

## Nucleotide sequence of Blackberry yellow vein associated virus, a novel member of the *Closteroviridae*<sup>☆</sup>

Ioannis E. Tzanetakis<sup>a,\*</sup>, James Susaimuthu<sup>b</sup>, Rose C. Gergerich<sup>b</sup>, Robert R. Martin<sup>a,c</sup>

<sup>a</sup> Department of Botany and Plant Pathology & Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331, USA

<sup>b</sup> Department of Plant Pathology, University of Arkansas, Fayetteville 72701, USA

<sup>c</sup> USDA-ARS, Horticultural Crops Research Laboratory, Corvallis, OR 97331, USA

Received 6 July 2005; received in revised form 10 October 2005; accepted 10 October 2005

Available online 5 December 2005

### Abstract

The complete nucleotide sequence of a novel member of the genus *Crinivirus* (family *Closteroviridae*), isolated from blackberry and tentatively named Blackberry yellow vein associated virus, was determined. The virus possesses a bipartite genome. RNA 1 is 7801 nucleotides in length and papain-like protease, methyltransferase, RNA helicase and RNA-dependent RNA polymerase motifs have been identified in the proteins coded for by this molecule. The polymerase is probably expressed via a +1 ribosomal frameshift, a common feature among members of the *Closteroviridae*. RNA 2 is 7917 nucleotides long and encodes nine open reading frames, similar in size and position to orthologous genes of other criniviruses with the exception of a second hydrophobic peptide found near the 5' terminus of the molecule. Phylogenetic analysis revealed a close relationship between Blackberry yellow vein associated virus and *Beet pseudo yellows virus*, another crinivirus that infects small fruit crops.

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**Keywords:** *Crinivirus*; *Closteroviridae*; *Rubus*; Nucleotide sequence; Yellow vein disease

*Rubus* spp. (blackberry and raspberry) is affected by more than 30 virus and virus-like diseases (Converse, 1987; Halgren et al., 2003; Tzanetakis and Martin, 2004a; Martin and Tzanetakis, 2005). In the last four years, a yellowing disease of blackberry has become apparent in the southern and southeastern United States. The symptoms include leaf yellowing, mottling, vein banding, and in several cases, plant death. Similar symptoms have been associated with *Tobacco ringspot virus* (TRSV) (Stace-Smith, 1987), but detection tests failed to associate TRSV with the disease. Double-stranded RNA (dsRNA) was extracted from diseased plants and cloned. Sequence information revealed the presence of an undescribed virus (Martin et al., 2004).

The new virus belongs to the *Closteroviridae*, the family with the largest positive-strand RNA plant viruses ranging from 15 to 20 kilobases (Karasev, 2000). The family consists of three

genera: *Closterovirus*, *Ampelovirus*, and *Crinivirus*. Aphids, mealybugs, and whiteflies transmit members of the three genera, respectively (Martelli et al., 2002). Sequence analysis indicated that the newly identified virus was most closely related with members of the genus *Crinivirus* (Martin et al., 2004).

This study presents the complete nucleotide sequence of the novel virus associated with the vein banding and chlorosis of blackberry, designated as Blackberry yellow vein associated virus (BYVaV). Phylogenetic analysis using the conserved motifs of the polymerase, the heat shock protein 70 homolog (HSP70h) and the major coat protein (CP) of the virus, verified the results of sequence analysis placing the virus in the *Crinivirus* genus.

The nucleotide sequence of BYVaV was obtained from an 'Apache' blackberry from South Carolina. dsRNA was extracted from 20 g of leaf tissue as described previously (Tzanetakis et al., 2005a). cDNA synthesis was performed using the methods of Jelkmann et al. (1989) or Tzanetakis et al. (2005a) using dsRNA extracted from the equivalent of 4 g of tissue as template and cloned into a TOPO pCR 4.0 vector (Invitrogen, Carlsbad, CA) as described (Tzanetakis et al., 2005a). The recombinant plasmids were screened using the polymerase chain reaction (PCR)

<sup>☆</sup> The nucleotide sequence data reported in this paper have been submitted to the GenBank database and have been assigned the accession numbers AY776334 and AY776335 for RNA 1 and 2, respectively.

\* Corresponding author. Tel.: +1 541 7384041; fax: +1 541 7384025.

E-mail address: [yannis@orst.edu](mailto:yannis@orst.edu) (I.E. Tzanetakis).

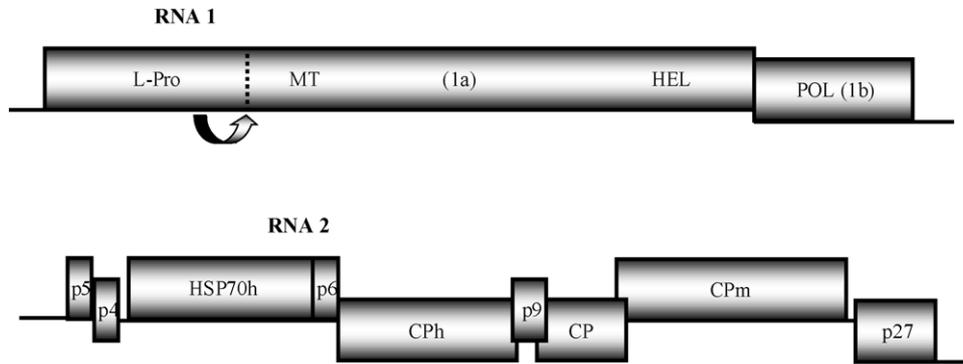


Fig. 1. Genome organization of Blackberry yellow vein associated virus. Abbreviations: L-Pro: leader papain-like protease; MT: methyltransferase; HEL: helicase; POL: RNA-dependent RNA polymerase; HSP70h: heat shock protein 70 homolog; CPh: coat protein homolog; CP: major coat protein; CPm: minor coat protein. Arrow indicates the putative protease cleavage site. Open reading frames are not to scale.

with the M13 forward and reverse primers and Taq polymerase (New England Biolabs, Beverly, MA). The PCR program 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 at the Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer.

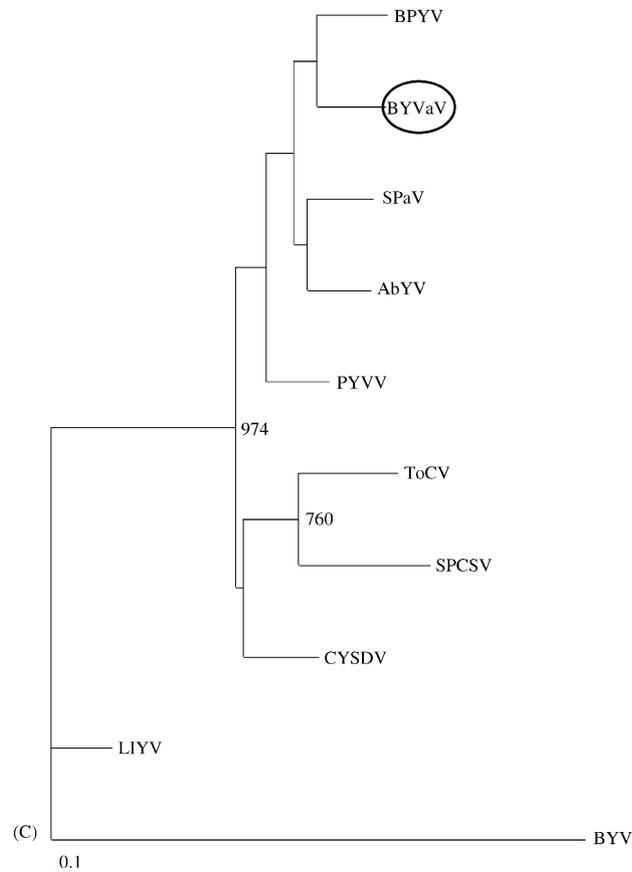
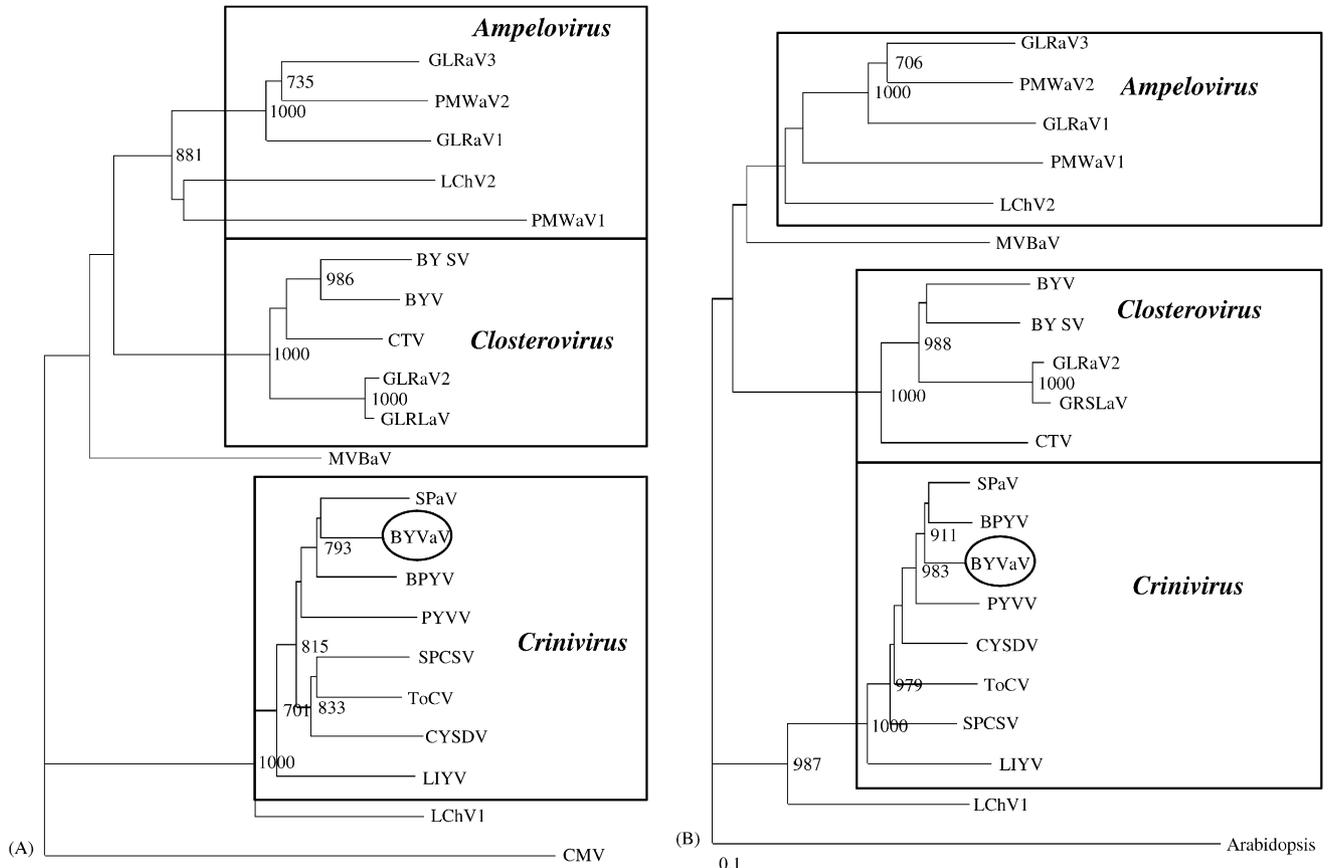
The sequence data obtained from shotgun cloning was utilized to develop oligonucleotide primers for reverse transcription-PCR (RT-PCR) amplification of BYVaV genomic regions as described previously (Tzanetakis et al., 2005b). The 5' and 3' termini of the virus were obtained after adenylation of dsRNA and RT-PCR (Tzanetakis and Martin, 2004b). In addition to the sequences from the shotgun cloning, at least four individual clones or PCR products were sequenced for all parts of the virus, for a minimum of a 4× coverage. The full-length sequence of the genome was obtained with the CAP3 software (Huang and Madan, 1999). Phylogenetic analyses of the conserved polymerase motifs, the HSP70h and CP of BYVaV and other members of the *Closteroviridae* were performed on 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). RNA secondary structure was predicted with mfold (Zuker, 2003) and the putative transmembrane domains of proteins with TMHMM (Krogh et al., 2001). Comparison of crinivirus proteins was performed on MatGat (Campanella et al., 2003).

BYVaV RNA 1 encodes the replication-related proteins and is 7801 nucleotides (nt) long. RNA 2 has an array of five genes that are involved in movement and are found in all members of the family in addition to several open reading frames (ORF) of unknown function (Fig. 1). The 5' untranslated regions (UTR) are 291 and 357 nt long for RNA 1 and RNA 2, respectively, and share no significant similarity other than the first seven nt that are identical. The 3' UTR are 202 and 190 nt long for RNA 1 and RNA 2, respectively, and are 93% identical.

The first ORF of RNA 1 (ORF 1a) codes for a polyprotein similar to that found in other members of the family with papain-like protease, methyltransferase, and RNA helicase motifs. The puta-

tive protein starts at position 291 and terminates at position 6083 with the start codon being in good context for translation (Kozak, 1986). The predicted protease domain is found at the N-terminus of the protein and the catalytic cysteine and histidine residues are located at positions 327 and 375, respectively. The protease cleavage site is predicted between Gly<sub>394</sub> and Val<sub>395</sub>, identical to the cleavage sites of BPYV (Tzanetakis and Martin, 2004b), *Cucurbit yellow stunting disorder virus* (CYSDV; Aguilar et al., 2003) and *Sweet potato chlorotic stunt virus* (SPCSV; Kreuze et al., 2002). The putative BYVaV protease with mass of 45 kDa is closely related to BPYV protease with 42% amino acid (aa) sequence identity and 64% similarity. The *Crinivirus* methyltransferase conserved motifs (Tzanetakis et al., 2005c) are also present in the orthologous domain of BYVaV. A region without any predicted enzymatic motifs of about 600 residues separates methyltransferase and helicase domains. The two transmembrane domains found in all members of the genus in this region, speculated to anchor the protein and the replication complexes to cellular membranes, are found between residues 1184–1206 and 1294–1316. The helicase domain is found at the C-terminus of the polyprotein between residues 1627 and 1930 and is the most conserved domain of the polyprotein with 65% aa sequence identity and 81% similarity with the orthologous region of BPYV.

The virus polymerase (1b) is probably expressed via a +1 ribosomal frameshift, a typical feature of members of the family (Martelli et al., 2002). Analysis of the RNA secondary structure of the region flanking the putative frame shift (ten Dam, 1995; Zuker, 2003), did not reveal structures that could enhance the putative frameshift, as was the case with other criniviruses sequenced to date (Aguilar et al., 2003; Kreuze et al., 2002; Tzanetakis et al., 2005c; Wintermantel et al., 2005). The conserved UUGA sequence (amber stop codon underlined), found in all criniviruses sequenced to date except for CYSDV, that may be involved in the frameshift was also identified in BYVaV. The 505 aa long polymerase has predicted mass of 59 kDa and contains the conserved motifs of other criniviruses identified previously (Tzanetakis et al., 2005c). The protein shares more than 70% aa sequence identity with the orthologous regions of BPYV and Strawberry pallidosis associated virus (SPaV).



RNA 2 is 7917 nt long and has nine ORFs. The first two ORFs (nt 358–495 and 509–616, respectively) encode two putative peptides of 5 and 4 kDa, respectively. Both proteins contain transmembrane helices (Krogh et al., 2001). The third ORF of RNA 2 codes for the virus HSP70h, the hallmark gene of members of the family *Closteroviridae*. The multifunctional protein contains the conserved enzymatic motifs identified in the HSP70h of other criniviruses (Tzanetakis et al., 2005c) and is involved in virus movement. The protein was also found associated with plasmodesmata and virions in the case of other members of the family (Dolja, 2003; Satyanarayana et al., 2000). The gene starts at nt 661 and terminates at position 2328, and the protein has predicted mass of 62 kDa. The gene has more than 80% aa sequence identity with the HSP70h of BPYV and SPaV and 75% similarity with all members of the genus sequenced to date other than *Lettuce infectious yellows virus* (LIYV). The next ORF (nt 2238–2499) encodes a putative protein of 6 kDa of unknown function with similarity to the C-terminus of *Little cherry virus-1* HSP70h and similar ORFs encoded by BPYV, SPaV, CYSDV and *Tomato chlorosis virus*. ORF 5 (nt 2493–4046) codes for a protein with molecular mass of 59 kDa orthologous to the coat protein homolog (CPh) of other closteroviruses (Napuli et al., 2003). The recognition of this protein as a homolog of coat proteins is reinforced by the homology of crinivirus CPhs with coat proteins of viruses of the family *Flexiviridae* (Tzanetakis, unpublished data). The conserved Arg and Asp, involved in the assembly process of virions (Dolja et al., 1991; Jagdish et al., 1993) are found at positions 434 and 471, respectively. A 9 kDa protein of unknown function identified in all criniviruses is found between nt 4028 and 4273. The two structural proteins of BYVaV, involved in genome protection and movement, follow with the 28 kDa CP encoded between nt 4266 and 5033 and the 77 kDa minor coat protein (CPm) found between nt 5005 and 7020. Both proteins show similarity with orthologous proteins of other criniviruses at their C-termini whereas the N-termini are diverse. The conserved residues of filamentous virus CPs (Ser, Arg and Asp; Dolja et al., 1991) are found at positions 127, 174 and 211 for the CP and 545, 589 and 630 for the CPm, respectively. The final ORF of RNA 2 (nt 7038–7727) encodes a putative 27 kDa protein of unknown function, but similar to the 3' proximal proteins found in RNA 2 of other members of the genus. Recently, the LIYV p27-like pro-

tein has been associated with plasmalemma deposits in infected plants (Medina et al., 2005).

Phylogenetic analysis using three genomic regions of BYVaV clearly places the virus within the genus *Crinivirus* (Fig. 2). BYVaV adds to the genome organization diversity seen in the other criniviruses that have been sequenced to date (Aguilar et al., 2003; Kreuze et al., 2002; Klaassen et al., 1995; Livieratos et al., 2004; Tzanetakis and Martin, 2004b; Tzanetakis et al., 2005c; Wintermantel et al., 2005). BYVaV encodes only the replication-associated proteins in RNA 1, as is the case with Cucumber yellows virus (Hartono et al., 2003), a strain of BPYV, although a strawberry isolate of BPYV has a small hydrophobic protein at the end of RNA 1 (Tzanetakis and Martin, 2004b). All criniviruses sequenced to date except for the most divergent member of the genus, LIYV, encode a small hydrophobic protein with a transmembrane domain at or near the 3' terminus of RNA 1 while all the members of the group have the first 5' proximal ORF of RNA 2 encoding a small protein with a transmembrane domain. BYVaV has two such peptides at the beginning of RNA 2. This finding may provide insight into the evolution of the *Closteroviridae*. There are indications that the monopartite members of the family predated criniviruses since there is more diversity between the monopartite closterovirus genera than within the genus *Crinivirus* (Karasev, 2000). In addition, Mint vein banding associated virus, a member of the family sharing characteristics of all three genera and a potential progenitor of the family is monopartite (Tzanetakis et al., 2005b). Studies suggest that the defective RNAs of *Citrus tristeza virus* (CTV) are analogous to the genomic RNAs of criniviruses (Che et al., 2003). If the evolution of criniviruses from one or more monopartite closteroviruses happened by a mechanism similar to the large defective RNAs of CTV, it may be that the two hydrophobic proteins in RNA 2 of BYVaV are the result of a recombination event that added the second peptide with a transmembrane domain in the same genomic molecule. This hypothesis is supported by the fact that several monopartite closteroviruses encode transmembrane domains in ORF 1a. Criniviruses, other than BPYV have relatively limited host ranges (Wisler et al., 1998). The close relationship of BPYV, SPaV, and BYVaV may be an indication that the common ancestor of all three viruses was a *Rubus* virus or a rosaceous host virus, given that SPaV is mainly restricted to rosaceous hosts (Tzanetakis, 2004).

Fig. 2. (A) Phylogram of the conserved polymerase motifs of Blackberry yellow vein associated virus and other members of the family *Closteroviridae*. Abbreviations and GenBank accession numbers: BPYV, *Beet pseudo yellows virus*, NP940796; BYV, *Beet yellows virus*, NP733949; BYSV, *Beet yellow stunt virus*, AAC55659; BYVaV, Blackberry yellow vein associated virus, AAV40963; CTV, *Citrus tristeza virus*, NP733947; CMV, *Cucumber mosaic virus*, NP049324; CYSDV, *Cucurbit yellow stunting disorder virus*, AAM73639; GLRaV 1, *Grapevine leafroll associated virus 1*, AAF22738; GLRaV 2, *Grapevine leafroll associated virus 2*, AAC40856; GLRaV 3, *Grapevine leafroll associated virus 3*, AAC40705; GRSLaV, *Grapevine rootstock stem lesion associated virus*, NP835244; LIYV, *Lettuce infectious yellows virus*, AAA61798; LChV 1, *Little cherry virus 1*, NP733945; LChV 2, *Little cherry virus 2*, AAP87784; MVBaV, *Mint vein-banding associated virus*, AAS57939; PMWaV 1, *Pineapple mealybug wilt-associated virus 1*, AAL66709; PMWaV 2, *Pineapple mealybug wilt-associated virus 2*, AAG13939; PYVV, *Potato yellow vein virus*, CAD89680; SPaV, *Strawberry pallidosis associated virus*, AY488137; SPCSV, *Sweet potato chlorotic stunt virus*, NP733939; ToCV, *Tomato chlorosis virus*, AY903447. CMV is used as the outgroup. (B) Phylogram of the heat shock protein 70 homolog of BYVaV and other members of the family *Closteroviridae*. GenBank accession numbers: *Arabidopsis thaliana* putative heat shock protein 70, AAN71949; BPYV, AAQ97386; BYSV, AAC55662; BYV, NP041872; BYVaV, AAV40966; CTV, NP042864; CYSDV, NP851572; GLRaV 1, AAF22740; GLRaV 2, AAR21242; GLRaV3, NP813799; GRSLaV, NP835247; LIYV, NP619695; LChV 1, NP045004; LChV 2, AF531505; MVBaV, AAS57941; PMWaV 1, AAL66711; PMWaV 2, AAG13941; PYVV, CAD89682; SPaV, AAO92347; SPCSV, NP689401; ToCV, AY903448. The *Arabidopsis* protein is used as the outgroup. (C) Phylogram of the coat proteins of BYVaV and other criniviruses. GenBank accession numbers: AbYV, *Abutilonyellows virus*, AAR00224; BYV, NP041875; BPYV, NP940792; BYVaV, AAV40970; CYSDV, NP851576; LIYV, NP619697; PYVV, CAD89686; SPaV, AAO92342; SPCSV, NP689404; ToCV, AY903448. BYV coat protein is used as the outgroup. Bootstrap values are shown as percentage value and only the nodes over 70% are labeled. The bars represent 0.1 amino acid changes per site.

## Acknowledgements

We thank Dr. G. Fernandez (North Carolina State University) for providing the isolate used for sequencing. We also thank Dr. R. Mumford and Mrs. A. Skelton (Central Science Laboratory, Sand Hutton, York) for helpful suggestions on the study. The project was funded by the U.S. Department of Agriculture.

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