Characterization of a Pepper Vein Banding Virus from Chili Pepper in India

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ABSTRACT

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A survey conducted in pepper-growing tracts of Karnataka State, covering 165 fields in 33 villages, revealed the occurrence of many pepper mosaic diseases. Based on reactions on selected test plants, the viruses were identified as pepper vein banding virus (PVBV), pepper vein mottle virus, potato virus Y, cucumber mosaic virus, and tobacco mosaic virus. Among these, PVBV was the most prevalent. PVBV was purified from infected leaves of *Capsicum annuum* cv. California Wonder. Electron microscopy revealed flexuous rod-shaped particles in the purified preparations. The coat protein (CP) molecular weight was 35,000, which is similar to members of the *Potyvirus* group. As in other potyviruses, the CP underwent proteolytic degradation to a fragment with a molecular weight of 31,000. Both of these bands cross-reacted with antibodies against tobacco etch virus in Western blots. Polyclonal antibodies were produced against PVBV. Cross-reactivity studies with other potyviral antisera showed that PVBV is serologically closer to peanut mottle virus than to peanut stripe virus or sorghum potyvirus. N-terminal sequence analysis of the intact CP and trypsin-resistant core revealed that PVBV is a distinct member of the *Potyvirus* group.

One of the major constraints to the production of pepper, which is an economically important crop in India, is infection by viruses. There are about 10 different viruses reported to infect pepper in India (2,5,9). Identification of these viruses has been done based on the symptoms produced on a set of test plant species and, in some instances, the serological relationships of the virus with other well-characterized group members. However, molecular characterization has not been reported for any of these isolates.

In the current paper, we report the occurrence of pepper vein banding virus (PVBV), pepper vein mottle virus (PVMV), potato virus Y (PVY), cucumber mosaic virus (CMV), and tobacco mosaic virus (TMV) in the pepper-growing tracts of Karnataka State, India. Further, we report the purification and characterization of PVBV, which is the most prevalent virus among those infecting peppers, and show that it is a distinct member of the *Potyvirus* group.

MATERIALS AND METHODS

Survey and collection of pepper mosaic isolates. To determine the prevalence of chili mosaic viruses in nature, a survey was undertaken around the Kolar and Shimoga districts of Karnataka State during 1987 to 1988. The age of the pepper crop surveyed varied from 2 to 4 months. At the village level, every fifth field was selected for observation and collection of virus isolates. In each field, five rows were randomly selected, and the total number of plants and the number of plants showing mosaic and other associated symptoms were recorded to calculate the percentage of diseased plants. Simultaneously, two plants in each of these five rows were selected randomly for collection of leaf samples. The samples collected were used subsequently to inoculate healthy seedlings of *Capsicum annuum* cvs. California Wonder and Byadagi Kaddi that were maintained under insect-proof conditions for symptom expression. Further, the isolates were inoculated to a set of test plants, as described by Prasad Rao and Yarguntaiah (9), to group the viruses.

PVBV virion purification. Leaves harvested from infected pepper (*C. annuum* cv. California Wonder) (Fig. 1) at 3 weeks post-inoculation were ground in 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (1:2 wt/vol), pH 7.5, containing 1 M urea, 1% Triton X-

100, 10 mM diethyl dithiocarbamate, and 0.1% β -mercaptoethanol. The extract was passed through cheesecloth, and the filtrate was clarified with 10% ice-cold chloroform. The virus was precipitated by adding 6% polyethylene glycol 6000 and 0.2 M NaCl. The precipitate was suspended in resuspension buffer (0.02 M HEPES, pH 7.5, containing 0.5 M urea and 0.02 M sodium sulfite), layered over 10 to 40% preformed sucrose density gradient prepared in the same buffer, and subjected to centrifugation at $92,500 \times g$ for 3 h at 4°C in a SW 28 rotor. Fractions collected at the end of the run were monitored for absorbance at 280 and 260 nm. The peak fractions were pooled and pelleted by centrifugation at $120,000 \times g$ for 3 h at 4°C. The final pellet was resuspended in a minimum volume of resuspension buffer and was used for further studies. Purified PVBV samples applied on Formvar-coated grids were stained with 1% uranyl acetate (4) and examined under a JEOL (Tokyo) 100S transmission electron microscope operating at 80 to 100 kV.

Production of polyclonal antisera and enzyme-linked immunosorbent assay (ELISA). Antisera were produced in rabbits against purified virions of PVBV by giving a series of intradermal injections: 500 μ g of virus in complete adjuvant for the first injection and 250 μ g of virus in incomplete adjuvant for three subsequent injections at 1-week intervals. A booster dose of 250 μ g of virus in PBS (0.1 M potassium phosphate buffer containing 0.9% sodium chloride, wt/vol) was administered intramuscularly 10 days prior to bleeding. The presence of PVBV-specific antibodies in the serum was tested by direct antigen coating-ELISA (DAC-ELISA) (8). Flat-bottomed polystyrene microtiter plates (Nunc-Immuno plate, Polysorp, Roskilde, Denmark) were precoated with 100 ng of purified PVBV in 0.05 M sodium carbonate buffer (pH 9.6) per well. The binding of antibodies in the serum to the antigen was assayed with goat anti-rabbit immunoglobulin (Ig) conjugated to alkaline phosphatase. The absorbance was read at 405 nm in a Bio-Tek (Winooski, VT) microplate auto-reader EL 311. All the assays were carried out in duplicates with normal rabbit serum as the negative control. The average of the duplicates is presented.

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Western blot analysis. The PVBV coat protein (CP) was subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6). After electrophoresis, the proteins were transferred to nitrocellulose membrane with Pharmacia (Uppsala, Sweden) Novoblot apparatus at a constant current of 0.75 mA/cm² of membrane for 2 h. The unoccupied sites were blocked with 5% milk powder for 1 h. Polyclonal antiserum to tobacco etch virus (TEV) (1:1,000 dilution) was added and incubated at 37°C for 2 h. Peroxidase conjugated to goat anti-rabbit Ig was used

as the secondary antibody for detection (12).

N-terminal sequencing of PVBV CP. Purified virions of PVBV were treated with trypsin (2 µg of virus per ml) at 20°C for 30 min to obtain the trypsin-resistant core (TRC) of the CP. The intact PVBV CP and the TRC, separated on 12.5% SDS-PAGE, were transferred to polyvinylidene difluoride membrane (7), and the N-terminal sequences of the proteins were determined by loading the cut membrane directly onto an automated gas phase sequencer, Shimadzu PSQ-1 (Shimadzu Corporation, Kyoto, Japan).

Fig. 1. Vein banding symptoms on leaves of *Capsicum annuum* cv. California Wonder induced by pepper vein banding virus (PVBV). (A) healthy leaf; (B and C) PVBV-infected leaves.

Fig. 2. Electron micrograph of purified pepper vein banding virus virions stained with 1% uranyl acetate. Bar = 250 nm.

RESULTS

Survey of pepper mosaic diseases in Karnataka State. The survey conducted in pepper-growing tracts of Karnataka State, covering 165 fields in 33 villages, revealed the widespread occurrence of pepper mosaic diseases. In different taluks of the Kolar District, under irrigated conditions the incidence of mosaic disease ranged from 15 to 31%, with an average of 25%, of the plants surveyed. In the Shimoga District, incidence under rain-fed conditions ranged from 37 to 58%, with an average of 47%, of the plants surveyed. Of the 66 pepper plants with mosaic collected from the fields (Kolar and Shimoga districts), 10% were not sap-infective, and 13% had mixed virus infections. Based on their reactions on selected test plants, sap-transmissible virus isolates of PVBV (27%), PVMV (18%), CMV (15%), PVY (8%), and TMV (6%) were identified.

Characterization of PVBV. In host-range studies, 56 species or cultivars be-

Table 1. Reactions of plants from the Solanaceae family to pepper vein banding virus

Host	Symptoms ^a
<i>Capsicum annuum</i> L. 'Byadagi Kaddi'	VB, RT, EP, RLS
<i>C. annuum</i> 'California Wonder'	VB, MM
<i>Datura metel</i> L.	MM, LD, RLS
<i>D. stramonium</i> L.	...
<i>Lycopersicon esculentum</i> Mill.	MM, VB
<i>Nicotiana bigelovii</i> S. Wats.	...
<i>N. debneyi</i> Domin.	...
<i>N. glutinosa</i> L.	MM, LD
<i>N. hybrid</i> L.	CS, VB, RLS
<i>N. nudicaulis</i> S. Wats.	...
<i>N. nesophila</i> Roxb.	...
<i>N. occidentalis</i> L.	VC, RLS, LD, EP, SG
<i>N. plumbaginifolia</i> Viv.	VB, MM
<i>N. rependa</i> Wild. Ex Lm.	LCR/S
<i>N. rosulata</i> L.	...
<i>N. rustica</i> L.	SNLL, VN
<i>N. simulans</i> N.T. Burbidge	LCS, LD
<i>N. sylvestris</i> Speg. & Gomes	...
<i>N. tabaccum</i> L. 'Anand 119'	VB, LD
<i>N. tabaccum</i> 'Candel'	VB, MM
<i>N. tabaccum</i> 'Delcrest'	VB, MM, LD
<i>N. tabaccum</i> 'FCV special'	VB, MM
<i>N. tabaccum</i> 'GT-4'	VB, MM
<i>N. tabaccum</i> 'GT-6'	VB, MM, LD
<i>N. tabaccum</i> 'Hicks 103'	VB, MM
<i>N. tabaccum</i> 'Hicks special'	VB, MM
<i>N. tabaccum</i> 'PCT-7'	VB, MM, LD
<i>N. tabaccum</i> 'PL-5'	VB
<i>N. tabaccum</i> 'Prabha'	VB, MM
<i>N. tabaccum</i> 'Samsun'	VB, MM
<i>N. tabaccum</i> 'Sona'	VB, MM
<i>N. tabaccum</i> 'Swarna'	VB, MM
<i>N. tabaccum</i> 'White Burley'	VB, MM, LD
<i>N. tabaccum</i> 'Xanthi'	VB
<i>N. unulata</i> L.	NLL, VN

^a ... = No reaction; RT = rat tailing; VB = vein banding; RLS = reduction in leaf size; CS = chlorotic specks; LCR/S = local chlorotic rings/spots; MM = mosaic mottling; SNLL = systemic necrotic local lesions; NLL = necrotic local lesions; LD = leaf distortion; VN = veinal necrosis; EP = elongation of petiole; SG = stunted growth.

longing to five families (Solanaceae, Chaenopodiaceae, Cruciferae, Cucurbitaceae, and Leguminaceae) were tested for infectivity by PVBV. Susceptibility to PVBV was confined to the family Solanaceae (Table 1). Symptoms produced in *C. annuum* cv. California Wonder included vein banding and mosaic mottling (Fig. 1).

Purified virion preparations gave a yield of 8 to 10 mg of virus per kg of leaves. The major problem encountered during purification was the aggregation of the virion particles, which was minimized by use of 1 M urea, 0.1% β -mercaptoethanol, and 1% Triton X-100 in the extraction buffer. Electron micrography of purified virions of PVBV showed that the particles were flexuous rods with an average length of 900 nm (Fig. 2). Using SDS-PAGE, the CP of PVBV was shown to have an approximate molecular weight of 35,000. In addition to this band, another band with an approximate molecular weight of 31,000 was observed in most of the preparations. This second band could be a degradation product of the intact CP, because the potyviral CPs are extremely susceptible to proteolytic degradation (11). Such a possibility was confirmed by Western blot analysis (Fig. 3B). Both bands reacted with antibodies to TEV in Western blot, confirming that PVBV is a potyvirus that is serologically related to TEV. The antibody dilution curve obtained with homologous antiserum identified a dilution end point of 1:128,000. In cross-reactivity studies, PVBV reacted well with peanut mottle virus (PeMoV) and peanut stripe virus (PStV) antisera but failed to react with sorghum potyvirus antiserum (Fig. 4).

The N-terminal sequence of the intact CP (35K) and the TRC (30K) could be determined up to 16 and 18 amino acid residues, respectively. These sequences were compared with other potyviral CP sequences available in the protein databank (data not shown). The N-terminal amino acid sequence of intact CP showed not more than 30% identity with other potyviruses. However, the N terminus of the TRC had a significant homology with other potyviruses. A comparison of the N-terminal sequences of the intact and TRC of the PVBV CP with the corresponding region in the CP sequences of other potyviruses infecting pepper is presented (Fig. 5). The N-terminal sequence of the PVBV CP encompassed the DAG sequence, which is present in aphid-transmitted potyviruses.

DISCUSSION

Our survey of the incidence of mosaic diseases in pepper in the extensively cultivated areas of southern Karnataka State revealed that PVBV was the predominant mosaic virus. This survey represents the usual disease incidence that is prevalent in this region, and the incidence was not particularly high during the year(s) of the survey. Based on an analysis of the symp-

Fig. 3. Analysis of the pepper vein banding virus (PVBV) coat protein (CP). (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing the PVBV CP. Lane 1, standard molecular weight markers. Lane 2, intact CP. Lane 3, trypsin-resistant core (TRC) of the CP. (B) Western blot analysis of the PVBV CP. Lane 1, intact CP. Lane 2, TRC of the CP.

Fig. 4. Cross-reactivity of pepper vein banding virus (PVBV) with other potyviral antisera. The antigen was 100 ng of purified PVBV virions. Antisera were used at different dilutions. The antigen was detected by direct antigen-coating enzyme-linked immunosorbent assay.

A

PVBV	A G E S V <u>D A G</u> S V K G E D S S	(this study)
PVY - D	A N D T I D A G E S S K K D A R	(10)
PeMV	A N D T I D T G G N S K K D V K	(3)
TEV - HAT	S G T V D A G A D A G K K K D Q	(1)
PepMoV	S S S R S D T L D A G E E K K K	(13)

B

PVBV	D R D V N V G T S G T F T I P R L K	(this study)
PVY - D	D K D V N A G T S G T H T V P R I K	(10)
PeMV	E K D V N A G T S G T H T V P R I K	(3)
TEV - HAT	D R D V N A G T S G T F S V P R I N	(1)
PepMoV	D S D V N A G T V G T F T I P R I K	(13)

Fig. 5. N-terminal amino acid sequence comparison of (A) intact and (B) the trypsin-resistant core of coat proteins of pepper vein banding virus (PVBV); potato virus Y (PVY-D); pepper mottle virus, PVY strain (PeMV); tobacco etch virus (TEV-HAT); and pepper mottle virus (PepMoV) infecting pepper. References are given in parentheses. Blocked regions indicate the conserved amino acid residues. The DAG sequence in PVBV is underlined.

toms produced in *C. annuum* cv. California Wonder, the host-range experiments, and transmission studies, it was apparent that the isolate causing vein banding symptoms could be a potyvirus. Subsequently, glass-house-grown *C. annuum* cv. California Wonder was inoculated mechanically with this isolate, and infected leaves were used for purification. The purified virus appeared as flexuous rods with an average length of 900 nm, which is consistent with the morphology of potyviruses. The size of the CP was in the range expected for potyviruses. The CP of PVBV cross-reacted with antibodies against TEV (in Western blots), which is a well-characterized member of the *Potyvirus* group. A high-titer polyclonal antiserum for purified virions of PVBV was produced. The cross-reactivity studies with other potyviral antisera suggested that PVBV is closer serologically to PeMoV than to PSTV. On the other hand, PVBV showed no cross-reactivity to sorghum potyvirus antiserum.

The N-terminal sequence of potyviral CPs varies in length as well as in sequence. However, strains of a distinct member show at least 90% homology (14). The N-terminal sequence of PVBV CP was compared with all the potyviral CP sequences available in the protein databank. The PVBV

N-terminal sequence did not have more than 30% similarity with any of the reported potyviral CP sequences. This suggested that PVBV may be a distinct member of the *Potyvirus* group. However, the DAG sequence conserved in all aphid-transmitted potyviruses also was present in the N-terminal sequence of the PVBV CP. Also, the N-terminal sequence of the TRC of PVBV showed that this region was extremely homologous with other potyviral CPs, confirming the identity of PVBV as a member of the *Potyvirus* group. The results presented in this paper demonstrate that PVBV is a distinct member of the *Potyvirus* group.

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