*Petunia vein banding virus: Characterization of a New Tymovirus from *Petunia* × *hybrida**

M. A. V. Alexandre, L. M. L. Duarte, E. B. Rivas, C. M. Chagas, and M. M. Barradas, Instituto Biológico, Av. Cons. Rodrigues Alves, 1252, CEP 04014-002, São Paulo, SP, Brazil; and R. Koenig, Biologische Bundesanstalt für Land-und Forstwirtschaft, Institut für Pflanzenvirologie, Mikrobiologie und Biologische Sicherheit, Messeweg 11/12, D-38104, Braunschweig, Germany

ABSTRACT

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Petunia plants from a nursery in the State of Rio Grande do Sul, Brazil, showed pronounced vein banding and contained isometric particles with diameters of approximately 45 and 30 nm. The larger ones apparently represent a caulimovirus, while the smaller ones, which included both empty shells and full particles, were identified as those of a new tymovirus for which we propose the name *Petunia vein banding virus* (PetVBV). Originally, PetVBV was transmitted only with difficulty to healthy *petunia* plants. However, from an experimentally infected *petunia*, it was later readily transmitted also to *Nicotiana benthamiana* and *Nicandra physalodes*, but not to other species in the Solanaceae or other plant families. It produces cytopathic effects typical for tymovirus infections. Its coat protein shows approximately 65% amino acid sequence identity with those of *Eggplant mosaic* and *Andean potato latent* viruses, to which it is also serologically more closely related than to any other tymoviruses.

Plants from the genus *Petunia* are economically important bedding and balcony ornamentals, especially the new hybrids which display intensively colored flowers. In Brazil, two viruses have been reported to infect *petunias* naturally: a species of the genus *Tobamovirus* (2) and a putative species of the genus *Caulimovirus* (3). Plants infected with the latter virus, which has a diameter of approximately 45 nm, also contained smaller particles of about 30 nm which consisted either of empty protein shells or intact particles (1). They were now identified as those of a new tymovirus for which we propose the name *Petunia vein banding virus* (PetVBV).

MATERIAL AND METHODS

Virus source and mechanical transmission. Leaves of *Petunia* × *hybrida* Vilm. with vein banding (Fig. 1) were collected in a government-run nursery at Gramado in the State of Rio Grande do Sul, Brazil. Extracts from infected leaves

were prepared in phosphate buffer (0.05 M, pH 7.1) and mechanically inoculated on 36 species.

Electron microscopy. Virus particles were visualized after negative staining with 2% uranyl acetate. For cytological studies, small pieces of healthy or virus-infected leaf tissue were fixed overnight at 4°C in 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), postfixed for 2 h at 4°C in 1% osmium tetroxide in the same buffer,

and stained overnight at 4°C in 0.5% uranyl acetate. After dehydration in graded dilutions of acetone, samples were embedded in Spurr's medium, sectioned with an LKB III ultratome, and double stained with uranyl acetate and lead citrate.

Insect transmission. *Myzus persicae* Sulz., *Aphis gossypii* Glov., and the chrysomelid beetle *Diabrotica speciosa* Germ. were tested as possible vectors. The aphids were given acquisition feeding periods of 30 min on infected *petunia* plants, then transferred for inoculation feeding for 30 min to 10 healthy plants (10 aphids/plant). In transmission tests with beetles, the acquisition and inoculation feeding times were 48 h.

Purification and antiserum production. The virus was purified by the Triton X-100/urea method of Hull et al. (8). The final purification was by centrifugation through a sucrose density gradient (10 to 40%) at 35,000 × *g* for 60 min. Rabbits were immunized by intramuscular or lymph nodal injections of purified virus emulsified with an equal volume of Freund's adjuvant complete (first injection) or incomplete (second and third injections).

Sequence analyses. The immunocapture reverse transcription polymerase chain

Fig. 1. Leaf of *Petunia* × *hybrida* showing vein banding.

Corresponding author: M. A. V. Alexandre
E-mail: alexand@biologico.br

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reaction (IC-RT-PCR) was performed as described previously (12) using an antiserum to *Eggplant mosaic virus* (EMV) for trapping the virus particles. For amplifying the coat protein gene and the 3' terminal part of the replicase gene of PetVBV, we used two primers which were derived from parts of the nucleotide sequence of EMV genomic RNA which are more or less conserved in the RNAs of other tymoviruses. EM1 corresponds to nucleotides 5,235 to 5,258 upstream and within the coding region for the RNA-dependent RNA polymerase motif VIII; EM3 is complementary to nucleotides 6,296 to 6,272 in the 3' untranslated region of EMV genomic RNA. The PCR product was purified from

agarose gels using the Jetsorb Gel Extraction Kit (Genomed GmbH, Bad Oeynhausen, Germany) and cloned into the pGEM-T vector (Promega Corp., Madison, WI) for sequencing, which was performed by a commercial company (SeqLab, Göttingen, Germany). Sequences were analyzed by the University of Wisconsin Genetics Computer Group (UWGCG) software, version 8 (5). The gene bank accession number of the partial sequence of PetVBV RNA is AF210709.

RESULTS

Host range, symptomatology, and transmission behavior. *Petunia* leaves showing vein banding (Fig. 1) contained

45- and 30-nm virus-like particles. After several unsuccessful attempts, we succeeded once in transmitting the smaller particles by mechanical inoculation to a healthy *petunia* plant from which they were then further transmitted to *petunia*, producing local chlorotic and necrotic spots and systemic vein banding. They were also transmitted to *Nicotiana benthamiana*, producing local necrotic spots and systemic chlorotic mosaic, and to *Nicandra physalodes*, producing systemic vein clearing and mosaic. These symptoms were observed only during autumn and winter. No infections were obtained on other solanaceous plants (i.e., *Capsicum annuum*; *Datura stramonium*; *Lycopersi-*

Fig. 2. (A) Electron micrograph of a negatively stained purified *Petunia vein banding virus* (PetVBV) preparation from *Petunia × hybrida* showing particles with clustering of protein subunits. Bar = 100 nm. (B–F) PetVBV-infected mesophyll cells from *Petunia × hybrida*. (B) Chloroplasts showing peripheral double-membraned vesicles (arrow head). (C) Tangentially sectioned chloroplast with double-membraned vesicles containing fibrillar material. (D) Distorted mitochondrion close to a chloroplast. (E) Mitochondrion with a double-membraned vesicle. (F) Virus-like particles associated with cytoplasmic dense amorphous masses. Ch = chloroplast, M = mitochondria, O = osmiophilic globules, S = starch, Ve = vesicles, and VLP = virus-like particles. Bars = 300 nm.

con esculentum “Kada gigante” or “Santa Cruz”; *Nicotiana debneyi*; *N. glutinosa*; *N. rustica*; *N. tabacum* “Samsun”, “TNN”, “Turkish”, or “White Burley”; *Solanum luteum*; *S. melongena*); and 16 other plant species belonging to the families Amaranthaceae, Apocynaceae, Asteraceae, Balsaminaceae, Brassicaceae, Chenopodiaceae, Cucurbitaceae, and Fabaceae.

No seed transmission was observed in tests with 500 seeds each from naturally and experimentally infected petunia plants. *M. persicae* and *A. gossypii* failed to transmit the virus. Transmission by the chrysomelid beetle *D. speciosa* could not be tested because the insects did not feed on petunia and died.

Electron microscopy. In negatively stained preparations of PetVBV, the clustering of coat protein subunits typical of tymoviruses was clearly visible (Fig. 2A). Examination of thin sections from infected leaves revealed remarkable cytopathic effects in chloroplasts comprising peripheral double-membraned vesicles (Fig. 2B) occasionally containing fibrillar material (Fig. 2C). Clumps of somewhat rounded chloroplasts were also noted. Mitochondria were sometimes distorted (Fig. 2D) and showed vesicles (Fig. 2E) similar to those found in chloroplasts. Virus-like particles were observed free in the vacuoles as well as in the cytoplasm and were sometimes associated with electron-dense amorphous masses (Fig. 2F).

Serology. In the agar gel double diffusion test, PetVBV did not react with antisera to *Cocoa yellow mosaic virus* (CYMV), *Clitoria yellow vein virus* (CYVV), *Desmodium yellow mosaic virus*, *Okra mosaic virus*, *Passion fruit yellow mosaic virus*, *Physalis mottle virus*, *Plantago mottle virus*, *Poinsettia mosaic virus*, *Scrophularia mosaic virus*, or *Turnip yellow mosaic virus* (TYMV), which were all adjusted to a homologous titer of 1:128. Strong reactions were, however, observed with antisera to all viruses of the *Andean*

potato latent virus (APLV)-EMV cluster (9,10) (i.e., the type, AI, and the tomato strains of EMV and the Col, Col2, Caj, and Ay strains of APLV). However, PetVBV was not identical to any of these viruses, because pronounced spur formation was observed when PetVBV and these viruses were tested in adjacent wells with antiserum to PetVBV (Fig. 3).

Molecular properties. Analyses of the deduced amino acid sequence of the coat protein and of the nucleotide sequence of the 3'-terminal part of the replicase gene of PetVBV both revealed the highest percentages of identity (i.e., 65% each) with the corresponding sequences of EMV and APLV (Fig. 4A and B). The percentages of sequence identity with other tymoviruses from solanaceous hosts were lower and with those from other hosts (shown in Fig. 4 only for TYMV) were even less. The groupings seen in Figure 4A and B were

also confirmed by neighbor joining analyses (15; *results not shown*). The molecular mass of the PetVBV coat protein was calculated to be 19.6 kDa.

DISCUSSION

The host range, serological, molecular, and cytopathological studies described in this article clearly indicate that the virus with 30-nm particles found in petunia is a distinct tymovirus which has its closest relationships to APLV and EMV. This new virus infects only three solanaceous hosts (i.e., *Petunia* spp. *N. benthamiana*, and *N. physalodes*), whereas the various strains of APLV and EMV have much wider host ranges (6,7,17). Also, the two EMV-like viruses described in Brazil, *Tomato white necrosis virus* (4) and an EMV isolate from tobacco (14), infect hosts belonging to several other families in addition to the Solanaceae family.

Fig. 3. Agar gel double diffusion test using sap from *Petunia vein banding virus* (PetVBV)-infected (1) and healthy (2) petunia leaves; and *Eggplant mosaic virus* type-strain from infected *Datura* spp. leaves (3) in the outer wells and antiserum to PetVBV in the central well (A).

Fig. 4. Percentages of identities in the sequences of (A) the amino acids of the coat proteins and (B) the approximately 370 3'-terminal nucleotides of the replicase genes of tymoviruses infecting solanaceous hosts, including *Belladonna mottle virus* (BelMV; X54529), *Dulcamara mottle virus* (DuMV; AF035634), *Andean potato latent virus* (APLV; AF035402), *Eggplant mosaic virus* (EMV; J04374), *Petunia vein banding virus* (PetVBV; AF210709), *Physalis mottle virus* (PhyMV; Y16104), and *Turnip yellow mosaic virus* (TYMV; X16378). The respective gene bank accession numbers are given in parentheses.

PetVBV produces several cytopathic effects typical for tymovirus infections (11); in particular, double-membrane bounded vesicles at the periphery of the chloroplasts. Some more specific alterations were also observed (i.e., distorted mitochondria with vesicles similar to those found in chloroplasts) which have so far been seen only in plants infected with CYVV (13). The presence of particles free in the vacuoles as well as in the cytoplasm and their occasional association with electron-dense amorphous masses had up to now been observed only in CYMV infections (11).

The PetVBV coat protein shows only 65% amino acid sequence identity with the coat proteins of EMV and APLV, the two tymoviruses to which PetVBV has its closest relationships, serologically as well as on the molecular level. Percentages of coat protein sequence identity below 90% have been suggested to be an important character for demarcating virus isolates as distinct species in the families *Poty-* and *Geminiviridae* (16). Similar considerations should also be applicable to plant viruses in other families and genera, including *Tymovirus*. The identification of a new virus species is suggested because the sequence identity is far below 90%, as is the case for the coat proteins of PetVBV and its closest known relatives. Further differentiating characters which should be taken into account are stressed in the *Guidelines for the Demarcation of Virus Species* (16), i.e., the existence of differences in the reactivities with key antibodies, in natural host ranges, in transmission properties, and in pathogenicity, including cytopathogenicity. The properties clearly differentiate PetVBV from other tymoviruses. The most reliable differences would be from nucleotide sequence comparisons for the entire genomes. Percentages of total sequence identity <85 are considered sufficient for demarcating poty- and geminiviruses as distinct viruses when supported by the other traits (16). Similar criteria apply to other virus genera. For PetVBV, we analyzed the coat protein gene sequence of the 370 3'-terminal nucleotides in the replicase gene. This sequence showed only 65% identity with corresponding sequences

in the genomic RNAs of APLV and EMV and even lower percentages of identity with the RNAs of other tymoviruses. Based on an alignment of the genomes of tymoviruses for which the entire nucleotide sequences are known (e.g., EMV, *Ononis yellow mosaic virus*, *Physalis mottle mosaic virus*, *Chayote mosaic virus*, *Kennedya yellow mottle virus*, *Turnip yellow mosaic virus*, and *Erysimum latent virus*), the 3'-terminal sequence of the replicase gene is a fairly conserved region of the tymovirus genome (*data not shown*). Even lower percentages of sequence identity might be expected in other parts of the genome, such as in the 5'- and 3'-untranslated regions or in the central part of the replicase gene between the coding sequences for the methyltransferase and helicase domains. Our data support the conclusion that PetVBV should be considered as a distinct tymovirus.

Following the recently published guidelines for the demarcation of virus species (16), PetVBV should be regarded as a distinct virus species. Its coat protein as well as the C-terminal part of its replicase show only about 65 to 70% amino acid sequence identity with the corresponding parts of EMV and APLV, the two tymoviruses to which PetVBV has its closest relationships—serologically as well as on the molecular level. Sequence identities below 85 to 90% have been suggested to be indicative for a distinct virus species in the above mentioned guidelines.

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