Evidence of Mixed Virus Infections Causing Severe Symptoms and Decline of Blackberries

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Abstract

Blackberry yellow vein disease has emerged in blackberries in the last five years in the southern and southeastern United States causing significant losses and in some cases, plant death. Blackberry yellow vein associated virus (BYVaV) has been associated with the disease but since asymptomatic plants infected with the BYVaV have been discovered it was necessary to investigate what caused the observed symptomatology associated with the disease. The complete nucleotide sequence of an isolate from a symptomatic plant has been obtained in addition to partial sequence of three isolates from plants showing different degrees of disease severity. No significant sequence variability was observed and the possibility of additional agents involved in disease development was examined using laboratory techniques and field observations. Three additional viruses have been isolated from symptomatic plants. Detection tests for the novel viruses were developed. The most common of the novel viruses was a potyvirus, designated as Blackberry virus Y, a virus distantly related to members of the genus Potyvirus that is lacking key motifs involved in aphid transmissibility. None of the three viruses were found consistently in symptomatic plants, leaving BYVaV the only consistent component of the disease.

INTRODUCTION

Blackberry yellow vein disease is a significant disease that has emerged recently in the southern and southeastern United States and Blackberry yellow vein associated virus (BYVaV) was found associated with the disease (Martin et al., 2004). The latent infection of some blackberry cultivars infected with the virus prompted us to investigate if sequence variability or other agents are involved in blackberry yellow vein disease development. The complete nucleotide sequence of a South Carolina isolate of BYVaV was obtained in addition to the partial sequence of three Arkansas isolates from plants showing different degrees of disease severity. The possibility that additional agents were involved in blackberry yellow vein disease was examined using electron microscopy and molecular methods. This paper reports the finding of three viruses in plants showing blackberry yellow vein disease in addition to BYVaV. Detection protocols were developed for these viruses. In addition, field experiments using asymptomatic plants infected with BYVaV, showed that at least one of the new viruses can induce blackberry yellow vein symptoms in these plants. None of the three viruses was found consistently in diseased plants, leaving BYVaV as the only consistent component of blackberry yellow vein disease thus far.

MATERIALS AND METHODS

The sequences of four BYVaV isolates and three novel viruses were obtained
essentially as described elsewhere (Tzanetakis et al., 2006) from plants obtained from Arkansas, South Carolina and Oregon. Detection of all viruses were performed as described previously (Martin et al., 2004) using the primers listed in Table I. Plants ('Chickasaw') used in the disease development assessment were obtained from a nursery and were tested for the presence for BYVaV, Tomato ringspot, Tobacco ringspot, Strawberry necrotic shock and Raspberry bushy dwarf viruses using ELISA (Martin et al., 2004).

For electron microscopy purposes, small leaf pieces (1–2 mm²) were placed in modified Karnovsky’s fixative (Karnovsky, 1965) for 2 h at room temperature under vacuum. The tissues were postfixed in 1% OsO₄ for 2 h then prestained in 0.5% aqueous uranyl acetate at 4°C. The tissues were dehydrated in an ethanol series, embedded in Spurr’s medium and sectioned. Sections were double-stained in 2% aqueous uranyl acetate for 5 min and lead citrate for 2 min before examination using a JEOL 100 CX electron microscope.

Phylogenetic analyses of the novel viruses were performed with ClustalW (Thompson et al., 1994) using the neighbor-joining method and Kimura's correction. The bootstrap analysis consisted of 1000 pseudoreplications and the trees were visualized with TreeView (Page, 1996).

RESULTS

The complete nucleotide sequence of an isolate of BYVaV from an ‘Apache’ plant from South Carolina (Genbank accession No AY776334 and AY776335) was obtained (Tzanetakis et al., 2006). RNA 1 and RNA 2 consist of 7801 and 7917 nucleotides, respectively. The genome organization is similar to that of Beet pseudo yellows virus and Strawberry pallidosis associated virus, two criniviruses closely related to BYVaV (Martin et al., 2004), except for an additional putative peptide near the 5’ terminus of RNA 2. Three kilobases of RNA 2 of BYVaV, including the heat shock protein 70 homolog and the major coat protein of the virus, from three Arkansas isolates (‘Navaho’ and ‘Chickasaw’, Genbank accession No AY873918-23) were also sequenced. These isolates showed different degrees of disease severity from dramatic yellow vein banding to asymptomatic. Sequence comparisons of these isolates with the ‘Apache’ isolate disclosed that sequence variability was minimal exceeding 96% identity in the nucleotide level. The three Arkansas isolates had 99% nucleotide sequence identity.

Electron microscopy indicated that some symptomatic plants have inclusion bodies indicative of potyvirus infection (Fig. 1). Cloning from dsRNA from plants with inclusion bodies and others that were not infected with other known viruses other than BYVaV revealed the presence of additional viruses. A member of the family Flexiviridae was the first to be identified and was designated as Blackberry virus X (BVX; Genbank accession No DQ378057). The second was a member of the Potyviridae, named Blackberry virus Y (BVY; Genbank accession No AY994084), while a third virus with similarities to insect infecting picorna-like viruses was identified almost simultaneously in raspberry (Martin and Tzanetakis, in press) and blackberry and was named Blackberry virus Z (BVZ; Genbank accession No DQ378058).

BVX does not share significant similarities with members of the any genera of the Flexiviridae and at this point should be considered an unassigned member of the family. BLAST searches disclosed that BVY is distantly related to members of the genus Potyvirus, the aphid-borne members of the family Potyviridae. Sequence analysis indicated that the virus is lacking motifs that are involved in virus-vector association (Urcuqui et al., 2001). Phylogenetic analysis showed that the virus is not clustering with any of the existing genera of the family. BVZ properties are analyzed in detail in another presentation of this meeting (Martin and Tzanetakis, in press).

The involvement of the new viruses in disease development was tested using asymptomatic BYVaV infected ‘Chickasaw’ plants. Ten of the 150 plants used in this study developed symptoms (Fig. 2). Testing for the novel viruses in these plants revealed that all ten were infected with BVY. Further testing of several symptomatic plants from
North and South Carolina and Arkansas failed to identify the virus in the majority of these plants.

**DISCUSSION**

Several viruses infect blackberry causing significant losses (Converse, 1987; Strik and Martin 2004). Blackberry yellow vein is a disease with symptoms similar to those attributed to Tobacco ringspot virus, which may be the reason that it was not studied extensively until recently (Martin et al., 2004). While BYVaV was found associated with the disease, the presence of the virus in plants that appear asymptomatic prompted us to investigate the basis of disease symptoms. Sequence analysis of four isolates for Arkansas, North and South Carolina did not reveal variability that could explain the symptom diversity and therefore the involvement of other agents involved in symptom development was examined.

The identification of novel viruses in diseased blackberries revealed that unknown viruses may be involved in the disease. Field experiments showed that one of the novel viruses, BVY, is involved in symptom development but it is not the causal agent, as it was not identified in all symptomatic plants. Attempts to transmit BYVaV with whiteflies and BVY with aphids were unsuccessful (Susaimuthu, unpublished data). The latter comes as further evidence of the uniqueness of BYV, as foreseen by sequence and phylogenetic analyses. Blackberry yellow vein disease causes significant losses where present and our results indicate that it is probably caused by co-infection of at least two viruses. The only consistent component thus far is BYVaV and it is highly probable that the three new viruses presented here operate synergistically in disease development. A recent study on blackberry viruses in southeastern United States (Guzman-Baeny, 2003) has confirmed the high incidence of four other viruses in the crop. The high incidence of many previously unidentified viruses in Rubus (this study; Martin and Tzanetakis, in press; Halgren et al., in press; Guzman-Baeny, 2003; Tzanetakis and Martin, 2004) makes assessment of disease development in Rubus crops a highly demanding task. Unless pure cultures of the viruses are obtained, it cannot be stated that any of these viruses are the causal agent of the variety of symptoms that have emerged in the last decade in Rubus plantations in the United States.

**ACKNOWLEDGMENTS**

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**Literature Cited**


**Tables**

Table 1. List of primers used for RT-PCR detection of Blackberry yellow vein associated virus (BYVaV), Blackberry virus X (BVX), Blackberry virus Y (BVY) and Blackberry virus Z (BVZ).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BYVaV F</td>
<td>AATCAACGGGAGAATGTTAT</td>
<td></td>
</tr>
<tr>
<td>BYVaV R</td>
<td>GGATTTGGCAACGTCCG</td>
<td>458bp</td>
</tr>
<tr>
<td>BVX F</td>
<td>CACCTAGCAGCCTTGCA</td>
<td>511 bp</td>
</tr>
<tr>
<td>BVX R</td>
<td>TGGTTTGACCACGGGAT</td>
<td></td>
</tr>
<tr>
<td>BVY F</td>
<td>TCGTTGAGGGGACCAGT</td>
<td></td>
</tr>
<tr>
<td>BVY R</td>
<td>CTCGCTCTCCCATTC</td>
<td>357 bp</td>
</tr>
<tr>
<td>BVZ F</td>
<td>GCCATCCTAACAGGCA</td>
<td></td>
</tr>
<tr>
<td>BVZ R</td>
<td>CTCGCTGACGGGTGCA</td>
<td>452 bp</td>
</tr>
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</table>
Fig. 1. Cytopathology of ‘Chickasaw’ blackberry attributed to *Blackberry virus Y* (BVY). Bar represents 250 nm; PW- pin wheel, LA- laminated aggregates.

Fig. 2. Symptoms development after infection of an asymptomatic ‘Chickasaw’ infected with *Blackberry yellow vein associated virus* with *Blackberry virus Y* (BVY). A. Plant before field exposure, free of BVY; B. ‘Chickasw’ plant after a field exposure and infected with BVY.