Real-time RT-PCR (TaqMan®) Assays and Low Density Array Detection of Viruses Associated with Rugose Wood Complex of Grapevine

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Real time TaqMan® RT-PCR assays and Low Density Arrays (LDA) have been developed to detect the viruses associated with Rugose wood complex of grapevines. These viruses included *Rupestris stem pitting-associated virus* (RSPaV) in the genus *Foveavirus*, *Grapevine virus A* (GVA), *Grapevine virus B* (GVB) and *Grapevine virus D* (GVD) in the genus *Vitivirus*. The coat protein (CP) gene of these viruses was found to be the most conserved gene, therefore, the primers and probes for TaqMan® RT-PCR assays were designed from the CP sequence pile up of various isolates of each virus. Comparisons were also made between the conventional one step RT-PCR, real-time TaqMan® RT-PCR for the detection of these viruses using four fold serial dilutions of both purified RNA and crude extract. Results showed that real-time TaqMan® RT-PCR was more sensitive and could detect viruses at 32 and 256 fold higher dilutions for purified RNA and crude extract, respectively, compared to RT-PCR. In addition, high throughput detection of these viruses using LDAs was compared to RT-PCR and real time TaqMan® RT-PCR. The efficiency of different RNA extraction methodologies and buffers were compared for use in low density array detection.

Rugose wood (RW) complex is a term used to describe a group of graft-transmissible diseases which are affecting grapevines (*Vitis spp*) worldwide (Martelli, 1993). RW is of great economic importance causing severe reduction of growth and yield of affected grapevine plants and accordingly having a great impact on the grape industry (Golino et al., 2000). The RW complex is classified into different diseases based on symptomatology on specific indicator hosts and can be divided into four components: Kober stem grooving (KSG), LN 33 stem grooving (LNSG), corky bark (CB) and rupestris stem pitting (RSP) (Martelli, 1993). Up-to-date three different phloem-restricted viruses belonging to two different genera have been identified to be associated with the etiology of RW including two viruses in the genus *Vitivirus*, *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), and one virus in the genus *Foveavirus, Rupestris stem pitting-associated virus* (RSPaV) (Meng et al., 1998; Zhang et al., 1998; Martelli et al., 1997). Furthermore, another vitivirus i.e. *Grapevine virus D* (GVD) (Abou Ghanem et al., 1997) was detected in a vine (*V. vinefera)* showing corky Rugose wood symptoms, but its role in the RW is unclear. Diagnostic tools for the detection of grapevine viruses have evolved through the years to include highly sophisticated and sensitive detection methodologies, starting from biological indexing using woody indicators and herbaceous hosts, Enzyme-Linked Immuno Sorbent Assay (ELISA), Polymerase Chain Reaction (PCR), real-time TaqMan® RT-PCR and Low density array (LDA) . Detection of RW is based on bioassays, ELISA, and RT-PCR. Bioassays are widely used, but they are time and labor intensive. The low concentration of RW-related viruses and their uneven distribution in infected plants along with the seasonal titer variation, make detection by ELISA methods difficult and sometimes unreliable. Further investigations have shown the existence of large sequence variability among different isolates of RSPaV (Meng et al., 1998 and Lima et al., 2006) GVA (Murolo et al., 2008) and GVB (Shi et al., 2004). In addition, high concentrations of phenolic compounds and polysaccharides that exist in grapevine tissues inhibit the function of enzymes used in RT-PCR and thus prevent virus detection. Real-time PCR is increasingly being used for the detection and quantification of pathogens in plant tissue (Osman et al., 2007 and 2008). TaqMan® PCR is a sensitive method that substantially increases the reliability of virus detection in host plants. Primers and probes used in TaqMan® RT-PCR assays for the detection of viruses constituting the RW complex were designed from the CP regions that were proven to exhibit high degree of sequence conservation. Therefore the TaqMan® assays developed were able to detect more isolates than conventional RT-PCR. Two sample preparation methods were compared in standard and real-time TaqMan® RT-PCR assays using purified RNA and crude extract. TaqMan® Low-Density Arrays (LDA, Applied Biosystems, Foster City, CA, USA) has recently been introduced as a novel approach for pathogen detection. LDA is a modified method of real-time TaqMan® PCR that uses micro plates with 384 wells. Similar to real time RT-PCR, these arrays enable a more focused and sensitive approach for the detection of plant pathogens while offering higher

throughput compared to RT-PCR. In this study, the LDAs have been evaluated as a diagnostic tool for detecting grapevine viruses. The objective of this study was to design sensitive TaqMan® PCR and LDA assays for the detection of RSPaV, GVA, GVB and GVD with the capability to detect large sequence variation within the viruses. The results presented here clearly show that TaqMan® RT-PCR and LDA are robust and reliable methods for the detection of viruses constituting the RW complex of grapevine whether using purified RNA or crude extract as the starting template.

Results showed that more viruses were detected in tested samples when total RNA prepared by AB method was used (Table 1). This automated approach for nucleic acid extraction has also the advantages of allowing a rapid (<1 h), high throughput sample preparation and reduced possibilities of cross contamination between samples, therefore, suitable for processing large number of samples which usually required in diagnostic laboratories. In addition, RNA extracted by the AB method was quite clean, contained negligible amount of inhibitors and had appropriate C_T values when tested by the internal control of 18S rRNA TaqMan® assay (Osman *et al*., 2007). In contrast, GES is a crude method of preparing samples that includes inhibitors of RT-PCR enzymes. As a result, the amplification of low tittered viruses in the samples is inhibited. In this study, the comparison of the real-time TaqMan® RT-PCR assay to conventional RT-PCR showed that real-time TaqMan®RT-PCR detected viruses in grapevine samples that were previously tested negative by conventional RT-PCR (Table 2). The 4 fold serial dilution experiments of the viruses associated with RW revealed that TaqMan RT-PCR was 32 and 256 fold more sensitive than RT-PCR, respectively, when samples prepared by AB and GES methods were used (Fig.1). In addition the results showed that the TaqMan RT-PCR was 4 fold more sensitive in detecting samples prepared by AB method compared to GES method and 32 fold more sensitive when RT-PCR was used (Fig.1). Similar results were obtained for all four viruses used in this investigation. The reliability of TaqMan® RT-PCR assays for routine diagnostics was improved by adding the 18S rRNA TaqMan® assay as an internal control. In summary, the TaqMan® RT-PCR assays described here were designed in order to advance routine virus diagnostics in grapevines. No validated routine molecular assay has previously been reported for high-throughput testing of grapevine diseases associated with the RW complex. The TaqMan® RT-PCR assays developed were found to be sensitive, specific and robust in that a range of isolates of RSPaV, GVA, GVB and GVD from geographically diverse regions were detected. Also, the testing process which starts from AB nucleic acid extraction to TaqMan® RT-PCR final results can be achieved in less than 3 hours. In addition, in this method, the results are evaluated quantitatively based on C_T values, therefore, it eliminates the use of gel electrophoresis and gel documentation, saving time and labor, especially when large number of samples are involved. To further improve the diagnostic method, the very sensitive, high capacity LDA system was investigated for the simultaneous detection of RW viruses in infected grapevines. In this study the LDA C_T values were compared with results obtained from conventional RT-PCR and real-time TaqMan® RT-PCR. A comparison between conventional diagnostic methods and the TaqMan® RT-PCR and LDA showed that the latter two diagnostic techniques were very sensitive in detecting viruses. The designed TaqMan® RT-PCR assays and LDA had a broad range which could detect virus isolates collected from wide geographical regions where many of them were undetectable by conventional RT-PCR (Table 2). Quantification of the viruses is also possible by TaqMan® RT-PCR in extracts of total plant RNA as well as crude extract in GES buffer (Osman et al, 2006). In summary, LDA analysis is a molecular diagnostic method that is primarily based on the real time TaqMan® RT-PCR assays and this is the first report of its use in the detection of plant viruses. It is rapid, reliable, very sensitive, easy to perform and applicable to use for testing large number of samples. The results showed that the LDA technology is a promising and time-saving tool in detecting of plant pathogens and allowing simultaneous analyses of different pathogens in the same sample in a single reaction set up. LDA system produces comparable and often better results than those produced by TaqMan® RT-PCR.

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Table 1 Comparison between conventional RT-PCR and real-time TaqMan RT-PCR for the detection of *Rupestris stem pitting associated virus* (RSPaV), *Grapevine virus A* (GVA), *Grapevine virus* B (GVB) and *Grapevine virus* D (GVD). Virus isolates from different regions of the world were selected. Two different sample preparation methods of GES and total RNA extraction (RNA) were compared. '-'indicates negative result.

	RSPaV					GVA					GVB					GVD				
Origin	# of grape variety tested	RT-PCR RSP CP 48F-49R primers		TaqMan® PCR RSPaV TaqMan assay		# of grape variety tested	RT-PCR GVA C1 - V1 primers		TaqMan® PCR GVA TaqMan assay		# of grape variety tested	RT-PCR GVB C1 V1 primers		TaqMan® PCR GVB TaqMan assay		# of grape variety tested	RT-PCR GVD CP 7V - 471C primers		TaqMan® PCR GVD TaqMan assay	
		RNA	GES	RNA	GES		RNA	GES	RNA	GES		RNA	GES	RNA	GES		RNA	GES	RNA	GES
Afganistan	3	3	2	3	3	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Australia	7	5	4	7	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Belgium	1	1	–	1	1	1	1		1	1	1	1	–	1	–	1	1	–	1	–
Chile	–	–	–	–	–	2	2	1	2	2	2	–	–	2	–	2	–	–	–	–
China	2	2	1	2	2	–	–	–	–	–	1	–	–	1	1	1	–	–	–	–
Egypt	–	–	–	–	–	2	2	1	2	1	2	1	–	1	–	2	–	–	–	–
France	38	32	25	36	33	9	5	1	5	–	9	4	1	6	4	7	1	–	1	–
Germany	7	3	2	6	5	4	4	2	4	4	–	–	–	–	–	–	–	–	–	–
Greece	1	–	–	1	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–
Hungary	2	2	–	1	1	3	1	–	1	–	–	–	–	–	–	–	–	–	–	–
India	1	1	–	1	1	1	1	–	1	1	1	–		1	–	–	–	–	–	–
Italy	18	15	10	16	13	8	7	3	8	5	8	5	2	5	3	8	3	1	5	2
Israel	3	2	1	2	2	3	1	–	1	1	3	–	–	1	–	–	–	–	–	–
Mexico	1	1	–	1	–	1	1	–	1	1	–	–	–	–	–	2	–	–	1	1
Portugal	6	6	4	6	5	5	3	1	4	4	5	–	–	4	3	5	–	–	1	–
South Africa	3	1	–	3	2	2	2	1	2	2	2	–	–	1	–	2	–	–	1	1
Spain	2	2	–	2	2	2	1		2	2	–	–	–	–	–	2	–	–	–	–
Russia	12	8	5	10	7	11	4	1	6	5	11	4	1	8	3	10	–	–	–	–
USA	2	2	1	2	2	9	4	1	5	3	9	4	1	8	5	9	2	–	4	2
Yugoslavia	2	2	1	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Unknown	12	10	8	11	9	23	15	8	17	11	23	12	6	18	9	23	8	4	16	6
Total	123	98	65	104	96	87	54	20	63	43	77	31	11	57	28	75	15	5	30	12
% of detection		80%	53%	85%	78%		62%	23%	72%	49%		40%	14%	74%	36%		20%	7%	40%	16%

Table 2, A comparison between the efficiency of RT-PCR, TaqMan® RT-PCR and low density arrays (LDA) for the detection of Rupestris stem pitting associated virus (RSPaV), Grapevine virus A (GVA), B (GVB) and D (GVD). Total of 29 plants with multiple virus infections were selected for this experiment. Number of plants tested positive for each virus by different detection methods are recorded in the table.

Virus	RT-PCR	TaqMan® RT-PCR	Low Density Arrays using 2X AB RNA
RSPaV	15	24	25
GVA	8	12	13
GVB	12	17	20
GVD	4	5	6

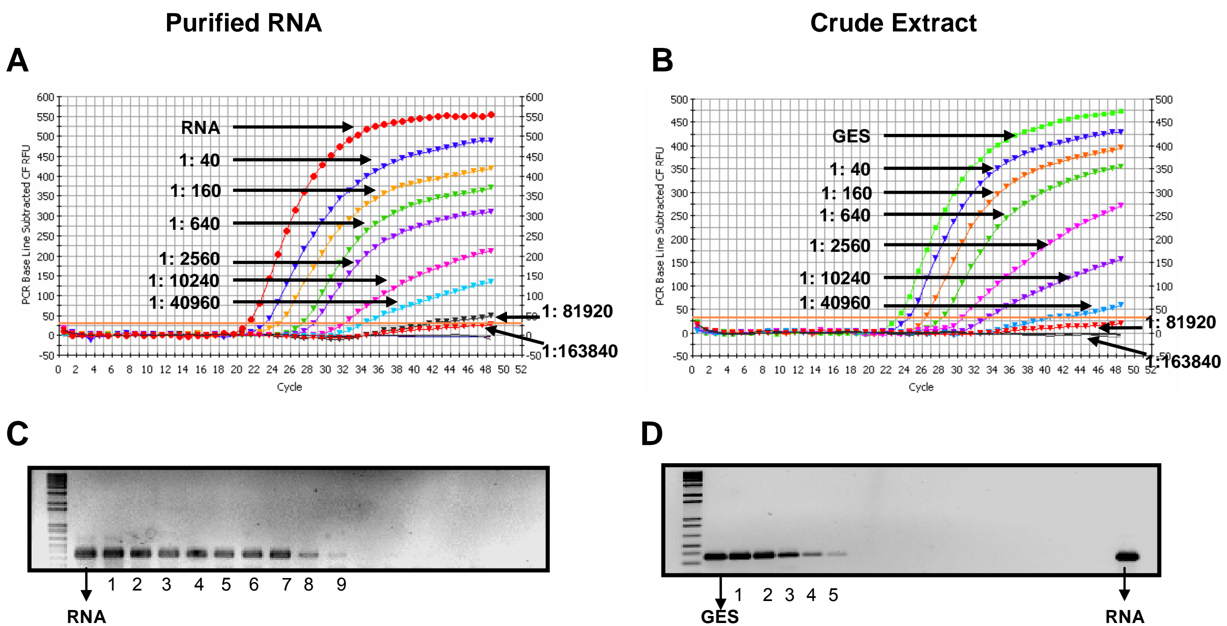

Fig. 1. Sensitivity comparison between conventional RT-PCR and TaqMan and assays for the detection of GVB using three replications of dilution series ranging from 1 to 1:163.840) from purified RNA (A and C) and infected grapevine crude extract (B and D). A and B Plots of the RNA dilution series against the threshold cycles values showing the dynamic range of detection of real-time RT-PCR assay. (B) Agarose gels showing the amplification products obtained by conventional RT-PCR with primers GVB V1 and GVB C1. Lane 1: 1 kb+ molecular weight marker (Invitrogen); lane 2: undiluted infected tissue; lane 3: 1:20 dilution, lane 4: 1: 40 dilution; 1:80 dilution; lane 5: 1: 160 dilution; lane 6: 1: 320 dilution; lane 7: 1:640dilution; lane 8: 1:1280 dilution, lane 9: 1: 2560 dilution.