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Biological and Molecular Characterization of *Potato virus Y* Infecting Potato (*Solanum tuberosum*) in India

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ABSTRACT

A survey conducted in potato growing tracts of Rajasthan (India), covering 25 fields in 15 villages, revealed the occurrence of potato mosaic disease. A *potyvirus* (*Potato virus Y*, PVY) causing severe mosaic mottling in potato (*Solanum tuberosum*) was identified. The aim of this study to characterize this *potyvirus* on the basis of biological, serological and partial nucleotide sequence properties from different locations of Rajasthan. Electron microscopy of this virus revealed flexuous filamentous virus particles of 750×15 nm and showed positive result against antiserum specific for PVY. RT-PCR assay was assessed using *potyvirus* specific primers designed against the core region of the Nib and Coat Protein (CP). cDNA fragments of Nib and CP gene, 323 and 353 bp were obtained, respectively. Sequencing and analysis of the amplicons showed that the virus is closely related to *Potato virus Y*. Sequence analysis reveals that virus isolate (PVY-PS) showed 93-97% similarity with other worldwide PVY isolates. Nucleotide sequences have been submitted to NCBI data base under the accession number-KC753451and KC753450.

Key words: *Potato virus Y*, *Potyvirus*, RT-PCR, coat protein, core region

INTRODUCTION

The genus *potyvirus* (Family Potyviridae) includes a large group of plant pathogen viruses that encompasses 111 recognized and 86 tentative species infecting more than 30 plant families (Fauquet *et al*., 2005). *Potyvirus* particles are flexuous rods of ~700-900 nm in length, contain single-stranded RNA genome (~10 kb). Genomic RNA has a 5' untranslated region (5' UTR), a single Open Reading Frame (ORF) and a 3' UTR which has a polyadenylated (poly A) tail. The whole genome encodes a single large polyprotein that is subsequently processed into ten functional proteins (Adams *et al*., 2005). Potyviruses are mainly transmitted by aphids in a non-persistent manner and infect a wide range of plants in which they causes significant losses, making the *potyvirus* genus interesting in a worldwide agricultural concern. Although, they are most prevalent in tropical and subtropical countries (Shukla *et al*., 1988).

Potato (*Solanum tuberosum*, Family solanaceae) a most important tuber crop cultivated in India is very rich in starch contents. In Mediterranean country, one of the most important aphid transmitted viruses is *Potato virus Y* (PVY), the type member of the *potyvirus* group. *Potato virus Y* causes a severe green mosaic on the leaves and stunting in infected plants. This

virus also causes serious diseases worldwide on pepper, tomato, tobacco and other alternate crops. The predicted crop losses due to PVY infections range from 10-80% (De Bokx and Huttingah, 1981). Conserve amino acid motifs of Nib and coat protein gene have recently becomes a useful tool for taxonomic classification of distinct potyviruses and related isolates (Zheng *et al*., 2010).

This current study focused on the characterization of geographically contiguous *Potato virus Y* Rajasthan (Northern India) isolate PS with morphological, serological methods. Concurrently molecular characterization done by cloning and sequencing of core Nib and CP genes to compare with other potato virus isolates described from other areas of the world.

MATERIALS AND METHODS

Survey and collection of leaves samples: The youngest leaves from 25 potato plant fields exhibiting typical *potyvirus* infection symptoms i.e., leaf curling, mosaic and stunted growth (Fig. 1a) were collected during Oct-2012 to Dec-2012 from Rajasthan, India. The samples were cleaned, cut, rolled in a piece of tissue study and then stored at -20°C in a deep-freezer till further use.

Mechanical inoculation: Virus inoculum was prepared by grinding 1 g of young diseased leaves of potato in 5 mL of 0.04 M $NaHPO_4$ containing 0.2% sodium diethyldithiocarbamate. Prior to inoculation, 75 mg mL^{-1} of carborundum and of activated charcoal were added to the sap extract (Morel *et al*., 2000). For an experiment near about 40 *N. benthamiana* plant grown on insect-free green house was taken and first three leaves of approximately four week old plants were mechanically inoculated.

Virus purification and electron microscopy: Leaves harvested from naturally infected potato and mechanically inoculated *N. benthamiana* at four week post-inoculation were used for virus particle purification as described by (Chen *et al*., 2003). Purified PVY samples applied on Formvar-coated grids were stained with 1% uranyl acetate (Christie *et al*., 1987) and examined under a JEOL 100S electron microscope operating at 80-100 KV (50,000×magnification).

Fig. 1(a-b): (a) Potato plant showing characteristics mosaic and stunting growth and (b) Healthy plant

Serological tests: Serological relationships of this virus isolate using antisera against *Potato virus Y* specific antiserum were investigated by employing plate trapped antigen enzyme-linked immunosorbant assay (PTA-ELISA) (Mowat and Dawson, 1987). In PTA-ELISA, leaf antigens were extracted in coating buffer (1:10 w/v; 200 mL well^{-1}) and purified virus (200 ng well^{-1}) was used. The primary antibodies were used at a dilution of 1:500 and Protein A conjugated to alkaline phosphatase at 1:1000 and horseradish peroxidase (HRP) conjugate at 1:1000 dilution were used as the secondary antibody. The reaction was developed using P-Nitrophenyl Phosphate (PNP) and 3, 3`, 5, 5`-tetramethyl benzidine (TMB) as a substrate. The absorbance was measured at 405 and 450 nm in a Labtech LT-4000 ELISA Reader, respectively.

RNA extraction and RT-PCR: Total RNA (Host and Viral) was extracted from 100 mg of symptomatic and healthy leaves using the TRIZOL method and resuspended in 30 μL nuclease free water. The RNA was subjected to 25 μL reaction mixture of cDNA containing 5 μL of RNA, 1 μL of oligo $d(T)_{16}$, 5 μL of 5X reaction buffer, 2 μL of 2.5 mM dNTPs (2.5 mM), 0.5 μL of AMV Reverse (20 U μL^{-1}) Transcriptase and 1 μL of $MgCl_2$ (25 mM). cDNA synthesis was carried out at 42°C for 1 h followed by 72°C for 10 min in a thermal cycler. Simultaneously PCR performed in a 25 μL reaction mixture containing 3 μL of cDNA, 2.5 μL 10X reaction buffer, 2.5 μL of dNTPs (2.5 mM) and 1 μL $MgCl_2$ (25 mM), 1 μL of 20 pmol each of *potyvirus* group specific published primers against coat protein and Nib protein gene. These primers are MJ1(F)-5'-TGGTHTGGTGYATHGARA AYGG-3' and MJ2(R)-5' TGCTGCKGCYTTCATYTG-3' (Chen *et al*., 2001) for CP gene and Nib(F)-5'-GTITGYGTIGAYGAY TTYAAYAA-3' and Nib(R)-5'-TCIACIACIGTIGAIGG YTGNCC-3' (Babu *et al*., 2012) for Nib gene. PCR was performed in thermal cycler with the programme 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 1 min (with CP specific degenerate primer) and 57°C for 1 min (with Nib specific degenerate primer), 72°C for 1 min and a final extension of 72°C for 10 min. The amplified products were analyzed on 1% agarose gel, stained with ethidium bromide.

Cloning and sequencing: The amplified PCR products were purified using the Gel Extraction Kit (QIAGEN) and the product was cloned into the pGEM-T Easy vector (Promega, USA) following the manufacture's protocols. The ligated mix were used to transformed *Escherichia coli* DH5α. Resulting recombinants clones were selected on Luria agar medium containing Ampicillin (100 μg mL^{-1}) and X-gal/IPTG (50 μg mL^{-1}-40 mM) (Sambrook and Russell, 2001). The clones were then subjected to sequenced by automated ABI sequencer.

Phylogenetic analysis: The nucleotide sequence was compared with other sequences from NCBI databases using BLASTn; (http://www.ncbi.nlm.nih.gov/blast). Highest and lowest match scores and closest matching sequence from different isolates of *Potato virus Y* was considered. From the blastn sequences phylogenetic trees were constructed with the neighbor-joining method with 1,000 bootstrap value to determine the reliability of tree using MEGA 4.0 software.

RESULTS

Particle morphology and biological properties: Results of the electron microscopy (Fig. 2) showed that particles were flexuous rods with an average dimension of 750×15 nm, similar to the members of Family potyviridae. Out of 40 *N. benthamiana* plants 29 were shown typical curling and mosaic symptoms caused by virus inoculums. This resulted in the development of same kind of symptoms as observed in naturally infected potato plants in the fields.

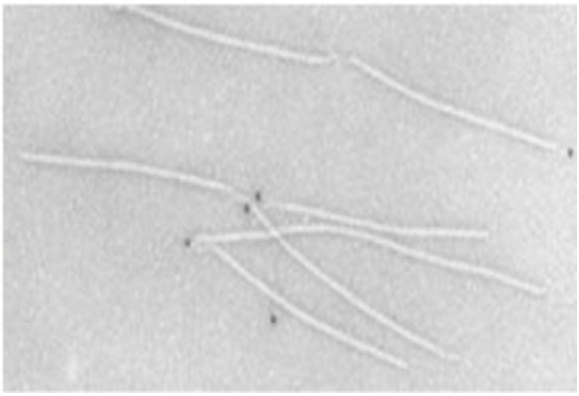

Fig. 2: Electron micrograph of purified virus particles causing mosaic disease on potato were stained with 1% uranyl acetate

Table 1: Absorbance values obtained by PTA-ELISA with 2 protein A enzyme conjugates and the reaction of their substrates with adsorbed components from sample extracts

		A_{405} values[b]		A_{450} values[b]	
Virus and antiserum (host plant)	Sample and dilution[a]	PA-ALP[c]	NPP[d] only	PA-HRP[c]	TMD[d] only
Potyvirus group (*Solanum tuberosum*)	I	1.75	0.12	0.75	0.16
	I/10	1.68	0.14	0.84	0.20
	H	0.23	0.12	0.15	0.12
	B	0.15	0.13	0.10	0.10
Potyvirus group (*N. benthamiana*)	I	1.28	0.19	0.75	0.16
	I/10	1.04	0.14	0.72	0.18
	H	0.21	0.15	0.21	0.18
	B	0.16	0.13	0.12	0.10

[a]I: Sap from infected leaf extracted in 5 mL of carbonate buffer (pH 9.8) g^{-1} of tissue; 1/10 = I diluted 1.10 with extraction buffer; H: Sap from uninfected leaf extracted as I; B: Extraction buffer control, [b]A_{405} values were recorded after overnight incubation of substrate at 5°C. A_{450} values were recorded after 20 min incubation of substrate at ambient temperature. Absorbance values are the means of 3 wells. [c]PA-ALP: Protein A-alkaline phosphatase conjugate; PA-HRP: Protein A-horseradish peroxidase conjugate. Wells received sample followed by detecting antiserum, protein A enzyme conjugate and substrate, [d]NPP: p-nitrophenyl phosphate, TMB: 3,3`, 5, 5`-tetramethyl benzidine; wells received successively sample and substrate

Detection and identification of plant viruses by PTA-ELISA: *Potato virus Y* a species of *potyvirus* group were detected by PTA-ELISA using the procedure described above (Table 1). Absorbance values were accepted as positive when the reading was greater than twice the mean absorbance of the virus-free control sample. The absorbance values of the wells receiving sample extract and substrate were only similar to those of wells containing extracts from virus-free tissue and extraction buffer controls. Serological results shown absorbance values at least twice the mean for virus-free samples were obtained with both dilutions of each sample therefore method was particularly valuable in identifying isolates of PVY.

Characterization of viral genome: PCR amplification products (323 and 353 bp) were observed from all the infected samples tested using Nib specific primers and CP specific primers (Fig. 3b).

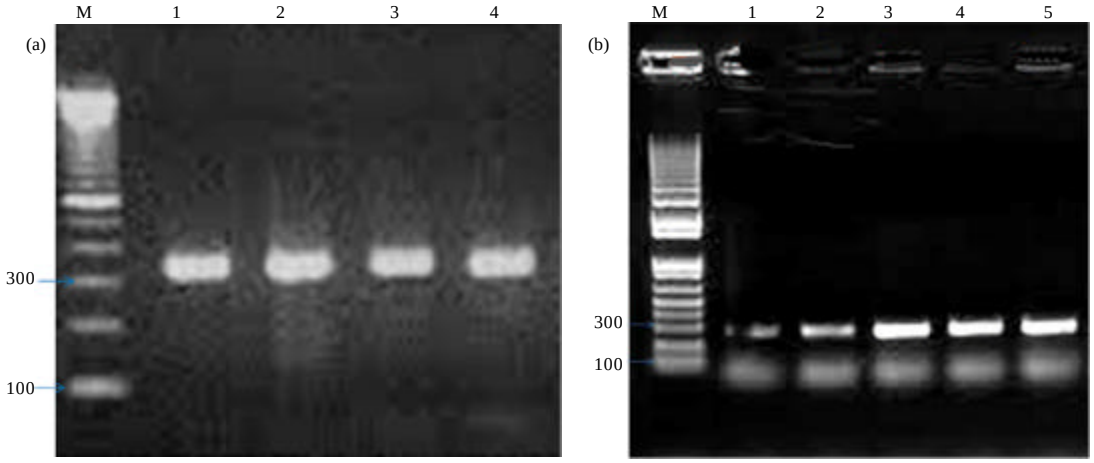

Fig. 3(a-b): (a) RT-PCR results of infected *S. tuberosum* plants with *potyvirus* group specific primers of Nib protein gene and (b) RT-PCR results of infected *S. tuberosum* plants with *potyvirus* group specific primers of coat protein gene

Table 2: Percentage identities of nucleotide sequences of the partial Nib gene of *Potato virus Y* infecting with other *Potato virus Y* isolates

Accession No.	Isolates	Nucleotide identity (%)	Country
AB461460	*Potato virus Y*-SYR-III-2-5	95	Syria
AB711146	*Potato virus Y*-NTNTK1	94	Japan
JN936442	*Potato virus Y*-Z26	94	SouthAfrica
KC634005	*Potato virus Y*-11439	94	UK
JN936436	*Potato virus Y*-SS607_31	94	SouthAfrica
JF927759	*Potato virus Y*-IUNG-II	94	Poland
JQ924287	*Potato virus Y*-ALF-VI	94	Brazil
EF026075	*Potato virus Y*-PB312	94	USA
HQ631374	*Potato virus Y*-HN1	94	China
KC296440	*Potato virus Y*-1108	93	China
AB331515	*Potato virus Y*-NTND6	93	Japan

The recombinant plasmids harboring cDNA inserts were sequenced as described in the methods section. The authenticity of these clones was confirmed by sequenced using T7 and SP6 universal primers and the sequences were deposited in the GenBank (KC753451 and KC753448). After sequencing, the consensus sequence was compared to the sequences registered in the NCBI database using blastn (http://www.ncbi.nlm.nih.gov/blast) which confirmed the presence of *Potato virus Y*.

Table 2 and 3 summarize the relative nucleotide sequences similarities between Nib and CP gene of viral isolate under study with other viral isolates available in GenBank. It showed that the sequences of our isolate (PVY-PS) have 93-97% identity with different isolates of *Potato virus Y* worldwide. The phylogenetic trees were constructed from the sequences of CP gene and Nib gene (Fig. 4b) reveals our isolate (PS) positioned in a separate monophyletic cluster.

The result suggested that potato samples act as a natural host for *Potato virus Y*. This is the first time that *Potato virus Y* has been isolated and characterized from Rajasthan (India).

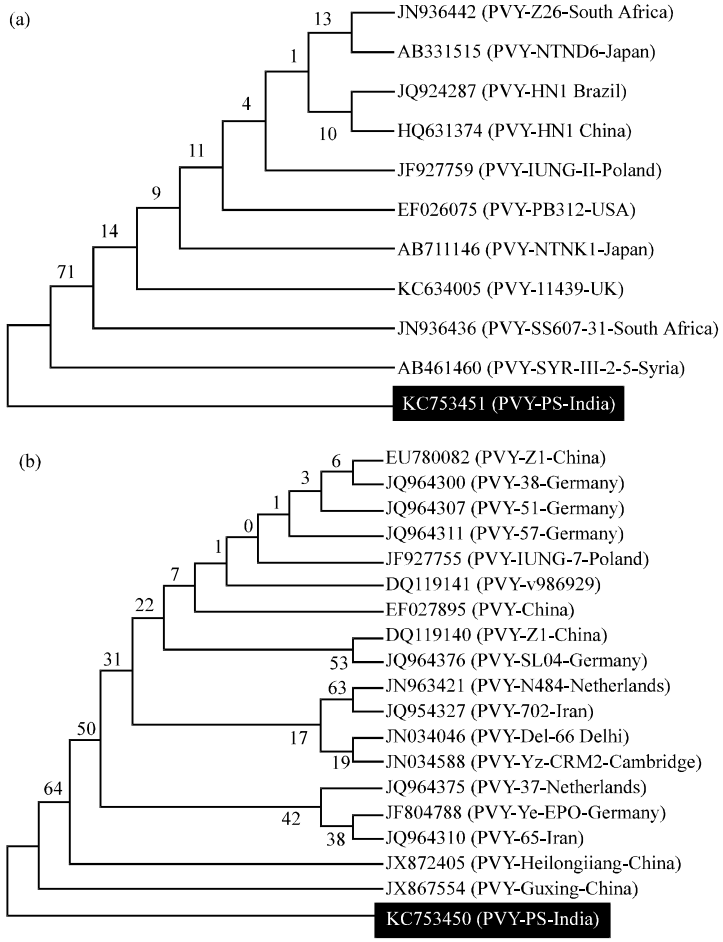

Fig. 4(a-b): (a) Phylogenetic tree representing our *Potato virus Y* isolate (PS, Nib gene) positioned in a separate monophyletic cluster with different viral isolates using neighbor-joining method with 1000 bootstrap replicates of Mega 4.0 Software and (b) Phylogenetic tree representing our *Potato virus Y* isolate (PS, CP gene) positioned in a separate monophyletic cluster with different viral isolates using neighbor-joining method with 1000 bootstrap replicates of Mega 4.0 Software

Table 3: Percentage identities of nucleotide sequences of the partial coat protein gene of *Potato virus Y* infecting with other *Potato virus Y* isolates

Accession No.	Isolate	Nucleotide identity (%)	Country
JN936421	*Potato virus* Y-N484	97	South Africa
JN034046	*Potato virus* Y-Del-66	97	NewDelhi, India
JF927755	*Potato virus* Y-IUNG-7	97	Poland
JX872405	*Potato virus* Y-Heilongiiang	96	China
JF804786	*Potato virus* Y-YE Epo	93	Poland
JQ954327	*Potato virus* Y-PB-702	93	Netherlands
DQ119140	*Potato virus* Y-PVY-A1	93	Iran
EU713856	*Potato virus* Y-ABRII1	93	Iran

Table 3: Continue

Accession No.	Isolate	Nucleotide identity (%)	Country
JX867554	*Potato virus Y*-Guxing	93	China
JQ954375	*Potato virus Y*-37	93	USA
JQ954310	*Potato virus Y*-65	93	Germany
DQ119141	*Potato virus Y*-PVY-Z1	93	Iran
EF027895	*Potato virus Y*-V986929	93	UK
EU780082	*Potato virus Y*	93	China
JQ954376	*Potato virus Y*-SL04	93	Solvenia
JQ954311	*Potato virus Y*-57	93	Germany
JN034568	*Potato virus Y*-CRM2	93	UK
JQ954300	*Potato virus Y*-38	93	Germany
JQ954307	*Potato virus Y*-51	93	Germany

DISCUSSION

A virus naturally infecting potato was characterized at biological, serological and molecular levels. Structure analysis reveals the purified virus appeared as flexuous rods with an average dimension of 750×15 nm which is consistent with the morphology of potyviruses. Subsequently, glasshouse-grown *Nicotiana* spp., was inoculated mechanically with purified isolate from infected leaves, approximates 80% of plants show mosaic symptoms showed occurrence of potyviruses. The virus showed a serological relationship with PVY specific antiserum while when used a ranges of other *potyvirus* specific antiserum were not give any absorbance with same substrate as above (data not shown). The existence of many conserved domains in the viral Nib and CP region facilitates the designing of numerous degenerate primers for RT-PCR based detection of the virus genome (Chen *et al*., 2001). The amplified products were cloned in pGEMT Easy vector, sequenced using T7 and SP6 universal primers. The sequences were deposited in the GeneBank (KC753451 and KC753450). BLASTn showed a similarity of the PVY-PS with other *Potato virus Y* with a highest similarity of 97% (Table 2-3). Viral sequences also showed similarity with all other *Potato virus Y* isolates, with a similarity ranging from 93-97% to confirm the presence of *Potato virus Y* isolate. The phylogenetic tree was constructed by using the neighbor-joining method of the MEGA 4.0 Software. The constructed trees from the sequences of Nib and CP gene reveals our isolate (PVY-PS) positioned in a separate monophyletic cluster which forms a clade, meaning that it consists of an ancestral species and all its descendants.

The group specific PCR and subsequent molecular analysis of amplified regions has been used for rapid detection and identification of potyviruses. Thus RT-PCR with degenerate primers designed to amplify a short conserved region of the potyviruses seem to be useful for the detection and identification of the *potyvirus* infecting *S. tuberosum* Rajasthan, India. This is the first time biological, sequence characterization and identification of the viral isolate infecting *S. tuberosum* in Rajasthan, India which is essential for the timely management of the disease and for the development of viral resistant strategies. This survey of the incidence of mosaic diseases in potato plants in the extensively cultivated areas of Rajasthan province revealed that PVY was the predominant mosaic virus.

CONCLUSION

It can conclude that the diseased plants showed typical symptoms of severe mosaic of the leaves, stunted and retardation of the plant growth. Positive results were also obtained by biological,

serological and RT-PCR methods to detect and identify the *potyvirus*. The sequences of RT-PCR fragments confirmed that a *potyvirus* associated with mosaic disease on potato plants in Rajasthan (India). *Potyvirus* infecting potato plants in Rajasthan are closely related to *Potato virus Y* (PVY). This survey represents the usual disease incidence that is prevalent in this region.

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