

## Yellow vein-affected blackberries and the presence of a novel *Crinivirus*

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During the last 5 years, blackberry plants in Arkansas and North and South Carolina exhibited virus-like symptoms of vein yellowing and mosaic, followed in some cases by death. Diagnostic tests for known blackberry viruses failed to identify a causal agent. Double-stranded RNA was extracted from affected plants and cloned. A new member of the *Closteroviridae* was identified and designated Blackberry yellow vein associated virus (BYVaV). Molecular and immunological assays have been developed for BYVaV, and examination of plants with symptoms revealed a close association of disease symptoms with the presence of BYVaV, although the virus was also found in symptomless plants. Molecular characterization of isolates from plants exhibiting different degrees of disease severity indicated that sequence diversity is probably not the cause of the observed phenotypic differences.

**Keywords:** *Closteroviridae*, *Crinivirus*, *Rubus*, yellow vein disease

### Introduction

Blackberry (*Rubus* spp.) production area in the United States exceeds 10 000 acres with an estimated production value of over US\$30 million (Anonymous, 2004). Much of the production is threatened by several virus and virus-like agents that infect *Rubus* spp. (Converse, 1987), some of which can cause significant losses of over 50% (Halgren *et al.*, 2003; Strik & Martin, 2003). A disease with symptoms similar to those associated with *Tobacco ringspot virus* in blackberry (Stace-Smith, 1987) has emerged in the south and southeastern USA in the last 5 years. Symptoms include leaf distortion, vein-banding, mottling and, in several cases, plant death. The disease was observed to spread rapidly, causing significant losses in the affected areas. Immunological tests and mechanical inoculations to a range of herbaceous hosts failed to identify any virus consistently associated with the observed symptoms. Double-stranded RNA (dsRNA) extractions disclosed the presence of high MW bands. Cloning and sequence analysis revealed the presence of an undescribed virus, related to criniviruses, designated as Blackberry yellow vein associated virus (BYVaV) (Martin *et al.*, 2004).

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Criniviruses form one of the three genera of the *Closteroviridae*, the family with the largest positive-strand RNA plant viruses, ranging from 15 to 20 kb (Karasev, 2000; Martelli *et al.*, 2002). Members of the genus are transmitted by whiteflies in a semipersistent manner. Criniviruses have become an emerging problem in world agriculture due to expanding ranges of the whitefly vectors into new areas (Wisler *et al.*, 1998; Wintermantel, 2004) and the movement of plant material between countries and continents.

This communication presents the nucleotide sequence of two genomic regions of the virus and compares them with those of other closteroviruses. Sequence data from three isolates of the virus that showed different degrees of symptom severity were acquired to evaluate the role of diversity in symptomatology. Molecular and immunological tests for BYVaV were developed and used to determine the presence of the virus in blackberry-producing areas exhibiting yellow vein disease symptoms and BYVaV association with the observed symptoms.

### Materials and methods

#### Plant material

Three Arkansas-BYVaV isolates from plants of cvs Navajo and Chickasaw grown at the Horticultural Crops Research Laboratory (HCRL), Corvallis, OR, and which exhibited different degrees of disease severity were

partially sequenced to investigate whether virus sequence diversity is associated with different symptomatology. At least 30 plants exhibiting symptoms from North and South Carolina (cvs Chester, Kiowa, Navajo, Apache, Arapaho and Chickasaw) and 10 from Arkansas (cvs Chester, Apache and Chickasaw) were assayed for the presence of the virus. In addition to these affected plants, more than 50 symptomless plants of cvs Chester, Apache, Chickasaw and Navajo from nurseries and breeding parental populations were also assayed for BYVaV.

### Mechanical transmission

Ten plants of each of the following nine species, *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Nicotiana benthamiana*, *N. clevelandii*, *N. occidentalis*, *N. tabacum* cv. 'samsun', *Phaseolus vulgaris* and *Tetragonia tetragonioides*, were inoculated with BYVaV infected leaf tissue in a 1:20 ratio (w/v) in 0.05x phosphate-buffered saline (PBS), pH 7.4, with 2% nicotine (v/v). Carborundum (600 mesh) was sprinkled on the leaf surface to assist transmission of the virus to indicator plants.

### Electron microscopy

Small leaf pieces (1–2 mm<sup>2</sup>) from yellow vein-affected blackberry leaves were placed in modified Karnovsky's fixative (Karnovsky, 1965) for 2 h at room temperature (–20°C) under vacuum. The tissues were postfixed in 1% osmium tetroxide 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.

### DsRNA extraction and cloning

DsRNA was extracted from 20 g of leaf tissue from 10 BYVaV-infected plants (five with and three without symptoms from Arkansas, two symptomless plants from South Carolina) and two virus-free plants from the HCRL, as described previously (Martin *et al.*, 2004). Complementary DNA synthesis was performed according to the methods of Jelkmann *et al.* (1989) or Tzanetakis *et al.* (2005a) using dsRNA extracted from 4 g of tissue (Tzanetakis & Martin, 2005) as template. The cDNA was cloned into the plasmid vector TOPO pCR 4.0 vector (Invitrogen) as described (Tzanetakis *et al.*, 2005a). The recombinant plasmids were screened using the polymerase chain reaction (PCR) with the M13 forward and reverse primers and *Taq* polymerase (New England Biolabs). The PCR programme consisted of 8 min denaturation at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 3 min at 75°C, concluding with 10 min extension at 75°C. Sequencing of the recombinant plasmids was performed by MacroGen Inc. using an ABI3730 XL automatic DNA sequencer.

### Sequence analysis

The sequence data obtained from shotgun cloning were compared against sequences found in GenBank using BLAST (Altschul *et al.*, 1997) and BYVaV-specific clones were utilized to design oligonucleotide primers (Table 1) for reverse transcription-PCR (RT-PCR) amplification of BYVaV genomic regions as described elsewhere (Tzanetakis *et al.*, 2005b). PCR products were cloned into pCR 4.0 (Invitrogen). Sequence was assembled with CAP3 (Huang & Madan, 1999) using at least four individual clones or the PCR products for a minimum of a fourfold coverage. The GenBank accession numbers are AY873918–873920 for region A and AY873921–873923 for region B of RNA 2 of the Chickasaw and Navajo isolates, respectively. The putative transmembrane domains of proteins were predicted with the TMHMM software (Krogh *et al.*, 2001). The similarity between the heat shock protein 70 homologues (HSP70h) and the major coat proteins (CP) of closteroviruses was calculated using MatGat (Campanella *et al.*, 2003).

### Detection

Both molecular and immunological tests were prepared for detection of BYVaV. For RT-PCR detection, total RNA was extracted according to a modified Spiegel & Martin method (1993). Briefly, 100 mg of leaf tissue was ground in 1 mL of extraction buffer containing 200 mM Tris pH 8.5, 1.5% lithium dodecyl sulphate, 300 mM lithium chloride, 10 mM EDTA, 1% deoxycholic acid, 1% Nonidet P40 (NP-40) and 1% mercaptoethanol. The extract (750 µL) and an equal amount of 6 M potassium acetate (2.8 M potassium, 6 M acetate), pH 6.5, were centrifuged at 16 000 g for 10 min. The supernatant was collected, chilled at –20°C for 30 min before centrifugation at 16 000 g for another 10 min. An equal amount of isopropanol and supernatant (750 µL) were mixed by inversion and centrifuged for 20 min at 16 000 g. The pellet was washed twice with 70% ethanol and dried briefly with a speed-vac (Savant Integrated Speed Vac System ISS110). First-strand cDNA synthesis, using the extracted RNA at a maximum volume of

**Table 1** List of oligonucleotide primers used for reverse transcription-polymerase chain reaction detection and sequencing of the two regions of RNA 2 of Blackberry yellow vein associated virus (BYVaV)

| Primer name               | Nucleotide sequence (5'–3')       |
|---------------------------|-----------------------------------|
| <i>Detection primers</i>  |                                   |
| BYVaV CP F                | AATCAACGGGAGAATGTTAT              |
| BYVaV CP R                | GGATTGGCAACGTCCG                  |
| <i>Sequencing primers</i> |                                   |
| RNA 2 beg F               | CTTTAGTCTGTCTCCTAGTTTCCTC         |
| RNA 2 mid R               | CCTATACAGAGTCGATAGTCTGACGGCAC     |
| HSP int F                 | CATTGGGTTTCTCACTTCGCAGGATCATA     |
| CP F                      | AGACGAAATAGAGTGTGAGCAATGGATATTACG |
| CP R                      | CTAGATCTCTAGATTCTATACTAGAGTCCG    |

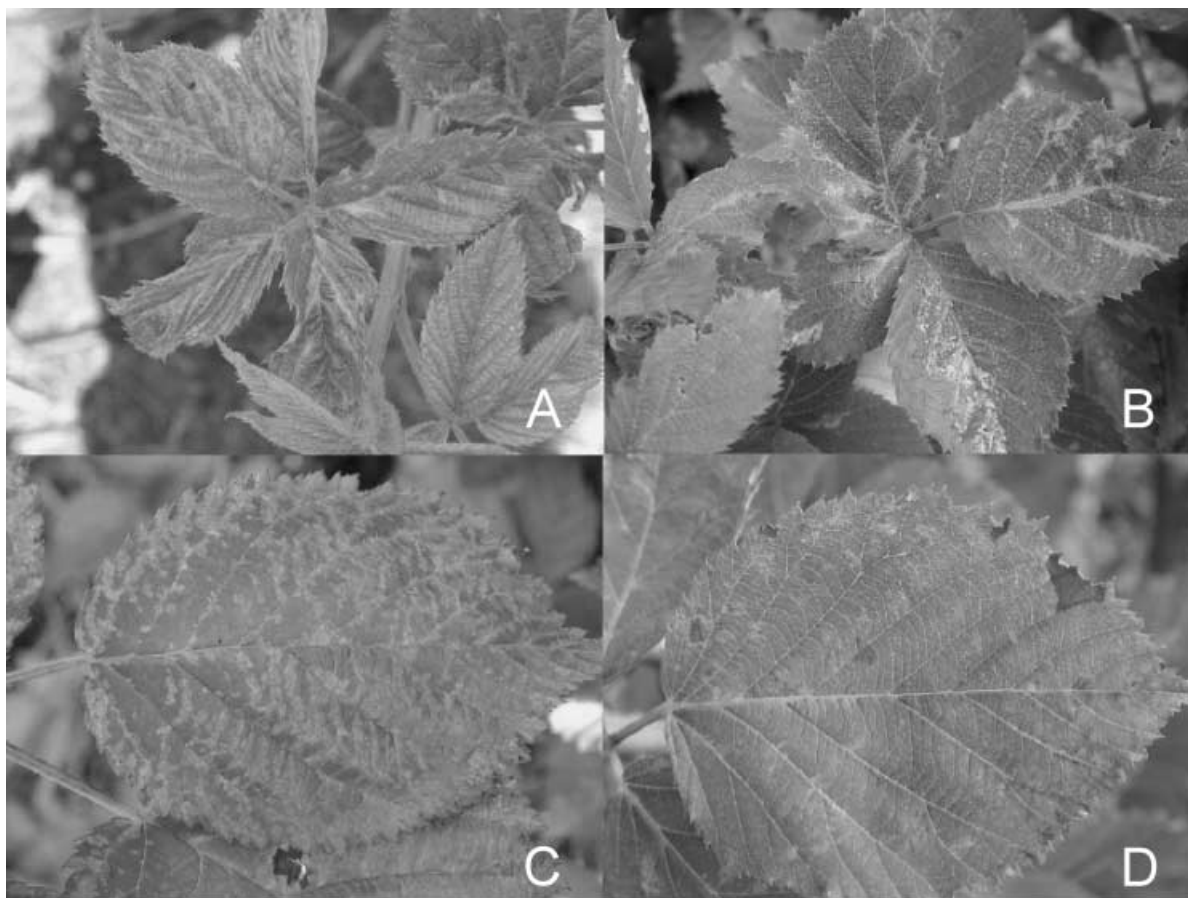
10% of the RT reaction, with Superscript III® reverse transcriptase (Invitrogen), was performed according to the manufacturer's instructions. The reaction was heat-inactivated for 10 min at 95°C and digested with RNase H (Invitrogen). Primers BYVaV CPF and BYVaV CPR that amplify a 458-base fragment of the CP were used for routine detection of BYVaV. Platinum Taq (Invitrogen) was utilized in the PCR reactions according to the manufacturer's instructions. The cDNA template from the RT reaction made up 4% of the final reaction volume because inhibition of the PCR was observed when higher amounts were used. The PCR programme consisted of 5 min denaturation at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 20 s at 68°C. The programme concluded with an extension step of 10 min at 68°C.

Antibodies were developed against the synthesized peptide SDGHLAAKHGTTSQFWGATSDFTNG (Genemed Synthesis Inc), which shares more than 90% amino acid identity between the Strawberry pallidosis associated virus (SPaV), *Beet pseudo yellows virus* (BPYV) and BYVaV CP genes. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed as described elsewhere (Converse & Martin, 1990).

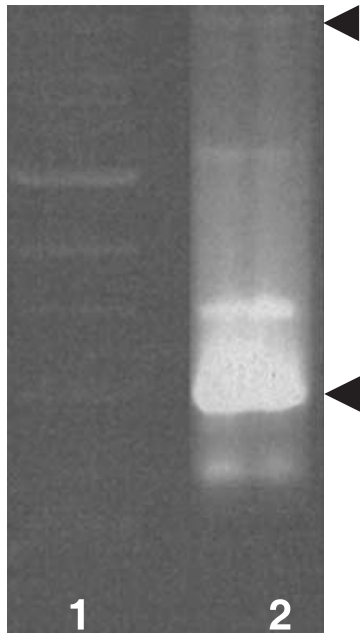
## Results

### Virus identification

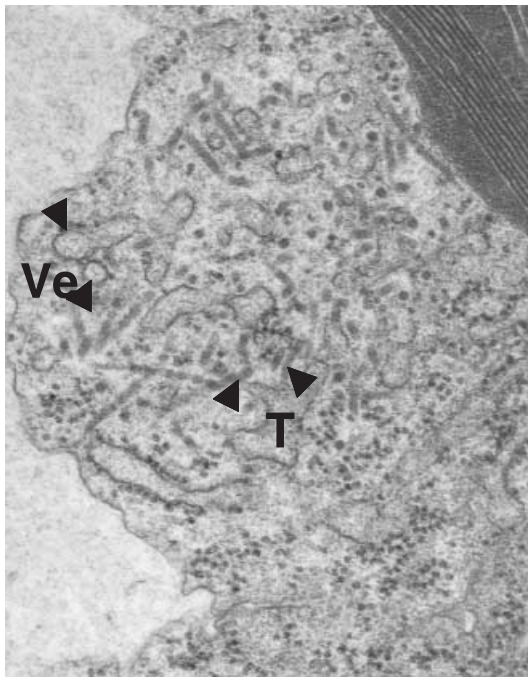
The size and number of dsRNA molecules extracted from affected plants showing yellow vein-banding, mosaic and mottling (Fig. 1) indicated that a crinivirus may be associated with disease symptoms. The amount of dsRNA extracted from blackberry leaf tissue is generally low (IET, personal observation), however, all 10 infected plants assayed yielded dsRNA bands of ~8 kb and 800 bases (Fig. 2), similar to those observed with SPaV (Tzanetakis *et al.*, 2004), while the virus-free plants did not yield any bands (data not shown). Additionally, the cytopathology of plants exhibiting symptoms observed under the electron microscope was similar to that caused by other criniviruses (Medina *et al.*, 2003). Phloem parenchyma cells contained vesicles (80–90 nm diameter) and tubules (45 nm diameter) (Fig. 3) which were not observed in healthy leaf mesophyll cells (data not shown). Ten plants from each of the eight species used for mechanical inoculations were tested by RT-PCR 1 month after the transmission efforts. None of these plants tested positive for BYVaV, similar to the results obtained with all



**Figure 1** Typical symptoms associated with yellow vein-affected blackberries. (A) Distortion and chlorosis of new growth in cv. Apache; (B) yellow vein banding in cv. Arapaho; (C) mottling in cv. Navajo; and (D) leaf necrosis in cv. Arapaho.



**Figure 2** Double-stranded RNA (dsRNA) extracted from blackberry leaf tissue showing yellowing symptoms infected with Blackberry yellow vein associated virus (BYVaV). Lane 1, 1 kb marker (New England Biolabs); lane 2, dsRNA extracted from BYVaV-infected blackberries cv. Apache. Arrowheads indicate bands of 8kb and 800 bases present in all plants tested positive for BYVaV.



**Figure 3** Cytopathology of blackberries cv. Chickasaw attributed to Blackberry yellow vein associated virus. Arrowheads indicate vesicles (Ve) and tubules (T).

previous attempts to transmit criniviruses mechanically (Martelli *et al.*, 2002).

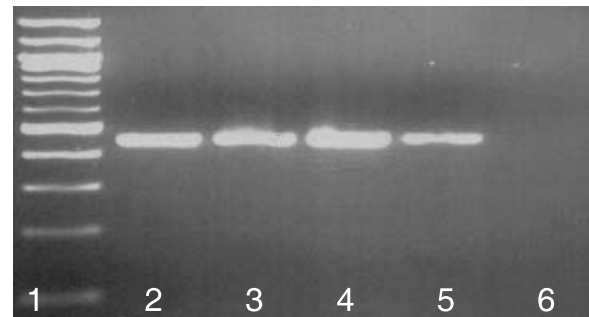
### Sequence analysis

Three kilobases of RNA 2 of BYVaV were sequenced. Region A contained three open reading frames (ORFs): a small hydrophobic protein with a transmembrane domain (Krogh *et al.*, 2001); a HSP70h, the hallmark of viruses in the *Closteroviridae*; and a small ORF similar to those found in other criniviruses in the same position. Region B contained two ORF: a 9 kDa peptide similar to those encoded by all sequenced criniviruses upstream of the coat protein (CP) ORF; and the CP of the virus. The nucleotide sequence identity among the three isolates exceeded 99%.

The HSP70h and CP ORF were compared with orthologous genes of other closteroviruses (Table 2), revealing a close relationship between BYVaV and BPYV, the latter being another crinivirus that was recently identified in blackberry (Tzanetakis & Martin, 2004a).

### Detection

All plants with symptoms tested positive for BYVaV when tested by RT-PCR (Fig. 4). Of the more than 50 symptomless plants, 30 were cv. Chickasaw and eight cv. Navajo. All cv. Chickasaw plants tested positive for the virus, while four of the Navajo plants gave amplicons. More than 20 amplicons from both plants with and without symptoms obtained in both laboratories were sequenced and were all BYVaV-specific. One of the symptomless plants was the source of the Arkansas Navajo isolate that was studied further at the molecular level to determine if sequence variability may be involved in different expression of disease symptoms. Difficulties in the routine extraction of criniviruses from small fruit crops (IET, personal observation) led to the development of an immunological test using antibodies directed against a synthetic peptide corresponding to part of the CP. The antibodies were used successfully in DAS-ELISA, but sensitivity was lower than RT-PCR and there was a strong



**Figure 4** Reverse transcription-polymerase chain reaction detection of Blackberry yellow vein associated virus (BYVaV). Lane 1, 100 base pairs marker (New England Biolabs); lanes 2–5, blackberry cvs Apache and Chickasaw exhibiting yellow vein banding symptoms infected with BYVaV; lane 6, virus-free blackberry cv. Chickasaw.

**Table 2** Amino acid sequence identity and similarity (in parentheses) of the heat shock protein 70 homologue (HSP70h) and major coat protein (CP) of Blackberry yellow vein associated virus and other viruses of the *Closteroviridae*. Abbreviations and GenBank accession numbers (HSP70h, CP): AbYV, *Abutilon yellows virus* (N/A, AAR00224); BPYV, *Beet pseudo-yellow virus* (AAQ97386, NP940792); BYSV, *Beet yellow stunt virus* (AAC55662, AAC55665); BYV, *Beet yellows virus* (NP041872, NP041875); BYVaV, Blackberry yellow vein associated virus (AAW67738, AAW67741); CTV, *Citrus tristeza virus* (NP042864, NP042867); CYSDV, *Cucurbit yellow stunting disorder virus* (NP851572, NP851576); GLRaV 1, *Grapevine leafroll associated virus-1* (AAF22740, AAF22742); GLRaV 2, *Grapevine leafroll associated virus-2* (AAR21242, AAC40861); GLRaV 3, *Grapevine leafroll associated virus-3* (NP813799, NP813801); GRSLaV, *Grapevine rootstock stem lesion associated virus* (NP835247, NP835250); LIYV, *Lettuce infectious yellows virus* (NP619695, NP619697); LChV 1, *Little cherry virus-1* (NP045004, NP045006); LChV 2, *Little cherry virus-2* (AF531505, AAM96228); MV 1, *Mint virus 1* (AAW32895, AAV58835); MVBaV, *Mint vein banding associated virus* (AAS57941, N/A); PMWaV 1, *Pineapple mealybug wilt-associated virus-1* (AAL66711, AAL66713); PMWaV 2, *Pineapple mealybug wilt-associated virus-2* (AAG13941, AF283103); PYV, *Potato yellow vein virus* (CAD89682, CAD89686); SPaV, *Strawberry pallidosis associated virus* (AAO92347, AAO92342); SPCSV, *Sweet potato chlorotic stunt virus* (NP689401, NP689404); ToCV, *Tomato chlorosis virus* (AF024630, AAR15080)

|                      | HSP70h    | CP        |
|----------------------|-----------|-----------|
| <i>Crinivirus</i>    |           |           |
| AbYV                 | N/A       | 40 (61)   |
| BPYV                 | 73 (86)   | 50 (68)   |
| BYVaV                | 100 (100) | 100 (100) |
| CYSDV                | 70 (84)   | 43 (62)   |
| LIYV                 | 49 (70)   | 24 (43)   |
| PYV                  | 68 (82)   | 47 (66)   |
| SPaV                 | 70 (84)   | 46 (63)   |
| SPCSV                | 64 (82)   | 26 (51)   |
| ToCV                 | 59 (76)   | 30 (50)   |
| <i>Closterovirus</i> |           |           |
| BYV                  | 26 (46)   | 16 (30)   |
| BSV                  | 28 (47)   | 20 (33)   |
| CTV                  | 27 (46)   | 17 (35)   |
| GLRaV 2              | 28 (49)   | 19 (33)   |
| GRSLaV               | 27 (48)   | 18 (33)   |
| MV 1                 | 27 (46)   | 17 (32)   |
| <i>Ampelovirus</i>   |           |           |
| GLRaV 1              | 27 (47)   | 21 (38)   |
| GLRaV 3              | 28 (49)   | 18 (36)   |
| LChV 2               | 28 (49)   | 16 (32)   |
| PMWaV 1              | 26 (45)   | 17 (39)   |
| PMWaV 2              | 28 (49)   | 21 (40)   |
| <i>Unassigned</i>    |           |           |
| LChV 1               | 26 (41)   | 18 (30)   |
| MVBaV                | 28 (51)   | N/A       |

background reaction, so the test was not used for routine detection of BYVaV.

## Discussion

A new virus, BYVaV, associated with yellowing and decline symptoms in blackberry has been identified in the southeastern United States. Sequence analysis of the

HSP70h and CP encoding genomic regions of BYVaV places the virus within the *Crinivirus* genus. Disease symptoms usually develop 6–8 weeks after bud break under glasshouse conditions, with symptom intensity changing during the growing period.

While the virus was found closely associated with yellow vein disease, several symptomless plants were infected with BYVaV. The majority of these plants were plants of cv. Chickasaw that originated from a single nursery. These plants were probably mass propagated from a single or very few plants that were infected with the virus. The Navajo plants came from different sources, with the four positives originating from a single blackberry breeding parental population. These plants were monitored for three growing seasons and symptoms did not develop under glasshouse conditions. DsRNA extractions and sequencing of two regions of RNA 2 of one Navajo isolate (GenBank accession numbers AY873920 and AY873923) revealed no significant sequence differences compared with the two isolates from plants with symptoms. While there are reports associating low levels of virus replication with lack of symptoms (Fraser, 1990), dsRNA extractions and RT-PCR tests failed to suggest significant differences between plants with and without symptoms.

Closteroviruses exhibit considerable variability (D'Urso *et al.*, 2003; IsHak *et al.*, 2003; Sambade *et al.*, 2003; Theilmann *et al.*, 2004) and appear to be able to maintain indel mutations as shown for *Beet pseudo yellows virus* (Tzanetakis & Martin, 2004b). The lack of diversity of the BYVaV sequenced regions, also observed in a few other members of the family (Offei *et al.*, 2004; Marco & Aranda, 2005; Turturo *et al.*, 2005), may be characteristic of this virus or it may be due to the relatively recent emergence of the virus in the USA. The possibility that a region or gene of BYVaV outside the area sequenced is highly variable or unique between isolates needs to be examined. This will require sequencing of the entire genomes of isolates from plants with and without symptoms.

The hypothesis that blackberry yellow vein symptoms are caused by a virus complex was investigated by shotgun cloning of dsRNA extracted from several affected plants that tested negative for any of the known *Rubus* viruses other than BYVaV. Sequence information revealed that, in addition to BYVaV, there were at least three other unknown viruses present in this pooled dsRNA (Susaimuthu *et al.*, 2005; IET & RRM, unpublished data). None of these three agents, however, was associated consistently with the yellow vein symptoms observed in blackberry. It is possible that development of yellow vein disease requires a mixed infection of BYVaV plus at least one of these other viruses.

The detection of BYVaV based on RT-PCR and DAS-ELISA tests will be useful for identification of infected plants both with and without symptoms. This is especially important in the case of the latter, to prevent the introduction of the virus into new areas. It is highly probable that BYVaV has been propagated in nursery material and introduced into commercial plantations and where yellow

vein-affected plants have emerged due to environmental conditions or coinfection with other viruses. Efforts are currently under way to identify a possible vector for BYVaV.

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