Engineering resistance against physalis mottle tymovirus by expression of the coat protein and 3′ noncoding region

C. T. Ranjith-Kumar*, M. Manoharan†, S. Krishna Prasad*, Shoba Cherian*, M. Umashankar*, G. Lakshmi Sita† and H. S. Savithri*,‡

*Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India
†Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

A 748 nucleotides cDNA fragment corresponding to the 3′ terminal of physalis mottle virus, PhMV (formerly known as belladonna mottle virus) (#Y16104) genomic RNA encompassing the tymobox, coat protein ORF and 3′ noncoding region was cloned into the binary vector pKYLX 71 35 S^2 and introduced into *N. tabacum* cv. Havana plants using *Agrobacterium*-mediated transformation. The R0 transgenic plants showed accumulation of coat protein which self-assembled into capsids *in vivo*. The transgenic R1 and R2 plants showed delay in symptom expression and virus accumulation upon challenge with PhMV. 55 and 65% of the plants showed no detectable symptoms in the R1 and R2 transgenic plants respectively, when challenged with 10 µg/ml virus. Further, no detectable symptoms were observed in 75% and 25% of the R1 and R2 transgenic plants respectively, after 50 days of post infection when challenged with 10 µg/ml RNA. Thus the expression of PhMV coat protein and 3′ noncoding sequence confers a high level of resistance against PhMV infection.

SANFORD and Johnston[1] suggested that engineered resistance to insect, fungal and viral parasites of plants could be achieved by utilizing portions of the pathogen's own genome. This concept of pathogen-derived resistance was first demonstrated in transgenic tobacco plants expressing the coat protein gene of tobacco mosaic tobamovirus (TMV)[2]. Since then, genetically engineered resistance has been reported for a number of plant viruses involving virus-derived genes or genome fragments[3–6].

Physalis mottle tymovirus (PhMV) (formerly known as belladonna mottle virus) belongs to the tymovirus group of plant viruses[7]. It consists of a 6.67 kb RNA genome (gene bank #Y16104)[8] encapsidated in a protein shell of 180 identical subunits (MW 20 kDa). The first attempt to generate transgenic plants resistant to tymovirus was made by Zaccomer *et al.*[9] by introducing 3′ terminal 100 nucleotides of turnip yellow mosaic tymovirus (TYMV) genome into rapeseed plants. These transgenic plants expressing the sense transcripts showed partial protection against TYMV infection, which was overcome when the inoculum concentration was increased. It was proposed that the competition of the sense RNA transgene transcript with the viral RNA for the viral replicase conferred resistance to viral infection in these plants[9].

In an earlier study, using an *in vivo* protoplast assay system we have shown that effective inhibition of virus multiplication was observed when protoplasts were coinoculated with PhMV genomic RNA (gRNA) and a transcript corresponding to 748 nucleotides from the 3′ end of the genome[10]. This 3′ terminal sequence encompassed a conserved 16 nucleotide sequence called the tymobox[11] upstream of the coat protein (CP) open reading frame (ORF), the CP gene and the entire 3′ noncoding (NC) region of PhMV RNA. In order to assess whether the expression of CP and NC would confer resistance to virus infection, we have developed transgenic tobacco plants by introducing the 3′ terminal 748 nucleotides of PhMV into the *N. tabaccum* genome. The transgenic plants not only showed expression of the CP gene but also exhibited a high level of resistance against intact virus as well as RNA infection in the R1 and R2 generation plants.

[α-^{32}P]dATP (3000 Ci/mmol) was obtained from Amersham International. Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs and Amersham International. All other chemicals were of analytical grade. The oligonucleotide primers were purchased from Bangalore Genei Pvt. Ltd, India.

PhMV was maintained on the systemic host *N. tabacum* cv. Havana. Transformation and regeneration experiments were also performed using the same host. PhMV was purified as described earlier[12] and gRNA was isolated according to Jacob *et al.*[7].

The 3′ terminal segment of PhMV genome encompassing the tymobox-CP–NC sequence (TCN) was specifically PCR amplified using primers,

T1 – GAATTCGAGTCTGAATTGCTTCAC and
N2 – TGGTTTCCGTTACCCACGGAAGGGGGG

from cDNA clone TA51 (ref. 13). The amplified TCN product was gel purified following low melting temperature agarose electrophoresis, end filled and cloned at the *Sma*I site of pBlueScript to obtain the clone pBSTCN[14]. The TCN insert was then released from pBSTCN by *Xba*I/*Hin*dIII double digestion and cloned at the same sites into the binary vector pKYLX 71 35 S^2 (ref. 15) to generate pKYTCN (Figure 1). This binary vector has a duplicated cauliflower mosaic virus 35 S promoter upstream of the multiple cloning site, followed by a ribulose-1,5-bisphosphate carboxylase/oxygenase subunit gene transcription terminator. In addition it also harbours a neomycin phosphotransferase II gene which confers resistance to kanamycin.

pKYTCN was introduced in *Agrobacterium tumefaciens* strain C58C1 by the direct DNA transfer method[16]. Leaf discs of greenhouse-grown *N. tabacum* cv. Havana plants were surface sterilized using standard procedures and

‡For correspondence. (e-mail: bchss@biochem.iisc.ernet.in)

transformed with C58C1 harbouring pKYTCN. The transformed shoots were regenerated on Murashige and Skoog (MS) medium containing vitamins, 0.8% (w/v) agar and 3% (w/v) sucrose supplemented with 1 mg/l 6-benzylaminopurine, 100 mg/l kanamycin and 250 mg/l carbenecillin and were subsequently rooted. Roots were developed on the MS medium supplemented with 1 mg/l indole-3-butyric acid or indole-3-acetic acid, along with 100 mg/l kanamycin and 250 mg/l carbenecillin. The putative transgenic plants in the four-leaf stage were transplanted into pots and used in all the further studies.

Segregation analysis was carried out by germinating the R1 and R2 seeds on plates containing MS medium in the absence or presence of 100 mg/l kanamycin medium. The number of seeds that germinated were counted to evaluate the presence of the transgene.

The transgenic and control plants were mechanically inoculated with either purified virus (10 µg /ml) or PhMV RNA (10 µg/ml) using a sterile glass rod. The appearance of symptoms was monitored on a daily basis until 50 days post inoculation.

Genomic DNA (50 ng) from the control and transformed plants which was isolated by the method of Dellaporta *et al*.[17] was used as template in the PCR reaction. The PCR was performed with CP primers, Ph-N (5′ GCGCCCATGGCCTCCATCCCCGCCCCT 3′) starting with a *Nco*I site (underlined nucleotides) followed by the coding sequences of CP from the 12th amino acid onwards in the sense orientation and Ph-A (5′ CCGGGATCCTTAGTTGGCTATCAG 3′) encompassing the stop codon and a *Bam*HI site (underlined nucleotides) in the antisense orientation. The reaction was carried out using Taq DNA polymerase under the following conditions: 94°C, 1 min for denaturation; 55°C, 1 min for annealing; 72°C, 1 min for elongation (30 cycles). The reaction products were analysed on 1% agarose gel.

Plant genomic DNA samples (25 µg) after restriction digestion with *Eco*RI were analysed on 0.8% agarose gel. The DNA was then transferred to nylon membrane by capillary transfer method and probed with random primer labelled CP gene as described by Sambrook *et al*.[14].

Leaves from transgenic and nontransgenic plants were homogenized in 0.05 M citrate buffer, pH 5.5 and the crude extract (200 µg total protein/well) was subjected to SDS-12% polyacrylamide gel electrophoresis. After electrophoresis the proteins were transferred to a nitrocellulose membrane and Western blot analysis was carried out using the mouse monoclonal antibody PA3B2 to native PhMV as the primary antibody and horseradish peroxidase conjugated goat antimouse IgG as the detecting antibody with 3,3′-diaminobenzidine as the substrate[18].

Empty capsids were isolated from the leaves of transgenic plants following the procedure described earlier for the isolation of top component of PhMV from infected *N. glutinosa* plants[12]. Capsids isolated from control, infected and transgenic plants were loaded onto 10–40% sucrose gradients and centrifuged at 35,000 rpm for 3 h in SW 41 rotor. At the end of the run 0.5 ml fractions were collected and the absorbance was measured at 280 nm. 0.1 mg/ml empty capsids isolated from transgenic plants were applied onto formvar-coated carbon-shadowed copper grids. The particles were visualized by negative staining with 1% (w/v) uranyl acetate and examined by high resolution JEOL 200X electron microscope at a magnification of 68,000 ×.

The pKYTCN construct harbouring the 3′ terminal 748 nucleotides of PhMV genome was obtained as described earlier (Figure 1). The leaf discs of *N. tabacum* cv. Havana plants were transformed with *A. tumefaciens* strain C58C1 harbouring binary vector pKYTCN. A total of 80 putative transformed plants were obtained upon regeneration using the protocol described earlier. Twelve of these plants were found to harbour CP gene by the PCR analysis of DNA isolated from these plants (Figure 2). The remaining plants could be false positives. The reasons for such escapes are unclear. One of these plants (Figure 2, lane 2) did not give a prominent PCR product. Genomic DNA extracted from the

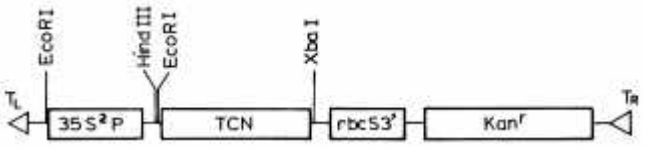

Figure 1. Schematic representation of PhMV sequence in pKYTCN. Duplicated cauliflower mosaic virus 35 S promoter, 3′ end of the gene encoding for the small subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase (rbcS), Kan^r – neomycin phosphotransferase II gene, T_L and T_R – T-DNA left and right borders, respectively are shown along with the PhMVTCN sequence. The *Xba*I, *Hin*dIII and *Eco*RI sites are indicated by arrows.

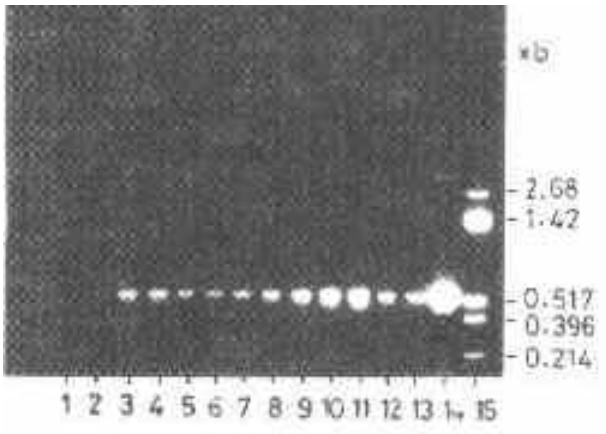

Figure 2. PCR analysis of transgenic plants. 50 ng of genomic DNA extracted from plants were used for PCR amplification with primers corresponding to CP gene. The PCR products were visualized on 1% agarose gel after ethidium bromide staining. Lane 1, nontransgenic plant; lanes 2–13, transgenic plants; lane 14, product obtained when pKYTCN was used as template; lane 15, DNA molecular weight makers.

remaining 11 plants T2–T12 were further subjected to Southern blot analysis (Figure 3). The putative transgenic lines T3–T6 and T8 gave a similar pattern, T9 showed four bands (lane 9) and T2, T7, T11 and T12 gave two bands. No bands were visible in lane 10 corresponding to T10. The similar banding pattern observed in T3–T6 and T8 could be because these putatively transgenic plants were generated from the same explants. Further analysis was carried out with 10 plants T2–T9 and T11–T12 which were confirmed positive by Southern analysis. These plants were transferred to pots and Western blot analysis was performed using the protein extract from the leaves of these plants. All the plants showed similar levels of CP accumulation (Figure 4).

Recombinant PhMV CP expressed in *Escherichia coli* has been shown to self assemble into capsids[19]. In order to examine whether the expressed CP in the transgenic plants was also capable of forming empty capsids, 75 g of leaves pooled from the transgenic R0 plants (shown positive by Southern and Western analysis) and non- transgenic plants were used for isolation of empty capsids formed, if any. A distinct peak co-migrating with the top component (empty capsids formed *in vivo* upon PhMV infection) was seen only in the extracts from transgenic plants and not in the control plants (Figure 5). The inset shows the electron micrograph of the particles isolated from the transgenic plants. Empty capsids of approximately 30 nm diameter with stain penetration in the center could be observed. Similar self-assembly of CPs expressed in transgenic plants has been observed in the case of Norwalk virus[20], TMV[21], arabis mosaic virus[22] and alfalfa mosaic virus (AlMV)[23].

Inoculation of PhMV to *N. tabacum* cv. Havana results in systemic infection leading to severe yellow mottle and leaf distortion within a week. The dilution end point for PhMV (previously named belladonna mottle virus (I)), is 10^{-6} to 10^{-7} (ref. 24). All the 10 R0 plants showed delay in symptom expression while 3 of them showed no detectable symptoms even after 30 days post infection (Figure 6). One of these three R0 plants (T9) which showed no detectable symptoms was self-pollinated and seeds thus formed were used for the production of R1 generation plants. Similarly, one of R1 generation plants that showed no detectable symptom expression upon challenge inoculation with purified virus was self-pollinated and the seeds thus obtained were used for generation of R2 plants. These R1 and R2 seeds (100 each) were germinated in the presence of kanamycin. 80 and 76 out of 100 seeds germinated in the presence of kanamycin in the case of R1 and R2 plants, respectively. The segregation ratio of 3 : 1 is suggestive of the stable integration of transgene and Mendelian pattern of inheritance. R1 and R2 progeny plants (18 each) that were selected on kanamycin medium were allowed to grow and transferred to pots. The total protein was extracted from each of these plants prior to inoculation and Western analysis was performed. It is apparent from Figure 7 *a* and *b* that the level of expression of the CP gene was variable when an equal amount of total protein (200 µg) was loaded from these plants. With the exception of one plant in each group, all of them expressed CP. These groups of 18 R1 and R2 plants which showed the expression of CP were used for evaluation of resistance.

Nine of the R1 and R2 progeny plants along with 9 non transgenic plants were inoculated with 10 µg/ml virus and 10 µg/ml RNA and the symptom expression was monitored over a period of 50 days. As apparent from Tables 1 and 2, all the transgenic plants showed delay in symptom expression. Further, 7 and 2 plants out of 9 R1 and R2 transgenic plants respectively did not show any symptoms even after 50 days post inoculation when inoculated with 10 µg/ml RNA. Similarly, 2 plants out of 9 R1 and R2

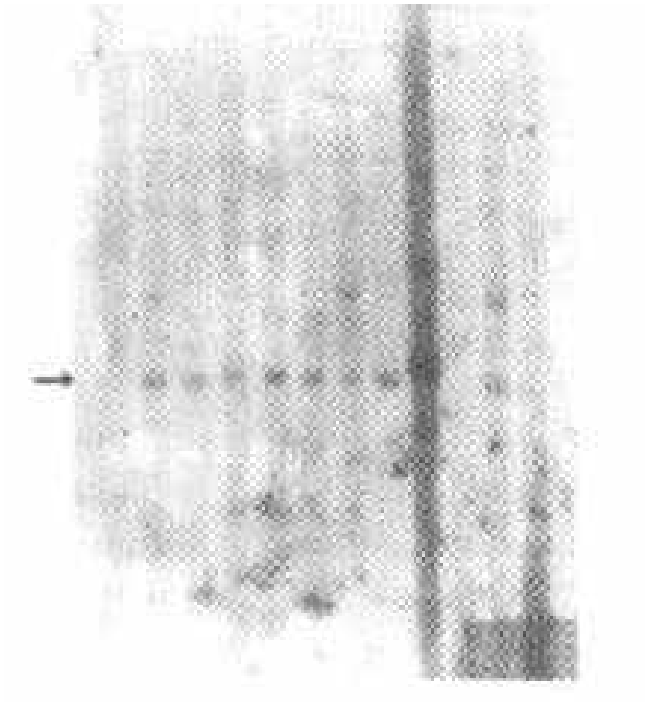

Figure 3. Southern blot analysis of putatively transgenic R0 plants. 25 µg of genomic DNA extracted from plants was digested with *Eco*RI and used for Southern blot analysis with labelled CP gene as probe. Lanes 1 and 13, non-transformed plants. Lanes 2–12, putatively transgenic R0 plants (T2–T12).

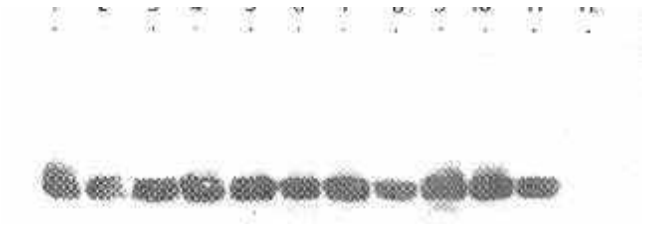

Figure 4. Western blot analysis of R0 transgenic plants. The crude protein extracted from the transgenic R0 plants (1 : 4 w/v) (25 µl) was loaded on SDS 12% PAGE and Western blot analysis was performed using monoclonal antibody PA3B2 against CP as described in text. Lane 1, PhMV control; lanes 2–12, T2–T9 and T11–T12 transgenic plants, and lane 12, non-transgenic plant.

transgenic plants did not show any symptoms 50 days post inoculation with 10 μg/ml virus. 100% infection was observed in all non-transgenic plants on day 4 itself. Interestingly, 4 and 6 of the R1 and R2 plants inoculated with virus showed recovery after 28 days.

The 3′ terminal 748 nucleotides of PhMV genome was introduced into *N. tobaccum* cv Havana plants and the expression of the CP gene was observed in the R0, R1 and R2 transgenic plants. Further, the expressed CP could assemble into capsids *in vivo* (Figure 3). Although the level of expression was similar in the R0 plants it was variable in the R1 and R2 transgenic plants. Further, plants which showed negligible expression exhibited high level of resistance even 30 days after inoculation (example – Figure 7 *a* and *b*, plants 1 and 11 in R1 trangenic plants and plants 5 and 6 in R2 transgenic plants). As evident from Table 1, nearly 80% of the R1 plants inoculated with RNA showed negligible infection. In the group that was challenged with virus, most of the plants showed recovery after 30 days. Thus in the present case, the observed resistance may not be just due to CP-mediated protection. The level of protection was better in the RNA inoculated samples. Even when as high as 10 μg/ml virus was used as inoculum, 2 out of 9 plants showed no detectable symptoms in both R1 and R2 progeny plants from day 1 to day 50. Interestingly, R2 progeny plants seemed to be more susceptible than R1 progeny plants although all of them showed delay in symptom expression. Further, many of the plants which showed mild symptoms also recovered after two months.

The results obtained when the transgenic plants were challenged with genomic viral RNA suggest that inhibition of virus multiplication might have occurred after the uncoating step. The transgenic nucleic acid could compete with the viral genome to redirect host- or viral-encoded

Figure 7. Western analysis of R1 and R2 transgenic plants. ***a***, Equal amount of total protein from each of the plants (200 μg) was loaded and Western analysis carried out using monoclonal antibody PA3B2 against coat protein as described in the text. Lane M, markers (stained with ponceau S and marked as shown prior to Western analysis. Molecular weights are as indicated). Lane H, total protein (200 μg) from control non-transgenic plant. Lanes 1–18 correspond to R1 plants 1–18. ***b***, R2 transgenic plants. Lane M, molecular weight markers; Lane H, non-transgenic control plants; Lanes 1–18 corresponds to R2 transgenic plants 1–18.

Table 1. Disease development in R1 transgenic plants inoculated with PhMV or PhMV RNA

Inoculum	Plant no	Disease development during various days post inoculation							
		4	6	8	10	12	14	28	50
PhMV RNA (10 μg/ml)	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0
	4	0	0	0	0	1	1	2	0
	8	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	1	2	2
	13	0	0	0	0	1	2	2	2
	14	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0
	C	3	5	5	5	5	5	5	5
PhMV (10 μg/ml)	5	0	0	0	0	1	1	2	0
	6	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	2	2	0
	10	0	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	2	1
	12	0	0	0	0	1	1	2	1
	16	0	0	0	0	0	1	2	1
	17	0	0	0	0	1	1	2	1
	18	0	0	1	1	2	2	3	3
	C	3	5	5	5	5	5	5	5

0: No sign of infection.
1: Chlorotic lesion but no systemic infection.
2: Chlorotic lesion with mild systemic infection.
3: Yellow mottling in the newly developing leaves only.
5: Severe systemic yellow mottling with leaf distortion.
C: Control non-transgenic plants (9 each).

Figure 5. Sucrose density gradient analysis of virus-like particles in transgenic plants. Capsids were isolated from infected (▲), R0 plants (■) and non-transgenic (●) plants and separated on 10–40% sucrose density gradient. 0.5 ml fractions (from the bottom of the gradient) were collected and absorbance was monitored at 280 nm. The *y*-axis corresponds to $A_{280\ nm}$ and the *x*-axis corresponds to the fraction number. T, top component (empty capsids), and B, bottom component (intact virus particles). The inset shows the electron micrograph of empty capsids isolated from transgenic plants visualized by negative staining with 1% (w/v) uranyl acetate, at a magnification of 68,000 ×.

Figure 6. Evaluation of infection in R0 transgenic plants. The R0 transgenic and [illegible] mechanically with 10 μg/ml virus. [illegible] 7 days post inoculation. The [illegible] correspond to non-transgenic plants, the lower four plants (5–8) correspond to R0 transgenic plants.

Table 2. Disease development in transgenic R2 plants inoculated with PhMV or PhMV RNA

Inoculum	Plant no	Disease development during various days post inoculation							
		4	6	8	10	12	14	28	50
PhMV RNA (10 μg/ml)	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	1	1	3	3
	3	0	0	0	0	1	1	3	3
	6	0	0	0	0	0	0	0	0
	7	0	0	0	0	1	1	2	1
	8	0	0	0	0	1	1	2	2
	9	0	0	1	1	2	2	3	1
	12	0	0	0	0	1	1	2	2
	13	0	0	0	0	1	1	2	1
	C	3	5	5	5	5	5	5	5
PhMV (10 μg/ml)	4	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0
	10	0	0	0	1	1	1	3	1
	11	0	0	0	0	1	1	3	0
	14	0	0	1	1	2	2	3	1
	15	0	0	1	2	2	2	3	1
	16	0	0	0	1	2	2	3	0
	17	0	0	0	0	1	1	2	0
	18	0	0	0	0	0	1	2	0
	C	3	5	5	5	5	5	5	5

0: No sign of infection
1: Chlorotic lesion but no systemic infection
2: Chlorotic lesion with mild systemic infection
3: Yellow mottling in the newly developing leaves only
5: Severe systemic yellow mottling with leaf distortion
C: Control non-transgenic plants (9 each)

proteins into nonproductive interactions for replication or spread of the virus in the infected plant. It was suggested that resistance from a transgenically expressed 3′ noncoding region of TMV RNA was conferred through direct competition with the viral genome[9]. Inhibition of virus multiplication was observed in protoplasts, when they were co-inoculated with PhMV gRNA and transcripts corresponding to 3′ NC region of PhMV[10]. It is possible that even in the whole plant, the RNA transcript encompassing the 3′ NC region might be interfering with the virus multiplication. Similar observations were also made by Yie *et al*.[25] who found elevated resistance to cucumber mosaic virus (CMV) in transgenic tobacco expressing both a CMV CP gene and a satellite RNA of CMV attenuated viral symptom expression. The mechanism of resistance in the present case is not clear and it could be due to the combined effect of expression of CP and 3′ non-coding sequence. Further experiments are in progress to delineate the roles of CP and 3′ NC in conferring resistance to PhMV.

In many of the positive single-stranded RNA plant viruses the CP gene is 3′ proximal. Hence the strategy of using the construct harbouring CP gene and 3′ NC region could be useful in the generation of transgenic plants with elevated levels of resistance against economically important viral diseases.

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