ABSTRACT

Verbena ‘Taylortown Red’ plants showed virus-like mottling symptoms. Virus purifications disclosed the presence of elongated and spherical particles, evidence of mixed virus infections, whereas double-stranded RNA analysis revealed the presence of several bands absent in healthy plants. After shotgun cloning, three viruses were identified in ‘Taylortown Red’: Broad bean wilt virus-1, Coleus vein necrosis virus, and a previously undescribed potyvirus. Given the importance of verbena to the ornamental industry, we studied the viruses found in ‘Taylortown Red’ and, in this article, we present our findings on the new potyvirus, provisionally named Verbena virus Y (VVY), VVY belongs to the Potato virus Y subgroup in the genus Potyvirus, has solanaceous plants, including potato, as alternative hosts, and can be transmitted by a ubiquitous pest in the ornamental industry, the green peach aphid.

MATERIALS AND METHODS

Ornamentals, worldwide, are a multibillion-dollar industry. Many commercially valuable plant species are clonally propagated, which heightens the risk of the accumulation of undetected viruses during propagation. This can lead to significant losses because of undesirable changes in the plant phenotype. Verbena (Verbena × hybrida) is a popular ornamental propagated from cuttings, in which several new viruses and viroids have been identified in recent years (4,6,9,13,28,29).

Verbena ‘Taylortown Red’ plants displaying mottling symptoms (Fig. 1) were examined for the possibility of viral infection. Virus and double-stranded RNA (dsRNA) purifications disclosed the presence of multiple viruses in Taylortown Red. Shotgun cloning followed by sequencing revealed three viruses infecting ‘Taylortown Red’: Broad bean wilt virus-1 (BBWV-1), Coleus vein necrosis virus (CVNV; 27) and a new Potyvirus sp., provisionally named Verbena virus Y (VVY), the subject of this article.

Members of the family Potyviridae are the most abundant plant viruses, with several hundred members identified to date. They can be transmitted mechanically, by seed, pollen, or vectors, including aphids, whiteflies, eriophyid mites, or plasmodiophorids. The mode of transmission of potyviruses and the vast number of potential vectors for VVY, in combination with the vegetative propagation of verbena, made characterization of this virus a necessity. Here, we present the sequence of the virus, detection protocols, and potential plant host and aphid vector species.

Corresponding author: I. E. Tzanetakis
E-mail: itzaneta@uark.edu

* The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 appears in color in the online edition.

Accepted for publication 3 June 2010.

Fig. 1. Symptoms of verbena ‘Taylortown Red’ infected with Verbena virus Y, Broad bean wilt virus-1, and Coleus vein necrosis virus. Left: infected and right: healthy material.
correction, and bootstrap consisting of 1,000 pseudoreplicates.

Detection. Three immunological techniques were used for VVY detection: Western blot, dot blot, and triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Western blot and dot blot were performed using universal Potyvirus (Agdia, Elkhart, IN) and Potato virus Y (PVY) polyclonal (Phyto Diagnostics Co. Ltd., North Saanich, BC, Canada) antibodies whereas, for ELISA, PVY polyclonal and monoclonal antibodies (Phyto Diagnostics Co. Ltd.) were used. VVY-infected 'Taylortown Red', healthy 'Twilight Blue with Eye', and pepper tissue infected with Tobacco etch virus were used in the tests. Western blots were performed as previously described (33). For dot blot, leaf tissue was ground in phosphate-buffered saline (PBS), pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4), and blotted on a nitrocellulose membrane using the Bio-Rad Bio-Dot microfiltration apparatus. After a brief wash with PBS, the protocol described by Tzaneakis et al. (34) for tissue blot was used. Buffers described by Clark and Adams (9) were used in a triple-antibody sandwich ELISA. A polyclonal antibody was used for capture and a monoclonal antibody was used in detection as per the manufacturer’s recommendation. Rabbit anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research, West Grove, PA) at 0.3 µg/ml was added after the monoclonal antibody. Following the final washing step, p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis) was added at 0.5 mg/ml. The colorimetric reaction was measured at an absorbance of 405 nm with a BioTek plate reader (BioTek, Winooski, VT).

Primers VVYF (5′-CCATCGAAGAAC TTCCAGGA) and VVYR (5′-GCTTCG AGTCCAGTTCAC) that amplify a 289-bp region of the VVY genome were selected for RT-PCR detection. BBWV-1 detection was done using primers BBWF (5′-CTCTATGGCTCCCTTGCAT) and BBWR (5′-CAATCACAACCTCACAGCA) whereas primers CVNVF (5′-TAAGGGTGACACTTCTGAT) and CVNVR (5′-CGCAATGGTCTAATCTCA CG) (24) were used for detection of CVNV. The quality of RNA of all samples was tested with primers ICF/ICR (39). The PCR program on a Stratagene Robocycler using Genescript Taq polymerase consisted of denaturation for 3 min at 94°C, followed by 40 cycles at 94, 56, and 72°C for 30 s each; and terminated with 5 min of incubation at 72°C. At least 10 amplicons were sequenced and all were VVY specific, validating the protocol.

Transmission. VVY host range was determined after mechanical inoculation using ‘Taylortown Red’ tissue homogenized in PBS (1:10 wt/vol) and rubbed onto 600-grit Carborundum-dusted leaves. The inoculated species were: Brassica rapa var. rapa, Chenopodium quinoa, Capsicum annuum, Cucumis sativus, Cucurbita pepo, Gomphrena globosa, Solanum lycopersicum, Nicotiana benthamiana, N. sanderana, S. tuberosum, Spinacea oleracea, and Vigna unguiculata subsp. dekindiana. In all, 5 to 10 plants of each species were inoculated and tested for VVY transmission using RT-PCR about 1 month post inoculation. The green peach aphid (GPA; Myzus persicae Sulz.) was tested as a vector of VVY. A colony was established in an insect-proof cage on dsRNA-free and BBWV-1, CVNV, and VVY RT-PCR-negative ‘Twilight Blue with Eye’ verbenas. Apterous individuals were transferred to ‘Taylortown Red’-infected material, where they fed for approximately 16 h. A group of at least 20 individuals were placed on ‘Twilight Blue with Eye’ plants and left to feed for 7 days before the application of a systemic insecticide. Two experiments for a total of 10 plants were performed and plants were tested for VVY, BBWV-1, and CVNV 1 month after the application of the insecticide.

RESULTS

VVY characterization. Unusual symptoms observed on Taylortown Red plants led us to investigate the possibility of a virus infection. Analysis of dsRNA extracts disclosed the presence of multiple bands and, through shotgun cloning, Kraus et al. (21) identified BBWV-1, CVNV, and a new potyvirus. We performed purifications in an effort to separate the viruses but they always co-purified, as indicated by the spherical and flexuous elongated particles in a triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Western blot and dot blot analyses confirmed the presence of multiple viruses (data not shown).

A combination of shotgun cloning, RT-PCR, and RACE was used to obtain the genome of VVY. The virus genome is 9,742 nucleotides (nt) excluding the A-tail and is most similar to isolates of PVY and Tobacco etch virus (TEV) orthologs; with about 40% amino acid sequence identity and between 56 and 58% similarity. The virus contains conserved P1 motifs between residues 193 and 261, including the catalytic protease residues His397-Asp217-Ser226 (1,41).

Table 1. Location of the putative cleavage sites and molecular mass of Verbena virus Y proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kDa)</th>
<th>Protein coordinates</th>
<th>Cleavage site (amino acid sequence –4/+3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>32.4</td>
<td>1–285</td>
<td>P1/HC-Pro (MQQF/SNA)</td>
</tr>
<tr>
<td>HC-Pro</td>
<td>51.7</td>
<td>286–741</td>
<td>HC-Pro/P3 (YRMGG/GLL)</td>
</tr>
<tr>
<td>P3</td>
<td>44.2</td>
<td>742–1,131</td>
<td>P3/VEH/Q/NKD</td>
</tr>
<tr>
<td>P3N/PPO</td>
<td>31.4</td>
<td>...</td>
<td>P3/VIH/Q/STG</td>
</tr>
<tr>
<td>6K1</td>
<td>6.0</td>
<td>1,132–1,183</td>
<td>6K1/VIH/Q/LDD</td>
</tr>
<tr>
<td>CI</td>
<td>7.1</td>
<td>1,184–1,817</td>
<td>CI/VIH/Q/LDD</td>
</tr>
<tr>
<td>6K2</td>
<td>5.7</td>
<td>1,818–1,869</td>
<td>6K2/VIH/Q/LDD</td>
</tr>
<tr>
<td>Vpg</td>
<td>21.5</td>
<td>1,870–2,157</td>
<td>Vpg/VEH/SGK</td>
</tr>
<tr>
<td>Na</td>
<td>28.0</td>
<td>2,058–2,303</td>
<td>Na/Nla/VEH/SGK</td>
</tr>
<tr>
<td>NIB</td>
<td>60.0</td>
<td>2,304–2,822</td>
<td>NIB/VEH/AAE</td>
</tr>
<tr>
<td>CP</td>
<td>31.8</td>
<td>2,823–3,105</td>
<td>CP/VEH/Q/AAE</td>
</tr>
</tbody>
</table>

The highly structured 134-amino acid peptide downstream of P1 and contains all signature HC-Pro Potyvirus motifs (40), including the tetrapeptide KMTC50.53, involved in virus-host interactions and essential for vector transmission, and the tripeptides ING248–250, CCC290–292, and PTK308–310, involved in genome amplification, systemic movement, and vector transmission, respectively. The protease active site residues are Cys342 and His415. The protein is related most closely to the HC-Pro of PVY and Pepper severe mosaic virus (PSMV), with 67% amino acid sequence identity and 82% similarity. The 390-aa P3 is one of the least conserved Potyvirus proteins and functions as a pathogenicity and host determinant (15,31). The VVY P3 shows about 40% amino acid sequence identity (60% similarity) to the PVY and PSMV orthologs. The slippery consensus sequence Ga246(248)–249(250) was identified at the 5′ end of the putative VVY pretty interesting Potyviridae open reading frame (PIPO; 8) whereas the PIPO stop codon is located at nucleotides 3,188 to 3,190. A 52-aa peptide downstream P3 is the 6K1 ortholog of VVY and is most similar to that of the PepMoV and PVV counterparts.
with 73 and 67% amino acid sequence identity (88 and 83% similarity), respectively. The 634-aa virus helicase, the cylindrical inclusion body protein (CI), includes signature RNA helicase motifs between residues 79 and 360 (25). The protein is closely related to the CI proteins of *Wild potato mosaic virus* (WPMV), *Peru tomato mosaic virus* (PTMV), PVV, and PVY, with about 70% amino acid sequence identity and over 80% similarity. A second 52-aa peptide is found downstream of CI and contains a transmembrane domain between residues 20 and 42 (22). Orthologs of this protein (6K2) anchor the virus replication complex to the membranes of the endoplasmic reticulum (40). The most similar proteins in the database are the 6K2 peptides of PSMV and PVY, with 57 and 53% amino acid sequence identity (75 and 83% similarity), respectively. The 188-aa genome-linked protein (Vpg) is most similar to the orthologous proteins of PepMoV and WPMV, with 82 and 78% amino acid sequence identity and 89% similarity, respectively. The third protease of the virus, the 246-aa nuclear inclusion bodies A protein (NIa), is more similar to the Nla of PepMoV and PVY, with about 69% amino acid sequence identity (84 and 81% amino acid similarity), respectively, and contains the His-Ser-Asp-Cys-Glu-Cys-Ser-Glu cysteine protease catalytic tetrad. The 519-aa virus polymerase, nuclear inclusion body B protein (Nb), is the most conserved protein of potyviruses and contains the eight conserved motifs identified by Koonin (20) between residues 180 and 431. VVY Nb is related more closely to the Nb of PTMV, with 73% and PepMoV, PVY, and PSMV with 70% amino acid sequence identity, whereas the similarity to all four viruses exceeds 85%. The 283-aa coat protein (CP) is related most closely to the CPs of PVY and PepMoV, with 80 and 73% amino acid sequence identity and 89 and 82% similarity, respectively. The protein contains the characteristic DAG8_10 motif involved in aphid transmission (5). The Arg173 and Asp217 residues are probably involved in the assembly process of virus particles (11,17). The 3′ UTR of the virus is 290 nt long, excluding the A-tail. Phylogenetic analysis of the polymerase conserved motifs and the CP of members of all recognized genera of the family *Potyviridae* clearly places the virus within the PVY subgroup in the genus *Potyvirus* (Fig. 2).

**Detection.** VVY was detected successfully with the three immunological techniques using universal *Potyvirus* monoclonal (18) or PVY-specific polyclonal antibodies (Fig. 3). In the ELISA test, performed twice, VVY-infected material typically gave readings that were at least five times higher than the control when using PVY polyclonal antibodies and more than twice the readings of the control using *Potyvirus* monoclonal antibodies. The virus was detected by RT-PCR after 30 cycles, although we chose to use 40 cycles for routine detection because the VVYF/VVYR primers produced no background at the higher number of PCR cycles (Fig. 4).

**Transmission.** Several herbaceous plants were tested as potential alternate hosts of VVY and, given the similarity of the virus to PVY, one of the most important viruses for agriculture worldwide, we included several solanaceous hosts in our studies. *N. benthamiana* (6/10) and *S. tuberosum* (2/8) were confirmed as hosts of VVY by RT-PCR and sequencing of the amplification products. Plants of both species were stunted compared with buffer control plants. The ability of the GPA to transmit the virus was also tested. The aphid is a vector for VVY, with one of the test plants becoming infected, as verified by RT-PCR amplification of the virus and sequencing of the products (Fig. 4).
DISCUSSION

Virus-like mottling symptoms were observed on 'Taylortown Red' plants. A study was initiated to detect the virus or viruses that could be associated with or cause the observed symptoms. Three viruses were found in all symptomatic 'Taylortown Red' plants. This was not a surprise given the clonal propagation of verbena. We have studied and characterized two of the viruses, CVNV (21) and VVY. VVY was characterized and the complete nucleotide sequence was obtained. VVY is a new Potyvirus, belonging to one of the most important groups of plant viruses for agriculture and floriculture, the PVY subgroup.

The close relationship of VVY to PVY led to the investigation of the serological relationships between the two viruses. Monoclonal universal Potyvirus antibodies readily detected the virus but, most importantly, polyclonal antibodies against PVY cross-reacted very strongly to VVY. Those findings suggest that VVY could have been mislabeled as PVY in verbena or other plants. This was not a surprise given the serological relationship of VVY with PVY, it is possible that plants thought to be infected with PVY are truly infected with VVY.

ACKNOWLEDGMENTS

This study is dedicated to the late Dr. Jennifer Kraus, who initiated this project but was unable to complete it due to an incurable illness. The National Floral Research Initiative and start-up funds provided by the Arkansas Agricultural Experiment Station to J. E. Tzanetakis funded this work.

LITERATURE CITED


Fig. 3. Western and dot-blot detection of Verbena virus Y (VVY) using Potato virus Y polyclonal or universal Potyvirus monoclonal antibodies. TEV: Tobacco etch virus, a potyvirus used to test antibody cross-reactivity. Control: healthy verbena tissue.

Fig. 4. Reverse transcription polymerase chain reaction detection verifying Verbena virus Y (VVY) transmission using the green peach aphid. BBWV-1: Broad bean wilt virus 1; CVNV: Coleus vein necrosis virus. Lane 1: ‘Taylortown Red’ infected with VVY, BBWV-1, and CVNV; lane 2: ‘Twilight Blue with Eye’ used in transmission study; lane 3: ‘Twilight Blue with Eye’ control; lane 4: water control.


