First Molecular Evidence of the Occurrence of a Pea Mosaic Strain of Bean Yellow Mosaic Virus in Verbena × Hybrida

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Abstract

Verbena × hybrida is an ornamental annual used in rock gardens, as an edging plant, and in hanging baskets. It comes in a variety of colors and grows approximately 6 to 10 inches tall. Verbena 'Lavender Shades' plants from California showing leaf mosaic symptoms tested positive for potyvirus in an antigen-coated plate ELISA using our potyvirus broad spectrum reacting PTY-1 monoclonal as the detecting antibody. The virus was transmitted mechanically to Nicotiana benthamiana from infected verbena plants by sap inoculation. Further serological analysis with our panel of Bean yellow mosaic virus (BYMV)-specific, BYMV-subgroup, and potyvirus cross-reactive monoclonal antibodies suggested that the verbena potyvirus might be a member of the BYMV subgroup. Total RNA extractions from infected verbena and tobacco leaves were used in RT-PCR assays with generic potyvirus-specific primers, which amplify highly conserved 700bp or 1600bp fragments from the 3' terminus of most potyviruses. This region includes the potyviral coat protein (CP) and the 3' non-coding region (3'NCR). The PCR amplified fragments were cloned using standard TA cloning procedures and sequenced using dye-terminator chemistry. The cloned nucleotide and putative coat protein amino acid sequences from the infected verbena and tobacco plants were compared to the corresponding regions of other potyviruses. Amino acid comparison of the CP region of the verbena potyvirus showed 95-96% identity to four pea mosaic strains (PMV) of BYMV, 85-89% identity to 20 other strains of BYMV, 74-76% identity with six strains of Clover yellow vein virus (CYVV), and only 50-64% identity with 28 other potyviruses. Additionally, similar pairwise analysis of the 3'NCR of the verbena potyvirus showed 98-99% identity to PMV strains, 81-94% to other BYMVs, 68-75% to CYVV, and 52-64% with other potyviruses. These and other phylogenetic analysis of the CP and 3'NCR sequences of PMV, BYMV, CYVV and other potyviruses confirmed the designation of the verbena potyvirus isolate as a pea mosaic strain of BYMV.

INTRODUCTION

Verbena, the common name for some members of the Verbenaceae, is a family of herbs, shrubs, trees, and vines (often climbing forms) of warmer regions of the world. Well-known wild and cultivated members of the family include species of the shrubby Lantana and of Verbena. Many cultivated verbenas have fragrant blossoms and leaves that are sometimes used as condiments, for distillation of oils or for tea.

Verbena × hybrida is an ornamental annual that offers both upright and trailing habits and is useful as a groundcover or specimen plant used in rock gardens, as an edging plant, and in hanging baskets. It comes in a variety of colors and grows approximately 6 to 10 inches tall. Greenhouse and nursery crops in the U.S. constitute a third of the total farm cash receipts from horticulture crops and were estimated at $14.3 billion in 2003 (USDA, 2004). Viruses infecting ornamentals are a serious threat to the industry as they reduce vigor of infected plants and decrease foliage and flower quality.

Previously documented viruses of verbena include Alfalfa mosaic virus, Apple mosaic virus, Arabis mosaic virus, Broad bean wilt virus, Carnation ringspot virus, Chrysanthemum virus B, Clover yellow mosaic virus, Cucumber mosaic virus, Impatiens mosaic virus, and Bean yellow mosaic virus.
necrotic spot virus, Melon necrotic spot virus, Melilotus mosaic virus, Prunus necrotic ringspot virus, Ribgrass mosaic virus, Tobacco etch virus, Tobacco mosaic virus, Tobacco ringspot virus, Tobacco streak virus, Tomato aspermy virus, Tomato ringspot virus, Tomato spotted wilt virus, and Verbena latent virus (Agdia, 2003; Baker et al., 2004; Breman et al., 1995; Brunt et al., 1996, 1997; Cohen et al., 2003). Chrysanthemum stunt viroid and Iresine viroid have also been detected singly- or doubly-infected in Verbena (Bostan et al., 2004).

Here we report the results of our investigations to determine the identity of a potyvirus detected by our potyviral broad-spectrum monoclonal antibody in various Verbena × hybrida ‘Lavender Shades’ plants showing leaf mosaic symptoms. We have used electron microscopy, serology and reverse transcription polymerase chain reaction (RT-PCR) methods, and cloning and sequence analysis, to identify this potyvirus as a pea mosaic strain of Bean yellow mosaic virus. Preliminary results have been reported (Guaragna et al., 2004).

MATERIALS AND METHODS

Plant Sources, dsRNA Analysis, Electron Microscopy, Inoculations and ELISA

Symptomatic plants of Verbena × hybrida ‘Lavender Shades’ were provided to us by a commercial grower from California. Plant samples were tested for the presence of viruses by electron microscopy and dsRNA analysis as previously described (Jordan and Dodds, 1985; Jordan et al., 2002). Samples were tested in a modified indirect ACP-ELISA using potyvirus-specific and broad-spectrum monoclonal antibodies (Jordan and Hammond, 1991; Jordan, 1992), or purchased polyclonal antisera (Agdia, Inc., Elkhart, IN; DSMZ, Braunschweig, Germany) as previously described (Jordan and Hammond, 1991). Representative samples from symptomatic and asymptomatic Verbena were also sent to Agdia, Inc for DAS-ELISA testing. Samples from symptomatic, PTY 1 positive Verbena leaves containing elongated particles were homogenized in 1% K2HPO4 and the sap extracts were mechanically inoculated to seedlings of Chenopodium quinoa, Cucumis sativus, Cucurbita maxima, Cucurbita moschata, Cucurbita pepo, Helianthus annuus, Hordeum vulgare, Lactuca sativa, Lolium perenne, Lycopersicon esculentum, Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. tabacum, Phaseolus lunatus, Phaseolus vulgaris, Raphanus sativus, Tagetes erecta.

Sample Preparation and RT-PCR

One hundred mg of fresh leaf tissue was ground in liquid nitrogen to a fine powder and total RNAs were isolated using the RNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA) following the manufacturer’s recommendations with minor modifications. The reverse transcription (RT) synthesis and PCR reactions were done using the RETROscript® First Strand Synthesis Kit for RT-PCR (Ambion, Inc., Austin, TX) according to manufacturer’s instructions. cDNA was synthesized (42°C for 1 hr) from ca. 1 ug total RNA using random hexamers or an oligo(dT)17(A/C/G)-3’ primer (PV1) (Pappu et al., 1993).

For amplification of a 335 bp fragment representing a 3’ potyvirus conserved central core (ca. one-third) of the coat protein (CP) gene, 5 ul RT mix was added to 45 ul of a PCR mix [final = 1X PCR Buffer (Ambion), 125 μM each dNTP, 2.5 μM forward primer, 2.5 μM reverse primer, and 2.5 U of SuperTag™ Plus (Ambion)] containing the primers U335 and D335 (Langeveld et al., 1991). The following PCR amplification regime was used: 94°C for 2 min; followed by 35 cycles of 94°C for 1 min, 50°C for 3 min, 72°C for 4 min; and finally 72°C for 10 min and overnight at 4°C. For amplification of a ca. 700 bp fragment representing the 3’ half of the potyviral CP gene and the 3’ non-coding region (3’ NCR), a PCR mix using a PV1-primed RT reaction contained the PV1 and U335 primers and was amplified following the regime above. For amplification of a ca. 1600 bp fragment representing the 3’ terminal portion of potyviral Nb gene, all of the CP gene and 3’ NCR, the PV1-primed RT reaction PCR mix contained the PV1 primer
and a conserved Potyviridae N Ib sequence primer, Poty-S-For (Chen et al., 2001; Gibbs et al., 2003) and followed the amplification regime above, but with an annealing temperature of 47°C for 3 min. DNA amplification products were fractionated by agarose gel electrophoresis, stained in ethidium bromide, and photographed in UV-light.

Cloning of PCR Fragments, Gene Sequencing and Computer Analyses
Specific-sized amplification fragments were concentrated and purified using Amicon Microcon-PCR centrifugal filters (Millipore, Bedford, MA), ligated into either the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA) or the pGEM-T EasyVector (Promega, Madison, WI) and transformed into TOP10 competent cells (Invitrogen) per manufacturer’s instructions. Recombinant plasmids purified from transformants were sequenced using the ABI Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Model 310 Sequencer. Assembled sequences were compared with databases using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). Sequence data was compiled and analyzed using programs at the Biology Workbench 3.2 web site (http://seqtool.sdsc.edu/CGI/BW.cgi) (San Diego Supercomputer Center, UCSD, San Diego, CA). Pairwise comparisons were done using ALIGN (Pearson, 1990) and CLUSTAL W (Thompson et al., 1994). Phylogenetic analyses were done using PHYLIP version 3.5c (Felsenstein, 1993), the phylogenetic trees were constructed by CLUSTALTREE and the resulting trees were displayed using Treeview (Page, 1996).

RESULTS AND DISCUSSION

Visual Symptoms, dsRNA Analysis and Electron Microscopy
Leaves of Verbena × hybrida ‘Lavender Shades’ plants grown in a containment growth chamber are smaller, slightly rugose and usually show a mild to severe mosaic. No dsRNA was detected in any of the Verbena plants using standard procedures. In initial experiments, none of the virus indicator hosts inoculated with sap extracts from symptomatic Verbena plants showed symptoms and all were negative in ACP-ELISA using the potyvirus broad-spectrum monoclonal antibody PTY 1 (data not shown), except for N. benthamiana. Electron microscopy of negatively stained verbena sap preparations contained small numbers of flexuous rod-shaped potyvirus-like particles measuring 700-750 nm in length (data not shown). No other virus-like particles were initially observed. In later host range experiments, verbena sap-inoculated N. benthamiana and C. quinoa plants did show severe mosaic leaf symptoms, apical necrosis and rapid death. These plants were also PTY 1 positive, had flexuous rod-shaped potyvirus-like particles, as well as small 30-32nm isometric particles (data not shown). DsRNA analysis on these plants revealed a complex dsRNA pattern (data not shown).

Serology
All symptomatic Verbena plants initially tested with PTY 1 were positive to this potyvirus broad-spectrum monoclonal antibody. None of the verbena samples or verbena sap-inoculated N. benthamiana and C. quinoa plants tested positive to other viruses in assays conducted by Agdia, Inc. The 15 negative tests were for: Alfalfa mosaic virus, Apple mosaic virus, Arabis mosaic virus, Chrysanthemum virus B, Cucumber mosaic virus, Impatiens necrotic spot virus, Prunus necrotic ringspot virus, Tobacco mosaic virus, Tobacco necrosis virus, Tobacco ringspot virus, Tobacco streak virus, Tomato aspermy virus, Tomato mosaic virus, Tomato ringspot virus, and Tomato spotted wilt virus. Samples were later also tested with antisera to Broad bean wilt viruses (BBWV) 1 and 2. The symptomatic verbena and inoculated N. benthamiana and C. quinoa reacted to BBWV 1 antisera, suggesting a double infection of verbena plants by BBWV 1 and a potyvirus.

In subsequent tests, using our panel of potyvirus-specific and broad-spectrum reacting monoclonal antibodies, the Verbena plants and inoculated N. benthamiana plants reacted positively to several BYMV-subgroup specific monoclonal antibodies (Table 1).
These results suggested that the potyvirus was probably a member of the BYMV subgroup (Hampton et al., 1992; Shukla et al., 1994).

**RT-PCR, Cloning and Sequence Analysis**

To further confirm the identity of the verbena potyvirus, total nucleic acid extracts from *Verbena* plants #48 and #70 and PTY 1 positive *N. benthamiana* plants were used as templates for reverse transcriptase polymerase chain reaction (RT-PCR) with the various potyvirus specific primers described above. The resultant 335, 700 and 1600 bp PCR amplicons were subsequently cloned and sequenced. One unique set of potyviral sequences were found in both *Verbena* and *N. benthamiana*. In all cases the 335 bp segments were 5'-coterminal with and included in the first 335 bp of the sequenced corresponding products from the expected ~700 bp PCR fragments and had 100% identity; in addition, these sequences had 100% identity within the corresponding 1600 bp amplicons.

Diversity of the CP sequences of potyviruses occurs predominately at the N-terminal regions while the C-terminal half is more highly conserved (Shukla et al., 1994). All of the molecular data compiled to date has shown that the sequences of the capsid protein and 3' NCR are the most useful regions of the potyviral genome for taxonomic analysis and that the 3' terminal region may be used as a reliable criterion in distinguishing the strains of a virus from distinct potyviruses (Berger et al., 2000; Shukla et al., 1994).

The cloned nucleotide and putative coat protein amino acid sequences from the infected verbena and tobacco plants were compared to the corresponding regions of other potyviruses (representative examples shown in Table 2). Amino acid comparison of the CP region of the verbena potyvirus showed 95-96% identity to four pea mosaic strains (PMV) of *Bean yellow mosaic virus* (BYMV), 85-89% identity to 20 other strains of BYMV, 74-76% identity with six strains of *Clover yellow vein virus* (CYVV), and only 50-64% identity with 28 other potyviruses (Table 2; not all data shown). Pairwise comparisons among and between the CP sequences of PMV, BYMV, CYVV and other potyviruses revealed identities of 92-99% for the homologous BYMV::BYMV, PMV::PMV, and CYVV::CYVV comparisons; 84-89% for the heterologous BYMV::PMV, 69-78% for BYMV::CYVV and PMV::CYVV comparisons, and 50-64% for all other potyvirus combinations (Table 2; not all data shown). Additionally, similar pairwise analysis of the 3'NCR of the verbena potyvirus showed 98-99% identity to PMV strains, 81-94% to other BYMVs, 68-75% to CYVVs, and 52-64% with other potyviruses (Table 2; not all data shown). Other 3'NCR pairwise comparisons generally revealed the same identity trend as described for the CP.

Phylogenetic analysis was performed to further clarify the relationship of the verbena potyvirus with 33 other BYMV subgroup members based on the coat protein amino acid sequence by using CLUSTAL W and PHYLIP and viewed in Treeview. The data set was subjected to 1000 bootstrap replicates. All nodes supported by >50% confidence values are shown (Fig. 2). It shows that the verbena potyvirus clusters with the pea mosaic strains of BYMV which branches out from those clades containing the other BYMV strains or CYVV isolates. These results together suggest that the verbena potyvirus is a pea mosaic strain of BYMV.

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**Literature Cited**


Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and


Tables

Table 1. Summary of ELISA results of infected Verbena samples using selected Bean yellow mosaic virus-specific, bean yellow mosaic virus subgroup-reactive and broad spectrum reacting potyvirus monoclonal antibodies in an antigen-coated plate assay.

<table>
<thead>
<tr>
<th>Potyvirus sample</th>
<th>PTY Monoclonal Antibody 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Verbena</td>
<td>-2</td>
</tr>
<tr>
<td>Verbena #48</td>
<td>+++</td>
</tr>
<tr>
<td>Verbena #70</td>
<td>+++</td>
</tr>
<tr>
<td>BYMV-PMV 204-1</td>
<td>+++</td>
</tr>
<tr>
<td>BYMV-GDD</td>
<td>+++</td>
</tr>
<tr>
<td>CYVV</td>
<td>+++</td>
</tr>
<tr>
<td>TVMV</td>
<td>+++</td>
</tr>
</tbody>
</table>

1 PTY 1 reacts with "most all" aphid-transmitted potyviruses; PTY 13 detects 9/9 BYMV, 2/2 BYMV-PMV, CYVV, TVMV; PTY 14 detects 9/9 BYMV, 2/2 BYMV-PMV, CYVV; PTY 17 detects 9/9 BYMV, BYMV-PMV 204-1, CYVV; PTY 21 detects 8/9 BYMV only (not BYMV-PMV, CYVV); PTY 24 detects 8/9 BYMV only (not BYMV-PMV, CYVV); PTY 43 only detects BYMV-GDD (Jordan and Hammond, 1991).

ELISA OD405 results: -- = <0.03; + = 0.1 - 0.8; ++ = 0.9 - 1.5; +++ = >1.5

Table 2. Percent identities of coat protein amino acid (above diagonal) and 3'NCR nucleotide (below diagonal) sequences in pairwise comparisons of the Verbena potyvirus with selected strains of Bean yellow mosaic virus (BYMV), including pea mosaic virus, and Clover yellow vein virus (CYVV) 1.

<table>
<thead>
<tr>
<th>BYMV</th>
<th>BYMV</th>
<th>Verbena</th>
<th>BYMV</th>
<th>BYMV</th>
<th>CYVV</th>
<th>CYVV</th>
</tr>
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<tbody>
<tr>
<td>-D</td>
<td>94</td>
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<td>87</td>
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<td>75</td>
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<td>-GDD</td>
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<td>Verbena potyvirus</td>
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<td>---</td>
<td>96</td>
<td>95</td>
<td>77</td>
</tr>
<tr>
<td>BYMV-CS</td>
<td>87</td>
<td>88</td>
<td>98</td>
<td>---</td>
<td>97</td>
<td>77</td>
</tr>
<tr>
<td>BYMV-PMV</td>
<td>88</td>
<td>87</td>
<td>99</td>
<td>99</td>
<td>---</td>
<td>75</td>
</tr>
<tr>
<td>CYVV-30</td>
<td>76</td>
<td>76</td>
<td>74</td>
<td>75</td>
<td>74</td>
<td>---</td>
</tr>
<tr>
<td>CYVV-P180</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>99</td>
<td>---</td>
</tr>
</tbody>
</table>

1 GeneBank sequence accession numbers are noted in Figure 1.
2 --- = 100% homologous identity; Identities greater than 90% are in bold.
Fig. 1. Phylogenetic analyses of Bean yellow mosaic virus subgroup coat protein amino acid sequences using CLUSTAL W and PHYLIP and viewed in Treeview. The data set was subjected to 1000 bootstrap replicates. All nodes supported by >50% confidence values are shown. GenBank sequence sources are listed by each virus.