protein in the plant was first experimented with cauliflower mosaic virus, a dsDNA plant virus [7]. Thereafter, mastreviruses (ssDNA viruses) with monopartite genome, maize streak virus and begomoviruses with bipartite genome, tomato golden mosaic virus and African cassava mosaic virus (ACMV) were attempted as gene expression vectors [10, 32]. The genus *Begomovirus* currently containing 322 virus species is the largest of all genera of the family *Geminiviridae*. Begomoviruses have a broad host range, they multiply at a high level in the infected plant cells and they have a small genome, which is easily maneuverable for developing highly infectious clone. These features make begomovirus as an attractive candidate for using as gene expression vector. Based on genomic constituent begomoviruses may be bipartite or monopartite. Initial attempts with few bipartite begomovirus [tomato golden mosaic virus (TGMV) and African cassava mosaic virus (ACMV)] as a gene expression vector were encountered with the structural instability of vector, which undermined the potentiality of geminivirus as an efficient vector for expression of foreign gene in plant. However, the change in the design of vector, where the viral genome was used as replicon by deleting coat protein or/and movement protein encoding sequences and by using the 35S promoter to drive the foreign gene expression brought back geminivirus as a potential candidate for the high level expression of foreign protein in plant [4, 12]. However, geminivirus based efficient gene expression vector is under the developmental stage. Replicon based strategy has been used to design expression/silencing vector for a few mastreviruses [bean yellow dwarf virus (BeYDV), maize streak virus (MSV), wheat dwarf virus (WDV)], curtoviruses [beet curly top virus (BCTV)], bipartite begomoviruses [ACMV, cabbage leaf curl virus (CaLCuV), abuliton mosaic virus (AbMV), tomato leaf curl New Delhi virus (ToLCNDV)] and monopartite begomoviruses [tobacco curly shoot virus (TbCSV), ageratum yellow vein virus (AYVV)] [11, 16, 20, 25, 32]. Of all there geminiviruses, bean yellow dwarf virus (BeYDV) has been extensively used as replicon vector for expressing various foreign proteins in the plant [4]. Besides this, geminiviral replicon vector has been utilized for plant genome engineering [23].

In this study, we have developed an infectious construct of a monopartite begomovirus, CYVMV and shown that CYVMV alone or in the presence of betasatellite, CroYVMB efficiently induce leaf curl disease in the three species of tobacco. The infectious construct of CYVMV was utilized to design an episomal replicon vector by deleting V1 and V2 genes. We demonstrate that the CYVMV replicon vector is useful in expressing green fluorescences protein (GFP) in the tobacco plant.

Materials and methods

Preparation of infectious constructs of CYVMV and CroYVMB

Previously complete genome of an isolate of CYVMV was cloned (M4A, Accession No. JX270684) in the pUC18 vector at *BamH*I site [28]. A partial tandem repeats (PTR) construct was developed from M4A clone in binary vector pCAMBIA 2300 (Fig. 1a). M4A clone was digested with *BamH*I and *Xba*I to generate a 1.0 kb (containing an origin of replication) and 1.7 kb fragments. The 1 kb fragment obtained by digestion with *BamH*I–*Xba*I was purified and cloned into the pCAMBIA2300 vector to generate pCAM-M4A-A0.4mer. The full-length (1.0-mer) CYVMV genome was released from M4A clone with *BamH*I digestion and was re-cloned into *BamH*I-linearized pCAM-M4A-A0.4mer to generate a partial dimeric construct, designated as pCAM-M4A-A1.4mer (Fig. 1a).

A CroYVMB molecule (Accession No. JX270685) was earlier cloned (M4β) [28]. The genome of CroYVMB was amplified with a mutated forward primer β-01* (5′tgtaccactacgctacgag3′, T in place of G in restriction site region of *Kpn*I of the universal β-01 primer) and β-02 universal primer [2] using the M4β clone as a template. This amplified product was then cloned into pGEMT easy vector through blunt end ligation to create pGEMT-M4β. The 1.3 kb betasatellite was released from pGEMT-M4β clone through digestion with *Sac*I and *Kpn*I and cloned into pCAMBIA 2300 vector in between the same restriction site, and designated as pCAM-M4β-1.0mer. For inserting another copy of betasatellite, a full-length betasatellite (ca. 1.3 kb) was released as *Kpn*I fragment from M4β and was re-cloned in *Kpn*I-linearized pCAM-M4β-1.0mer. The resulting complete dimeric construct was then designated as pCAM-M4β-2.0mer (Fig. 1b). The tandem orientation of pCAM-M4A-A1.4mer and pCAM-M4β-2.0mer was confirmed by restriction digestion with *Xba*I, and *Eco*RI, respectively.

Agroinoculation

The pCAM-M4A-A1.4mer and pCAM-M4β-2.0mer was mobilized into the EHA105 strain of *Agrobacterium tumifacience* by freeze–thaw method [33]. Similarly, pCAMBIA 2300 were also transformed. *Agrobacterium* containing these constructs were grown separately in LB broth containing kanamycin and rifampicin at 28 °C for

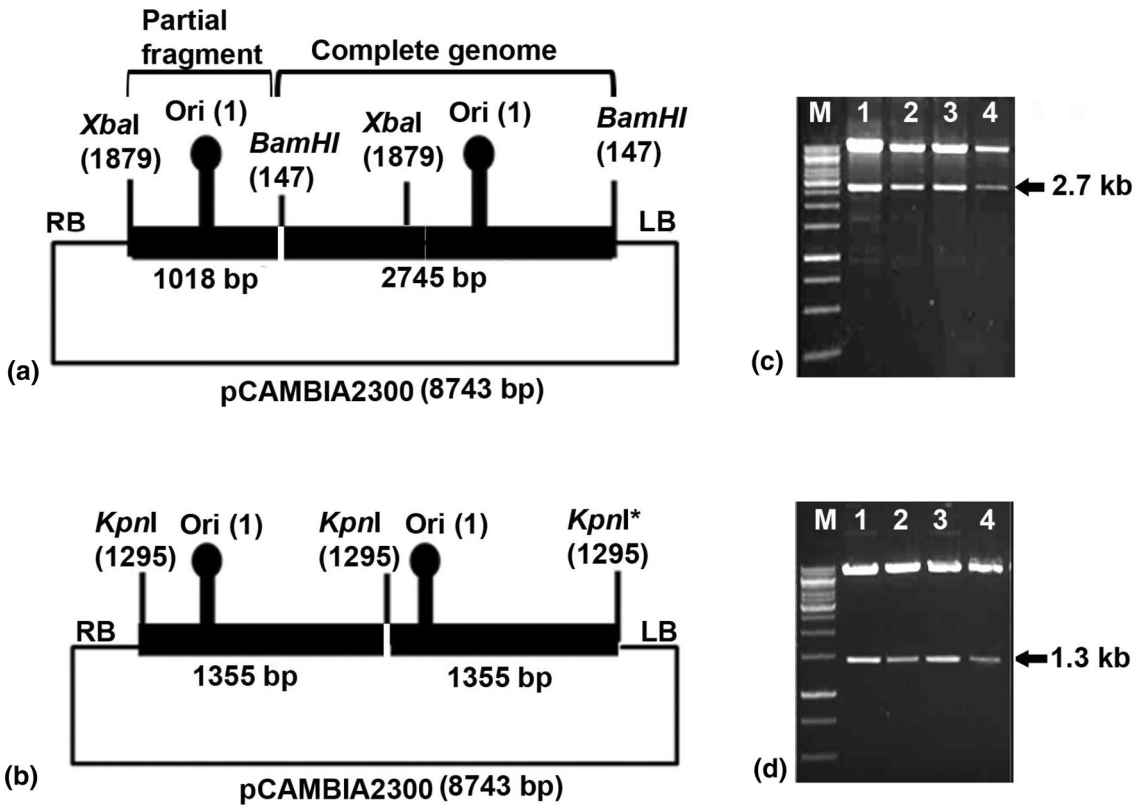

Fig. 1 Schematic diagram showing design of infectious constructs. **a** Partial tandem repeat construct to genome of croton yellow vein mosaic virus (CYVMV), **b** dimeric construct to genome of croton yellow vein mosaic betasatellite (CroYVMB). Confirmation of the constructs by restriction digestion for **c** CYVMV using *Xba*I, and **d** CroYVMB using *Eco*RI

24 h with 150 rpm. The cells were harvested and re-suspended in agroinfiltration buffer [150 µM Acetosyringone, 10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino) ethane sulfonic acid (MES, pH 5.7)]. The resuspended culture was further incubated at 28 °C for 1–3 h with 150 rpm to obtain a final O.D of 0.6. The culture containing pCAM-M4A-A1.4mer was infiltrated either alone or co-inoculated with pCAM-M4β-2.0mer into the lower surface of leaves of *N. benthamiana*, *N. glutinosa* and *N. tabacum* plants using 2 ml syringe by pressure infiltration method [13]. All the inoculations were performed in three independent sets and in each case, a mock-inoculation with a culture containing pCAMBIA2300 alone was included as negative control. The agroinoculated seedlings were grown in an insect-free glasshouse for 30 days and observations were recorded periodically.

Detection of viral DNA

Total genomic DNA was extracted by CTAB method [5] from newly emerged leaves of agroinoculated plants of *N. benthamiana*, *N. glutinosa* and *N. tabacum* showing typical symptoms. To detect the presence of CYVMV genome in the agroinoculated plants, a specific primer set [BM90F and BM82R] [27], which is expected to produce ca. 750 bp amplicon, was used. To detect the betasatellite in CroYVMB co-inoculated plants, a CroYVMB specific primer set [BM534F and BM535R] designed by the multiple alignments of full-length betasatellite sequences of CroYVMB isolates and other betasatellite sequences available in the database. The specificity of the primers was validated through online Primer Blast tool [34].

Designing of CYVMV-replicon vector

A multiple cloning sites (MCS) cassette containing the double 35S promoter, restriction enzyme sites (*Sma*I, *Kpn*I, *Bam*HI, *Eco*RI) and NOS terminator was amplified from the pRI101 vector (Takara, Japan) with the primers BM 638F and BM 639R. The primers were designed in such a way that the 5′ end of forward primer (BM 638F) contains *Spe*I and *Mlu*I while that of reverse primer (BM 638F) contains *Hin*dIII and *Bsr*GI restriction enzyme sites. The PCR mastermix contained 4 µl of 5× reaction buffer, 1.6 µl of 2.5 mM dNTP mix, 0.8 µl each of forward and reverses primers (1.0 micromoles), 1.0 U of Phusion *Taq*

Table 1 Details of the primers used for amplification of different components of CYVMV, CroYVMB, MCS cassette and GFP

Primer name	Primer sequence (5′-3′)	PCR template	Location	Nucleotide co-ordinates	Amplicon size (kb)
BM90F	ATGTCGAAGCGTCCAGCAGAT	CYVMV genome	V2	302-322	0.75
BM82R	TACAGAATCGTAGAAGTAA	Do	V1	1045-1063	
BM 638F	***CGACGCGT***TGTGCGCAATACACTAC	Do	IR	122-141	1.70
BM 639R	***GGACTAGT***TTAATAAAGATTGAATTTTATTG	Do	C3	1075-1094	
BM 640RF	***GGTGTACA***ATTGAATTGGGGACACTCA	Do	IR	2613-2631	0.20
BM 641FR	***CCCAAGCTT***TGTGCGCAATACACTACTTG	Do	IR	122-141	
BM 652F	ACTATCACCCTCAATCACTATAC	Do	C1	1944-1966	3.70
BM 653R	AGAACGGGCAAGACGATG	Do	C1	1926-1943	
BM 534F	CARTCATATCCTCCTSYTTGAATTC	CroYVMB	Beta C1	312-336	0.26
BM 535R	CATATATCAGAATGAGACGGGKTTG	Do	AT rich region	745-770	
BM 642F	***ACGCGTGGACTAGT***TTGCATGCCTGCAGGTCC	pRI101	35S Promoter	9009-8991	1.30
BM 643R	***TGTACACCCAAGCTT***CAGGAAACAGCTATGACCAT	pRI101	NOS terminator	7701-7682	
BM 650F	***CGGGGTACC***GTAGATCTGACTAGTAAAGG	pCAMBIA1302	GFP	4-23	0.77
BM 651F	***CGGAATTC***GCTAGCTTTGTATAGTTCAT	pCAMBIA1302	GFP	713-733	
BM AC3F	CAACCCCTATGTGTTTACAA	CYVMV genome	C3	1284-1303	

Bold and italic indicate position of a restriction enzyme site

polymerase (NEB, USA) and DNase-free water to make up the volume of 20 µl reaction mixtures. The PCR was performed in a thermocycler, Verti (Invitrogen, USA) with the following thermocycling programme: denaturation at 98 °C for 30 s followed by 40 cycles, each consisting of denaturing at 98 °C for 10 s, primer annealing at 52 °C for 20 s and synthesis at 72 °C for 40 s/kb followed by one cycle of final extension at 72 °C for 10 min. The amplified product was purified with Wizard® SV Gel and PCR Cleanup System (Promega, USA). The purified amplicon was cloned into pGEM®-T Easy Vector Systems (Promega, USA) as per manufacture's protocol. The clone (pGEM-MCS) was confirmed by restriction digestion and sequencing.

Partial genome of CYVMV [par-CYVMV, containing intergenic region (IR), C1, C2, C3 and C4 ORFs] was amplified from the CYVMV infectious clone using primers BM 640F (with *Spe*I site) and BM 641R (with *Mlu*I site). The IR was also amplified from the CYVMV infectious clone using primers BM 642F (with *Hin*dIII site) and BM 643R (with *Bsr*GI site). The amplified products were cloned separately into the pGEM®-T Easy Vector Systems (Promega, USA) as per manufacture's protocol. Partial genome clone (pGEM-par-CYVMV) and intergenic region clone (pGEM-IR) were confirmed by restriction digestion and sequencing.

To assemble all these clones, first the internal MCS of pCAMBIA 2300 vector was removed by digestion with *Eco*RI and *Hin*dIII and the sites were made blunt end using Quick blunting kit (NEB, USA). The new MCS-cassette was re-amplified from pGEM-MCS using Phusion *Taq* Polymerase and the amplicon was cloned into the blunt ended pCAMBIA2300 vector to generate pCAM-MCS. The intergenic region was released from pGEM-IR clone with *Hin*dIII, *Bsr*GI enzymes and cloned into pCAM-MCS to generate pCAM-MCS-IR. Finally, the partial CYVMV genome was released from the pGEM-par-CYVMV clone with *Spe*I and *Mlu*I digestion and cloned into pCAM-MCS-IR at *Spe*I and *Mlu*I site to generate the replicon vector (pCro). Finally the orientation of different sub-fragments of pCro clone was confirmed by sequencing with AC3F and M13R primer (Table 1). The schematic diagram (Fig. 2) illustrates the detail cloning steps.

Cloning of GFP in CYVMV replicon vector

The GFP gene was amplified from the pCAMBIA1302 using primers BM 650F (with *Kpn*I site) and BM 651R (with *Eco*RI site) following the PCR conditions mentioned earlier. The amplified GFP gene was cloned into pCro replicon vector at *Kpn*I and *Eco*RI sites to generate pCro-GFP.

Plant inoculation and GFP expression analysis

The pCro and pCro-GFP were mobilized separately into the EHA105 strain of *A. tumifacience* and agroinfiltration was carried out in *N. tabacum* as described earlier. The plant genomic DNA was isolated from the pCro and pCro-

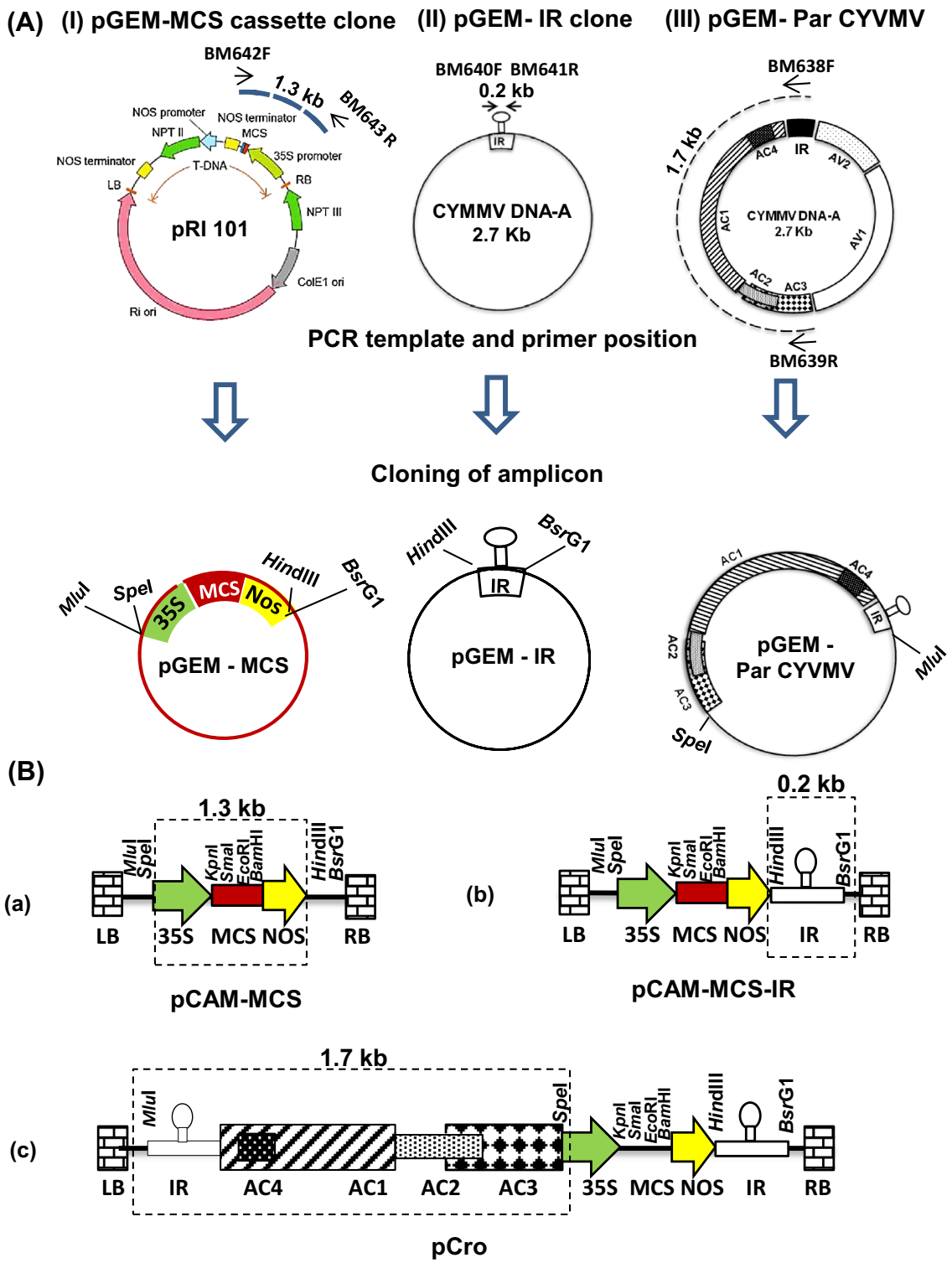

Fig. 2 Schematic representation of the design of pCro replicon vector based on genome of croton yellow vein mosaic virus (CYVMV). **a** Amplification and cloning of *i* MCS cassette, *ii* intergenic region (IR) and *iii* partial genome of CYVMV (par-CYVMV) in pGEMT-easy vector. Template for PCR, primer name, their positions and expected amplicon sizes were depicted in the *upper panel*. **b** Sequential sub-cloning of *i* MCS cassette, *ii* IR and *iii* par-CYVMV from pGEMT vector to a blunted pCAMBIA2300 vector to develop pCro vector

Table 2 Agroinfectivity analysis of the cloned DNA of croton yellow vein mosaic virus (CYVMV) and croton yellow vein mosaic betasatellite (CroYVMB) on different *Nicotiana* spp.

Construct[a]	Plant	No. of symptomatic plants out of ten no. inoculated			dpi	Systemic symptoms
		E1	E2	E3		
CYVMV	NB	10	8	8	10–13	Leaf curling, leaf rolling and vein thickening
	NG	6	7	5	15–20	Mild puckering on leaves
	NT	2	1	4	30–35	Vein clearing
CYVMV + CroYVMB	NB	10	10	9	8–10	Severe leaf curling, leaf rolling, vein thickening and stunting
	NG	4	8	7	10–12	Severe leaf curling with puckering, smalling of leaves, stunting
	NT	4	7	6	25–30	Puckering on leaves and stunting
pC2300 (mock inoculation)	NB	0	0	0	–	No symptoms
	NG	0	0	0	–	No symptoms
	NT	0	0	0	–	No symptoms

NB, *Nicotiana benthamiana*; NG, *N. glutinosa*; NT, *N. tabacum*; dpi, days post inoculation; E, experiment number

[a] CYVMV: a partial dimer was used, CroYVMB: croton yellow vein mosaic betasatellite dimer was used. Constructs were mobilized into *Agrobacterium tumefaciens* EHA105 strain

GFP-infiltrated *N. tabacum* leaf using DNeasy Plant Mini Kit (Qiagen, USA) as per manufacturer's protocol. After plant inoculation, episome formation from the agro-constructs of pCro and pCro-GFP was assessed through inverse PCR using outward primer pairs BM 640R/BM 642F and BM 652F/BM 653R, respectively (outward arrows in Fig. 5a). 200 ng of total plant DNA from the agroinfiltrated plants was used in inverse PCR keeping all other PCR parameters same as described earlier. Expression of green fluorescent protein (GFP) was observed through the epi-fluorescence analysis of infiltrated leaf at 5 days post inoculation (dpi) under the UVL-56 illuminating ultraviolet (UV) light at 365 nm (UV product, Upland, CA) in the dark room and photographed with a digital camera.

Results

Infectivity analysis of the agro-constructs

Restriction digestion of pCAM-M4A-A1.4mer with *Xba*I and pCAM-M4β-2.0mer with *Eco*RI, released 2.7 and 1.3 kb fragments, respectively (Fig. 1c, d) and thus confirmed the tandem orientation of the constructs. The result of the infectivity experiment was summarized in Table 2. In every set of experiment, more number of plants of *N. benthamiana* developed symptom compared to other two species of *Nicotiana*. Inoculation of pCAM-M4A-A1.4mer alone produced leaf curling, leaf rolling and vein thickening symptoms on *N. benthamiana* at 10–13 dpi (Fig. 3d). In *N. glutinosa* and *N. tabacum* it produced mild puckering and vein clearing symptoms, respectively (Fig. 3e, f). The incubation time in *N. tabacum* is more (30–35 dpi) than that in *N. glutinosa* (15–20 dpi) (Table 2). Co-inoculation of pCAM-M4A-A1.4mer with pCAM-M4β-2.0mer on *N. benthamiana*, *N. glutinosa* and *N. tabacum* resulted in severe leaf curl and stunting symptoms (Fig. 3j–l). The incubation time was 8–10, 10–12 and 25–30 dpi for *N. benthamiana*, *N. glutinosa* and *N. tabacum*, respectively when both the constructs were co-inoculated. Plants inoculated with pCAMBIA2300 alone, however, did not produce any symptoms.

Detection of CYVMV and CroYVMB in agroinoculated plants

Newly developed symptomatic leaves of plants agroinoculated either with the construct of CYVMV alone or in combination with CroYVMB showed expected 750 bp amplicon specific to CYVMV (Fig. 4a), indicating replication and systemic distribution of the virus. Plants, which were co-inoculated with betasatellite produced CroYVMB specific 260 bp amplicon (Fig. 4b). However, no amplification was observed in the plants inoculated either with CroYVMB alone (data not shown) or plants inoculated with binary vector, pC2300.

CYVMV-based replicon vector

PCR amplification of MCS cassette, IR and partial CYVMV genome yielded 1.3, 0.2 and 1.7 kb amplicons, respectively. The strategy adopted to construct CYVMV replicon has been described in detail in the "Materials and methods" section. After sequential cloning of these components into a blunt pCAMBIA2300 vector, orientations of

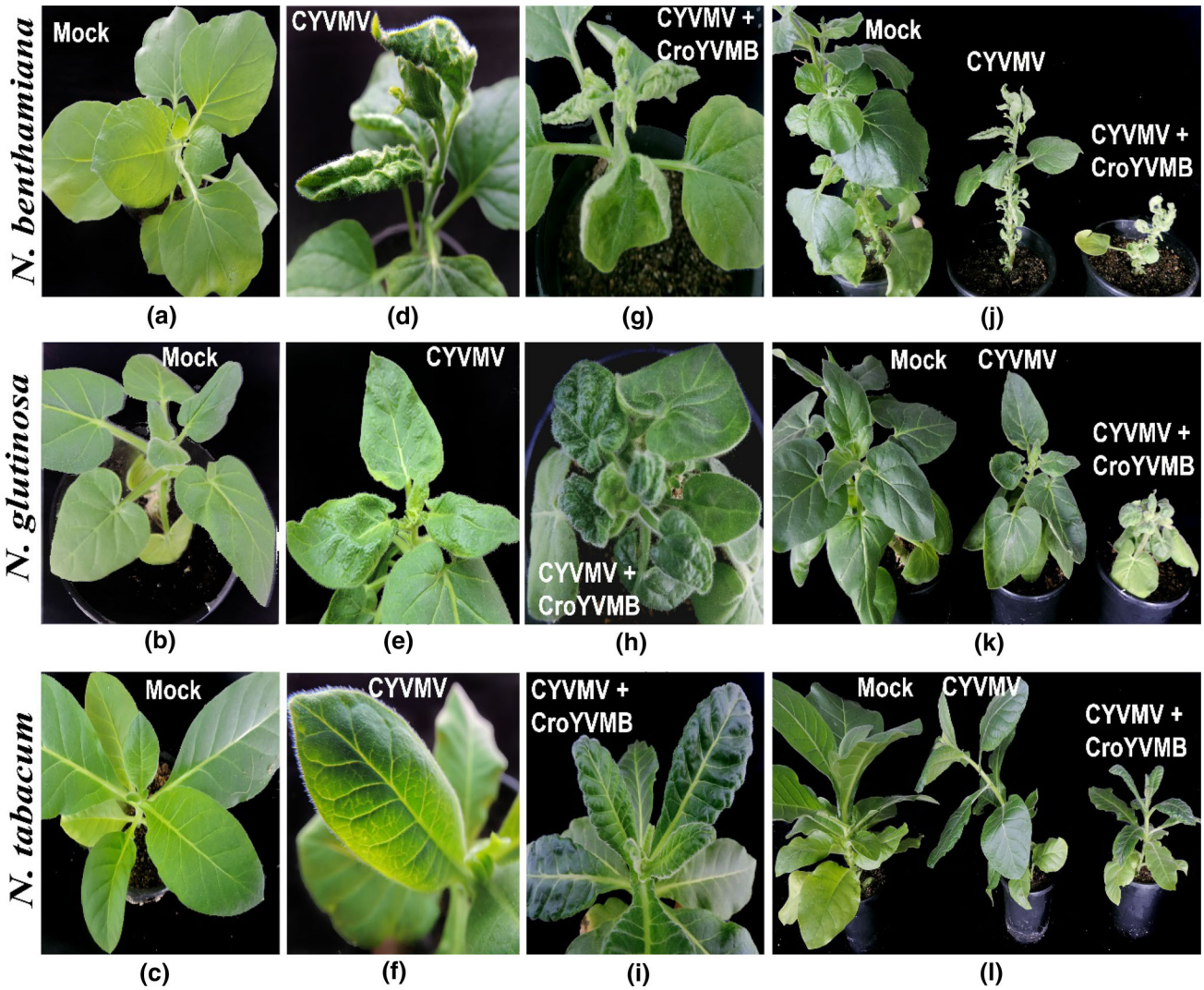

Fig. 3 Agroinfectivity of croton yellow vein mosaic virus (CYVMV) and croton yellow vein mosaic betasatellite (CroYVMB) on *Nicotiana benthamiana*, *N. glutinosa* and *N. tabacum*. **a–c** Mock inoculated plants showing no symptom, **d–f** inoculated plants with CYVMV construct alone showing different symptoms in the different species of tobacco, **g–i** inoculated plants with CYVMV DNA-A and betasatellite CroYVMB showing severe symptoms, **j–l** comparison of overall plant growth and stunting symptoms in the different tobacco species

these fragments were confirmed by appropriate restriction digestion and by PCR using appropriate primers (data not shown). The final resulting plasmid, designated as pCro, is depicted in Fig. 2b(iii), showing the various restriction sites and the positions of the cloned fragments.

In-planta expression of GFP through CYVMV replicon

Amplification of GFP gene resulted in an amplicon of 0.77 kb, which was cloned in the pCro vector. After agroinoculation of pCro plasmid either alone or with GFP (pCro-GFP) into plants, no visible symptom of the virus is produced. However, a circular episome of 3.0 kb for pCro and 3.7 kb for pCro-GFP were excised out (Fig. 5a) and replicates independently in leaves for a period of time. These replicon episomes were detected by inverse PCR, which resulted in 1.3 and 3.7 kb amplicons for pCro and pCro-GFP, respectively in only the inoculated portion of the leaf at 5 dpi and no such amplicon was observed in healthy control (Fig. 5b, c). The epifluorescence of GFP was observed in the pCro-GFP inoculated leaves under the UV lamp at 5 dpi (Fig. 5d).

Discussion

Highly infectious binary vector based constructs to genome of CYVMV and CroYVMB were developed and agro infectivity of these constructs was demonstrated in this

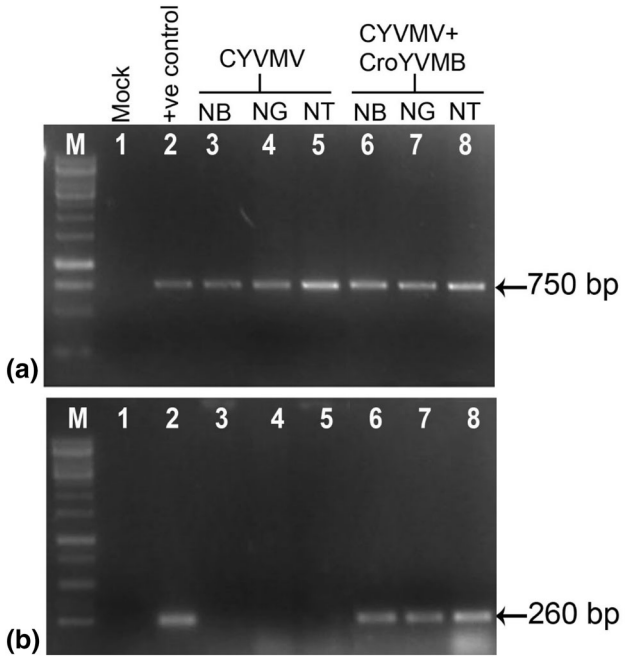

Fig. 4 PCR confirmation of the presence of genome of **a** CYVMV and **b** CroYVMB in agroinoculated *Nicotiana benthamiana* (NB), *N. glutinosa* (NG) and *N. tabacum* (NT) plants. *M* marker; *lane 1* mock inoculated plant; *lane 2* positive control for CYVMV and CroYVMB; *lane 3–5* amplification of CYVMV (for **a**) or CroYVMB (for **b**) from NB, NG and NT plants, respectively inoculated with only CYVMV; *lane 6–8* amplification of CYVMV (for **a**) or CroYVMB (for **b**) from NB, NG and NT plants, respectively inoculated with CYVMV and CroYVMB

study. Infectivity of the agro-mobilized constructs of CYVMV and CroYVMB was assessed on *N. benthamiana*, *N. glutinosa* and *N. tabacum* based on symptom production and PCR analysis. Partial tandem repeat construct of genome of CYVMV is infectious and could produce leaf curl symptoms alone. Co-inoculation with the betasatellite reduced the incubation time for symptom development and enhanced the symptom severity. This study indicated that CYVMV was a typical monopartite begomovirus and the betasatellite was involved only to modulate the symptom severity. However, earlier studies indicated that both CYVMV and CroYVMB are essential for production of yellow vein mosaic symptom in *C. bonplandianum* [27]. There are two types of betasatellite associated monopartite begomoviruses depending upon whether or not the cognate betasatellite molecules are essential for inducing symptoms [8]. In the majority of these begomoviruses, betasatellite molecules are necessary for symptom development [3, 8, 14, 29]. In the second group, betasatellite is dispensable [17, 30]. The present study and earlier observation suggested that in the case of CYVMV, the requirement of betasatellite for induction of symptom is host dependent and CYVMV is capable of inducing symptom alone in at least three different tobacco species.

In this study, a CYVMV genome-based replicating shuttle vector, pCro, was constructed and tested for its ability to express GFP in the plant. The addition of an MCS under the control of a double 35S promoter increases the opportunity of cloning different genes and directs their translation. Efforts to develop geminiviral replicons for gene expression vector began long back, but the genome size constraints have hindered the use of full viruses for heterologous protein expression. Virus genome size constraints are imposed by cell-to-cell movement through plasmodesmata and not by replication [6]. Therefore, virus genes involved in encapsidation and cell-to-cell movement can be deleted to increase the cargo capacity for large heterologous sequences. Deletion of coat protein in some bipartite begomoviruses has been employed to insert foreign sequence up to 800 nt [22]. Geminivirus replicon vector was also developed by deleting AV2/V2 and CP genes of other geminiviruses [11, 21, 24, 25]. In this study, we deleted the V2 and V1 gene of CYVMV and retained the elements required for rolling-circle replication. This replicon based vector enabled us to simultaneously amplify and expresses foreign gene in high-copy number by rolling-circle replication. Due to the absence of the V2 and V1 genes, the vector is incapable of spreading and developing disease throughout the host plant. The V2 gene has been shown to be involved in movement and it acts as a potent silencing suppressor in other begomoviruses [26, 35]. Deletion of V2 may hinder the rate of virus multiplication which needs to be studied in future. Additionally, lack of CP eliminates any potential for insect-based transfer of the geminivirus replicon between plants, and thus ensures the biosafety of such replicon vector.

In summary, we have established that agro construct of CYVMV genome alone is highly infectious in the different tobacco species. Using this infectious construct, a CYVMV-based replicon vector was developed for transient expression of GFP in plants. Such monopartite begomovirus based vector is rare and will help in the production of vaccine and other pharmaceuticals in the plant. As the CYVMV has a large host range, the system that we are presenting here will be suitable for expressing proteins in many plant species. This replicon vector has obvious applications in the area of functional genomics either as an overexpression or as a silencing system. Based on this basic replicon vector, in future, improvement can be made

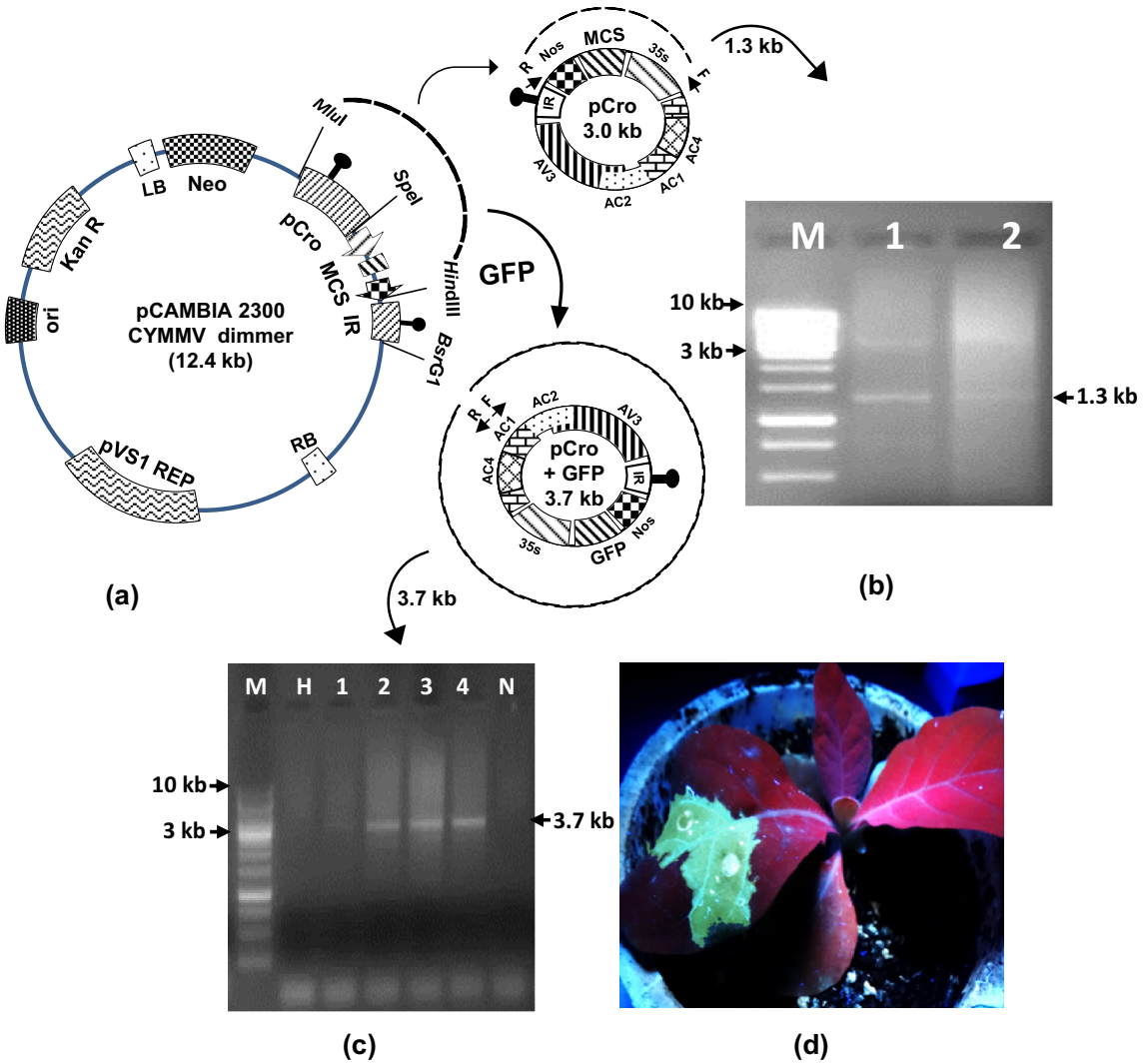

Fig. 5 Expression of GFP in *N. tabacum* plant using pCro vector. **a** Schematic representation of episomal release of pCro and pCro-GFP from binary vector backbone. Inverse primer positions are indicated as *small arrows* within the episomal fragments, **b** detection of pCro episome from agroinoculated plants by inverse-PCR which yielded a 1.3 kb amplicon, **c** detection of pCro-GFP episome from agroinoculated plants by PCR which yielded a 3.7 kb amplicon. *M* 1 kb ladder, *N* negative control, *H* healthy control; **d** epifluorescence of GFP from *N. tabacum* under UV light

to increase its efficiency and distribution throughout the plants.

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