Journal of Virological Methods 248 (2017) 191–194

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Development of reliable detection assays for blueberry mosaic- and blackberry vein banding- associated viruses based on their population structures

Thanuja Thekke-Veetil, Ioannis E. Tzanetakis*

Department of Plant Pathology, Division of Agriculture, University of Arkansas, Fayetteville, AR, 72701, USA

ARTICLE INFO

Keywords:
Detection
Sensitivity
Specificity
Reliability
Certification

ABSTRACT

Blueberry mosaic associated virus (BlMaV), the presumed causal agent of the homonymous disease and blackberry vein banding associated virus (BVBaV), a component of the blackberry yellow vein disease complex, are recently characterized RNA viruses. There is a need for efficient and sensitive detection protocols for the two viruses, not only for screening during the nursery propagation process but also in commercial fields to better understand virus epidemiology and minimize disease spread. RNA viruses display significant nucleotide variation forming quasi-species. Therefore, sequence-based detection methodologies, even though sensitive, may lead to false negative results. For this reason, information on the genetic diversity of virus populations is essential to develop diagnostic assays that have the potential to detect all variants. Detection assays for BlMaV and BVBaV were developed based on existing genetic diversity data and were validated by screening samples from different geographical areas in the United States. These detection tests provide sensitivity and specificity and will serve as the protocols of choice for virus screening in *Vaccinium* and *Rubus* certification programs in the United States and elsewhere. Given the increasing global trade of both blueberry and blackberry these tests will be valuable in avoiding virus introductions to new areas.

1. Type of research

i The genetic information of the putative disease agent of blueberry mosaic, blueberry mosaic associated virus (BlMaV), was recently unveiled (Thekke-Veetil et al., 2014). BlMaV is a tripartite negative-sense RNA virus in the *Ophioviridae* family encoding viral replicase and a 24 kDa protein of unknown function in RNA 1, the movement protein in RNA 2 and the nucleoprotein in RNA 3.

ii Blackberry vein banding associated virus (BVBaV), a member of subgroup I of the genus *Ampelovirus* in the *Closteroviridae* family, is a component of blackberry yellow vein disease and found widespread in wild and cultivated blackberries (Thekke-Veetil et al., 2013).

iii RNA viruses exhibit higher rates of sequence variation due to their error prone replication mechanisms (Lauring and Andino, 2010). The genetic variation in BlMaV and BVBaV populations were studied in depth (Thekke-Veetil et al., 2013; Thekke-Veetil et al., 2015) and sequence information obtained from these studies were utilized in designing PCR-based detection assays for both viruses.

2. Time required

Whole protocol: 7 h

a Total nucleic acid extraction: 2 h
b Reverse transcription: 1.5 h
c PCR: 1.5 h
d Gel electrophoresis and imaging: 2 h

3. Materials

3.1. Special equipment

No special equipment is required. Any microcentrifuge, vortexer, conventional thermocycler, gel electrophoresis apparati and gel imaging system could be used for performing the steps mentioned in the protocols.

* Corresponding author.
E-mail address: itzaneta@uark.edu (I.E. Tzanetakis).

http://dx.doi.org/10.1016/j.jviromet.2017.07.014
Received 5 April 2017; Received in revised form 19 May 2017; Accepted 24 July 2017
Available online 25 July 2017
0166-0934/

Table 1
Details of the isolates used for designing detection primers for blueberry mosaic associated virus. Isolates AR1 through OR22 are U.S. isolates.

Isolate	State/Country	GenBank accession Number
AR1	Arkansas	KJ849098
AR2	Arkansas	KJ849099
AR3	Arkansas	KJ849100
AR4	Arkansas	KJ849101
AR5	Arkansas	KJ849102
KY1	Kentucky	KJ849127
KY2	Kentucky	KJ849128
KY3	Kentucky	KJ849129
KY4	Kentucky	KJ849130
MI1	Michigan	KJ849092
MI2	Michigan	KJ849093
MI3	Michigan	KJ849094
MI4	Michigan	KJ849095
MI5	Michigan	KJ849096
MI6	Michigan	KJ849097
NJ1	New Jersey	KJ849131
NJ2	New Jersey	KJ849132
NJ3	New Jersey	KJ849133
NJ4	New Jersey	KJ849134
NJ5	New Jersey	KJ849135
NJ6	New Jersey	KJ849136
NJ7	New Jersey	KJ849137
NJ8	New Jersey	KJ849138
NJ9	New Jersey	KJ849139
NJ10	New Jersey	KJ849140
NJ11	New Jersey	KJ849141
OR1	Oregon	KJ849103
OR2	Oregon	KJ849104
OR3	Oregon	KJ849105
OR4	Oregon	KJ849106
OR5	Oregon	KJ849107
OR6	Oregon	KJ849108
OR7	Oregon	KJ849115
OR8	Oregon	KJ849121
OR9	Oregon	KJ849113
OR10	Oregon	KJ849109
OR11	Oregon	KJ849112
OR12	Oregon	KJ849116
OR13	Oregon	KJ849125
OR14	Oregon	KJ849117
OR15	Oregon	KJ849118
OR16	Oregon	KJ849119
OR17	Oregon	KJ849124
OR18	Oregon	KJ849111
OR19	Oregon	KJ849110
OR20	Oregon	KJ849126
OR21	Oregon	KJ849122
OR22	Oregon	KJ849114
BC1	Canada	KJ849120
BC2	Canada	KJ849123
Slo1	Slovenia	KJ849142
Slo2	Slovenia	KJ849143
Slo3	Slovenia	KJ849144
Slo4	Slovenia	KJ849145
Slo5	Slovenia	KJ849146
Slo6	Slovenia	KJ849147
Slo7	Slovenia	KJ849148
Slo8	Slovenia	KJ849149
Slo9	Slovenia	KJ849150

NA- Not available

3.2. Chemicals and reagents

a Total nucleic acid extraction: RNA extraction buffer (200 mM Tris-HCl [pH 8.5], 300 mM lithium chloride, 1.5% sodium dodecylsulphate, 10 mM ethylene diamine tetra-acetic acid [EDTA], 1% sodium deoxycholate, 1% NP-40, and 1% 14 M β-mercaptoethanol solution [vol/vol] added just before use), 5.8 M potassium acetate (pH 6.5), 100% isopropanol, RNA wash buffer (10 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 50 mM NaCl, and 50% ethanol), silica, and Tris-EDTA (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

b Reverse transcription: Maxima® reverse transcriptase, RiboLock™ RNase inhibitor and random hexamer primer, UltraPure™ DNase/RNase-Free distilled water (Thermo Fisher Scientific, USA).

c PCR: *Taq* DNA polymerase and dNTP Mix (Genscript, USA), detection primers synthesized by Integrated DNA Technologies (USA).

d Gel electrophoresis and imaging: sodium borate (SB) agarose gel buffer, 1X (10 mM NaOH, 66 mM boric acid, pH8.2) and GelRed (Biotium, USA).

Table 2
Details of the isolates used for designing detection primers for blackberry vein banding associated virus.

Isolate	Location	GenBank accession number
AR1	Arkansas	KC912179
AR2	Arkansas	KC912188
GA1	Georgia	KC912181
GA2	Georgia	KC912185
GA3	Georgia	KC912186
GA4	Georgia	KC912182
GA5	Georgia	KC912183
GA6	Georgia	KC912187
GA7	Georgia	KC912184
MS1	Mississippi	KC912165
MS2	Mississippi	KC912189
NC1	North Carolina	KC912171
NC2	North Carolina	KC912170
NC3	North Carolina	KC912166
NC4	North Carolina	KC912169
NC5	North Carolina	KC912168
NC6	North Carolina	KC912172
NC7	North Carolina	KC912173
NC8	North Carolina	KC912167
NC9	North Carolina	KC912178
NC10	North Carolina	KC912175
NC11	North Carolina	KC912176
NC12	North Carolina	KC912177
SC1	South Carolina	KC912180
SC2	South Carolina	KC912174

4. Detailed procedure

4.1. Assay design and standardization

Sequence information obtained from GenBank (61 BlMaV and 25 BVBaV isolates; Tables 1 and 2) were aligned using MUSCLE (Edgar, 2004) and conserved regions were identified. Degenerate primer sets and PCR assays were designed for each virus. After preliminary experiments primers that provided sensitive and reliable results were selected and PCR conditions were optimized. For BlMaV, the detection primers BlMaVMPdegF (5′-CCWGTATCAAGCATAGTYACAAG-3′) and BlMaVMPdegR (5′-AAGAAGGTRGTGATTGAGA-3′) target a 254- base region of the MP gene, and for BVBaV, primers BVBaVdetF (5′-CTGCTRTAYAGCACRGTTAAACA-3′) and BVBaVdetR (5′-TACGTGCGGACTTTGTTAGTG-3′) amplify a 157- base region of the minor coat protein gene. Reaction mixtures and conditions were optimized to also amplify a 721-base region of the NADH dehydrogenase ND-2 subunit transcript (Thekke-Veetil et al., 2016; Tzanetakis et al., 2007), to ensure the quality of the extracted nucleic acids.

4.2. Detailed steps

4.2.1. Total nucleic acid extraction

Total nucleic acids were extracted from 50 mg of leaf tissue following the steps essentially as described by Poudel et al. (Poudel et al., 2013).

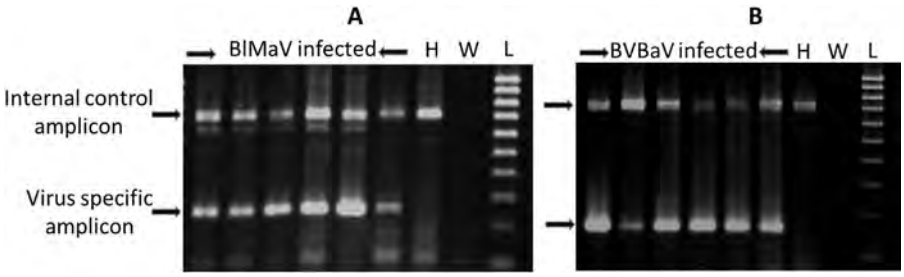

Fig. 1. Agarose gel electrophoresis of reverse transcription-PCR amplicons of blueberry mosaic associated virus (BlMaV) (A) and blackberry vein banding associated virus (BVBaV) (B). H- healthy control, W-water control, L–100 bp DNA marker. Internal control amplicon–721 bp, BlMaV amplicon–254 bp, BVBaV amplicon–157 bp.

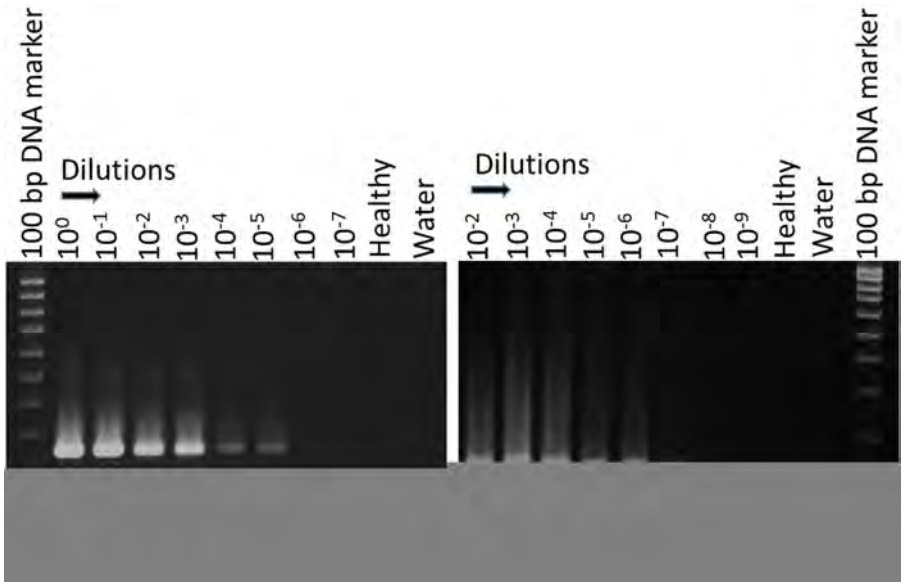

Fig. 2. Detection range of RT-PCR assays of blueberry mosaic associated virus (BlMaV) and blackberry vein banding associated virus (BVBaV). cDNAs obtained from 10 ng in vitro transcripts were serially diluted 10-fold and used as templates for PCR amplification. Note that for BVBaV dilutions initiate at 10-2.

4.2.2. Reverse transcription

The 50 µl reverse transcription (RT) reaction included 2.5 µl of extracted nucleic acids, 6 ng random primer, 50 U of Maxima® reverse transciptase, 1 x RT buffer, 6 U of RiboLock™ RNase inhibitor and 0.4 mM dNTPs. The reactions were incubated at 50 °C for 60 min for cDNA synthesis and enzymes were inactivated by heating at 85 °C for 5 min. The cDNAs were diluted two-fold in DNase/RNase-free distilled water to reduce the effect of inhibitors.

4.2.3. PCR

Amplification of BlMaV and BVBaV were performed in 25 µl duplex PCRs using 0.5 unit of *Taq* DNA polymerase in a final reaction mixture that contained *Taq* buffer, 200 µm dNTPs and 400 nm virus specific primers. The same concentration (400 nm) of the internal control primers were used in the BlMaV assays whereas for BVBaV internal control primers at 60 nm provided optimal results. BlMaV products were amplified using initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C–20 s, 54 °C–20 s and 72 °C-10s. The BVBaV program initiated with 3 min at 94 °C followed by 40 cycles of 94 °C–20 s, 54 °C–20 s and 72 °C-20 s. RTs and PCRs were performed on a C1000 Thermo Cycler® (Biorad, USA).

4.2.4. Gel electrophoresis and imaging

The products were electrophoresed through agarose gel (2%) in SB buffer, stained with GelRed, and visualized and documented under UV light using a KODAK Gel Logic 100 Imaging System (Eastman Kodak, USA).

4.3. Assay validation and sensitivity

The assays were validated using 58 blueberry samples collected from Arkansas (AR), Florida (FL), Georgia (GA), Michigan (MI) and Oregon (OR) between 2009 and 2015. Three hundred and fifty nine (359) blackberry samples were collected between 2008 and 2011 from AR, FL, GA, Mississippi (MS), North Carolina (NC), and South Carolina (SC). The nucleic acid extraction and RT-PCR were performed as mentioned above.

Sensitivity of the protocols was determined using *in vitro* transcripts. Virus fragments were amplified using the detection primers described above with the modification of the forward primers having the T7 promoter sequences (5′-TAGATTAATTAATACGACTCACTATAG-3′) at their 5'ends. PCR products were purified and subjected to RNA synthesis using the mMESSAGE mMACHINE ® kit (Thermo Fisher Scientific) according to the manufacturer instructions. The products were treated to remove the DNA template and purified (TURBO DNA-free kit; Thermo Fisher Scientific). After quantification, 10 ng of RNAs were used for reverse transcription. The cDNAs obtained were serially diluted 10-fold from 10 ng–0.001 fg of corresponding *in vitro* transcribed RNAs before PCR using the aforementioned conditions. RT and PCRs were conducted in four replicates to confirm the sensitivity range of the assays.

5. Results

Both detection assays successfully amplified virus-specific products, 254 bp for BlMaV and 157 bp for BVBaV, in combination with a 721 bp internal control product (Fig. 1A and B) from the NADH ND-2 subunit gene in a duplex PCR system. Only the internal control was amplified from healthy material. To use the assays for routine procedures, we validated the methods according to EPPO (EPPO, 2010) guidelines. The PCR detection assays were validated by screening multiple blueberry and blackberry samples, collected from various geographical locations in the U.S. The RT-PCR assay for BlMaV gave positive results in 14/58 archived blueberry samples and the BVBaV assay gave positive results

in 33/359 blackberry samples tested. Products were sequenced with either the forward or reverse detection primers to ensure their virus nature. Sensitivity of both assays were determined using serial dilution of cDNAs derived from *in vitro* transcripts (10 ng–0.001 fg). The RT-PCR assay for BlMaV detected as low as 100 fg of the *in vitro* transcripts, whereas the BVBaV assay detected as low as 1 fg of transcripts (Fig. 2A and B) in all four trials.

6. Discussion

The assays were designed to assure the quality of the extracted nucleic acids without affecting the amplification of the viral templates as the internal control amplicon is larger than the virus counterparts which, as such, are preferentially amplified ((Thekke-Veetil et al., 2016); Fig. 1A and B). The assays detected as low as 100 fg and 1 fg of RNA transcript respectively for BlMaV and BVBaV (Fig. 2) and were able to detect viruses in field-collected samples from different regions of the U.S.

6.1. Trouble-shooting

a The thermocycler conditions and the concentration of the virus- and internal control- specific primers have to be followed as described to amplify virus specific product preferentially and any changes may alter the ratio of virus/internal control amplicon yield.
b In PCR, cDNAs could be used undiluted or diluted depending upon the quality of samples under study.

6.2. Alternative and support protocols

If there is difficulty amplifying both virus and internal control templates simultaneously as mentioned here, the protocol could be used for the virus specific amplification alone. However, in such situation, absence of amplification of virus template could not be ruled out from the negative result due to poor quality RNA template unless verified by a separate amplification of internal control.

Acknowledgements

Funding for this study was obtained from USDA-APHIS 15–8130-0420-CA and USDA-APHIS 16–8130-0420-CA.

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Quick procedure

i. Extract total nucleic acids from 50 mg of leaf tissue following the steps described in Poudel et al. (Poudel et al., 2013).
ii. Synthesize cDNAs in a 50 µl reaction mixture (5% of extracted nucleic acids, 6 ng random primer, 50 U of Maxima® reverse transciptase, RT buffer, 6 U of RiboLock™ RNase inhibitor and 0.4 mM dNTPs) by incubating at 50 °C for 60 min followed by the inactivation of the enzymes at 85 °C for 5 min. Dilute the cDNAs two-fold with DNase/RNase-free water.
iii. Amplify BlMaV and BVBaV in 25 µl duplex PCR mixture (final reaction mixture contains 0.5 unit of Taq DNA polymerase, Taq buffer, 200 µm dNTPs and 400 nm virus specific primers and 400 nm internal control primers (60 nm internal control primers for BVBaV detection).
– For BlMaV perform PCR at 94 °C for 3 min for initial denaturation followed by 35 cycles of 94 °C-20 s, 54 °C-20 s and 72 °C-10 s.
– For BVBaV initiate the program with 3 min denaturation at 94 °C followed by 40 cycles of 94 °C-20 s, 54 °C-20 s and 72 °C-20 s.
iv. Electrophorese the products in SB buffer and 2% SB agarose gel and stain with GelRed to visualize.