Cloning and sequencing of potato virus Y coat protein gene from an Indian isolate and development of transgenic tobacco for PVY resistance

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Potato virus Y (PVY) coat protein gene has been cloned from an Indian isolate of PVY*. The coat protein gene from this isolate showed 93.2% nucleic acid sequence homology and more than 94% amino acid sequence homology compared to an American isolate. The coat protein gene has been sub-cloned into plant expression vector pBINPLUS and tobacco leaf discs were transformed using Agrobacterium tumefaciens. The transgenic tobacco plants analysed through PCR and Southern hybridization, confirmed the integration of coat protein gene into tobacco genome. The transgenic plants were hardened in the greenhouse and were mechanically infected with crude viral extract. The virus titre was analysed through ELISA and the transgenic plants showed less virus titre compared to non-transformed control plants. The cloned coat protein gene of PVY will be useful for imparting resistance against that virus in Indian potato cultivars.

Potato virus Y (PVY) is a type member of the potyvirus group, the largest group of plant viruses. It is a flexuous, helical, rod-shaped virus of approximately 700 nm length, 12 nm diameter and 3.4 nm helical pitch. It has a single-stranded positive sense RNA genome of approximately 10 kb length, encapsidated by about 2000 copies of a single coat protein of approximately 30 kD size. PVY infects potato and many other Solanaceous crops, including tobacco, tomato, etc. Infection of tobacco with PVY severely reduces leaf yield and quality. PVY infection in tobacco crop results in symptoms ranging from vein banding and mild mosaic to severe leaf necrosis and necrotic lesions on veins and stalks, depending on virus isolate and tobacco genotypes. Control of PVY is difficult, the most effective approaches to control PVY in tobacco field include the development of PVY-resistant varieties and the use of cross-protection or interference between strains of viruses. Sources of PVY resistance in N. tabacum germ plasm is rare and sometimes associated with negative attributes. Moreover, some of the virulent PVY strains could overcome certain resistance genes.

The recent progress in genetic engineering has opened up many avenues to develop viral disease-resistant plants. Different genetic engineering techniques applied to achieve viral disease resistance are cloning and expression of antisense RNA, plantibody, coat protein gene, viral polymerase, etc. Coat protein-mediated protection (CPMP) is potentially useful for developing PVY-resistant flue-cured and barley tobacco, which are commercially very important types of tobacco. Successful coat protein gene-mediated resistance against PVY in both potato and tobacco has already been reported4–7, but no source of PVY resistance among existing Solanaceous crop varieties is available in India. In this paper we report cloning and sequencing of PVY coat protein (CP) gene from an Indian isolate and its transfer to tobacco plants for developing PVY resistance.

Materials and methods

Isolation of virus and preparation of viral genomic RNA

PVY was isolated from infected tobacco plants as mentioned by Paul Khurana et al.8. It was further purified in 10–40% sucrose gradient at 26,000 rpm in Beckman SW 28.1 rotor. Purified viral particles were treated with proteinase K and SDS at final concentrations of 100 µg/ml and 0.5% respectively, in 50 mM Tris buffer and incubated for 30 min at 50°C. Protein was removed by phenol chloroform extraction and RNA was precipitated by alcohol and sodium acetate.
Cloning and sequencing of coat protein gene

From viral genomic RNA, cDNA was synthesized by AMV reverse transcriptase and oligo dT primer. Using cDNA as template, CP gene was PCR-amplified with Vent DNA polymerase. The following primers were used for amplification.

(I) PvyI 5′ CGGAATTCATGGCAATGACACATT

EcoRI

GATGCA 3′ 33-mer (5′ primer)

(II) PvyII 5′ CGGGATCCCTCAGATGTTCTTGACTCCAA

BamHI

GTAG 3′ 32-mer (3′ primer).

A 50 µl PCR reaction mix contained the primers (1 µM final concentration each), Vent DNA polymerase (1.0 unit), 200 µM of each dNTP, 1X PCR reaction buffer. Reactions were overlaid with 50 µl of mineral oil. PCR conditions were 94°C initial melting for 3 min followed by 35 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min, with a 72°C/10 min final extension. The amplified CP gene was cloned into the EcoRI–BamHI site of pUC 8 vector. The recombinant plasmid was called pCP4. Sequencing was done by Sanger’s dideoxy method by using USB Sequenase version 2 kit.

Computer analysis

PC-based program Sequaid II version 3.5 was used to compare and analyse the sequence data. The hydrophilicity–hydrophobicity determination of the individual amino acid residues of the PVY coat protein was done using the algorithm of Kyte and Doolittle. PVY CP gene sequences from one American isolate and one Canadian isolate were compared with the current PVY CP gene.

Cloning of PVY CP into binary vector and plant transformation

The CP gene was taken out from pCP 4 by NcoI–BamHI digestion and cloned into the NcoI–BamHI site of pBI525. An EcoRI–HindIII fragment containing PVY CP gene downstream of d35S promoter and AMV ribosome-binding site (RBS) and Nos terminator at the 3′ end was taken out and cloned into EcoRI–HindIII site of pBINPLUS binary vector. The recombinant binary plasmid was called pBINCP (Figure 1). The pBINCP plasmid was mobilized into Agrobacterium tumefaciens strain EHA 105 by electroporation. Leaf discs from in vitro-grown Havana 425 tobacco plants were used for transformation and regeneration, essentially as described by Horsch et al. The leaf segments were precultured in regeneration medium [MS + BA (1 mg/l) + NAA (0.1 mg/l)] for 48 h and then infected with Agrobacterium suspension (0.5 O.D.) for 30 min. The explants were then blotted dry with sterile Whatman No. 1 filter paper and returned to the regeneration medium for co-cultivation for 48 h. The explants were then transferred to fresh regeneration medium with cefotaxime (400 mg/l) and kanamycin (50 mg/l) for selection of transgenic shoots and inhibition of Agrobacterium growth. The regenerated kanamycin-resistant shoots were subcultured to MS basal medium with kanamycin (100 mg/l) for rooting.

Characterization of transgenic plants

The ability to form roots on media containing kanamycin (100 mg/l) was used as the first indicator of the transgenic nature of the regenerated plantlets, since untransformed control plants did not root at that concentration of kanamycin.

PCR amplification: Integration of the transgene was confirmed by PCR and Southern hybridization using DNA from one-month-old in vitro grown transgenic and control tobacco plants. DNA was isolated by CTAB method. PCR was used to confirm the presence of CP gene in the putative transgenics using the same set of primers and conditions as mentioned above for cloning of the CP gene, except that 10 ng of genomic DNA was used as template.

Southern hybridization of genomic DNA: About 10 µg of DNA from each sample was digested with NcoI enzyme.
and the resulting fragments were separated according to size by electrophoresis through an agarose gel. The DNA was then denatured in situ and transferred to nylon membrane. Then the membrane was processed as mentioned by Sambrook et al.\textsuperscript{16}. This membrane was then probed with $\alpha^{-32}$P-labelled random primed coat protein gene probe.

**Northern hybridization:** mRNA was extracted from transgenic and control leaves of tobacco using the method of Steikema et al.\textsuperscript{17}. About 10 µg of mRNA from each sample was separated by a denaturing formaldehyde-agarose gel, transferred to a nylon membrane and fixed and probed with the same probe as in case of DNA Southern hybridization.

ELISA: Transgenic and control plants were hardened in the greenhouse and fifteen days after transferring into soil, they were infected with PVY and after another fifteen days, leaves were plucked and ELISA test was performed using PVY ELISA kit (Catalogue No. 647 420) from Boehringer Manheim GmbH, Germany following the protocol suggested by the supplier.

**Results**

**PVY coat protein gene sequence**
The coat protein gene of this isolate (PVY\textsuperscript{a}) was found to be 801 nucleotides long, coding for 267 amino acids. Gene bank accession number is AY061994. The DAG sequence is underlined (Figure 2).

**Transformation of tobacco**
Each leaf disc produced many putative transgenic shoots as evidenced by adventitious shoot formation in the presence of inhibitory level of kanamycin (50 mg/l) and the ability of those shoots to successfully root in higher concentration (100 mg/l) of kanamycin. All of the transgenic tobacco plants carrying the PVY coat protein gene were morphologically similar to non-transformed or pBI 121-transformed controls with respect to appearance, flowering and seed set (data not shown). Seven putative transgenic plants were taken for further analysis and named T1 through T7.

**Molecular analysis of transgenic plants**

**PCR and Southern blot analysis:** Genomic DNA was isolated from seven putative transgenic plants and PCR products from these samples had the expected size of ~ 830 bp, representing the PVY coat protein gene only. The DNA from non-transformed control plants did not amplify any detectable PCR product (Figure 3), as expected. NcoI-digested genomic DNA from these putative transgenic tobacco plants hybridized with a $\alpha^{-32}$P-labelled 800 bp EcoRI-BamHI fragment representing the PVY CP gene in Southern hybridization. The autoradiogram from Southern hybridization (Figure 4) clearly shows that these are independent transgenic lines. No hybridization signal was observed with DNA from control plants (Figure 4). After repeated attempts, no noticeable amount of mRNA corresponding to the PVY CP gene was detected.

**In vitro assay of virus resistance**
To assay the ability of individual transgenic and control plants to resist viral attack, five replicates of each line of greenhouse-hardened transgenic and control plants were infected with virus and crude extracts from those plants before and after infection were used to perform ELISA tests. Data presented here are average (with standard error) of five replicates. In ELISA tests before infection, all transgenic and control plants showed very low level of signal, comparable to the negative control. All 7 transgenic lines showed good amount of resistance against PVY accumulation upon infection, but T2, T3, T4 and T6

\[ GCA\ AAT\ GAC\ ACA\ ATT\ GAT\ GCA\ GGA\ GAA\ ACG\ AGG\ AAA\ GAT\ GGG\ AAA\ CCG \] 
\[ A\ N\ D\ T\ I\ D\ A\ G\ E\ N\ S\ R\ K\ D\ K\ F\ ] 
\[ GGA\ CGG\ GST\ AGC\ ATC\ CAG\ CCA\ ATT\ CTT\ ARC\ AGC\ GGA\ AGT\ AGG\ GCT\ GTC\ ] 
\[ E\ Q\ S\ Q\ I\ Q\ P\ W\ F\ H\ G\ K\ K\ D\ K\ D\ V\ ] 
\[ AAT\ GCC\ GST\ ACA\ TCT\ GGA\ ACA\ CTT\ ACT\ GTA\ AGA\ AGA\ GCT\ ATT\ AGG\ ] 
\[ A\ G\ T\ G\ S\ A\ H\ T\ V\ P\ R\ I\ K\ A\ T\ I\ T\ ] 
\[ TCC\ AAA\ ATG\ AGA\ ATG\ CTT\ AAA\ AGC\ AAG\ GGA\ GGU\ GCT\ GTC\ CTA\ CAC\ CTA\ GAA\ ] 
\[ S\ K\ M\ R\ M\ P\ K\ S\ K\ G\ A\ A\ V\ L\ N\ L\ E\ ] 
\[ CAT\ TGG\ GAA\ TAT\ GAT\ CCT\ CCA\ CAA\ GAT\ ATG\ ATT\ TCA\ ATA\ ACT\ AGC\ GCA\ H\ L\ L\ E\ Y\ A\ P\ Q\ Q\ I\ D\ I\ S\ N\ T\ R\ A\ ] 
\[ ACT\ CTA\ CAG\ TTT\ GAT\ AGG\ TGG\ TGA\ GAA\ GCA\ GTC\ CCG\ CTA\ SCA\ TAC\ GAC\ T\ Q\ S\ Q\ D\ T\ W\ Y\ K\ T\ A\ R\ V\ L\ D\ Y\ ] 
\[ ATG\ GAA\ GAA\ GCT\ CAA\ ATG\ CCG\ GGT\ ATG\ GGG\ ATC\ TTG\ TGG\ GTC\ ] 
\[ T\ G\ E\ A\ M\ E\ P\ T\ V\ H\ N\ G\ L\ W\ V\ C\ ] 
\[ ATT\ AAA\ ATG\ GGA\ ACC\ TGG\ CCA\ ATT\ ATG\ AAG\ GTC\ GGA\ GGT\ GGG\ GAT\ ATG\ ] 
\[ I\ K\ N\ G\ T\ S\ P\ N\ V\ N\ G\ V\ W\ M\ M\ ] 
\[ GGG\ CAA\ GAA\ GAA\ GCT\ AGG\ AGT\ TTG\ GAT\ AGG\ TGG\ GAT\ GAT\ ] 
\[ G\ N\ E\ Q\ V\ E\ T\ P\ L\ K\ F\ I\ V\ E\ N\ K\ ] 
\[ AGG\ AAT\ CTT\ ACC\ ACA\ ACT\ GCA\ AAT\ TTC\ CAT\ GAT\ ACT\ GCA\ AAA\ G\ ] 
\[ C\ G\ A\ T\ C\ G\ A\ G\ C\ T\ C\ G\ C\ T\ C\ C\ G\ T\ C\ C\ G\ C\ T\ C\ T\ C\ C\ G\ C\ G\ ] 
\[ A\ T\ A\ G\ G\ T\ G\ C\ C\ AAC\ AAC\ AAA\ AAG\ GAA\ CCA\ CTA\ ATG\ CCA\ CTA\ GAT\ CTA\ TTT\ G\ ] 
\[ I\ E\ M\ R\ N\ K\ K\ K\ E\ P\ Y\ M\ P\ R\ V\ G\ L\ V\ ] 
\[ CGA\ AAT\ TGG\ CCG\ GAT\ GAT\ GGT\ GTA\ GCG\ CTT\ GAT\ TTG\ TAG\ GAT\ GAT\ ] 
\[ R\ N\ L\ R\ D\ V\ G\ V\ A\ R\ Y\ A\ F\ D\ F\ E\ Y\ ] 
\[ GTC\ ACA\ TCA\ CAA\ ACA\ CCG\ GTC\ AGG\ GCT\ AGG\ AAG\ GCG\ GAA\ CAC\ ATA\ CAA\ AAG\ ] 
\[ V\ T\ S\ R\ T\ P\ V\ R\ A\ E\ H\ A\ I\ Q\ M\ K\ ] 
\[ GCC\ GCA\ GCA\ TTA\ AAA\ TCA\ CTT\ CCA\ CCT\ CCA\ CTA\ GGT\ GCA\ AAC\ GCA\ AAA\ A\ ] 
\[ A\ A\ A\ L\ R\ A\ A\ Q\ P\ K\ R\ L\ F\ G\ D\ L\ G\ ] 
\[ ATC\ ACT\ ACA\ CAA\ GAG\ GAG\ ACC\ ACA\ AGG\ ACC\ ACC\ AGC\ GAG\ GCT\ TCT\ IT\ S\ T\ Q\ S\ E\ N\ T\ E\ A\ R\ S\ T\ T\ T\ E\ D\ V\ S\ ] 
\[CCA\ AGT\ CAT\ ACT\ CTA\ GCA\ GCT\ ATT\ AAC\ AMG\ AGG\ TGA\ P\ S\ M\ L\ T\ L\ L\ G\ V\ K\ H\ K\ <\ ]
plants showed very high level of resistance when compared with the level of PVY accumulation in non-transgenic control tobacco plants (Figure 5).

Discussion

In the present communication, the cloned PVY\textsuperscript{o} coat protein gene sequence was compared with that of American and Canadian isolates. It showed 93.2% nucleic acid sequence homology and 94.8% amino acid sequence homology with American isolate and 92.9% nucleic acid homology and 94.1% amino acid sequence homology with the Canadian isolate. Comparison with many other reported PVY CP gene sequences also gave similar results.

It was also observed that most of the amino acid differences (7 aa) between this sequence and other reported sequences of the same gene are concentrated within the first 25 N-terminal residues. The 30 N-terminal amino acids are known to be exposed on the virion surface and are reported to play a crucial role in aphid transmissibility\textsuperscript{18}. Remaining 7 to 9 amino acid differences are spread randomly in the protein. From the N-terminal, amino acid positions 27 to 34, 173 to 177 and 242 to 246 are stretches of hydrophilic regions of PVY coat protein and are highly conserved, not only among different PVY strains, but also among different potyviruses\textsuperscript{19}. Amino acid position 10 to 17, the other hydrophilic region highly conserved among potyviruses, shows a different sequence in our isolate and the hydrophilicity of the region is slightly less than other reported potyviral coat protein sequences.

It has been proved that virus encoded helper component (HC) protein effects transmission of potyviruses by binding the virus to aphid mouthparts\textsuperscript{18}. Probably, one or more domains of HC protein interact with aphid mouthparts and some other domains interact with certain amino acid residues, including the DAG sequence among the first 30 N-terminal amino acids of PVY CP, because that is the region of the coat protein which is exposed on the virion surface. The DAG sequence, which is a...
hallmark of aphid transmissibility, is also present in this protein.

From the current sequence data we can conclude that this is a distinct Indian isolate. Depending on the amino acid changes in the HC protein of the same virus, it may or may not have altered aphid transmissibility. Sequencing of the HC gene and further analysis of that sequence along with protein–protein interaction studies of HC and CP proteins may reveal the role of coat protein in aphid-mediated virus transmission.

The transgenic lines of Havana 425 variety of tobacco have shown resistance to PVY accumulation to a varying degree after mechanical inoculation of PVY<sup>o</sup>. Attenuation or absence of symptoms was also very clearly visible on the PVY<sup>o</sup>-inoculated transgenic plants (data not shown). The seven transgenic tobacco lines did not show any detectable level of corresponding mRNA. Even though there are many reports correlating the accumulation of viral transgenic coat protein to the level of resistance against the virus, the mechanism of resistance may be different in different viruses. There are a few reports indicating the resistance against PVY in tobacco without apparent expression of PVY coat protein in the transgenic plants<sup>20,21</sup>, for example by transferring the untranslatable or truncated CP gene to tobacco. In this case, since the gene was cloned under d35S promoter, the corresponding mRNA or protein is likely to be present, but mRNA could not be detected by Northern analysis.

Data presented in this communication clearly demonstrate that this distinct Indian isolate of PVY can be used in imparting coat protein gene-mediated resistance in tobacco and the level of resistance is not correlated to the level of expression of the PVY CP transgene in tobacco. Further, the same gene can be used for developing PVY resistance in Indian potato cultivars and work is being actively pursued in this direction.

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