RT-PCR Based Detection of *Rupestris stem pitting associated virus* Within Field-Grown Grapevines Throughout the Year

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ABSTRACT

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The presence of *Rupestris stem pitting associated virus* (RSPaV) can go unnoticed since symptoms appear only if additional viruses are present. Detection by reverse transcription-polymerase chain reaction (RT-PCR) is possible; however, this assay could be unreliable if the tissue that is being tested has detection-interfering compounds, or if the virus has a low titer. This paper reports on (i) use of a recently developed extraction method and internal control to determine which tissues from field-grown grapevines yield extracts that are reliable for virus detection by RT-PCR, and (ii) a survey for RSPaV of different tissues from the *Vitis vinifera* varieties Riesling, Chardonnay, Cabernet Franc, Merlot, Sauvignon Blanc, Pinot Noir, and Gamay, as well as from the rootstocks 3309 and Riparia, which were harvested in Ontario, Canada, at different times of the year. Amplifiable extracts were obtained from virtually all bud, shoot tip, seed, and cane samples tested. Detectable amounts of RSPaV were generally found in all tissues of infected plants except young buds collected in the summer. A combination of three single buds from dormant canes, less time-consuming than the preparation of cane shavings, was a reliable source for RSPaV detection.

Additional keywords: *Rupestris stem pitting*, virus distribution

Rupestris stem pitting, corky bark, Kober stem grooving, and LN33 stem grooving, collectively referred to as Rugose wood, are a group of graft-transmitted diseases of grapevines characterized by distortions of the woody trunk which can be found in all viticultural regions of the world (5). These diseases are responsible for graft incompatibility problems, delayed budburst, severe decline, and even death of vines (2,8). A combination of viruses and possibly phytoplasmas is thought to be the causative agent for the Rugose wood diseases (2). However, exactly which combinations of viruses and phytoplasmas are present in diseased plants, and which combinations of those pathogens cause which symptoms still need to be determined.

Rupestris stem pitting (RSP) appears to be the most widespread disease of the Rugose wood complex (10). *Rupestris stem pitting associated virus* (RSPaV), a foveavirus, is consistently detected in RSP-infected grapevines (9,22); however, RSPaV alone generally does not produce any symptoms and has no major impact on grapevine growth and yield (2,15). The presence of another virus, such as *Grape-*

vine virus A (GVA), *Grapevine fleck virus*, or *Grapevine leafroll associated virus-1*, in addition to RSPaV, is required for Rugose wood-type symptoms to occur (2). These viruses can be transmitted from infected vines to an RSPaV-infected vine via grafting (2,8) or, at least for GVA, via the vine mealybug *Planococcus ficus* (3,11). This means that cultivating symptomless but RSPaV-infected vines carries a risk for the development of RSP, and it is therefore of interest to know if a vine contains RSPaV.

The presence of RSPaV can be determined by reverse transcription-polymerase chain reaction (RT-PCR) using primers based on its genomic sequence (9,22) with apparent greater reliability than biological indexing on St. George (9). It is not known if the RT-PCR assay is reliable for all tissues and at all times of the year. Grapevines contain high amounts of compounds such as phenolics and polysaccharides, believed to interfere with RT-PCR (11,16,17,21), and it is likely that these amounts differ among different tissues at different times of the year. Also, several grapevine viruses appear to have an irregular distribution and/or low virus titer at certain times of the year (11–13,17,19,20), and such may also be the case for RSPaV. As a result, it is possible that its presence is not detected even though a plant is infected.

Several methods have been developed in an attempt to deal with inhibitors of RT-PCR assays (7,11,18). We reported on an improved RNA extraction method to minimize inhibitors of reverse transcriptase

and *Taq* polymerase, as well as controls that can identify plant extracts that contain such inhibitors and thus cannot be amplified by RT-PCR (14). In this study, we use the extraction method and control to survey extracts from grapevine tissues harvested from RSPaV-infected plants at different times of the year. Tissues shown to be amplifiable are tested for the presence of virus using a one-step RT-PCR assay, to determine its distribution.

MATERIALS AND METHODS

Plant material. RSPaV-infected samples came from six plants each of the *Vitis vinifera* varieties Riesling, Chardonnay, Cabernet Franc, Merlot, Sauvignon Blanc, Pinot Noir, and Gamay, as well as from the rootstocks 3309 and Riparia growing in fields in Niagara Peninsula, Ontario, Canada.

Buds, shoot tips, seeds, or cane samples were collected at different times in 1999 from six infected but symptomless plants of each variety, immediately put on ice for transport, and then stored at -80°C until they were extracted. Spring samples consisted of single buds from stages C (opening bud), D (leaf emergence), E (two to three leaves unfolded), and/or F (inflorescences visible), and the last two nodes from stages G (inflorescences separated) or H (flowers separated) (stages according to M. Baggiolini [1]). The spring stages collected from each plant depended on what stages were available at the collection date. Summer samples consisted of single buds from woody canes, single buds from green canes, and single shoot tips. Fall samples consisted of three or more seeds from one berry. The rootstocks Riparia and 3309 do not produce any seed, hence none were collected. Prior to storage at -80°C , seeds were cleaned with water to remove pulp. Some seeds were additionally treated with a 3.5% sodium hypochlorite solution for 5 min and then rinsed in water for 5 min. Winter samples were cane shavings and a single bud from each of three dormant canes. Cane shavings were obtained using a mechanical pencil sharpener (4).

Preparation of total RNA extract. Total RNA was extracted from samples according to a procedure modified from MacKenzie et al. (7), as described previously (14). Briefly, approximately 0.5 g of tissue was placed in a sample bag (Agdia Inc., Elkhart, IN) with 5 ml of lysis buffer composed of 4 M guanidium isothiocyanate, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 6% PVP-40, and 1% 2-

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mercaptoethanol (added just prior to use), and homogenized with a hand-held homogenizer (BIOREBA, South Bend, IN). Single buds, some weighing as little as 0.009 g, were extracted with a minimum of 2 ml of lysis buffer. A 1-ml aliquot of the lysate was further processed with a Qiagen RNeasy plant extraction kit according to the instructions outlined by the manufacturer.

The obtained total RNA extracts (100 µl) were stored in small aliquots at -80°C (stock sample) and at -20°C (working sample). Samples were thawed and refrozen no more than four times to minimize possible RNA degradation.

RT-PCR amplification. Target sequences were detected using target-specific primers (Table 1) in a single one-tube RT-PCR reaction as described in Nassuth et al. (14). The RT-PCR mixture (final volume of 25 µl) contained 2.5 µl total RNA extract, 10 mM Tris-HCl, pH 8.3, 100 mM KCl, 1.5 mM MgCl₂, 200 µM Mg balanced dNTPs, 5 mM dithiothreitol, 2% (wt/vol) sucrose, 0.1 mM cresol red (Sigma, Oakville, ON), 0.1 units AMV RTase (reverse transcriptase, Boehringer Mannheim/

Roche, Laval, QC), 2 units *Taq* polymerase (Gibco/BRL, Burlington, ON, or Fermentas, Burlington, ON), and 0.5 µM (unless otherwise stated) of both the complementary and homologous primers specific for the target sequence(s). Amplification was carried out in a thermocycler (Perkin Elmer 2400 or MJ Research PTC100HB-96V) using one 45-min step at 54°C (reverse transcription), followed by 35 cycles of the following: 94°C for 30 s, 54°C for 45 s, and 72°C for 60 s, with a final elongation step at 72°C for 5 min. Amplified products were stored at 4°C until analysis.

All combinations and concentrations of primer pairs used to detect the desired sequences were determined previously (14). Spring, summer, fall, and winter samples were first tested with a combination of primer pairs RubiscoL (control) and RSPaV (0.025 µM/0.5 µM) or malate dehydrogenase (MDH) (control), RSPaV and ArMV (0.1 µM/0.5 µM/0.6 µM). Samples initially testing positive for the control(s) but negative for RSPaV were retested with only the RSPaV primer pair to verify the result. We tested all samples also with a

combination of primer pairs GVA/GVB (0.5 µM/0.5 µM).

Analysis of amplified product. Aliquots (10 µl) of the PCR products were analyzed on a 1.5% agarose gel in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA) followed by staining with ethidium bromide (0.5 µg/ml). DNA fragments were visualized with a UV transilluminator, photographed with an IS-1000 digital imaging system (Alpha Innotech Corp., CA), and printed using a Seikosha VP-1500 II printer. DNA molecular weight markers (Fermentas) were used to determine the size of the amplified fragments.

RESULTS

Amplifiability of total RNA extracts. RubiscoL and/or MDH primer pairs amplified fragments of the expected size to clearly detectable amounts in essentially all total RNA extracts. Only one extract was nonamplifiable and thus discarded, and the remaining 419 extracts could be reliably tested for the chosen grapevine viruses.

Virus detection. RSPaV was detected in most extracts from RSPaV-infected plants (Table 2). Gel electrophoretic analysis showed fragments with sizes expected for amplification on Rubisco RNA or DNA (171 bp) and RSPaV RNA (355 bp) (data not shown) or MDH RNA (196 bp) and RSPaV RNA (355 bp), and larger fragments which represent MDH sequence amplified on DNA present in the extracts (14) (Fig. 1).

RSPaV could be detected at every stage in the spring; however, the virus was not detected in all spring samples. It was also generally detected in summer tissues except in buds collected from green canes. All seeds, untreated and bleach-treated, and dormant cane tissue contained detectable amounts of RSPaV. When three dormant buds per plant were tested individually, all three tested RSPaV-positive for all but one plant, for which two buds were positive (Table 3).

No plant extracts tested positive for *Arabis mosaic virus* (ArMV), GVA, or *Grapevine virus B* (GVB).

Table 1. DNA primers employed for reverse transcription-polymerase chain reaction (RT-PCR) amplification of grapevine viruses and control mRNA sequences

Target sequence	Primer name	Primer sequence (5' to 3')	Product size (bp)	Ref.
MDH ^a	MDH-H968	GCATCTGTGGTTCTTCGAGG	196	14
	MDH-C1163	CCTTTGAGTCCACAAGCCAA		
RubiscoL	RBCL-H535	CTTTCCAAGGCCCGCCTCA	171	14
	RBCL-C705	CATCATCTTTGGTAAATCAAGTCCA		
RSPaV ^b	RSP-H5638	AGGGATTGGCTGTTAGATGTT	355	6
	RSP-C5992	CTTCAGGCAACCCCAAAAA		
ArMV	ARMV-H428	GCGGCGGATTGGGAGTT	440	6
	ARMV-C867	CGATGGTAGGGGAGCGTATT		
GVA	GVA-H7038	AGGTCCACGTTTGCTAAG	236	6
	GVA-C7273	CATCGTCTGAGGTTTCTACTAT		
GVB	GVB-H6980	GTGCTAAGAACGTCTTCACAGC	460	6, 11
	GVB-C7439	ATCAGCAAACACGCTTGAACCG		

^a Controls: MDH, malate dehydrogenase, primer names based on *Vitis vinifera* MDH gb L34836; RubiscoL, large subunit of Ribulose biphosphate carboxylase oxygenase, primer names based on Rubisco sequence for *Triticum* (dbj D00206).

^b Viruses: RSPaV, *Rupestris stem pitting associated virus*, primer names based on RSPaV gb AF026278; ArMV, *Arabis mosaic virus*, primer names based on ArMV emb X55460; GVA, *Grapevine virus A*, primer names based on GVA emb X75433; GVB, *Grapevine virus B*, primer names based on GVB emb X75448.

Table 2. One-tube reverse transcription-polymerase chain reaction (RT-PCR) detection of *Rupestris stem pitting associated virus* (RSPaV) in samples collected at different times of the year from RSPaV-infected field-grown *Vitis vinifera* varieties and rootstocks

Variety	Spring			Summer			Fall Seeds	Winter Dormant canes
	Opening bud to leaf emergence	2-3 leaves unfolded to inflorescences visible	Inflorescences separated to flowers separated	Shoot tips	Buds from woody canes	Buds from green canes		
Cabernet Franc	1/1 ^a	3/3	8/8	6/6	5/6	5/6	6/6	6/6
Chardonnay	6/6	25/25	9/10	5/8	6/6	4/6	6/6	6/6
Gamay		8/8		6/6	6/6	6/6	6/6	6/6
Merlot	4/4	7/7	6/6	6/6	6/6	2/6	6/6	6/6
Pinot Noir		7/7	3/3	6/6	6/6	6/6	6/6	6/6
Riesling	2/2	10/11	2/5	6/6	6/6	4/6	6/6	6/6
Sauvignon Blanc	3/3	10/10		5/5	6/6	6/6	6/6	6/6
Riparia		4/4	6/6	4/4	4/4	2/4		4/4
3309		4/4	8/8	6/6	6/6	3/6		6/6
Total	16/16	78/79	42/46	50/53	51/52	38/52	42/42	52/52

^a RSPaV positive samples/total number of samples; blank spaces represent no sample tested.

DISCUSSION

The amplification of the internal controls to easily detectable amounts shows that this method for extracting total RNA is effective at reducing inhibitors in all grapevine tissues and at all times of the year, allowing our extracts to be amplified and tested for the presence of virus. A negative result would thus indicate that the amount of virus in the sample is low or nil.

RSPaV has been characterized only recently (10,22) and there are no reports on the distribution of this virus within infected plants at different times of the year. Our results indicate that RSPaV is present in nearly all tissues of infected plants in amounts high enough for detection by the RT-PCR procedure employed. RSPaV was

not detected in many of the buds collected in the summer from green canes, but it was detected in buds from woody canes collected at the same time. We suspect that since the buds on green canes are still young, the virus had not yet multiplied in them and thus the titer was too low to detect. There were no differences in RSPaV detection among the seven surveyed varieties or rootstocks 3309 and Riparia.

The fact that we did not have any problems finding RSPaV-infected vines in the Niagara region supports the claim that RSPaV is widespread (2,9). Since RSPaV can cause RSP symptoms if present in combination with another virus, we also tested for the presence of ArMV, GVB, and in particular GVA in cane samples, which were shown to be a reliable source for their detection (14). None of these viruses were detected.

The finding that all seeds tested positive for RSPaV, even after bleach treatment, suggests that virus is present inside the seed, even though RSPaV is not known to be seedborne (22). This has negligible importance for the industry since propagation of grapevines is via grafting.

Once grapevines become infected, RSPaV cannot be cured in the field. Control of the virus thus depends on the ability to detect and eliminate infected plants from nursery material, propagation stock, and imported material. For this to be successful, the most efficient sampling strategy to deal with fluctuations in virus titer and uneven distribution of virus within the plant has to be applied. Our results show that RT-PCR on dormant and actively growing tissue, but not on buds from green canes collected in the summer, can provide reliable data. Dormant canes must first be

ground into shavings before the nucleic acids can be extracted. Fortunately, this time-consuming task can be avoided by using dormant buds instead. We suggest using at least three samples to account for the uneven distribution of virus within a plant.

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Fig. 1. Agarose gel analysis of multiplex reverse transcription-polymerase chain reaction (RT-PCR) assays with the primers for malate dehydrogenase (MDH), *Rupestris stem pitting associated virus* (RSPaV), and *Arabis mosaic virus* (ArMV) on total RNA extracts from: lanes 1 (positive control), 4, and 5, dormant cane; lane 2, bud from woody cane; lane 3, seed; and lane 6, water (control). Lane M, molecular weight marker. Lanes with RSPaV-negative extracts are not shown.

Table 3. Distribution of *Rupestris stem pitting associated virus* (RSPaV) in individual infected grapevines in which at least one sample tested negative by one-tube reverse transcription-polymerase chain reaction (RT-PCR)

Variety	Spring													Summer				Winter			
	Opening bud to leaf emergence		2-3 leaves unfolded to inflorescences visible		Inflorescences separated to flower-seeds separated		Shoot tips		Buds from woody canes	Buds from green canes	Fall Seeds	Dormant canes	Dormant buds								
	1 ^a	2	1	2	1	2	1	2					1	2	3						
Chardonnay			+	+	-	+	+		+	+	+	+	+	+	+	+F ^b					
Chardonnay			+	+			-	+	+	-	+	+	+	+	+	+					
Chardonnay			+	+			-	-	+	-	+	+									
Riesling	+		+		+	-	+		+	+	+	+	+	+	+	+					
Riesling			-	+	+	-	+		+	+	+	+	+	+	+	+F					
Riesling			+				+		+	-	+	+	+	+	+						
Riesling			+				+		+	-	+	+									
Merlot	+		+				+		+	-	+	+	+	+	+	+					
Merlot			+				+		+	-	+	+									
Merlot			+				+		+	-	+	+									
Merlot	+	+			+	+	+		+	-	+	+	+	+	+	+					
Cabernet Franc	+		+		+	+	+		-	-	+	+	+	+	+F	-					
Riparia							+		+	-		+	+	+	+	+					
Riparia					+	+	+		+	-		+									
3309					+		+		+	-		+									
3309			+		+		+		+	-		+	+	+	+	+					
3309			+		+		+		+	-		+									

^a 1, 2, and 3 indicate different samples from the same vine.

^b F = faint band; blank spaces represent no sample tested.

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