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Association of *Grapevine fanleaf virus*, *Tomato ringspot virus* and *Grapevine rupestris stem pitting-associated virus* with a grapevine vein-clearing complex on var. Chardonnay

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Abstract A disease complex was observed on grapevine var. Chardonnay (*Vitis vinifera*) in a commercial vineyard in Missouri that destroyed the affected vineyard. Conspicuous vein-clearing symptoms on the leaves of originally diseased Chardonnay vines and bud-grafted Chardonnay, *V. vinifera* 'Cabernet Franc', *V. vinifera* 'Baco Blanc', and hybrid 'LN-33' vines are characteristics of the disease complex, which is referred to as the grapevine vein-clearing complex (GVCC). By applying reverse-transcription polymerase chain reaction (RT-PCR) using virus-specific primers, we detected combinations of *Grapevine fanleaf virus* (GFLV), *Tomato ringspot virus* (ToRSV) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV), in symptomatic Chardonnay vines. Sequencing of RT-PCR amplified DNA fragments confirmed the identity of each virus, indicating the occurrence of ToRSV yellow vein strain, and two distinct strains of GRSPaV in the GVCC-affected Chardonnay vines. This is the first report of the co-infection of two nepoviruses and GRSPaV in var. Chardonnay. This study demonstrated that mixed infections of grapevine viruses belonging to different taxonomic groups pose a great threat to vineyards under certain climatic and soil conditions.

Keywords Grapevine · Mixed infection · Nepoviruses · Reverse-transcription polymerase chain reaction

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Introduction

Grapevine is a host to 58 viruses that are assigned to nine families and seventeen genera in addition to the newly identified viruses that are yet to be classified (Martelli and Boudon-Padieu 2006). Grapevine degeneration and decline, leafroll disease and Rugose wood complex are the most prevalent and detrimental virus disease complexes in vineyards worldwide. The causal agents of grapevine degeneration and decline are members of the genus *Nepovirus* (nematode-transmitted polyhedral virus; family *Comoviridae*) including *Grapevine fanleaf virus* (GFLV) and *Tomato ringspot virus* (ToRSV) (Martelli and Boudon-Padieu 2006). Nepovirus-incurred symptoms vary from fan-like leaf, vein banding, chlorotic ring spots on leaves to severe decline in vigour and fruit production of vines (Martelli 1993). Genetically and serologically distinct viruses designated *Grapevine leafroll-associated virus 1–7, 9* (GLRaV 1–7, 9) in the genera *Closterovirus* and

Ampelovirus within the family *Closteroviridae* are associated with grapevine leafroll disease (Martelli and Boudon-Padieu 2006). They are widely epidemic in most vineyards and exist as populations of viral isolates that differ in nucleotide and amino acid sequences in naturally infected grapevines (Turturo et al. 2005). Rugose wood complex consists of rupestris stem-pitting, corky bark, Kober stem-grooving, and LN 33 stem-grooving, and is associated with *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus* A (GVA), *Grapevine virus* B (GVB), and other unidentified viruses (Martelli and Boudon-Padieu 2006). The existence of a large number of viruses and mixed infections with distinct viruses and viral isolates pose great challenges to diagnosis, detection, and confirmation of the disease-causal pathogens and effective management of viral diseases of grapevines (Rowhani et al. 2005).

A combination of viral variants of distinct sequences and virus species belonging to different genera greatly enhances the genetic complexity of the grapevine virus population in cultivated grapevines (Meng et al. 2006; Prosser et al. 2007). For instance, mixed infections of genetically diverse GFLV variants were reported in naturally infected grape varieties in California (Naraghi-Arani et al. 2001), France (Vigne et al. 2004), Tunisia (Fattouch et al. 2005), and Iran (Bashir et al. 2007). GRSPaV also exists as either complex populations that consist of two to four distinct viral variants in infected grapevine scion varieties or as a population of single viral variants with more sequence homogeneity in rootstock varieties (Meng et al. 2006). At cross-species levels, GRSPaV and GLRaV−1, −2, −3, and −5 were found in a single vine of the table grape var. Waltham Cross (Prosser et al. 2007). In another case, GLRaV-1, GVB, GRSPaV, and *Grapevine fleck virus* (GFkV) were all detected in the Chardonnay clone 75 (Komar et al. 2006). More recently, five different viruses were detected in a single vine of the table grape 'Kismis vankana' by enzyme-linked immuno-sorbent assay (ELISA) (Qiu, unpublished results). Previous surveys of commercial vineyards in Missouri also discovered that GLRaV-3, GFkV, and ToRSV were present in major grapevine cultivars (Kovacs et al. 2001; Milkus 2001; Milkus and Goodman 1999).

Many grapevine viruses spread from vine to vine or from alternative hosts to vine via insect and nematode vectors (Martelli 1993). They also spread from cultivated grapevine scions to rootstocks or vice-versa by grafting. Grafting of grapevine scions onto rootstocks is a routine practice in viticulture and provides a common means of introducing different viruses into a single grapevine. The transmission of grapevine viruses by insects, nematodes, and vegetative propagation as well as frequent national and international exchange of propagating materials significantly increase the diversity of grapevine viral populations (Golino 2000; Meng et al. 2006). Co-evolution of grapevine viruses with genetically diverse wild *Vitis* species may also provide complexity to the population structure of grapevine viruses (Golino 2000; Meng et al. 2006).

In 2004, a severe disease was discovered in the grape var. 'Chardonnay' (*Vitis vinifera*) in a commercial vineyard in Missouri. The characteristic symptom is the translucent vein-clearing on affected Chardonnay as well on grapevines Cabernet Franc, Baco Blanc, and hybrid LN-33 (Qiu et al. 2007). The causal pathogens were found to be graft-transmissible (Qiu et al. 2007). By applying reverse-transcription polymerase chain reaction (RT-PCR) using degenerate primers designed for detecting coat protein-encoding sequences of grapevine nepoviruses (Digiaro et al. 2007), we detected the presence of GFLV-specific sequences that share the highest identity with an Italian isolate, GFLV-SG11 (Qiu et al. 2007). However, we also detected GFLV in asymptomatic Chardonnay vines (Qiu et al. 2007). This raised the question of whether GFLV is the sole causal agent of the disease. The present study was conducted to determine whether other members of the *Nepovirus* genus as well as viruses belonging to different taxonomic groups are also present in the affected Chardonnay. Using virus-specific primers, we detected ToRSV and GRSPaV in addition to GFLV and discovered mixed infection of GRSPaV, ToRSV and GFLV in individual vines. The results suggested that mixed infections of these three viruses are associated with the vein-clearing complex on Chardonnay.

Materials and methods

Chardonnay grapevines

In 2004, diseased Chardonnay grapevines were found in a 0.8-h vineyard at Augusta, Missouri. Hardwood or green cuttings with three buds were collected from

Chardonnay grapevines with conspicuous vein-clearing symptoms. Vines were propagated and grown in Promix BX horticultural medium (Premier Horticulture, Inc., Quakertown, PA) in 3.785-l plastic pots under greenhouse conditions. For dsRNA analysis, cambium tissues were collected from hardwood canes obtained directly from the vineyard. Leaf samples for total RNA extraction were collected from symptomatic Chardonnay vines propagated and grown on their own roots in the greenhouse.

ELISA testing

Young leaves were collected for testing of nepoviruses in early spring. Leaf petiole and cambium tissues were sampled for detecting GLRaV-3 in the fall. The tissues were frozen in liquid nitrogen and homogenised in grapevine virus extraction buffer (0.1 M Tris-HCl buffer, pH 8.2, 1 l buffer containing 60.5 g Tris, 8.0 g sodium chloride, 20 g polyvinylpyrrolidone (40,000 MW), 10 g polyethylene glycerol (6,000 MW); 0.2 g sodium azide, 0.5 ml Tween-20) at a 1:5 ratio of tissue to buffer (w/v). Double antibody sandwich ELISA (DAS-ELISA) was performed for testing ToRSV, *Tobacco ringspot virus* (TRSV), *Arabis mosaic virus* (ArMV), and *Peach rosette mosaic virus* (PRMV) with a commercial ELISA testing kit (Agdia, Elkhart, Indiana), and for testing GLRaV-3 using the ELISA kit of Agritest (Tecnopolis, Italy). The reactions were scanned at 405 nm in the Labsystems Multiscan instrument (Labsystems Oy, Helsinki, Finland). The samples that had the absorbance value three times higher than the mean absorbance value of the negative and buffer controls were counted as positive.

RNA extraction, RT-PCR and cloning

Total RNA was extracted following the protocol as previously described (Fung et al. 2008). Briefly, 2 g leaf tissues were ground in 50 ml extraction buffer (2% hexadecyltrimethyl ammonium bromide, 2.5 M NaCl, 0.5 M Tris, 50 mM EDTA, 10% SDS, 5% β-marcaptoethanol, 3% polyvinyl poly-pyrrolidone), and dispensed into 2 ml centrifuge tubes for storage at −80°C. A total of 4 ml ground leaf tissue was centrifuged at 12,000*g* for 20 min at 4°C after thawing in a 45°C water bath for 10 min. Supernatant was extracted with an equal volume of chloroform, and followed by centrifugation at 12,000*g* for 15 min at 4°C. RNA was precipitated with 2 M LiCl for 2 h at −20°C, and collected by centrifugation at 12,000*g* for 30 min at 4°C. After the pellet was washed with 80% ethanol twice, RNA was dissolved in 20 µl RNase-free water. DNase treatment of total RNA and inactivation of DNase followed provider's instructions (Promega, Madison, Wisconsin). Subsequently, total RNA was purified by the RNeasy MinElute Cleanup Kit and eluted in 15 µl RNase-free water (Qiagen, Valencia, CA).

The dsRNA was isolated from cambium tissues scraped from infected hardwood canes of Chardonnay vines exhibiting severe symptoms following the protocol described previously (Meng et al. 1998). The synthesis of cDNA from total RNA or dsRNA was conducted with addition of random 50 µM hexamer primers by the Superscript™ III Reverse Transcriptase kit following the conditions as recommended by the manufacturer (Invitrogen, Carlsbad, California). The cDNA products were subjected to polymerase chain reaction (PCR) using the thermal cycling conditions as follows. Primers used in RT-PCR are listed in Table 1. For GFLV, PCR was performed using primers 433 V and 912C with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 58°C for 45 s, and extension at 72°C for 50 s, and a final extension at 72°C for 7 min. For ToRSV, nested PCR was used. In the primary PCR, cDNA templates were first denatured at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 56°C for 45 s, and extension at 72°C for 60 s, and a final extension at 72°C for 7 min. For the second round amplification, PCR products diluted at 1:50 from the primary PCR were first denatured at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s, and a final extension step at 72°C for 7 min. For GRSPaV, cDNAs were amplified using the following conditions: an initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 35 s, annealing at 54°C for 45 s, extension at 72°C for 50 s, followed by a final extension at 72°C for 7 min. The PCR products were cloned into plasmid vectors using the Zero-Blunt TOPO PCR cloning kit (Invitrogen) or the pGEM-T Easy vector (Promega, Madison, Wisconsin) following the manufacturer's instructions.

Table 1 Primers used in this study to specifically detect *Grapevine fanleaf virus* (GFLV), *Tomato ringspot virus* (ToRSV), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus* A (GVA), *Grapevine virus* B (GVB), *Grapevine fleck virus* (GFkV), and *Grapevine leafroll-associated virus* 3 (GLRaV-3)

Primer pair	Sequences (5′ to 3′)	Viruses	Genomic regions and PCR products	Reference
GFLV-F GFLV-R	CTGCAGGATTAGTCATGCCTAC GTCCACTTCACATGAAAACGTG	GFLV	Coat protein (203 bp)[1]	This study
GFLV-433 V GFLV-912C	GAACTGGCAAGCTGTCGTAGAAC GCTCATGTCTCTCTGACTTTGACC	GFLV	Coat protein (480 bp)	Izadpanah et al. (2003)
CP71V CP620C	GAGGAACGCTCTTGCACACTCT GGCAACGGATTGGCACTTAACTCA	ToRSV	Coat protein (550 bp)	Rowhani (personal communication)
CP6698 CP6697	GTAAGAGTATGAGTCTCCTAAGGTACAAG ATCAGAGAGACTGATAACATCAGTT	ToRSV	Coat protein (221 bp)	This study
RSP21 RSP22	GAGGATTATAGAGAATGCAC GCACTCTCATCTGTGACTCC	GRSPaV	Coat protein (441 bp)	Meng et al. (2003)
RSP35 RSP36	AGRYTTAGRGTRGCTAARGC CACATRTCATGVCCYGCAAA	GRSPaV	RdRP (476 bp)	This study
GVA-1 GVA-2	ATGGCACACTACGCCAAG AGTGCATAGCCTGTATCAC	GVA	Coat protein (517 bp)	This study
GVB-1 GVB-2	ATGGAAAATATATCCCGGATG GGCTTGTGCTGTGAAGACG	GVB	Coat protein (566 bp)	This study
GFkV-1 GFkV-2	CAGGTTGTAGTCGGTGTTGTC GTCCTCGGCCCAGTGAAAAAG	GFkV	Replicase (416 bp)	This study
GLRaV3-CP3D GLRaV3-CP3U	CGGCGCCCATAACCTTCTTACA ATGGCATTTGAACTGAAATTAGGGC	GLRaV-3	Coat protein (484 bp)	Turturo et al. (2005)

[1] Length in base-pairs of DNA fragments amplified in the reverse transcription polymerase chain reaction is given in parenthesis

Sequencing and sequence analysis

Recombinant clones were selected through PCR quick screen, purified using the plasmid miniprep kit (Qiagen, Valencia, CA) and sequenced at the College of Biological Science Genome Sequencing Facility, the University of Guelph, Guelph, Canada, or at the Nevada Genomic Centre, Reno, Nevada, USA. Sequences were analysed and compared to each other and to the corresponding regions of reference isolates using the MegAlign programme (DNAStar, Madison, Wisconsin).

Results

Vein-clearing is the diagnostic symptom

Symptoms of the disease on Chardonnay in the commercial vineyard and on three indicator grapevines *V. vinifera* ‘Cabernet Franc’, *V. vinifera* ‘Baco Blanc’ and hybrid ‘LN-33’ were described in detail in a previous publication (Qiu et al. 2007), and also are shown in Fig. 1. In the spring of 2005, hardwood cuttings of the symptomatic Chardonnay vines were propagated and grown in the greenhouse. Young leaves of new shoots consistently exhibited vein-clearing symptoms in the spring and early summer over the last three years (Fig. 1). Vascular and surrounding tissues along major and minor veins were translucent and chlorotic when symptomatic leaves were held against sunlight (Fig. 1a). Vein-clearing persisted through the development stages of the leaf and changed to vein chlorosis or severe mosaic on the fully expanded leaves (Fig. 1b). Mature shoots showed a zigzag pattern of growth with short internodes. There were variations of symptoms from vine to vine and at different stages of leaf development. Since conspicuous vein-clearing consistently appeared on originally diseased and bud-grafted Chardonnay vines as well as on

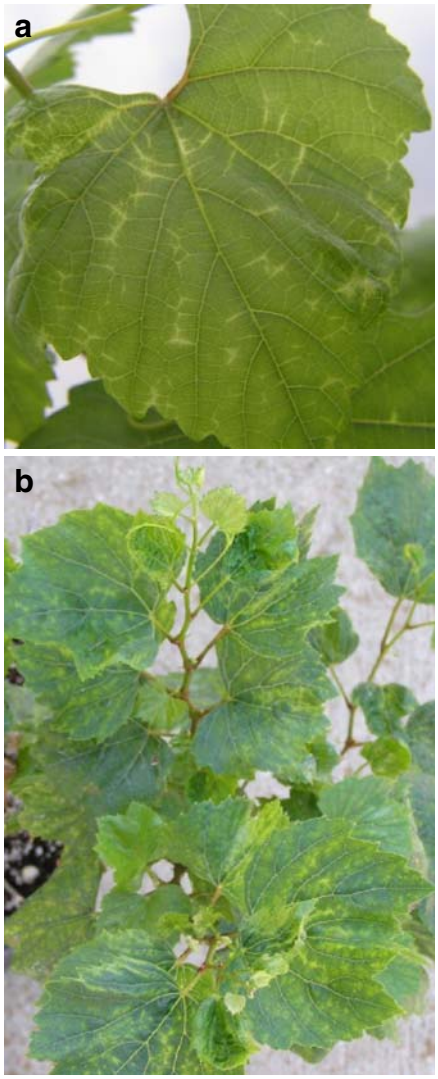

Fig. 1 Characteristic symptoms of grapevine vein-clearing complex (GVCC) on Chardonnay vines grown in potted soils under greenhouse conditions. **a**. Typical translucent vein-clearing symptom appeared on leaves of the newly emerged shoots of the GVCC-affected Chardonnay vine; **b**. Leaves of the mature and woody vine show severe mosaic and chlorotic symptoms and short internodes with a zigzag pattern

indicator grapevines 'Cabernet Franc', 'Baco Blanc' and hybrid 'LN-33' , we refer to this disease here as 'grapevine vein-clearing complex' (GVCC).

ToRSV and GFLV were detected in GVCC-affected Chardonnay grapevines

Symptoms of GVCC-affected leaves were reminiscent of those induced by infection of different nepoviruses. We expected that some of these viruses would be present in the diseased Chardonnay vines. Therefore, ELISA was applied to detect four nepoviruses: ToRSV, TRSV, ArMV, and PRMV in the beginning of this investigation. As shown previously, GLRaV-3, the type member of the genus *Ampelovirus* within the family *Closteroviridae,* is widely distributed in Missouri vineyards (Kovacs et al. 2001; Milkus 2001; Milkus and Goodman 1999). To test if GLRaV-3 is present in the affected Chardonnay plants, we also included GLRaV-3 in the ELISA tests. To our surprise, none of the above viruses was detected by ELISA in the GVCC-affected Chardonnay vines (data not shown).

It is not unusual to obtain false negative results by ELISA in woody plants due to low virus concentration and uneven distribution in different tissues. Therefore, we applied RT-PCR using virus-specific primers to determine if grapevine viruses that are widely distributed in Missouri vineyards were associated with GVCC. RT-PCR analysis using degenerate primers designed for discovering nepoviruses (Digiaro et al. 2007) detected GFLV sequence in these Chardonnay plants (Qiu et al. 2007). To confirm these preliminary data, RT-PCR was performed on samples of original GVCC-affected Chardonnay vines and of Chardonnay vines grafted with buds from original diseased Chardonnay vines using GFLV-specific primers (Table 1). The expected amplification product of 203 bp was obtained. To verify the identity of DNA fragments, the 203 bp PCR-amplified products were isolated and cloned into plasmid vectors. Three individual clones containing the 203 bp DNA fragment amplified from original and bud-grafted Chardonnay vines were sequenced (GenBank accession numbers: FJ577801, FJ577802, FJ577803, FJ577804). It was found that GFLV sequence derived from the GVCC-affected Chardonnay vines is 99% identical to GFLV-SG11 (GenBank accession number: DQ362924), an Italian isolate. To investigate if ToRSV was also associated with GVCC, RT-PCR was conducted using a pair of primers CP71V and CP620C designed to be specific for the ToRSV coat protein (CP) sequence (Table 1). The DNA fragments of the expected 550 bp were detected in leaf samples of both the original GVCC-affected Chardonnay vines and the Cabernet Franc vines grafted with buds of original GVCC-affected Chardonnay vines. One larger and two smaller bands were

also present (Fig. 2a), possibly resulting from non-specific amplification. To verify the identity of the bands, the 550 bp DNA fragments were isolated from the agarose gel, cloned and sequenced (GenBank accession numbers: FJ577795, FJ577796, FJ577797, FJ577798, FJ577799, FJ577800). We found that the 550 bp DNA fragment from GVCC-affected Chardonnay and Cabernet Franc shared 99% identical nucleotide sequences with the corresponding region of the yellow vein strain of ToRSV (GenBank accession number: AF135411). The 550 bp DNA fragments were then used as templates in a second round PCR with another primer pair (CP6698 and CP6697; Table 1), designed to amplify a 221 bp fragment nested within the 550 bp DNA fragment. As expected, the 221 bp DNA fragment was detected (Fig. 2b). This 221 bp DNA fragment was also isolated from the gel and sequenced, and shared 100% identity with the sequences derived from the 550 bp fragment.

We also tested if GRSPaV, GVA, GVB, and *Grapevine fleck virus* (GFkV) were present in GVCC-affected Chardonnay vines by using either virus-specific primers or by using degenerate primers for closteroviruses targeting the conserved HSP70 region (Prosser et al. 2007; Tian et al. 1996) (Table 1). We detected GRSPaV in the GVCC-affected Chardonnay vines, but did not detect GVA, GVB, and GFkV by RT-PCR analysis.

Two strains of GRSPaV were detected in GVCC-affected Chardonnay grapevines

Using broad-spectrum primers RSP21 and RSP22 targeting the CP gene of GRSPaV (Meng et al. 2003), the expected 441 bp DNA fragment was consistently detected in GVCC-infected Chardonnay vines using dsRNAs isolated from the cambium scrapings (data not shown). The same-sized DNA fragment was also detected from total RNAs isolated from leaves of Chardonnay vines. To determine the identity of GRSPaV viruses detected in Chardonnay vines, the resulting PCR products were cloned and five clones were sequenced (GenBank accession numbers: FJ577805, FJ577806. FJ577807, FJ577808, FJ577809). Resulting sequences were compared to the corresponding regions of the GRSPaV CP genes derived from the four reference strains to which genome sequences are available in GenBank (Lima et al. 2006; Meng et al. 2005, 1998; Zhang et al.

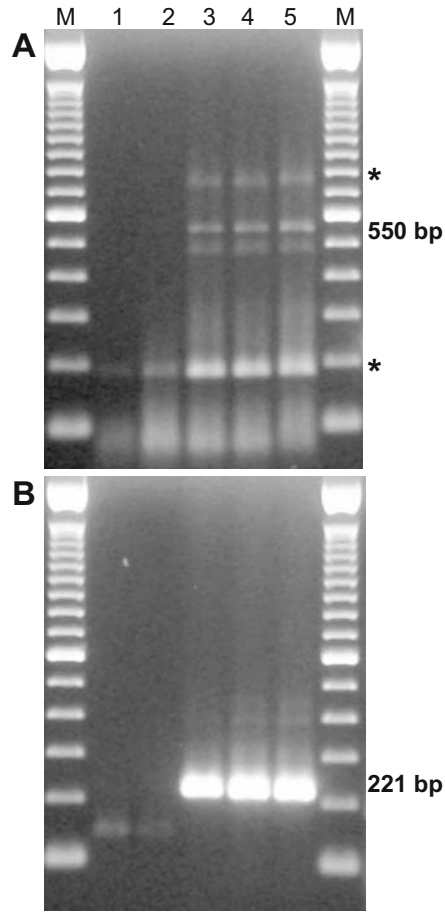

Fig. 2 Results of reverse-transcription polymerase chain reaction (RT-PCR) assay of *Tomato ringspot virus* (ToRSV) coat protein (CP) gene-specific fragment in grapevines. Top panel: DNA products (expected size of 550 bp) of RT-PCR using primer pair CP71V and CP620C. Bottom panel: RT-PCR amplified DNA products with expected 221 bp with primer pair CP6698 and CP6697 nested within the 550 bp fragment. Lane 1, negative control without cDNA template; lane 2, negative control using 2 µl of the reverse transcription reaction mix lacking reverse transcriptase (to rule out possibility that non-specific DNA could be amplified from residual genomic DNA in total RNA); lane 3, leaf sample of originally infected Chardonnay vine; lane 4, leaf sample of Cabernet Franc vine grafted with two buds of originally infected Chardonnay vines; lane 5, positive control using RNA of ToRSV-infected *Chenopodium quinoa* as template. The marker (M) is a 100 bp DNA ladder (Invitrogen). An asterisk indicates amplified DNA products that are not virus-specific. PCR-amplified DNA fragments were separated by electrophoresis on 2% agarose gel. Note: non-specific DNA fragments were also amplified, but sequencing of 550 bp fragments confirmed the CP gene specificity of ToRSV

1998). The five clones grouped into the two clusters. The first cluster was composed of three clones (GVCC-VC−5, −7 and −8) that had the highest sequence identity (96.4%) to the reference strain GRSPaV-SG1 (GenBank accession number: AY881626). The second cluster consisted of two clones (GVCC-VC-2 and −6) more closely related to the reference strain GRSPaV-BS (GenBank accession number: AY881627), with 90% nucleotide sequence identity. The sequence identity of GRSPaV CP genes in the sequenced portion between the two clusters was 84%.

GFLV, ToRSV and GRSPaV were detected in GVCC-affected Chardonnay grapevines

Initially, we detected GFLV, ToRSV, and GRSPaV in the GVCC-affected Chardonnay by using composite samples from a collection of diseased vines in the vineyard (Qiu et al. 2007) (Fig. 2). To investigate if GFLV, ToRSV, and GRSPaV were simultaneously present in individual vines and a possible correlation of different combinations of viruses with symptom variability, we sampled young leaf tissue from eleven individual Chardonnay vines with vein clearing symptoms and performed RT-PCR using CP gene-specific primers for each virus (Table 1). GRSPaV was detected in the eleven vines tested, GFLV in six vines, and ToRSV in three vines (Fig. 3). Three viruses were co-present in two individual vines. GFLV was detected in a Chardonnay vine with no conspicuous viral symptoms (Fig. 3), also found in a non-symptomatic Chardonnay vine in a previous study (Qiu et al. 2007), suggesting a latent infection of GFLV in this asymptomatic Chardonnay vine.

Discussion

A severe disease was discovered on Chardonnay vines in a 10 year-old commercial vineyard in Missouri. The fruit yield and vine vigour were reduced significantly (Qiu et al. 2007). Distinctive symptoms included translucent vein-clearing, chlorosis, and mosaic on the leaves (Fig. 1). The plants had short internodes and a zigzag pattern of shoot growth (Fig. 1b). This disease was given the provisional name ‘grapevine vein-clearing complex (GVCC)’. The chrome-yellow symptoms of GVCC observed on these Chardonnay grapevines were similar to those of the grape vein-banding disease originally described on grapevines of *V. vinifera* and rootstocks in 1962 (Goheen and Hewitt 1962), later shown to be caused by the vein banding strain of GFLV (Hewitt and Bovey 1979). Therefore, we do not consider GVCC as a new disease, rather a composite disease caused by a combination of different etiological agents and exhibits a range of variations in the intensity of symptoms including vein-clearing.

Results from this study demonstrated the presence of GFLV, ToRSV, and GRSPaV in the GVCC-affected Chardonnay vines and also in the GCVV-grafted Chardonnay and Cabernet Franc (Figs. 2 and 3), suggesting the association of these viruses with a spectrum of vein-clearing symptoms. The origins of GFLV, ToRSV, and GRSPaV were unknown. The Chardonnay vines were bought from two nurseries in California and Oregon. Therefore, one possibility would be that these viruses may have been introduced into Missouri along with the purchased propagation materials.

Based on the bio-indexing results on three indicator grapevines, grapevine leafroll-associated viruses are unlikely to be associated with GVCC since leaf-rolling and interveinal red tissues were not observed on Cabernet Franc that is a sensitive indicator for grapevine leafroll disease. Furthermore, we did not detect GVA, GVB, GFkV, and closteroviruses by RT-PCR. ELISA tests conducted by a commercial testing service (Agdia, Elkhart, Indiana) did not detect other nepoviruses including TRSV, ArMV, and PRMV (Qiu et al. 2007). In our previous report, ToRSV was not detected by ELISA or by RT-PCR (Qiu et al. 2007), but we were able to detect ToRSV using two sets of virus-specific primers in the nested PCR in this study (Figs. 2 and 3). To verify that ToRSV was present, the DNA fragments of the expected size amplified through RT-PCR were isolated from the agarose gels and sequenced. Sequence analyses confirmed the identity of a virus CP gene-specific fragment. This suggests that primer specificity and the sensitivity of nested PCR influence the detection results, commonly observed in the detection of grapevine viruses, especially nepoviruses (Rowhani et al. 2005).

It is well documented that GRSPaV is composed of a family of sequence variants which differ in nucleotide sequence by up to 25% (Meng et al. 1999, 2006; Nolasco et al. 2006). It is also common

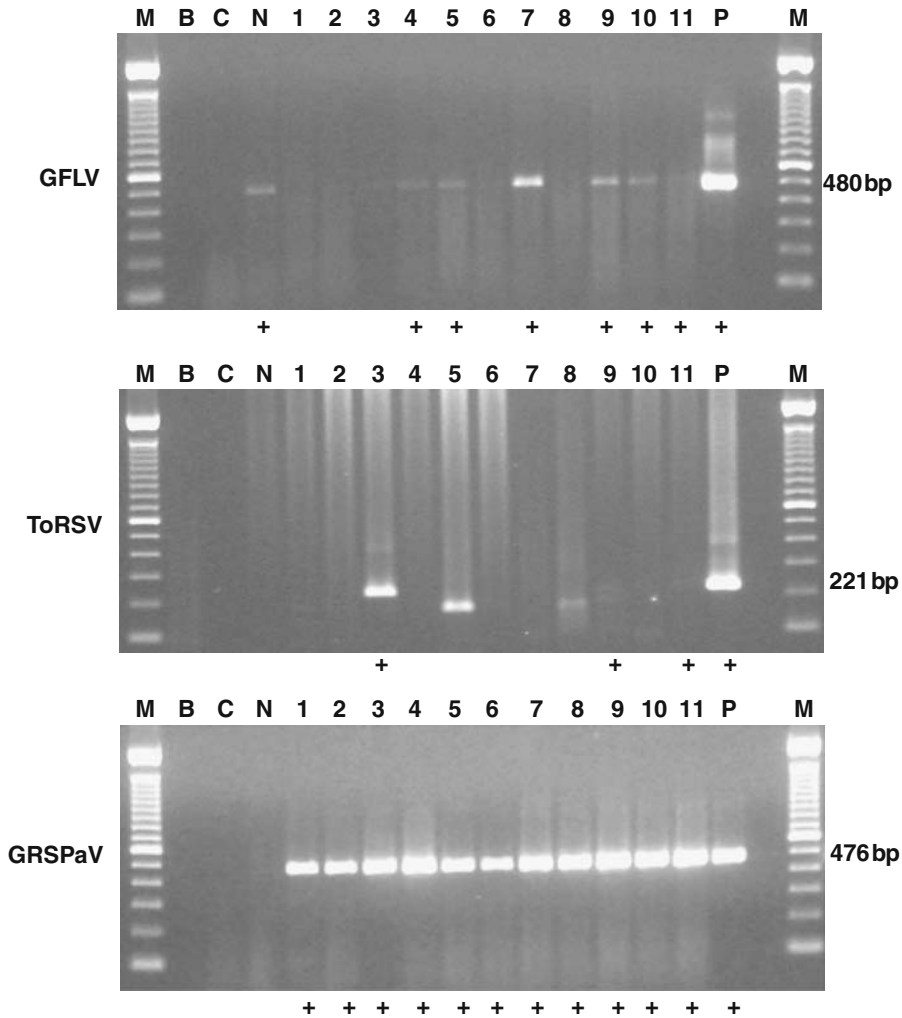

Fig. 3 Detection of *Grapevine fanleaf virus* (GFLV), *Tomato ringspot virus* (ToRSV), and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) in eleven individual vines by reverse-transcription polymerase chain reactions (RT-PCR). The specific virus to be detected and the expected size of the RT-PCR product are indicated on the left and right side of each panel, respectively. Lane B, negative control without DNA template; lane C, negative control with 2 µl of reverse transcription reaction mix lacking reverse transcriptase to rule out possibility that non-specific DNA could be amplified from residual genomic DNA in total RNA; lane N, RNA of asymptomatic Chardonnay vine as negative control; lanes 1 to 11, eleven individual Chardonnay vines; lane P, virus-specific DNA band as positive control. The marker (M) is a 100 bp DNA molecule marker (Invitrogen). PCR-amplified DNA fragments were separated on 1.2% agarose gel by electrophoresis. Presence of each virus as indicated by the '+' sign was determined by visibility of DNA bands on original agarose gel

to detect more than one viral variant in a single grapevine in field samples (Meng et al. 1999, 2006). To date, the genomes of four distinct strains of GRSPaV have been sequenced (Lima et al. 2006; Meng et al. 2005, 1998; Zhang et al. 1998). It has been demonstrated that infection with strain GRSPaV-SG1 (AY881626) is asymptomatic while infection with strain GRSPaV-1 (NC_001948) causes only mild symptoms in *V. rupestris* (Meng et al. 2005). Strain GRSPaV-SY (AY368590) is associated with the decline syndrome of Syrah grape in California (Lima et al. 2006). Although the pathogenicity of strain GRSPaV-BS (AY881627) is yet to be investigated, this strain can become severe in the mixed infection with other grapevine viruses. Two distinct strains of GRSPaV were detected in the GVCC-affected Chardonnay vines, one was closely related to strain GRSPaV-SG1 while the other to strain GRSPaV-BS.

The presence of two GRSPaV strains suggests different sources of original Chardonnay grapevines.

Populations of genetically diverse grapevine virus species and variants exist broadly in vineyards worldwide (Martelli and Boudon-Padieu 2006). It is not surprising that three distinctive viruses are associated with GVCC. It has been reported that mixed infections with more than one grapevine virus species or strains frequently augmented the severity of disease and intensified symptoms (Credi 1997). Our results from RT-PCR assay of eleven individual vines showed that GFLV, ToRSV and GRSPaV were present alone or in combination in individual vines (Fig. 3). It is possible that the appearance of severe symptoms on infected Chardonnay vines as well as the reduction of vigour and yield is a result of synergistic interactions among these three identified viral pathogens and perhaps other unidentified agents under Missouri climatic and soil conditions.

Infection with different viruses either produces visible symptoms or remains symptomless in grapevine varieties and rootstocks. Symptomless scions often develop symptoms once they were grafted onto a different rootstock and planted in commercial vineyards at a different location, as in the case of the *Grapevine leafroll-associated virus 2* Redglobe strain (Rowhani et al. 2005). Frequent exchanges of propagation grapevine materials and grafting of various combinations of scions and rootstocks create ample opportunities for different viruses and viral isolates to merge into a single vine. Afterwards, a viral complex would perpetuate in the progeny vines via large-scale vegetative propagation. This study provides another example of mixed infection and supports the imperative need to develop a network of virus-tested grapevines for national and international exchange of propagation grapevine materials (Golino and Savino 2008). Growing certified grapevines can reduce the incidence of mixed infections and thus prevent new disease outbreaks in grape-growing regions with diverse climatic, environmental and soil conditions.

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