



A member of a new genus in the *Potyviridae* infects *Rubus*

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Abstract

Blackberry yellow vein disease causes devastating losses on blackberry in the south and southeastern United States. Blackberry yellow vein associated virus (BYVaV) was identified as the putative causal agent of the disease but the identification of latent infections of BYVaV led to the investigation of additional agents being involved in symptomatology. A potyvirus, designated as Blackberry virus Y (BVY), has been identified in plants with blackberry yellow vein disease symptoms also infected with BYVaV. BVY is the largest potyvirus sequenced to date and the first to encode an AlkB domain. The virus shows minimal sequence similarity with known members of the family and should be considered member of a novel genus in the *Potyviridae*. The relationship of BVY with Bramble yellow mosaic virus, the only other potyvirus known to infect *Rubus* was investigated. The presence of the BVY was verified in several blackberry plants, but it is not the causal agent of blackberry yellow vein disease since several symptomatic plants were not infected with the virus and BVY was also detected in asymptomatic plants.

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1. Introduction

Several virus and virus-like agents infect *Rubus* spp. (Converse, 1987). In recent years, a new disease designated as blackberry yellow vein (BYVD) has emerged in the south and southeastern United States. The disease is characterized by the appearance of yellow vein symptoms on the older leaves of primocanes and vein-yellowing and die-back of floricanes. A new crinivirus, Blackberry yellow vein associated virus (BYVaV) has been associated with symptoms (Martin et al., 2004) but the identification of ‘Chickasaw’ plants that were asymptomatic suggested that additional agents may be involved in symptom development (Susaimuthu et al., 2006). Using standard tech-

niques we verified the presence of a novel potyvirus, designated as Blackberry virus Y (BVY).

The family *Potyviridae*, the largest plant virus family, is comprised of six genera of positive-sense single-stranded RNA viruses (Hull, 2002). Viruses in the family can be transmitted by aphids (genera *Potyvirus* and *Macluravirus*), eriophyid mites (genera *Rymovirus* and *Tritimovirus*), whiteflies (genus *Ipomovirus*) and plasmodiophorid fungi (genus *Bymovirus*) (Adams et al., 2005a). Potyviruses, with the exception of members of the *Bymovirus* genus, which are bipartite, have monopartite and monocistronic genomes, with a VPg attached to the 5′ end and a poly-adenosine tail at the 3′ end of the genome. The genome is expressed as a single polypeptide which is proteolytically processed to mature virus proteins, in a similar fashion to animal picornaviruses. A major difference between the two groups is that unlike picornaviruses, potyviruses have flexuous filamentous particles.

This communication presents molecular and epidemiological data for BVY, investigates the relationship of the virus with other members of the *Potyviridae* and the possibility BVY is an

Abbreviations: HC-Pro, helper component protease; CI, cylindrical inclusion bodies protein; VPg, genome-linked protein; NI, nuclear inclusion bodies protein; CP, coat protein.

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isolate of Bramble yellow mosaic virus (BrYMV), the only other potyvirus known to infect *Rubus* species (Engelbrecht, 1976).

2. Materials and methods

2.1. Plant material

The nucleotide sequence of BVY presented in this communication was obtained from a blackberry plant ('Chickasaw') from Arkansas (C3ARK) with BYVD symptoms. In order to study the spread of BVY in the field, an experiment was designed in which 10 BVY-free blackberry plants ('Chester') were placed on pedestals next to the symptomatic field plants ('Chickasaw') affected with BYVD. These sentinel plants were replaced at two-week intervals throughout the growing seasons (April–September) of 2004 and 2005 and were tested by reverse transcription-PCR (RT-PCR) and dot-blot hybridization for BVY-infection 6–10 months after field placement. Wild blackberry plants in Arkansas were sampled in 2005 from more than 50 locations throughout the state to determine if these plants serve as a source for the BVY found in cultivated blackberry plantings. Cultivated blackberry plants affected with BYVD from Georgia, Tennessee, Kentucky, South and North Carolina were also tested for the presence of BVY.

2.2. Detection

Double-sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Converse and Martin, 1990) with potyvirus-specific monoclonal antibodies (Agdia, Elkhart, IN) and RT-PCR using a universal potyvirus primer (Chen et al., 2001) were originally evaluated for detection of the virus.

BVY was detected by RT-PCR using BVY-specific primers BVY312F (5' CTGTGGGGAGATTTGGAGAA 3') and BVY695R (5' TCATTCCATGGGTGTGTC 3') that amplified a 383-base region of the genome. Leaf samples were used for RNA extraction and RT-PCR was performed as described by Susaimuthu et al. (2006) or Tzanetakakis et al. (2007).

Hybridization experiments were carried out to detect BVY from symptomatic and asymptomatic blackberry plants. The RNA blots were prepared by dotting RNA on a nylon membrane as described (Susaimuthu et al., 2007). Probe DNA was synthesized by RT-PCR with primers BVY312F and BVY1105R (5' TATCTCCCCTCCTGCTCTCA 3') specific for BVY. An established protocol was followed for the hybridization procedure (Sambrook et al., 1989), and the radioactive blots were exposed to BioMax MS X-ray film (Eastman Kodak Company, Rochester, NY) for 4 h.

2.3. Transmission studies

Seedlings of the following species were dusted with carborundum and mechanically inoculated with sap from BVY-infected blackberry leaves and petals: *Chenopodium quinoa* Willd., *C. amaranticolor* Coste and Reyn, *Nicotiana benthamiana* Domin, *N. rustica* L., *N. glutinosa* L., *N. clevelandii* Gray,

N. tabacum L. 'Kentucky-16', *Phaseolus vulgaris* L. 'Black Valentine', *Pisum sativum* L., *Vigna unguiculata* (L.) Walp. ssp. *unguiculata* 'Georgia 21', *Cucumis sativus* L. 'Boston Pickling', *C. melo* L., *Lycopersicon esculentum* Mill., *Petunia hybrida* Vilm. 'Dream Rose'. Two sets of inoculations were carried out with inoculation buffer (0.05 M phosphate buffer, pH 7.2) containing a 10 mM sodium sulfite or 2% nicotine. Test plants were inspected for symptom development and evaluated for virus infection using RT-PCR. To verify transmission of viruses from blackberry tissue, sap from *Tobacco ringspot virus* (TRSV)-infected raspberry leaves was used to inoculate *C. sativus* L. cv. 'Boston Pickling' as described above.

2.4. Electron microscopy

Leaf tissue from BVY-infected asymptomatic 'Chester' blackberry obtained by placement of sentinel 'Chester' plants in a BYVD affected production field was prepared for thin-section electron microscopy as described previously (Susaimuthu et al., 2006). Sections were double-stained for 15 min in a 2% aqueous solution of uranyl acetate (pH 5.0) and for 5 min in lead citrate (pH 12.0). Specimens were examined using a JEOL 100 CX transmission electron microscope.

2.5. Nucleic acid extractions and cloning

Double-stranded RNA was purified as described by Tzanetakakis and Martin (2005) from 20 g of tissue from symptomatic 'Chickasaw' plants from Arkansas. Complementary DNA was prepared using dsRNA as template and cloned as described (Tzanetakakis et al., 2005a) without the use of restriction endonucleases. Plasmids were screened by PCR and those with the largest inserts were sequenced at the Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer.

2.6. Sequence analysis

Sequences obtained were screened against sequences found in Genbank using blastn and blastx (Altschul et al., 1997) and the ones identified as BVY-specific were used for development of oligonucleotide primers for RT-PCR amplification of the genome (Tzanetakakis et al., 2005b). Acquisition of the 5' terminal region of the genome was done as described (Tzanetakakis and Martin, 2004b). The consensus of the sequence presented, deposited in Genbank under accession number AY994084, was obtained with CAP3 (Huang and Madan, 1999) and represents an at least a 3× sequence coverage. The putative polyprotein cleavage sites were determined after comparison of orthologous domains of BVY and with those of other potyviruses and the use of the PredictProtein server (Adams et al., 2005b; Rost et al., 2004). Phylogenetic analysis of the helicase and polymerase conserved motifs and the CP of potyviruses was performed with ClustalW (Thompson et al., 1994) using the neighbor-joining algorithm, Kimura's correction and bootstrap consisting of 1000 pseudoreplicates. Phylogenetic trees were visualized with Treeview (Page, 1996).

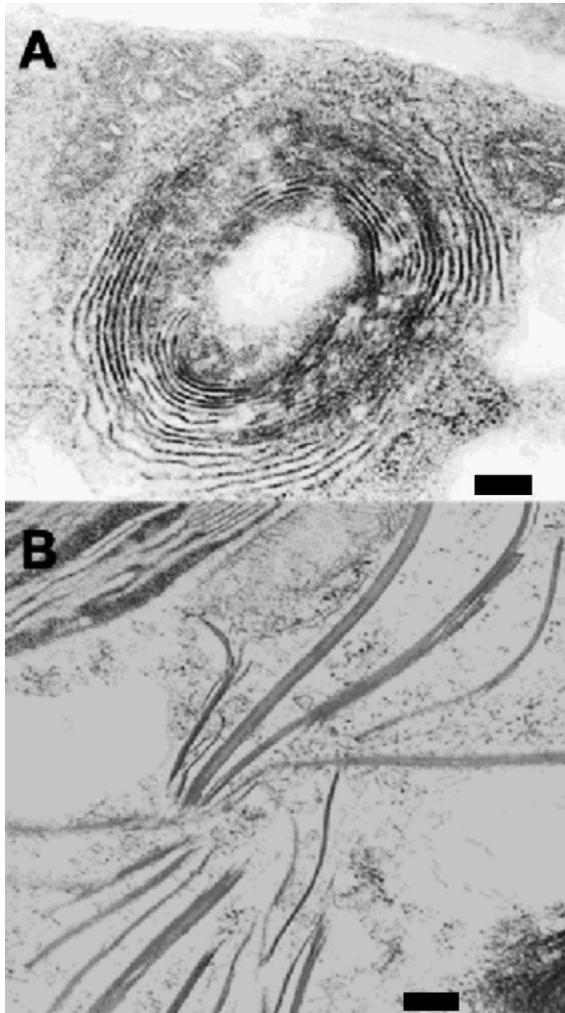


Fig. 1. Electron micrographs of leaf mesophyll cells in blackberry yellow vein disease affected plants having structures typical of potyvirus infection. (A) Proliferation of nuclear membrane and (B) laminated aggregates in the cytoplasm.

3. Results

Electron microscopic observation of blackberry plants ('Chickasaw') with BYVD revealed a variety of potyvirus-like inclusions (Fig. 1). The use of a universal monoclonal antibody against potyviruses (Jordan and Hammond, 1991) in ELISA and the universal potyvirus primer (Chen et al., 2001) in RT-PCR tests failed to detect the putative virus, indicating that it was not a typical *Potyvirus*. Four BYVD plants were used for dsRNA extractions. DsRNA from all four was cloned, but only C3ARK gave sequences that corresponded to the genome of BVY.

The complete nucleotide sequence of BVY genome was obtained and consists of 10,851 nucleotides (nt) excluding the poly-adenosine tail (43% GC content). The 176 nt 5' untranslated region (UTR) has low GC content (36%). The virus encodes a 3491 amino acid (aa) polyprotein of 394 kDa that is predicted to be processed to 10 mature proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, Vpg, NIa-Pro, NIb and CP from the N' to the C' terminus of the polyprotein (Fig. 2). The predicted cleav-

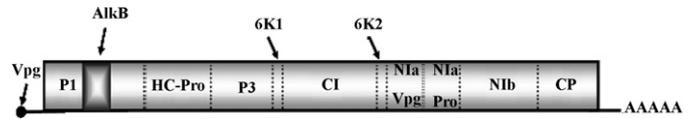


Fig. 2. Representation of Blackberry virus Y genome. The AlkB domain of the virus is highlighted.

age sites of the polyprotein are given in Table 1 (Adams et al., 2005b).

The BVY P1 ortholog, a serine protease, is 745 aa with MW of 84 kDa similar in size to *Ipomovirus* P1 proteins. The protein does not show significant similarities with orthologous proteins of other potyviruses, a feature not surprising since P1 orthologs are the least conserved proteins encoded by potyviruses (Adams et al., 2005a). P1 has a significant role in virus replication (Verchot and Carrington, 1995) probably due to the stimulation of the gene silencing suppressor HC-Pro (Kasschau and Carrington, 1998). P1 remains functional even after the insertion of a peptide in the coding region although in the case of *Potato virus A* an insertion was lost or altered 2 weeks post-inoculation (Rajamaki et al., 2005). The tolerance of P1 to allow insertions in its coding region is also observed in the BVY ortholog, where an AlkB domain was identified near its N' terminus. The conserved residues of the AlkB domain of BVY found between residues 180 and 280 of P1 (Bratlie and Drabløs, 2005) show similarity with orthologous domains of viruses that primarily infect rosaceous hosts, including *Little cherry virus-2* (Rott and Jelkmann, 2005) and *Black raspberry necrosis virus* (Halgren et al., 2007). The catalytic His–Asp–Ser residues of the protease are found at positions 643, 651 and 692, respectively (Adams et al., 2005b).

The 325 aa HC-Pro domain of BVY is about 120 aa smaller than the typical *Potyvirus* domain. The region missing from the BVY HC-Pro corresponds to the N-terminus of the *Potyvirus* orthologs and is involved in gene silencing and probably vector transmission (Revers et al., 1999; Stenger et al., 2006; Urcuqui-Inchima et al., 2001; Young et al., 2007). In addition to the much smaller HC-Pro mature protein, BVY HC-Pro is lacking conserved motifs involved in genome amplification and systemic movement (Urcuqui-Inchima et al., 2001). The cysteine protease activity of HC-Pro is found at the C' terminus of the mature protein and the conserved Cys and His residues are found at positions 211 and 284, respectively. The HC-Pro of BVY shows

Table 1
 Location of the putative cleavage sites of Blackberry virus Y polyprotein

Region	Position of amino acid at position – 1	aa sequence (–4/+3)
P1/HC-Pro	745	YLQY/SEE
HC-Pro/P3	1070	YRVG/GFA
P3/6K1	1420	IQFQ/SAD
6K1/CI	1476	IIFQ/AER
CI/6K2	2096	FVQQ/NPN
6K2/Vpg	2163	ITFE/ASG
Vpg/NIa	2353	IVPE/GRS
NIa/NIb	2590	ITFQ/NNR
NIb/CP	3143	SEPA/LQP

about 25% aa identity with orthologous proteins of members of the genus *Potyvirus*.

The 41 kDa P3 is believed to be involved in virus replication (Urcuqui-Inchima et al., 2001) and probably host range and symptom development (Hjulsager et al., 2006; Suehiro et al., 2004). Limited conservation is found between orthologs of P3 proteins of potyviruses, something also observed in BVY P3 which shares about 20% aa identity with orthologs in the *Potyvirus* and *Rymovirus* genera. The 6K1 shows similarity with orthologous proteins of members of the *Potyvirus* genus sharing about 35% identical residues (55% similar residues). The 69 kDa CI protein of BVY has RNA helicase motifs identified between residues 75 and 329 (Gorbalenya and Koonin, 1989). The region shows about 50% aa sequence identity with orthologous domains of viruses in the genus *Potyvirus*.

A small peptide of 8 kDa with minimal similarity to potyvirus proteins is found downstream of CI. Orthologs of this protein (6K2) in other potyviruses are believed to anchor the replication complex to the ER membranes (Urcuqui-Inchima et al., 2001). A transmembrane domain has been identified in this protein between residues 38 and 57 (Krogh et al., 2001).

The 22 kDa BVY Vpg has about 30% aa sequence identity with orthologous proteins of the *Potyvirus* and *Rymovirus* genera. The Vpg is attached to the 5' terminus of the genome and is indispensable for virus replication. The 26 kDa NIa is the fourth protease encoded by BVY and has less than 30% aa sequence identity with orthologous proteins of members of the genus *Potyvirus*. The catalytic residues of the cysteine protease, His–Asp–Cys–His are found at positions 47, 82, 151 and 167, respectively.

The 63 kDa virus polymerase NIb contains all conserved motifs identified by Koonin (1991) between residues 188 and 439. This region shares the greatest similarity between BVY and any member of the family with over 50% aa sequence identities and 70% aa similarities.

Potyviral CPs encapsidate the genome and they are involved in virus movement and genome amplification (Urcuqui-Inchima et al., 2001). BVY CP shows less than 30% aa sequence identity with CP of other members of the *Potyvirus* genus, and it is the missing motifs that are associated with aphid transmission. The 3' UTR preceding the adenosine tail is 199 nt long and is predicted to fold into three stem-loop structures of unknown function (data not shown).

Phylogenetic analysis using the helicase motifs of CI, the polymerase motifs of NIb and the CP of members of the *Potyviridae* failed to cluster BVY with any of the existing genera of the *Potyviridae* (Fig. 3).

Several herbaceous hosts were used for mechanical transmission of BYV, many of which were used in the transmission of BrYMV (Engelbrecht, 1976). None of these plants developed symptoms, and all were tested positive for BVY by RT-PCR while all *C. sativus* L. plants were successfully inoculated with TRSV in the control experiments.

Spread of BVY to sentinel 'Chester' blackberry plants in the field was detected by RT-PCR in 10 of the 120 plants placed in 2004, and 37 of the 100 sentinel plants became infected in 2005. For this reason we used aphids and mites for BYV transmission

with negative results (data not shown). BVY-infected sentinel plants were asymptomatic, and detection by RT-PCR revealed that BYVaV, the originally described agent of BYVD, was not spreading in the field (Susaimuthu et al., 2007).

Surveys of wild blackberry plants indicated that BVY was present in symptomatic leaf samples of some of the wild blackberry plants (*Rubus fruticosus*, identified by Dr. Johnnie L. Gentry, University of Arkansas, Fayetteville) from two of the 19 Arkansas counties surveyed (Susaimuthu et al., 2007). The symptomatic wild blackberry plants that tested positive for BVY were also positive for BYVaV (Susaimuthu et al., 2007), and these samples were from wild blackberry plants in northwest Arkansas where the BYVD affected production field is located. BVY was not detected in any of the other eight symptomatic cultivated blackberry plants from Georgia, Tennessee, Kentucky, South and North Carolina.

4. Discussion

BVY is a new virus recently identified in *Rubus*. The complete nucleotide sequence of the virus was determined and phylogenetic analyses, supported by high bootstrap values failed to cluster BVY with members of any of the existing genera of the *Potyviridae*. The aa sequence of the virus shows less than 30% identity to any of the species found in the databases. These properties place BVY in a new genus in the *Potyviridae* (Adams et al., 2005a) and we propose the name Blackyvirus for this genus.

There are several aspects of the BVY that are atypical for a potyvirus. BVY is the sole potyvirus identified to date that encodes an AlkB domain. Verification of the AlkB in the genome of BVY was done by performing three sets of RT-PCR reactions with six sets of primers corresponding to sequences inside and outside the domain (data not shown). AlkB orthologs are found in prokaryotes, eukaryotes and viruses and are involved in repair of nucleic acid alkylation (Aas et al., 2003). It has also been speculated that AlkB orthologs may be involved in suppression of gene silencing (Aravind and Koonin, 2001). The AlkB region of BVY shows about 30% aa identity with orthologous domains of several virus species that infect, primarily, rosaceous hosts. Since the BVY AlkB resembles orthologous domains of other viruses, a feasible explanation of its origin is that the domain was incorporated after a recombination event between BVY or a progenitor of the virus and a virus that carried the domain. The similarity of the BVY AlkB domain to orthologous domains of other viruses is higher than the similarity shared between the BVY P1 and its ortholog in other potyviruses. AlkB orthologs have enzymatic activity and it is expected that the conserved motifs are required for activity. Therefore it cannot be determined if the AlkB domain was acquired after speciation of BVY or if acquired in an event that involved a progenitor of the virus.

BVY lacks the N-terminus of a typical potyvirus HC-Pro. This region is involved in gene silencing and vector transmission, properties of utmost importance for a virus. If indeed, AlkB is involved in gene silencing it may be that BVY P1 is having a more active role in counterdefense other than that of assisting HC-Pro (Kasschau and Carrington, 1998). *Cucumber vein yellowing virus* lacks an HC-Pro ortholog but has two P1 copies,

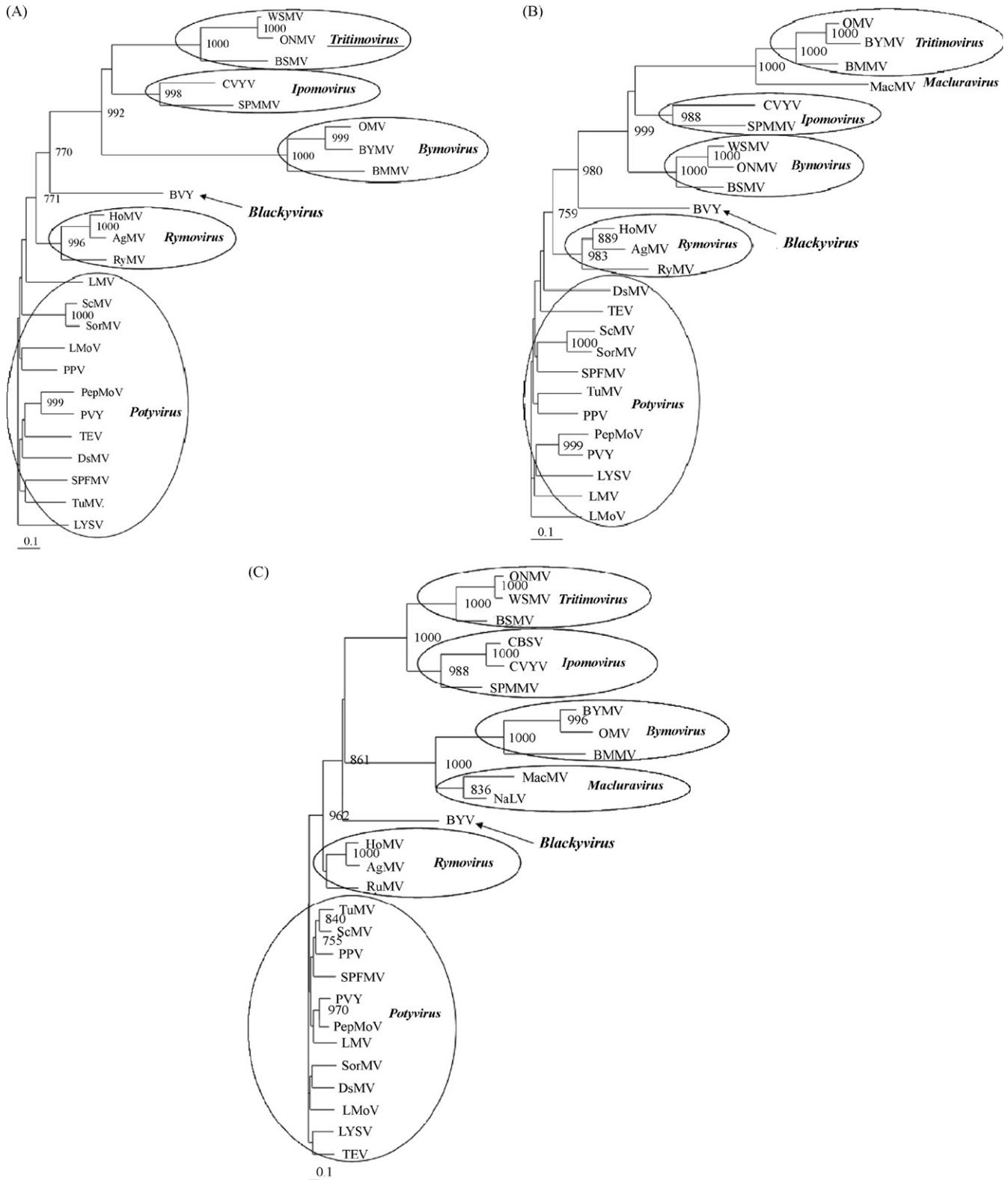


Fig. 3. Unrooted phylograms of the helicase (A), and polymerase (B) conserved motifs and coat protein (C) of Blackberry virus Y and other potyviruses. The sequences used for the construction of the phylogram were obtained from the Genbank accessions of the complete genome of the viruses unless otherwise noted. Abbreviations: *Agropyron mosaic virus*, AgMV; *Barley mild mosaic virus*, BMMV; *Barley yellow mosaic virus*, BYMV; *Brome streak mosaic virus*, BSMV; *Cucumber vein yellowing virus*, CVYV; *Dasheen mosaic virus*, DsMV; *Hordeum mosaic virus*, HoMV; *Leek yellow stripe virus*, LYSV; *Lettuce mosaic virus*, LMV; *Lily mottle virus*, LMoV; *Maclura mosaic virus*, MacMV, Genbank accession U58771; *Narcissus latent virus*, NaLV, Genbank accession U58770; *Oat mosaic virus*, OMV; *Oat necrotic mottle virus*, ONMV; *Pepper mottle virus*, PepMoV, *Plum pox virus*, PPV; *Potato virus Y*, PVY; *Ryegrass mosaic virus*, RyMV; *Scallion mosaic virus*, ScMV; *Sorghum mosaic virus*, SorMV; *Sweet potato feathery mottle virus*, SPFMV; *Sweet potato mild mottle virus*, SPMMV; *Tobacco etch virus*, TEV; *Turnip mosaic virus*, TuMV; *Wheat streak mosaic virus*, WSMV. The 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.

one of which is involved in gene silencing suppression (Valli et al., 2006), and it may be that BVY P1 with the incorporation of AlkB has evolved to have a similar function.

We have demonstrated that BVY is present in cultivated and wild blackberries exhibiting symptoms of BYVD in northwest Arkansas. Based on the results of our sentinel plant study, we conclude that BVY is transmitted by an aerial vector in commercial blackberry fields in northwest Arkansas.

There is a report from South Africa of another potyvirus infecting wild trailing blackberry, BrYMV (Engelbrecht, 1976). This virus is readily transmissible to herbaceous hosts. We have repeatedly tested several of the hosts that were reported as susceptible to BrYMV, but we were unable to transmit BVY to them, while all control experiments with TRSV were successful. No information is available on the serological properties, nucleotide sequence or vector transmission of BrYMV, therefore we are not able to determine if these two viruses are different species.

BVY-infected sentinel ‘Chester’ blackberry plants are symptomless. Since BYVaV has been shown to cause latent infections in different blackberry cultivars including ‘Chickasaw’ and wild blackberry plants in Arkansas (Susaimuthu et al., 2006), it can be speculated that BVY from wild blackberry plants moved into the ‘Chickasaw’ planting originally infected asymptotically with BYVaV and prompted the appearance of symptoms.

It was concluded that BVY may be a component of the observed symptomatology but is not the causal agent of the disease since only the symptomatic plants in Arkansas were infected with the virus. With the data that we have acquired over the 6 years of studying BYVD, we have concluded that the disease is caused by mixed infections of at least two viruses with BYVaV being a constant component of the complex. We are currently working on the other agents that were identified in diseased plants (Tzanetakis and Martin, unpublished) and *Beet pseudo-yellows virus*, a virus related to BYVaV (Tzanetakis et al., 2006) that has been recently identified in blackberry (Tzanetakis and Martin, 2004a). We also plan to graft BYVaV and BVY singly infected blackberry plants with the opposite virus to determine if co-infections of these viruses lead to BYVD symptoms.

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