A New *Potyvirus* sp. Infects Verbena Exhibiting Leaf Mottling Symptoms

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ABSTRACT

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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.

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 sev-

eral 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.

MATERIALS AND METHODS

Virus and nucleic acid purifications.

Virus purification was performed according to the Martin and Bristow (26) protocol. The procedure was repeated twice, with and without a 20% sucrose cushion during the ultraspeed centrifugations. Purified virus preparations were stained with

2% ammonium molybdate and viewed using a Philips CM-12 scanning transmission electron microscope. DsRNA extractions were performed using a modified Yoshikawa and Converse (42) protocol. Total RNA for reverse-transcription polymerase chain reaction (RT-PCR) detection purposes was extracted as previously reported (35).

Cloning, genome sequence, and analysis. Purified virus single-stranded RNA or dsRNA were used as template to obtain the sequence of VVY, with cloning and sequencing performed as described previously (36). BLAST was used to identify clones carrying virus sequences (3). Oligonucleotide primers were developed and used in RT-PCR amplification to obtain the sequence of the gaps between potyvirus contigs obtained from shotgun cloning (38). The 5′ end of the virus was obtained using random amplification of cDNA ends (RACE) after two tailing reactions using different nucleotides (37). The genome sequence, representing at least a 3× genome coverage, was assembled using CAP3 (16) and deposited in GenBank under accession number EU564817.

The putative cleavage sites of the polyprotein were identified using PredictProtein (30) and ClustalW (32), as well as comparison of the VVY putative cleavage sites to those of other potyviruses (1). Similarities between VVY protein orthologs were calculated using GATMA at default settings (7). Phylogenetic analysis was performed with ClustalW (32) using the neighbor-joining algorithm, Kimura’s

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.

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

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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 Tzanetakis 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 developed for 1 h at room temperature and the optical density with absorbance at 405 nm was determined using a BioTek plate reader (BioTek, Winooski, VT).

Primers VVYF (5' CCATTCGAAAC TTCGAGGA) and VVYR (5' GCTTCG AGTCGATTTTCAGG) that amplify a 289-bp region of the VVY genome were selected for RT-PCR detection. BBWV-1 detection was done using primers BBWVF (5'-CTTCATGGCTCCCTTGCAT) and BBWVR (5'-CAATCACACTCATCAAGC CAA) whereas primers CVNVF (5'-TAAGGGTGACACTTCGGATG) and CVNVR (5'-CGCAATGTGCTAACTTCA 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 Strategene 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. sanderae*, *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 a 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 observed under the electron microscope. Using RT-PCR, we verified purification of all three 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 *Pepper mottle virus* (PepMoV), sharing 63% nucleotide identity over the length of the genomes. The percent identity between

VVY and other potyviruses indicates that VVY is a new potyvirus (2).

The highly unstructured 134-nt 5' untranslated region (UTR) (dG <−6.6 kcal/mol; 40) probably accommodates easy access of the ribosomes to the putative start codon of the polyprotein, which is in excellent translational context (19,24). The putative 3,105-amino-acid (aa) polyprotein has a molecular mass of 352 kDa. The putative cleavage sites of the polyprotein and mass of the mature proteins are listed in Table 1. The first 285 aa of the polyprotein is the VVY P1 serine protease ortholog, the least-conserved region of the potyviral polyprotein (2). VVY P1 shows the highest similarity with PVY and *Potato virus Y* (PVY) orthologs; with about 40% amino acid sequence identity and between 56 and 58% similarity. The protein contains conserved P1 motifs between residues 193 and 261, including the catalytic protease residues His₁₉₃-Asp₂₀₂-Ser₂₃₆ (1,41).

The 456-aa helper component serine protease (HC-Pro) is found 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 conserved sequence G₂A₆₍₂₉₄₃₋₂₉₄₉₎ was identified at the 5' end of the putative VVY pretty interesting *Potyviriidae* 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

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

Protein	Molecular mass (kDa)	Protein coordinates	Cleavage site (amino acid sequence −4/+3)
P1	32.4	1–285	P1/HC-Pro (MQQF/SNA)
HC-Pro	51.7	286–741	HC-Pro/P3 (YRMG/GLL)
P3	44.2	742–1,131	HC-Pro/P3 (VEHQ/KND)
P3N/PIPO	31.4	...	...
6K1	6.0	1,132–1,183	6K1/CI (VHHQ/LDD)
CI	71.1	1,184–1,817	CI/6K2 (VHHQ/STG)
6K2	5.7	1,818–1,869	6K2/Vpg (VSHQ/GKR)
Vpg	21.5	1,870–2,057	Vpg/Nla (VEHE/SKS)
Nla	28.0	2,058–2,303	Nla/Nlb (VQEQ/GLS)
NIB	60.0	2,304–2,822	...
CP	31.8	2,823–3,105	Nlb/CP (VSHQ/AEE)

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 81% 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 NIa of PepMoV and PVY, with about 69% amino acid sequence identity (84 and 81% amino acid similarity),

respectively, and contains the His₄₈-Asp₈₃-Cys₁₅₁-His₁₆₇ cysteine protease catalytic tetrad. The 519-aa virus polymerase, nuclear inclusion body B protein (NIb), is the most conserved protein of potyviruses and contains the eight conserved motifs identified by Koonin (20) between residues 180 and 431. VVY NIb is related more closely to the NIb 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* mono-

clonal (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).

Fig. 2. Unrooted phylograms of the **A**, polymerase conserved motifs and **B**, coat protein (B) of Verbena virus Y and other potyviruses. Sequences used for the construction of the phylogram were obtained from the GenBank accessions of the complete genome of the viruses unless otherwise noted. *Agropyron mosaic virus*, AgMV; *Banana bract mosaic virus*, BBMV; *Barley mild mosaic virus*, BMMV; *Barley yellow mosaic virus*, BYMV; *Brome streak mosaic virus*, BSMV; *Cassava brown streak virus*, CBSV, GenBank accession number AF311052; *Cucumber vein yellowing virus*, CVYV; *Dasheen mosaic virus*, DsMV; *Hordeum mosaic virus*, HoMV; *Leek yellow stripe virus*, LYSV; *Lettuce mosaic virus*, LMoV; *Lily mottle virus*, LMV; *Maclura mosaic virus*, MacMV, GenBank accession number U58771; *Moroccan watermelon mosaic virus*, MWMV; *Narcissus latent virus*, NaLV, GenBank accession number U58770; *Oat mosaic virus*, OMV; *Oat necrotic mottle virus*, ONMV; *Peace lily mosaic virus*, PLMV; *Pepper mottle virus*, PepMoV; *Plum pox virus*, PPV; *Potato virus Y*, PVY; *Ryegrass mosaic virus*, RyMV; *Scallion mosaic virus*, ScMV; *Sorghum mosaic virus*, SorMV; *Squash vein yellowing virus*, SqYVV; *Sweet potato feathery mottle virus*, SPFMV; *Sweet potato mild mottle virus*, SPMV; *Teloma mosaic virus*, TelMV; *Tobacco etch virus*, TEV; *Tobacco vein banding mosaic virus*, TVBMV; *Turnip mosaic virus*, TuMV; *Verbena virus Y*, VVY; *Wheat eglid mosaic virus*, WEMV; *Wheat streak mosaic virus*, WSMV; *Wild tomato mosaic virus*, WTMV. Numerical values of the nodes with bootstrap values of less than 70% are not shown, as they are not considered significant. The bars represent 0.1 amino acid changes per site.

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 hosts thought to be infected by PVY. We tested the hypothesis that VVY can infect important solanaceous hosts by testing transmission of the virus to potato, tomato, pepper, and *Nicotiana* spp. Potato and *Nicotiana* spp. were found to be experimental hosts for the virus, and plants infected with the virus exhibited stunting.

GPA is a cosmopolitan pest and a vector of a number of potyviruses. These properties made it an excellent candidate to verify aphid transmissibility of VVY, a property deduced by phylogenetic analysis and the motifs present in HC-Pro and CP. GPA

is also one of the most noxious pests in greenhouse settings because it has developed resistance to a number of insecticides (13), making it a potentially important disseminator of the virus if verified as a VVY vector. VVY-infected material remained asymptomatic for a period of more than 2 years. Transmission rate was low but these experiments verified that VVY is aphid transmissible and, depending on the virus isolate, aphid clone, and optimal acquisition access period (12,23) on infected verbena, it can potentially be an important vector for the virus in greenhouse settings. The latency of the VVY infection indicates that symptoms on Taylortown Red are caused by BBWV-1, CVNV, or the synergy between the viruses. The presence and distribution of VVY in verbena is unknown but, given the serological relationship of VVY with PVY, it is possible that plants thought to be infected with PVY are truly infected with VVY.

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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.

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