Assocation of an unknown potyvirus isolate with a severe mosaic disease of *Narcissus tazetta* L.

Aminuddin, J A Khan & S K Raj

Plant Virus Laboratory, National Botanical Research Institute, Lucknow 226 001, India

Received 5 April 1999; revised 21 June 1999

An isolate of potyvirus was found to be associated with severe mosaic disease in *Narcissus tazetta* L. Electron microscopy, SDS-polyacrylamide gel electrophoresis of coat protein, serological relationship and RT-PCR were carried out to identify the virus isolate. Electron microscopy of purified virus preparation revealed the presence of flexuous particles of 730 x 13 nm. One band with a molecular weight of 33 kDa was obtained by SDS-polyacrylamide gel electrophoresis. Serological relationship of the virus isolate with bean yellow mosaic virus was established in electroblot immunoassay. Employing potyvirus specific primers in RT-PCR, a band of about 300 bp was obtained from the core region of the coat protein, thus confirming the association of a potyvirus with the mosaic disease of *N. tazetta*.

*Narcissus tazetta* L. cv. Paper White is an important ornamental plant cultivated in India for floriculture industry. Vegetative propagation through bulbs, leads to large scale dissemination of systemic viruses in *Narcissus* spp. stocks. Several reports have appeared on the natural occurrence of filamentous potyviruses 1,2 and a potexvirus3. *Narcissus* latent (Carlavirus) was found on *Narcissus*,4,5 *Nerine*6 and bulbous iris7. Other slightly flexuous Carlavirus like *Nerine* latent virus was isolated from *Nerine bowdenii*8,9 and Hippeastrum latent virus from *Hippeastrum hybridum*7. However, there is no report of potyvirus infecting *Narcissus* in India. We report here natural occurrence of a potyvirus on *Narcissus tazetta* L.

*N. tazetta* cv. Paper White plants growing in the nursery of the National Botanical Research Institute, Lucknow, exhibited severe mosaic symptoms accompanied with light-green to yellow stripes on leaves. Plants showed extreme reduction in growth and flowers as compared to apparently healthy plants. In view of identifying putative virus associated with the disease, virus purification and electron microscopy were undertaken followed by determination of molecular weight of the coat protein subunits and serological relationship analysis and RT-PCR.

Crude sap and purified virus preparations in phosphate buffer (0.1 M, pH 7.2) were mechanically inoculated on the plant species viz. Beta vulgaris, *Chenopodium album*, *C. amaranthicolor*, *C. quinoa*, *Cucurbita pepo*, *Cucumis sativus*, *Datura stramonium*, *Gomphrena globosa*, *Nicotiana benthamiana*, *N. rustica*, *N. tabacum* cv. Samsun N, *Petunia hybrida*, *Phaseolus vulgaris* cv. The Prince, *Solanum melongena*, *Vigna radiata* and *V. mungo*.

Virus was purified by clarification of buffered (phosphate buffer, 0.1 M, pH 7.2) *Narcissus* leaves extract with 15% n-butanol followed by precipitation with 6% polyethylene glycol (PEG) and sedimentation through 15% sucrose pad containing 0.1% Triton X-100. Purified preparations (3-4) were observed under transmission electron microscope (Philips 410) using 2% uranyl acetate (pH 4.2) as negative stain. Average size of the virus particles was calculated by measuring 90 particles with a magnifier, directly from electron micrograph negative. The calibration was done using tobacco mosaic virus as external standard.

Virus infected crude samples were mixed with an equal volume of denaturing solution (0.04 M, Tris-HCl; pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) (β-mercaptoethanol and 0.05% bromphenol blue) and heated in a boiling water bath for 3 min. Electrophoresis was carried out in 12% (separating) polyacrylamide gels according to the method described by Laemmli10 using Bio-Rad Protein II mini gel apparatus. The marker proteins (Pharmacia, Sweden) used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.2 kDa) and (α-laetalbumin (14.4 kDa). Sample (20 μl) was loaded to the slot of the gel.
Antiserum was raised in rabbits by three intramuscular injections of purified virus preparations mixed with complete Freund's adjuvant. Homologous and heterologous tests for antiserum titre and serological relationships were carried out by double diffusion tests.

Electroblot immunoassay (EBIA) was performed as described by Towbin et al.\textsuperscript{11} with some modifications. Infected crude sap (10 μl) was loaded on the gel. After electroblotting, nitrocellulose membrane was put in phosphate-buffered (pH 7.4) saline with 0.05% Tween-20 (PBS-Tween) containing 5% (w/v) non-fat milk / 1% haemoglobin as a blocking agent for 1 hr at 37°C. The membrane was then washed three times (10 min each) with PBS-Tween containing haemoglobin and incubated in alkaline phosphatase-conjugated antibody (Sigma), diluted 1:3000 in PBS-Tween at 37°C for 1 hr and washed as described earlier. A mixture of nitroblue tetrazolium chloride and 4-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma, USA) was used as substrate.

Total RNA was isolated from 50 mg infected Narcissus leaf as described by Langeveld et al.\textsuperscript{12} and final pellets were dissolved in 50 μl sterilized distilled water.

Potyvirus specific degenerate primers of U341 and D341 positioned in the core and degenerate oligo dT synthesized as earlier (Belgium) were used\textsuperscript{13,14}.

RT-PCR based amplification of coat protein gene (partial) was carried out. First strand cDNA synthesis was performed using 6 μl total RNA and degenerate oligo dT as primer. For amplification of cDNA representing the part of 3' NTR, the C-terminal region and core of coat protein, primers U341 and D341 were used. PCR was performed as described by Khan et al.\textsuperscript{14} using 5 μl of cDNA mix, 40 pmol of each primer and 2 unit Taq polymerase (Bangalore Genei, India).

Twenty five cycles were performed in Robocycler (Stratagene, Germany) with periods of 1.5 min for annealing at 50°C, 1 min for synthesis at 72°C and 1 min for melting at 94°C. Samples (5 μl) were analysed for 1.2% agarose gel electrophoresis containing ethidium bromide.

Several attempts to transmit the virus mechanically from N. tazetta to indicator plants failed, even when purified virus preparations were used for inoculation. No symptoms were observed upto 25 days after
inoculation and serological tests were also not positive.

Electron microscopy of purified virus preparations revealed the presence of flexuous particles of 730 x 13 nm size (Fig. 1). Morphology and size of the particles of our virus isolate were similar to those of typical potyviruses, which range from 690-900 nm long and 11-13 nm in diameter. The normal length of a particle population is one of the most useful...15,16.

The molecular weight of the viral protein subunits was calculated to be 33 kDa. Single band was observed when purified preparations were used (Fig. 2). A band of about 67 kDa was also observed when crude sap of infected Narcissus leaf samples were loaded. The minor band may be of host origin as it did not appear in Western blotting. Antiserum to the isolate reacted strongly in homologous combination. Also, it reacted with the antiserum to bean yellow mosaic potyvirus.

Amplification of the core region of the coat protein gene was done by employing potyvirus specific degenerate primers. A PCR amplified fragment of nearly 300 bp was obtained. This fragment covers the area of the core region of the coat protein. When total RNA from healthy leaves of Narcissus was used as template for making cDNA and subsequently RT-PCR, no such amplified fragment was obtained. Thus, it confirms the presence of a potyvirus in the sample (Fig. 3).

On the basis of similarities of particle morphology, presence of a 33 kDa protein subunit, strong serological relationship with BYMV and amplification of RNA template through RT-PCR employing potyvirus specific degenerate primers, the isolate from Narcissus was identified as a potyvirus isolate. This is the first report of a potyvirus occurring on Narcissus in India.

References