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Large scale evaluation of primers for diagnosis of rupestris stem pitting associated virus-1

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Abstract

The unavailability of adequate immunological reagents has prevented the use of ELISA for the diagnosis of rupestris stem pitting disorder of grapevines. In this work, the performance of five primer pairs for broad-scale detection of rupestris stem pitting associated virus-1 by RT-PCR using ds-RNA templates was compared and contrasted with biological indexing. The virus was widespread among the budwood of 35 Portuguese grapevine varieties assayed, with a prevalence of 85%. The biological assay proved to be unreliable as an index of infection due to the high number of false negatives. Five sets of primers were assayed and compared by means of their relative sensitivity and negative predictive value. The primer pair specific for the coat protein gene was excluded because of the difficulty in identifying the specific amplified product. From the other four primer pairs, those specific for the helicase domain of the putative polymerase gene had the highest sensitivity and negative predictive value. However, a high confidence in the assay, as desirable for a certification scheme, could not be obtained by the sole use of this primer pair. An additional pair should be used in a separate or in a multiplex RT-PCR reaction.

Introduction

Rugose wood is a complex of graft transmissible diseases of grapevine which occurs worldwide. Major symptoms include the development of pitting and grooving of the woody cylinder. No specific symptoms occur on the foliage. The disease may be present as a latent stage on *Vitis vinifera* cultivars and only manifests itself following grafting on to American rootstocks (Credi, 1997). Growth and yield may be severely affected, or premature death induced, especially if other decline diseases such as fanleaf are also present (Garau et al., 1985; Savino et al., 1985). Biological indexing using a panel of three indicators differentiates the four disorders associated with this complex: Kober stem grooving, grapevine corky bark, LN33 stem grooving and rupestris stem pitting (RSP)(Martelli, 1993). RSP disorder is so designated because of the characteristic stem pitting symptoms it induces on *V. rupestris* cv. St. George. Despite increasing evidence in recent years associating the Trichoviruses GVA and GVB with Kober stem grooving and corky bark, respectively (Boscia et al., 1997), the aetiology of RSP has remained obscure. Double-stranded RNA (ds-RNA) species of 0.359 kbp (Monette et al., 1989) or two about 5.5×10^6 and 4.4×10^6 Da (Azzam and Gonsalves, 1991; Walter and Cameron, 1991) were tentatively associated with RSP diseased grapevines on different occasions. Recently the larger ds-RNA species was cloned and sequenced, revealing a viral genome of 8726 nt with an organisation similar to that of apple stem pitting virus; this new virus was called rupestris stem pitting associated virus 1 (RSPaV-1) (Meng et al., 1998) or grapevine rupestris stem pitting associated virus

(GRSPaV) (Zhang et al., 1998b). RT-PCR with primers derived from these sequences consistently associated the presence of this virus with RSP diseased vines (Meng et al., 1998; 1999; Zhang et al., 1998b). Both sequences share a homology of 97%. Additionally, a putative open reading frame (ORF6) that is present in the isolate sequenced by Zhang et al. (1998b) has its counterpart in the sequence presented by Meng et al. (1998), although these authors did not report it. This suggests that both sequences represent very close isolates of the same virus. On the other hand, the existence of sequence variants have been suggested based on different amplification efficiencies between different primers (Meng et al., 1999; Zhang et al., 1998b).

Despite being a grapevine disease of major importance, the diagnosis of RSP has been problematic. The unavailability of antisera precludes the use of ELISA technique. Biological indexing may be in certain cases unreliable (Savino et al., 1985) and may take up to 3 years for a conclusive diagnosis. ds-RNA analysis provides useful broad-spectrum markers of infection but is difficult to implement as a viral specific diagnosis system due to low sensitivity for analysis of field vines and the occurrence of mixed infections (Boscia et al., 1997). RT-PCR appears then to be an interesting alternative for the diagnosis of RSP disease. Diverse primer pairs derived from American isolates have been designed with this purpose (Minafra et al., 1997; Meng et al., 1998; 1999; Zhang et al., 1998b). The primer pair 13 and 14 was designed with the purpose of being 'universal' (Meng et al., 1999). However the performance of these primers in large scale assays has not yet been compared.

In the present study, the feasibility of using ds-RNA as a template for the detection of RSPaV-1 was determined and the performance of a panel of 5 primer pairs assessed. It has been common in plant virology to compare the performance of diagnosis systems based on amplification of nucleic acids on the ability (usually called 'sensitivity') to detect serial dilutions of one or a few infected samples, normally maintained in the research laboratories for years. This procedure does not take in consideration the natural variability of plant viruses and cannot assess the performance of a method at a population level. To circumvent this problem, the analysis was based on a set of parameters that are commonly used for assessing diagnosis methods in medical fields: sensitivity as the ability to identify samples which are truly positive; specificity as the ability to identify samples which are truly negative; positive (negative) predictive value as the probability of being truly positive (negative) given a positive (negative) test result.

Materials and methods

Plant material

Dormant canes, from 35 diverse Portuguese varieties, totalling 288 samples, were assayed in these studies. Most of the material consisted of propagation budwood from mother plants maintained in field conditions. A few plants were previously indexed for RSP disease symptoms on Rupestris St. Georges. The dormant canes were maintained at 4 °C for up to several weeks until processing. The samples consisted of bark shavings obtained with knives or glass pieces. The external brown layer was scraped and discarded. Usually about 2.5 g of tissue, corresponding to a piece of cane of two or three buds was used but in several cases the amount of tissue was significantly less. The bark shavings were kept frozen at −20 °C until processed.

Double-stranded RNA extraction and RT-PCR

ds-RNA was extracted using standard phenol–chloroform and CF11 isolation procedures adapted from Hu et al. (1990). The ds-RNA was finally resuspended in 25 µl of MilliQ quality water and kept at −70 °C until used. RT-PCR was performed in a one-tube protocol. The ds-RNA template was diluted 1/20 in water, denatured for 5 min at 90 °C and 1 µl was added to a 50 µl RT-PCR reaction mix containing: 20 mM Tris–HCl pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 200 nM each primer (see below), 200 µM each dNTP and 8 units RNAse inhibitor (RNAguard Pharmacia), 10 units of MMLV reverse transcriptase (Perkin Elmer) and 1 unit of Taq polymerase (GIBCO-BRL). Thermal cycling consisted of 38 °C for 45 min, 94 °C for 2 min and 30 cycles of 92 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. An additional step of 72 °C for 5 min ended the procedure. Usually, one-tenth of the amplified products were analysed in 6% polyacrilamide gels and the DNA visualised after silver staining with standard procedures.

Oligonucleotide primers used in diagnosis assays

Five pairs of primers for different genomic regions were used (Table 1). The sequences for primer pairs 9 and

Table 1. Primers used for detection of RSPaV-1

Primer pair	Position*	Sequence	Amplicon (bp)
McK1U	5639	F: AGGGATTGGCTGTTAGATGTT	355
McK1D	5975	R: CTTCAGGCAACCCCAAAAA	
McK2U	5695	F: ATTTGACAACGAGCTTAGAGTAG	299
McK1D	5975	R: CTTCAGGCAACCCCAAAAA	
9 and 10	6243	F: GGCCAAGGTTCAGTTTG	498
	6724	R: ACACCTGCTGTGAAAGC	
13 and 14	4373	F: GATGAGGTCCAGTTGTTTCC	339
	4692	R: ATCCAAAGGACCTTTTGACC	
RSP 52 and 53	7709	F: TGAAGGCTTTAGGGGTTAG	905
	8595	R: CTTAACCCAGCCTTGAAAT	

*Relative to the genebank accession number AF57136.

10 and 13 and 14 were provided by Dr. Gonsalves (Cornell University, USA) and are specific respectively for a fragment encompassing the 3′ terminal part of the ORF1 and the beginning of the ORF2 and for a fragment inside the helicase-like domain present in the ORF1 of RSPaV-1 (Zhang et al., 1998b). Primers McK1U and 1D and McK2U and 1D whose sequence was provided by Dr. MacKenzie (Canada Food Inspection Agency, Canada), were previously referred to in Minafra et al. (1997); these primers lie in the RdRp region of the ORF1 (Zhang et al., 1998b). Primers RSP 52 and 53 comprise the putative coat protein gene and two short regions adjacent up- and downstream. These primers were provided by Dr. Rowhani (University of California, USA).

Cloning and analysis of the putative coat protein gene

The products of RT-PCR amplification with primers RSP 52 and 53 were TA-cloned in the pGem T easy vector (Promega) according to the instructions of the manufacturer. Clones harbouring inserts were selected by PCR with primers RSP 52 and 53. For this purpose, a portion of several colonies was picked with a pipette tip and scraped in the bottom of PCR tubes. The PCR mixture and cycling parameters were the same as described above except that the RNAse inhibitor, reverse transcriptase and initial step at 38 °C were omitted. Selected colonies were used to prepare minipreps according to standard procedures. The presence of the insert was confirmed by digestion with *Eco* R I. Specificity of the inserts was assessed by internal amplification with primers RSP 48 (5′-AGCTGGGATTATAAGGGAGGT-3′) and RSP 49 (5′-CCAGCCGTTCCACCACTAAT-3′) provided by Dr. Rowhani and which are specific for the fragment 8178–8507. A few amplifications of part of the coat protein gene were also performed with primer CP3 (5′-ACTCCCCGGTGAATCAAA-3′) as forward primer (position 7791–7808) and RSP 49 as reverse primer.

Evaluation of test performance

Computation of sensitivity, specificity, positive and negative predictive values and prevalence was carried out according to Schachter (1997). An example of how the calculations were performed is given in Table 4. Due to the intrinsic characteristics of the RT-PCR methodology, the biological indexing could not be considered as the gold standard. Instead the gold standard was taken to be the Boolean sum of the results of four pairs of primers (the results of primer pair 52 and 53 were not considered for diagnosis purposes): positive = positive by at least one primer set; negative = negative (no specific product) by all primer sets.

Results

Double-stranded RNA extraction

Although the routine protocol deals with 2.5 g of tissue, in several cases the amount of tissue available was considerably less, down to 0.4 g. This did not affect the quality of the results as the intensity of the bands was not in these cases especially reduced. Long storage periods at −70 °C did not produce any noticeable effect on the quality of the template for amplification. In fact, some results were confirmed from the same templates about 1 year later and most of the reactions with primers 13 and 14 were performed almost 2 years after

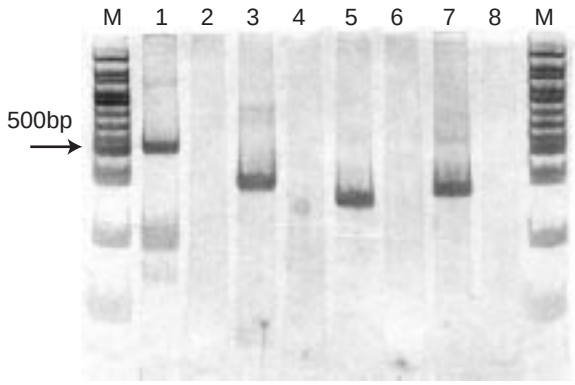

Figure 1. Typical aspect of the 6% PAGE separation of the RT-PCR products obtained with primers 9 and 10 (lanes 1 and 2), McK1U1D (lanes 3 and 4), McK2U1D (lanes 5 and 6) and 13 and 14 (lanes 7 and 8). Infected samples: lanes 1, 3, 5 and 7. Healthy sample: lanes 2, 4, 6 and 8. M, 100 bp ladder (Fermentas). Silver staining.

the extraction. However, when samples were exposed to repeated cycles of freezing and defrosting, as happened with samples used as positive controls, a progressive decrease in band intensity was noticed.

Analysis of the amplification products by PAGE

A typical result of the amplification with four primer pairs is presented in Figure 1. In most of the cases, it was easy to identify the specific amplified product even in the presence of non-specific bands. These were usually fainter than the specific product. Primers McK1U1D frequently gave rise to conspicuous slow migrating bands. Occurrence of non-specific bands in the absence of the specific band was rare, occurring in only 0.6% and 0.3% of the samples analysed with primers 9 and 10 and McK2U1D respectively. The use of silver staining enabled the detection of faint bands that would not be detected if the gels were visualised with UV light after ethidium bromide staining. This situation was more frequent with primers McK2U and 1D (9.6%) then with the other primer sets (2.3% with 9 and 10, 3.8% with McK1U and 1D and rare with 13 and 14).

Occurrence of variants differing in genome length

The primers RSP 52 and 53 were used to assay only 118 samples. Analysis of the amplification products was not straightforward. The expected product that should have 905 bp usually appeared accompanied by

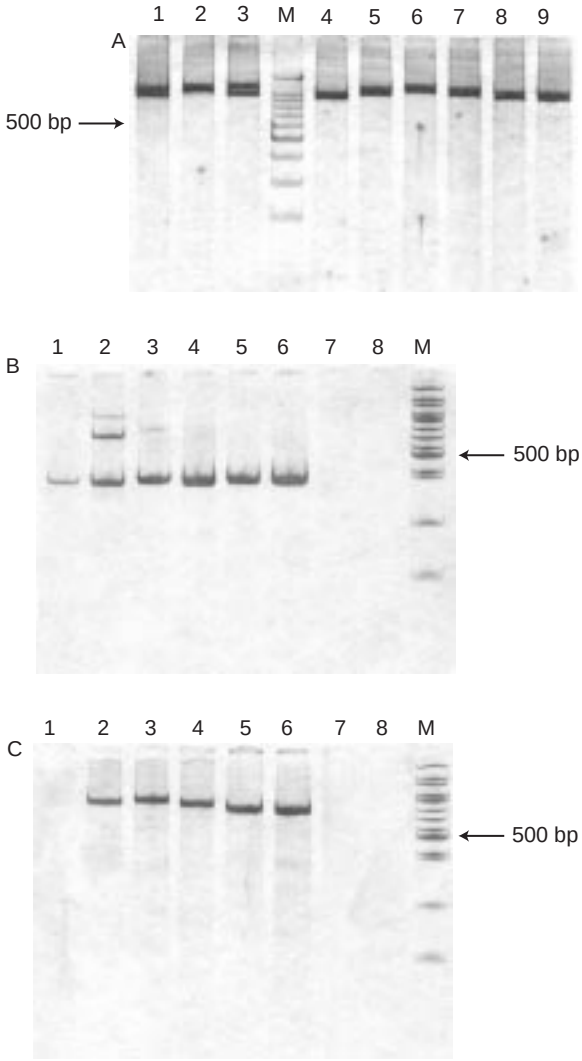

Figure 2. Analysis of amplification products obtained with primers specific for the coat protein region. Panel A: lanes 1–3, RT–PCR (primers RSP 52 and 53) from samples B11, B10 and B1; lanes 4–9, PCR (primers RSP 52 and 53) from selected colonies obtained by TA-cloning of the products from lanes 1–3, respectively, clones B1.1, B1.2, B10.1, B10.3, B11.1, B11.2; M, 100 bp ladder (Gibco BRL). Panel B: PCR with primers RSP 48 and 49; lanes 1–6, clones B1.1, B1.2, B10.1, B10.3, B11.1, B11.2; lanes 7 and 8, negative controls (no DNA); M, 100 bp ladder (Fermentas). Panel C: PCR with primers CP3 and RSP 49; same disposition of samples as in B. Silver staining.

other larger band(s) (Figure 2A). To further investigate this aspect, samples which gave rise to only one or the two bands, were selected and their amplicons were TA-cloned in *E. coli*. Colonies harbouring the larger

Table 2. Comparison of results obtained with 37 samples analysed by biological indexing and RT-PCR

Bioindex	RT-PCR					
	Positive total*	Negative	Positive by 13 and 14	Positive by 9 and 10	Positive by McK1U and 1D	Positive by McK2U and 1D
Positive	21	1	18	18	15	17
Negative	12	3	8	12	7	9

*Notice that some samples are positive for more than one pair of primers but are computed for the positive total only once.

or the shorter insert were selected and the inserts re-amplified with primers RSP 52 and 53. Differences in the size of the products could be seen in the gels. To further localise the cause of these discrepancies, the same inserts were amplified with the internal primers RSP 48 and RSP 49 and CP3 and RSP 49, which, based on published sequences, should originate amplicons of 330 and 717 bp lying inside of the coat protein gene. Fragments of very similar size were obtained with primers RSP 48 and RSP 49 but not with primers CP3 and RSP 49 (Figure 2B and C). In this case the differences in size paralleled those observed with primers RSP 52 and 53. One of the clones (B1.1) could not be amplified with CP3 and RSP 49. These observations suggested that the RSP 52 and 53 primers were amplifying a family of entities related to RSPaV-1 but differing in the length of the 5′ terminal half of the coat protein gene. As the relationship of these variants with the disease is not yet established it was decided not to include these primers in the comparison for diagnosis purposes.

RT-PCR detection versus biological indexing

Detection by RT-PCR revealed a very high frequency of infection by RSPaV-1: 246 out of the 288 samples assayed by RT-PCR (85% of the samples), were found to be positive with at least one pair of primers. On the other hand, from 37 samples that have previously been indexed on Rupestris St. George only 59% were found to be positive (by biological indexing). A comparison of the two methodologies is presented in Table 2. Almost the totality of the samples indexed positive (with the exception of 1 sample) were also found to be positive by RT-PCR. The four sets of primers did not show any marked difference in their ability to detect these kind of samples. On the other hand a significant proportion (80%) of the samples biologically indexed as negative were found to be positive by RT-PCR. This precluded the use of the biological indexing as the gold standard for evaluation of RT-PCR performance.

Comparison of the diagnosis performance of the primers

The ability of each primer pair to detect RSPaV-1 was variable as can be inferred from Table 3. Only 46% of the positive samples could be detected by any of the primer pairs. Besides that, each of the primers can detect only a specific range of isolates and there is only partial superposition of the spectra.

A more systematic comparison of the performances of the primers in RT-PCR assay and of the biological indexing assay was carried out by computing the sensitivity (defined as the ability to identify a sample that is truly positive) as explained in Table 4. The specificity (ability to identify a sample that is truly negative) of the RT-PCR assay was not computed as it would be meaningless (100%) as a consequence of the kind of gold standard that was used. The specificity of biological indexing was not as high as one would expect, and gave rise to 25% false positives, but these results should be interpreted with caution as the number of negative samples assayed was very low. On the contrary, the high number of false negatives causes an inadequate relative sensitivity (64%). The RT-PCR assay with each of the primer pairs originated a better sensitivity than the biological indexing, ranging from 69% to 84%. The higher value was obtained with primers 13 and 14. The negative predictive value (defined as the probability of a sample being truly negative given a negative test result) was also computed. As can be seen, these values are not satisfactory, as a negative result obtained with any of these primers gives only a 39–52% probability of corresponding to a true negative.

An alternative way to rise the confidence level on the RT-PCR assay would be to perform more than one

reaction (with different primers) and to combine the results. The data of Table 3 was then used to estimate the increase of sensitivity and negative predictive value that could be obtained in such way (Table 4). All the possible combinations of two primer pairs were considered.

Table 3. Distribution of occurrences regarding the amplification by each primer pair in the analysis of 288 samples

13 and 14	9 and 10	McK1U1D	McK2U1D	No. Samples
−	−	−	−	**42***
−	+	−	−	10
−	−	+	−	1
−	−	−	+	9
−	+	+	−	2
−	−	+	+	0
−	+	−	+	2
−	+	+	+	15
+	−	−	−	13
+	+	−	−	14
+	−	+	−	6
+	−	−	+	17
+	+	+	−	13
+	−	+	+	20
+	+	−	+	12
+	+	+	+	112
Positive by at least one primer pair				**246***

*Values in bold were taken as the 'gold standard': 42 samples negative, 246 samples positive.

For every combination, a sample was taken as positive when it was positive by at least one set of primers and negative when it was negative by both primer pairs. The best combinations were found to be primers 13 and 14, and 9 and 10 (sensitivity 96%, negative predictive value 84%) and 13 and 14 and McK2U1D (sensitivity 95%, negative predictive value 76%).

Discussion

In this work five primer pairs were evaluated for RSPaV-1 detection by RT-PCR using ds-RNA as a template and the results were partially compared with data from biological assays. A striking aspect of the detection of RSPaV-1 was its high prevalence (85%), in spite of the material tested being budwood currently in use for propagation purposes. A high prevalence has also been noticed by other authors (Zhang et al., 1998b; Minafra et al., 1997) who used RT-PCR detection. In addition, the virus was found in a high percentage of plants that were biologically indexed as negative for RSP disease. From the present results it can be concluded that biological indexing is not a reliable tool for diagnosis purposes, as the probability that a negative result by biological assay corresponds to a truly negative sample is low. The unreliability of biological indexing has previously been discussed by Savino

Table 4. Characteristics of the RT-PCR and biological indexing tests

Assay	Sensitivity[1]	NPV[1]	Prevalence (Number of samples)
RT-PCR (1 primer pair)			
9 and 10	73%	39%	85% (288)
McK1U1D	69%	35%	85% (288)
McK2U1D	76%	42%	85% (288)
13 and 14	84%	52%	85% (288)
RT-PCR (2 primer pairs)[2]			
13 and 14, and 9 and 10	96%	84%	85% (288)
13 and 14, and McK1U1D	91%	67%	85% (288)
13 and 14, and McK2U1D	95%	76%	85% (288)
9 and 10, and McK1U1D	84%	52%	85% (288)
9 and 10 and McK2U1D	92%	68%	85% (288)
McK1U1D and McK2U1D	85%	53%	85% (288)
Biological indexing	64%	20%	89% (37)

[1]See text for definition. NPV – negative predictive value. Example of the calculations made with the data for primer pair 13 and 14 (see Table 3): Sensitivity $= (13+14+6+17+13+20+12+112)/246\times100 = 84\%$; NPV $= 42/(10+1+9+2+0+2+15+42)\times100 = 51\%$; Prevalence $= 246/(246+42) = 85\%$.

[2]Two primer pairs used in separate reactions (see text). Calculations as above, made with the data from Table 3.

et al. (1985). Although the RT-PCR technique is considered to be a very sensitive technique, it seems likely that other factors could account for its better performance. There could exist mild strains that do not produce detectable symptoms in field conditions or in indicators. Due to the unavailability of alternative diagnostic methods, mild strains could have been spreading worldwide, eventually infecting the indicator plants. Cross protection phenomena could then prevent the accurate diagnosis of the disease. If this holds true, the sanitary status of indicator plants should be re-checked by RT-PCR.

The purpose of the present work was not to develop a routine extraction method for ds-RNA; nevertheless, without transforming the laboratory to a ds-RNA factory, 30–40 samples could usually be extracted each week by one operator. The protocol used for ds-RNA extraction, although time consuming and requiring the use of phenol and other noxious compounds, provided a good quality template that proved to be stable for longer than one year. This is important for this kind of assays in which there is sometimes the need to re-test exactly the same material or include additional primers in the comparison. Contrary to other recently presented methods that provide single-stranded RNA (ss-RNA) (MacKenzie et al., 1997), this method does not require expensive reagents or kits. Additionally, the ds nature of the template is beneficial, as its preparation does not require any special anti-RNAase care (no RNAase-free water was used in any of the experiments). This would be very advantageous if used in routine conditions. In view of the advantages of ds-RNA templates over ss-RNA, some more improvement of the routine protocols is needed, namely in simplifying the CF11 chromatography and the final purification steps. A small-scale protocol suitable for routine extraction was recently presented by Zhang et al. (1998a). However the use of very small amounts of tissue for diagnosis is not straightforward. It is necessary to be sure that the virus being tested has an even distribution in the infected tissues.

When introducing diagnosis methodologies based on nucleic acid amplification it has been frequently found that the new methodologies are by themselves more sensitive than the one previously used as a standard for comparison purposes i.e. the gold standard (Schachter, 1997). Higher sensitivity or other factors in the present study led the RT-PCR technique to detect a significant number of positives that were previously considered to be negative by biological assay. The possibility that this excess of positives could be due to contamination with amplified DNA was discarded because the negative controls remained negative. Due to the absence of an alternative technique we used as gold standard the Boolean sum of the results of four primer sets (9 and 10, 13 and 14, McK1U1D and McK2U1D), the results obtained with primers RSP 52 and 53 having been discarded due to difficulties in the interpretation of the results. The ‘gold standard’ used caused the specificity and positive predictive value of the primers to rise artificially to 100%, reason why these characteristics were not taken in account.

The reasons so far advanced to explain the inability of each primer set to detect a broad spectrum of isolates were the genomic variability of the virus which could be impairing an efficient priming or the unspecific breakage of the ds-RNA molecules during the extraction procedure. As unspecific breakage would affect the primers in a similar way, a similar value of sensitivity would be expected, which was not the case. The existence of sequence variants have been suggested based on different amplification efficiencies between different primers (Meng et al., 1999; Zhang et al., 1998b). The situation may reveal to be more complex if taking into account the results from the present study which additionally suggest the existence of length variants of the coat protein gene. The relationship of these RSPaV-1 variants and RSP disease is not clear and needs further elucidation. Sequencing of these variants is underway.

Based on evidence that the helicase-like domain could be highly conserved at the genomic level, Meng et al. (1999) designed primers 13 and 14 with the purpose of having a broad spectrum of detection. However, in our study in which a larger number of samples was used, these primers, although having the highest sensitivity (84%) did not perform as ‘universal’. The sensitivity for two primer pairs assayed by Zhang et al. (1998b) was also computed. From these, RSP2 and 21 has a high sensitivity (90%) that makes it interesting as a candidate for broad-spectrum detection, but there is not enough data regarding the variability of the population in which it was assayed. Taking the prevalence of 85% as representative for the propagating budwood actually available in Portugal the risk of missing infected samples in a certification programme is still considerable, even using primers 13 and 14. The task of finding a suitable genomic region to design broad-spectrum primers may be more difficult than expected. Meanwhile it should be possible to increase the confidence level of the RT-PCR diagnosis by using more

than one primer pair in parallel reactions. But which primer pairs? It is not evident that the best combination is composed by those primer pairs that alone show the highest sensitivities and negative predictive value. By computing the sensitivity and negative predictive value of all the possible combinations of two primer pairs we determined that the best one is composed by the primer pairs 13 and 14 and 9 and 10. This way, the sensitivity and negative predictive value increase to levels (96% and 84% respectively) that may be considered acceptable for a certification scheme. Additionally, preliminary assays have already shown that these two pairs of primers are compatible, thus allowing their joint use in a single multiplex reaction which may significantly lower the cost of the RT-PCR diagnosis of RSPaV-1.

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References

Azzam OI and Gonsalves D (1991) Detection of ds-RNA in grapevines showing symptoms of rupestris stem pitting disease and variabilities encountered. Plant Disease 75: 960–964

Boscia D, Minafra A and Martelli GP (1997) Filamentous viruses of grapevine: putative trichoviruses and capilloviruses. In: Monette PL (ed) Filamentous Viruses of Woody Plants. pp 19–28 Research Signpost, Trivandrum, India

Credi R (1997) Characterization of grapevine rugose wood disease sources from Italy. Plant Disease 81: 1288–1292

Garau R, Cugusi M, Dore M and Prota U (1985) Investigations on the yield of “Monica” and “Italia” vines affected by legno riccio (stem pitting). Phytopathologia mediterranea 24: 64–67

Hu JS, Gonsalves D and Teliz D (1990) Chraracterization of closterovirus – like particles associated with grapevine leafroll disease. Journal of Phytopathology 128: 1–14

MacKenzie DJ, McLean MA, Mukerji S and Green M (1997) Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription – polymerase chain reaction. Plant Disease 81: 221–226

Martelli, G.P. (1993) Rugose wood complex. In: Martelli GP (ed) Graft-Transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis (pp 45–54) FAO, Rome

Meng B, Pang S-Z, Forsline P, McFerson JR, Gonsalves D (1998) Nucleotide sequence and genome structure of grapevine rupestris stem pitting associated virus-1 reveal similarities to apple stem pitting virus. Journal of General Virology 79: 2059–2069

Meng B, Johnson R, Peressini S, Forsline PL and Gonsalves D (1999) Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. European Journal of Plant Pathology 105: 191–199

Minafra A, MacKenzie DJ, Casati, Bianco P, Saldarelli P and Martelli GP (1997) Detection of an unusual RNA in grapevines indexing positive for rupestris stem pitting. In: Extended abstracts 12th Meeting ICVG, Lisbon, Portugal, 29 September–2 October, (p 43)

Monette PL, James D and Godkin SE (1989) Double stranded RNA from rupestris stem pitting-affected grapevines. Vitis 28: 137–144

Savino V, Boscia D, Musci D and Martelli GP (1985) Effect of legno riccio (stem pitting) on ‘Italia’ vines grafted onto rootstocks of different origin. Phytopathologia Mediterranea 24: 68–72

Schachter J (1997) Evaluation of diagnostic tests – special problems introduced by DNA amplification procedures. In: Lee H, Morse S and Olsvik O (eds) Nucleic Acid Amplification Technologies – Application to Disease Diagnosis, pp 165–169 Eaton Publishing.

Walter MH and Cameron HR (1991) Double stranded RNA isolated from grapevines affected by rupestris stem pitting disease. American Journal of Enology and Viticulture 42: 175–179

Zhang Y-P, Uyemoto JK and Kirkpatrick BC (1998a). A small scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. Journal of Virological Methods 71: 45–50

Zhang Y-P, Uyemoto, Golino D and Rowhani A (1998b). Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytophatology 88: 1231–1237