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RESEARCH ARTICLE

Distribution of *Rupestris stem-pitting-associated virus* variants in two Australian vineyards showing different symptoms

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Abstract

The incidence of *Rupestris stem-pitting-associated virus* (RSPaV) in two vineyards in South Australia was studied by comparing symptoms with the results obtained from biological indexing and from virus detection by reverse-transcription-polymerase chain reaction (RT-PCR). Vineyard 1 was an experimental block comprising a number of varieties grafted onto *Vitis rupestris* cv. St George rootstocks for the detection of Rupestris stem pitting disease. No swelling at graft union or decline was observed at 3 years, but some vines showed pitting on the rootstock trunk. Vineyard 2 contained *Vitis vinifera* cv. Shiraz (Syn. Syrah) vines on Paulsen 1103 rootstock, showing a range of symptoms including swelling of the graft union, pitting on the rootstock, leaf reddening and vine decline, resembling those in Syrah Decline, a disorder known to occur in this variety. RT-PCR using the coat-protein-specific primers detected RSPaV in all samples from either vineyard including symptomless *V. rupestris* cv. St George, which is used as the biological indicator for the virus. In contrast, a pair of primers designed from the replicase gene detected the virus only in symptomatic vines, whereas the symptomless St George control and nonsymptomatic vines in both vineyards tested negative. An assay for 12 other major grapevine viruses showed that none were associated with either type of symptom. Comparison of a 628-bp amplicon on the replicase gene in 13 RSPaV isolates showed that while they had 96–99% nucleotide sequence identity to each other, their identity to the American isolates from California and New York was around 65%. This low homology may indicate that a different virus species is present. The variability was more pronounced between the two vineyards than among the virus isolates in the Shiraz vines within the same vineyard. However, even the vines of the same vineyard did not contain viruses with exactly the same sequence homology. We found no association between the type of symptom expressed and the sequence variation in the amplicons in either vineyard. The sequence variants in Vineyard 1 showed a closer cluster than in Vineyard 2.

Introduction

Rupestris stem pitting (RSP) is a widespread viral disease of the grapevine and has been reported from all over the world (Martelli, 1993). Flexuous virus particles of 800 nm were isolated from infected vines and grapevine extracts (Conti *et al.*, 1980; Terlizzi & Credi, 2002). Although the aetiology of RSP is not well understood, a virus, *Rupestris stem-pitting-associated virus* (RSPaV), has been isolated from diseased plants and fully sequenced by two independent laboratories in the USA (Meng *et al.*, 1998, 2005; Zhang *et al.*, 1998). In both labs, grapevine

sources of the virus had been biologically indexed positive on *Vitis rupestris* cv. St George, a standard indicator for RSP. It has been reported that healthy RSP indicators, *V. rupestris* cv. St George, tested positive for RSPaV by PCR (Meng *et al.*, 2000, 2005), an indication that other factors maybe associated with the RSP disease. RSPaV contains five genes and is very similar to *Apple stem pitting virus*, the type member of the *Foveavirus* genus (Martelli & Jelkmann, 1998).

In Australia, grafted vines showed severe symptoms of swelling and cracking at the graft union (Fletcher, 1995). It appears that the increase in these symptoms coincided with a gain in popularity and a high demand for planting *Vitis vinifera* varieties grafted on nonvinifera hybrid rootstocks of American origin. Most of these hybrid rootstocks are infected with viruses without showing symptoms (Golino, 1993). The symptoms may appear when virus-sensitive scions are grafted onto certain rootstocks, which are symptomless carriers of the virus.

Sequence variability in the RSPaV genome has been reported (Meng *et al.*, 1999; Nolasco *et al.*, 2006). However, to the best of our knowledge, there is no available report linking symptom variability with the nucleotide sequence analysis of this virus. Here, in order to examine a correlation between symptomatology and molecular variability of RSPaV, we compared the nucleotide sequence of a specific region on the RSPaV genome in *V. vinifera* cv. Shiraz showing different symptoms in two vineyards in South Australia (SA). The results may lead to better understanding of 'Syrah decline', an RSPaV-related viral disease, suspected to be present in our vineyards.

Materials and methods

Grapevine material

Mature cane samples were collected from two vineyards in SA. Vineyard 1 was an experimental block located at Loxton Research Centre, Riverlands, 250 km north of Adelaide, SA. In this vineyard, selected grapevine scions were grafted onto *V. rupestris* cv. St George, an indicator for RSP (Goheen, 1988), in a replicate of three, and the symptoms were recorded following pulling out the vines and inspecting the pitting on the rootstock trunks 3 years after graft inoculation. For healthy controls, self-grafted, symptomless St George vines were used. Following inspection for symptoms, the same vines were randomly sampled for the reverse-transcription-polymerase chain reaction (RT-PCR) analysis (Table 1). The name of the vine varieties and clones used in this report are summarised in Table 1.

Vineyard 2 was located at McLaren Vale, 40 km south of Adelaide. In this vineyard, 2-year-old *V. vinifera* cv. Shiraz (Syn. Syrah) grafted onto the rootstock Paulsen 1103 (*V. berlandieri* × *V. Rupestris*) showing a range of symptoms were also sampled (Table 1). The clonal source of Shiraz and Paulsen 1103 in each vineyard was different.

Table 1 Grapevine samples collected from two South Australian vineyards and a brief description of their symptoms

Sample Code[a]	Scion/Rootstock	Symptom Descriptor
RS5	Paulsen/St George	None
RS6	Richter/St George	None
RS7	Riesling/St George	Pitting on St George
RS8	Chenin Blanc/St George	Pitting on St George
RS9	Colombard/St George	Pitting on St George
RS10	Dolcetto/St George	None
RS11	Shiraz-8[b]/St George	Pitting on St George
RS12	Sangiovese/St George	Pitting on St George
RS13	Shiraz-12/St George	Pitting on St George
RS14	Cabernet Franc/St George	None
RS15	Shiraz-30/St George	Pitting on St George
RS16	Pinot Noir/St George	Pitting on St George
RS17	Cabernet Franc/St George	Pitting on St George
RS18	Rubired/St George	Pitting on St George
RS19	Shiraz-41/St George	Pitting on St George
912-1-1	Shiraz/Paulsen	Minor swelling[c]
912-2-1	Shiraz/Paulsen	Swelling, pitting, decline[d]
912-2-2	Shiraz/Paulsen	Swelling, pitting, decline
924-1	Shiraz/Paulsen	Swelling, pitting, decline
924-2	Shiraz/Paulsen	Swelling, pitting, decline
H1R73	Shiraz/Paulsen	Minor swelling
D5R78	Shiraz/Paulsen	Swelling, pitting, decline
H5R78	Shiraz/Paulsen	None
D6R78S	Shiraz/Paulsen	Swelling, pitting, decline

[a]RS samples were from the Vineyard 1 and the rest were from Vineyard 2.

[b]Indicates the clone of Shiraz used.

[c]Plants look normal except that a minor swelling at graft union was observed.

[d]Major swelling at graft union, reddening of leaves and pitting on rootstock and decline were observed in these plants.

Grapevine material used as positive control for a range of viruses was selected from infected vines grown in local vineyards.

Extraction of total nucleic acids

Phloem shavings from dormant canes were used as the source of RNA. A total of 0.2 g of dormant phloem shavings were soaked in 2 mL of the extraction buffer containing 4 M guanidinium hydrochloride, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA (ethylenediaminetetraacetic acid), 2% PVP-40 [polyvinyl pyrrolidone (mol.wt. 40,000)] and 1% freshly added sodium metabisulphite in a plastic bag. The tissue was homogenised using a Bioreba (Reinach, Switzerland) Homex 6 sap extractor. Total RNA was extracted using RNeasy Plant mini kit (Qiagen, Hilden,

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Germany), essentially as described by MacKenzie *et al.* (1997). Total nucleic acid extract from 0.2 g of tissue was suspended in 80 µL of water and stored at −20°C.

Primers and RT-PCR

For the detection of RSPaV by RT-PCR, two primer pairs were used. One pair RSP48 (5′-AGCTGGGATTA-TAAGGGAGGT-3′) and RSP49 (5′-CCAGCCGTTCCAC-CACTAAT-3′) (Zhang *et al.*, 1998; see also Nolasco *et al.*, 2000) amplified a 330-bp fragment from the coat protein gene. The other pair Sy9F (5′-AGGATTCCAAA CTGTAGAGCAA3′) and Sy8R (5′-TTGGTCGTCATCTTC CAGTT-3′) amplified a fragment of the replicase gene with an amplicon size of 628. This primer pair was obtained from the nucleotide sequence of RSPaV-SY strain, isolated from a diseased Shiraz vine in California (M.F. Lima, unpublished data). For primer designs, the DNAsis Max program package version 2.0 (Hitachi software Engineering Co., Middlesex, UK) was used. Specific primers for other grapevine viruses were derived as described previously (Habili & Randles, 2002). Single-tube RT-PCR and the visualisation of the amplicons in agarose gel were performed as described previously (Shi *et al.*, 2003).

Cloning and sequencing

The cloning of amplified complementary DNA fragments was carried out in the pGEM-T Easy vector system (Promega, Madison, WI, USA). The sequencing reaction was set up using two pGEM-T Easy specific primers, T7 and SP6, which are located on either end of the polylinker site. The PCR product from Sy9F and Sy8R were used in sequencing. Each DNA was sequenced on both strands using the TaqDye terminator cycle sequencing kit in an automated sequencer (ABI Prism 377, Applied Biosystems, Foster city, CA, USA).

The multialignment was performed using the ClustalX program, and the phylogenetic tree was constructed using Genedoc and Mega 2.1 programs. The nucleotide sequences were analysed using the BLAST program (Altschul *et al.*, 1990). The GenBank accession numbers for the American Isolates of RSPaV used in the construction of the tree are: RSPaV-1, AF057136 (Meng *et al.*, 1998); RSPaV, AF026278 (Zhang *et al.*, 1998); RSPaV-BS, AY881627 and RSPaV-SG1, AY881626 (Meng *et al.*, 2005).

Results

Vines and their symptoms

We studied the occurrence of RSP disease in two vineyards in SA. Vineyard 1 was an experimental block comprising 15 grapevine varieties and clones grafted onto St George rootstock for the detection of RSP (Table 1). No swelling or decline was observed after 3 years, but the diseased vines showed pitting on the rootstock.

In Vineyard 2, *V. vinifera* cv. Shiraz on Paulsen showed two types of distinct symptoms of variable severity. In the milder types, which comprised most plants, only a small swelling at the graft union was observed while the plants were growing normally. Besides, there was no significant difference between the diameter of scion and rootstock (Fig. 1). However, in 70 out of 14 000 (0.5%) vines, an enlarged swelling at the graft union was observed and the diameter of the rootstock was significantly less than that of the scion. Small pits were also observed under the bark of the Paulsen rootstock (Fig. 1), and the leaves showed a severe reddening, which was followed by occasional death of the vines. The severe symptoms in these plants resembled those described for Syrah Decline in France and in California (Renault-Spilmont *et al.*, 2003; Battany *et al.*, 2004).

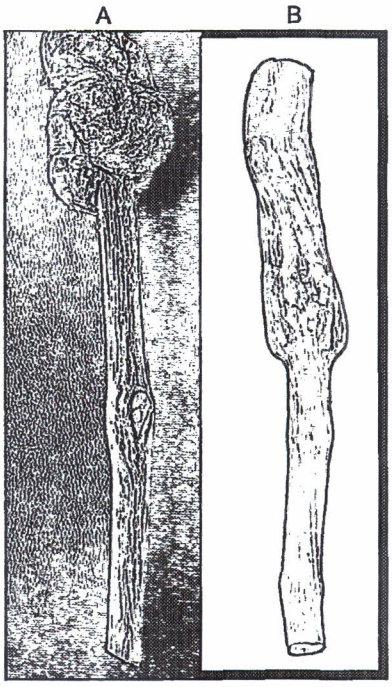

Figure 1 *Vitis vinifera* cv. Shiraz grafted on Paulsen 1103 rootstock, showing bulging at graft union and pitting on the rootstock (A on the left). This vine, which showed decline, tested positive for RSPaV by reverse-transcription-polymerase chain reaction (RT-PCR) using Sy9F/Sy8R primers, whereas the vine on the right, B, grew normally and tested negative by the PCR assay.

Detection of RSPaV

In an initial experiment, all vine samples were tested by RT-PCR for a range of viruses listed previously (Habili & Randles, 2002). The samples tested negative to all viruses except RSPaV. All vines in Vineyard 1 were grafted onto the St George indicator for the detection of RSP in which virus-positive vines showed specific symptoms described above.

The indexing results for 15 varieties and clones are shown in Table 2. A number of vines including healthy standards did not induce pitting. As a comparison, we carried out an RT-PCR assay for the specific detection of RSPaV to see if these results were reproducible.

For this purpose, the detectability of two different primer pairs targeting two distinct genes of the virus was compared. The results in Table 2 suggest that one primer pair, RSP48/RSP49, derived from the coat protein gene gave a positive reaction to all samples analysed including the apparently healthy St George indicator. This confirmed the results obtained by others that the St George used for indexing tested positive for RSPaV by RT-PCR (Meng *et al.*, 2000, 2005). The other pair (SY9F/SY8R) targeting the replicase gene was more specific as it gave no signal to the bioindexed RSP-negative samples (Table 2). However, three samples, which tested positive by bioindexing, gave a negative result by RT-PCR using these primers (Table 2). Sample, H5R78, with no symptoms tested negative using the more specific SY9F/SY8R primer pair (Table 2). It appears that this primer pair was useful for virus detection in symptomatic vines, and this prompted us to further analyse its PCR product.

Table 2 Results of biological indexing and RT-PCR assay on selected grapevine samples

Isolate[a]	Bioindexing[b]	RT-PCR[b] (Sy9F/Sy8R)	RT-PCR (RS48/RS49)
912-1-1	ND	Positive	Positive
912-2-1	ND	Positive	Positive
912-2-2	ND	Positive	Positive
924-1	ND	Positive	Positive
924-2	ND	Positive	Positive
H1R73-S1	ND	Positive	Positive
D5R78-S2	ND	Positive	Positive
H5R78	ND	Negative	Positive
D6R78S-S2	ND	Positive	Positive
R. vitis St G.[c]	Negative	Negative	Positive
RS5	Negative	Negative	Positive
RS6	Negative	Negative	Positive
RS7	Positive	Positive	Positive
RS8	Positive	Positive	Positive
RS9	Positive	Negative	Positive
RS10	Negative	Negative	Positive
RS11	Positive	Positive	Positive
RS12	Positive	Negative	Positive
RS13	Positive	Positive	Positive
RS14	Negative	Negative	Positive
RS15	Positive	Positive	Positive
RS16	Positive	Negative	Positive
RS17	Positive	Positive	Positive
RS18	Positive	Positive	Positive
RS19	Positive	Positive	Positive

ND, not determined; RT-PCR, reverse-transcription-polymerase chain reaction.

[a]See Table 1 for the relevant symptom observed in each isolate.

[b]The results of the RT-PCR using the primer pair Sy9F/Sy8R conformed to those of bioindexing except in samples shown in bold.

[c]Bioindexing was performed on *Vitis rupestris* St George.

Sequence analysis of RSPaV PCR products

Using the replicase-specific primers, SY9F/SY8R, a 627-bp RT-PCR product was obtained when 13 selected isolates in both vineyards were analysed. The fragments were sequenced and compared to the sequences from two American isolates available in the database. The sequence of all the Australian isolates had 96–99% nucleotide sequence and 83–97% amino acid identity to each other, regardless of the variety, type of the symptom and the location of the vineyard. A similar range of sequence identity was observed between American isolates (Meng *et al.*, 2005). However, the nucleotide and amino acid sequence identity between the Australian and the American isolates in this region of the gene did not exceed 65% and 71%, respectively (Table 3). This value is significantly lower than those reported for sequence variants of the same virus. This was confirmed by the phylogenetic analysis in Fig. 2, which showed two major branches each containing the American or the Australian virus isolates. In addition, it showed that the isolates from the St George trial in Vineyard 1 were more clustered than those from Vineyard 2.

Discussion

Grapevine samples from two vineyards in SA were subjected to the RT-PCR assay for the detection of 12 most commonly occurring grapevine viruses in which only RSPaV was detected. This virus has a high incidence in Australia, infecting every grapevine variety tested (Habili *et al.*, 2000), and it is known to be present in samples showing stem-pitting symptoms (Martelli, 1993). Using PCR, we confirmed the result of others (Meng *et al.*, 2000, 2005) that the virus is also present in healthy St George vines, a biological indicator for RSP. The high incidence of the virus may indicate that it is not the only factor associated with the pitting symptoms. It is also possible that a complementary factor or a totally different virus may induce these symptoms. It is important to note that the detection of RSPaV in healthy indicators

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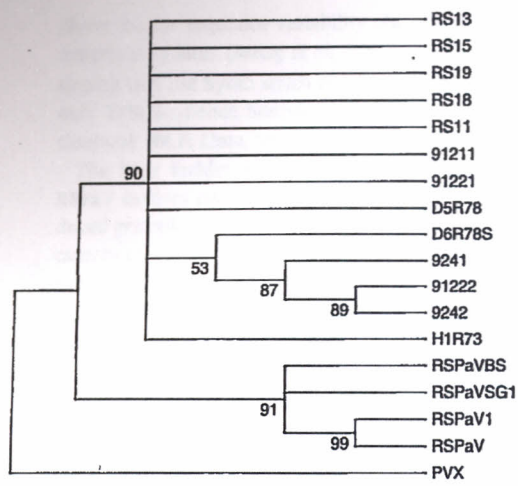

Figure 2 Phylogenetic tree constructed for the *Rupestris stem-pitting-associated virus* (RSPaV) isolates based on the multiple sequence alignments of a 628-nucleotide sequence from the replicase gene of RSPaV. The tree was constructed using Genedoc and Mega 2.1 programs. Bootstrap values are shown. The corresponding fragments of sequences available in GenBank: RSPaV (AF026278); RSPaV-1 (AF057136); RSPaV-SG1 (AY881626) and RSPaV-BS (AY881627) were used. PVX (NC_001455) was used as an out group.

was linked to the type of primer pairs used in the RT-PCR assay. Although the primer pair derived from the coat protein detected the virus in all samples tested, the pair derived from the replicase gene detected the virus in symptomatic vines only, whereas the healthy indicator vines tested negative. The latter pair could not discriminate between the types of symptoms present in each vineyard. It may be possible to design primers from a region of the virus genome for the specific detection of each type of symptoms showning in each vineyard. More work, especially a complete sequence analysis of the Australian virus isolates, will be required as a basis for evaluating this view.

Both the sequence analysis and the phylogenetic tree suggest that the Australian isolates are similar to each other and distinct from the USA isolates. As sequence homology between American and Australian isolates in this region of the replicase gene was only 65%, one may conclude that the Australian virus studied here are a different species. It has been suggested that distinct virus species have less than 72% sequence identity in their genome (Adams *et al.*, 2004). Additional sequence information will be needed to substantiate this claim. Analysis of the phylogenetic tree showed that the virus isolates in Vineyard 1, which showed less variability in symptoms (Table 1), were more clustered together than those from Vineyard 2. In the latter vineyard, which showed two distinct symptoms, no association between the types of expressed symptoms and sequence variation in the amplicon was observed. A complete nucleotide sequence of selected Australian isolates can provide more insight into the variability that may exist between the isolates present in the two countries.

As most vine varieties, currently grown in Australia, have been imported from the USA, the reason for this difference in sequence would be difficult to contemplate.

Table 3 Grapevine isolates and their nucleotide (nt) and amino acid (aa) identities (%) in the amplified region of the replicase using primer pair Sy9F/Sy8R

Sample Code/Virus Strain	Isolate	Nucleotide % Identity (628 nt)	Amino Acid % Identity (192 aa)
912-1-1	Shiraz/Paulsen	64	68
912-2-1	Shiraz/Paulsen	64	70
912-2-2	Shiraz/Paulsen	63	71
924-1	Shiraz/Paulsen	63	68
924-2	Shiraz/Paulsen	63	71
H1R73	Shiraz/Paulsen	65	68
D5R78	Shiraz/Paulsen	65	70
D6R78S	Shiraz/Paulsen	63	65
RS11	Shiraz 8828/St George	64	67
RS13	Shiraz 33/St George	65	69
RS15	Shiraz 1654/St George	64	69
RS18	Rubired/St George	66	70
RS19	Shiraz 8816/St George	63	70
RSPaV-1[a]	USA, Cornell	96	95
RSPaV[a]	USA, Davis (used as reference)	100	100
RSPaV-BS[a]	USA, Cornell	84	85
RSPaV-SG1[a]	USA, Cornell	82	83

[a]The GenBank accession numbers are: RSPaV-1, AF057136 (Meng *et al.*, 1998); RSPaV, AF026278 (Zhang *et al.*, 1998); RSPaV-BS, AY881627 and RSPaV-SG1, AY881626 (Meng *et al.*, 2005).

However, the sequence variability also exists among the American isolates (Meng *et al.*, 2005). It is worth mentioning that the Syrah strain of RSPaV from the USA has only 77% sequence homology to all those listed in the GenBank (M.F. Lima, unpublished data).

The high incidence and low variation in Australian RSPaV isolates could be explained by grafting of introduced germplasm onto infected St George biological indicator as part of the postquarantine indexing programs. We suggest using virus-free St George as an indicator for the biological assay of RSP disease. This is now possible in our laboratory as we have been able to eliminate RSPaV from several grapevine sources using a combination of thermotherapy and meristem tip culture (R. Davies & N. Habili, unpublished data).

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