The detection of avocado sunblotch viroid in avocado using a real-time reverse transcriptase polymerase chain reaction

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Abstract

The sunblotch disease is one of the diseases that affects avocado (*Persea americana* Mill) crops in particular, causing serious crop losses. The causal agent is the avocado sunblotch viroid (ASBVd), a circular RNA-based pathogen built of 247 nucleotides, which causes stem discoloration, bleaching, variegation, and distortion on the leaves and sunken yellow or red blotches on the fruits. In this study, a novel SYBR green-based real-time RT-PCR (RT-qPCR) assay specific for ASB viroid was developed. The assay was followed by a standard curve and a melting curve analysis for the detection, identification, and quantification of the full-length ASB viroid. The assay's lower limit of detection in the samples of avocado leaves was 8.8 copies/µl. The RT-qPCR was 100 times more sensitive to ASBVd than the conventional RT-PCR. The melting curve analysis showed a variation in the specific melting temperature (Tm) between positive samples, probably due to the alteration in the nucleotide sequences of viroids. In conclusion, the use of RT-qPCR assays established in this study, as a routine diagnostic method in breeding programs, could decrease the viroid incidence in avocado crops and its further accumulation in plants.

Key words: *Persea americana*, avocado sunblotch viroid, RNA-based pathogen, real-time RT-PCR, sensitivity, specificity.

Introduction

Avocado sunblotch viroid (ASBVd), a member of *Avsunviroidae* family (Flores et al., 2000a; Flores et al., 2000b; Flores et al., 2004), is a small, circular, globally distributed RNA-based pathogen of 247 nucleotides in length (Palukaitis et al., 1979; Hutchins et al., 1986; Daròs et al., 1994). The viroid induces the sunblotch disease (Dale et al., 1982; Desjardins, 1987) causing characteristic symptoms such as stem discoloration, bleaching, variegation, and distortion on the leaves and sunken yellow or red blotches on the fruits (Semancik and Szychowski, 1994). The pathogen reduces crop yields by 18–95% (Wallace and Drake 1962; Vargas et al., 1991; Lemus et al., 2005; Grozo et al., 2006). ASBVd is trans-mitted by the grafting of infected budwoods, pollen, and seeds, but it has no known vector (Mathews et al., 1997).

Currently, diagnostic techniques such as nucleic acid hybridization and reverse transcription coupled with polymerase chain reaction (RT-PCR) have become a common practice for the detection of viroids because of their relative simplicity and high sensitivity when compared with conventional diagnostic methods such as biochemical indexing, *in situ* hybridization, polyacrylamide gel electrophoresis (PAGE), and nucleic acid spot hybridization (Allen and Dale, 1981; Mackenzie et al., 1997; Bernard and Duran-Vila, 2006; Fekih Hassen et al., 2006; Hajizadeh et al., 2012). However, the con-

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ventional RT-PCR procedure for detecting ASBVd leads to false-negative results due to primer dimer formation, the presence of samples with low viroid levels, and unequal distribution of viroids throughout the plants. Because of these issues, certification programs that verify pathogen-free status are not maximally effective, and may thus permit the pathogen to infect new avocado plantations and areas.

A rapid, sensitive, and quantitative method for monitoring the viroid replication and translocation throughout plant tissues, with increased capacity to avoid false-negative results, is necessary to support both basic research and field applications. Real-time RT-PCR (RT-qPCR) has shown high sensitivity in the viroid detection in many previous studies (Boonham et al., 2004; Roenhorst et al., 2005; Tessitori et al., 2005; Ruiz-Ruiz et al., 2007; Rizza et al., 2009; Monger et al., 2010; Luigi et al., 2011; Parisi et al., 2011, Botermans et al., 2013). In this study, we developed a real-time RT-PCR assay based on SYBR Green I for the absolute quantification of ASBVd in avocado samples.

Methods

Sample collection

Plant samples used in this study consisted of Hass avocado (*Persea americana*, Mill) leaves collected from symptomatic and asymptomatic trees in Peruvian non-commercial avocado crops in La Libertad, Peru. Five leaves from each of 390 avocado trees (10 symptomatic and 380 asymptomatic) of 19 plots in 5 different areas were sampled. Leaf age can affect the symptom expression and prevent RNA extraction (Suzuki et al., 2001); therefore, leaves were taken from the middle to the top of the tree to avoid collection of older leaves near the bottom. Leaves from *in vitro* Hass avocado ASBVd-free plants were used as a negative control.

RNA extraction and cDNA synthesis

Viroid RNA from leaf samples (~250 mg) was isolated following a previously described protocol (Chang et al., 1993). The extracted RNA was eluted in 11 µl of nuclease-free water and stored at −80°C until use. A viroid RNA obtained from the sample of a symptomatic tree, previously confirmed by RT-PCR in a reference laboratory, was used as a positive control. The cDNA was synthesized in a 25 µl reverse transcription reaction containing 1 µg of RNA template, 1X RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2, 50 mM DTT), 1.25 µM of each primer (ViroidR, ASBVd2qPCR-R, and ActR), 0.5 mM of dNTPs, 20 U of Ribolock RNase Inhibitor (Fermentas, Thermo Scientific, Waltham, MA USA), 200 U of Maxima Reverse Transcriptase (Fermentas, Thermo Scientific, Waltham, MA, USA). A reverse transcription reaction was conducted at 55°C for 55 min.

### ABSVd detection using PCR and quantification by RT-qPCR

The specific primers taken from Schnell and coworkers (1997) and Schnell and coworkers (2001) were used for ASBVd detection by end-point PCR. The ASBVd2qPCR-F and ASBVd2qPCR-R primers for the quantification of ASBVd by RT-qPCR were designed using the online Primer3 version 4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/). Primer ActF and ActR of β-actin were used as internal controls (Brunner et al., 2004).

The end-point PCR was carried out in a 50 µl reaction mixture containing 1 µl cDNA, 25 µl PCR master mix (2×) (Fermentas, Thermo Scientific, Waltham, MA USA), 1 µl (0.4 µM) of both primers, and 22 µl of Ultra-pure water (Invitrogen, USA). cDNA from samples was amplified using the following conditions: 95°C for 4 min, then 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. All PCR reactions were conducted in triplicate and the products were analyzed on a 3% agarose gel in 1× TAE buffer to confirm the specificity of the reaction.

Real-time RT-PCR reactions were set up using a total volume of 15 µl containing 1 µl of cDNA, 7.5 µl 2× LightCycler 480 SYBR Green I Master (Roche Diagnostic GbH, Germany), 1.0 µl (0.6 µM) of each primer, and 4.5 µl of DNase/RNase-free distilled water (Life Technologies, USA). Real-time PCR experiments were run on a LightCycler 480 Instrument II (Roche Diagnostics, Mannheim, Germany) after activation of the hot start enzyme for 5 min at 95°C, followed by 40 cycles of 10 s at 95°C, 10 s at 62°C, and 10 s at 72°C. Amplification signals were detected during the annealing step of each cycle at 62°C, using a SYBR Green I (483–533 nm). For the melting curve analysis, after the PCR machine was heated, the reaction mixtures were kept at 95°C for 1 min, allowed to cool at 55°C for 1 min, and then slowly heated again to 95°C at a ramp rate of 0.06°C/s. Continuous fluorescence was acquired at the

Table 1. The sequences of primers used for ASBVd detection and quantification by RT-PCR and RT-qPCR

Primer name	Sequence (5′–3′)	References
Viroid-F	AAGTCGAAACTCAGAGTCGG	Schnell et al. (1997)
Viroid-R	GTGAGAGAAGGAGGAGT	
ASBVd2qPCR-F	TGTTCCGACTTTCCGACTCT	this study
ASBVd2qPCR-R	AAACCTTGCGAGACTCATCA	
ACT-2F	CCCATTGAGCACGGTATTGT	Brunner et al. (2004)
ACT-2R	TACGACCACTGGCATACAGG	
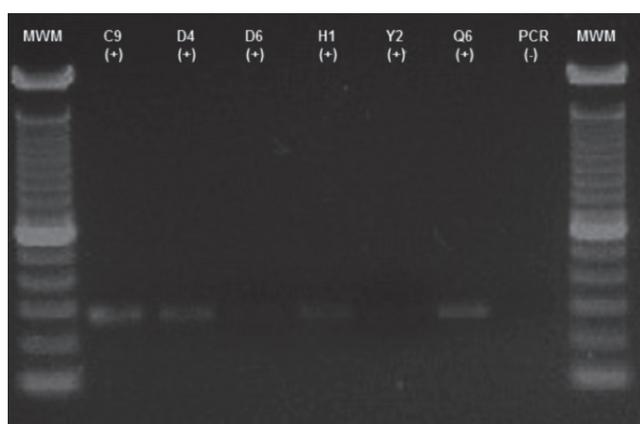
Fig. 1A. RT-qPCR assay for ASBVd detection in leaf samples; migration of positive detection of ASBVd by RT-qPCR in avocado leaves: D4, D6, H1, Y2 (asymptomatic), C9, Q6 (symptomatic) samples show the specific product 249 nt; PCR (−) – negative control (RNA from ASBVd-free plant); MWM – Molecular Weight Marker

Fig. 1B. β-actin amplification of different avocado leaves samples (D1–D7) used as an internal control (219 nt), CE – extraction negative control, CPCR – PCR negative control (RNA from a bacterial sample), MPM – Molecular Weight Marker

indicated wavelengths. The RT-qPCR products were analyzed on a 3% agarose gel in 1X TAE buffer to confirm their specificity.

Sequencing

Amplicons, obtained in the RT-PCR and RT-qPCR, were directly sequenced after purification. Aliquots of 40 µl of each amplicon were purified with the Qiamp PCR purification kit (Qiagen, Hilden, Germany) and sequenced using the BigDye Terminator Cycle Sequence Kit v3.1 in an ABI3130xl automated sequencer (Applied Biosystems, USA) using ViroidR and ASBVd2qPCR-R primers. The obtained nucleotide sequences were subjected to the basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/blast).

Detection limit and the standard curve

A log-10 dilution series of ASBVd artificial template (plasmid) was done in Ultrapure water (Thermo Fisher Scientific, USA) containing an initial concentration of $6.37\,E+08$ copies/µl. Serial dilutions were then evaluated in triplicate by using the optimized RT-qPCR assay in order to determine its analytical sensitivity and repeatability. PCR standard curves were generated by analyzing the crossing point (Cp) values acquired from the LightCycler 480 instrument II with LightCycler 480 software release 1.2.0.0625, version 1.2.0.169.

The detection limit of the RT-qPCR and RT-PCR assays was determined by testing 2 serial 10-fold dilutions of the *in vitro* transcribed viroid RNA ranging from 10^5 to 10^0 copies/µl. The results from both assays were compared to determine the most sensitive assay.

Detection of ASBVd from avocado leaves

Leaves samples from 380 asymptomatic avocado trees (*Persea americana* Mill) located in La Libertad, Peru, were evaluated for the detection and quantification of ABSVd using the RT-qPCR assay described above.
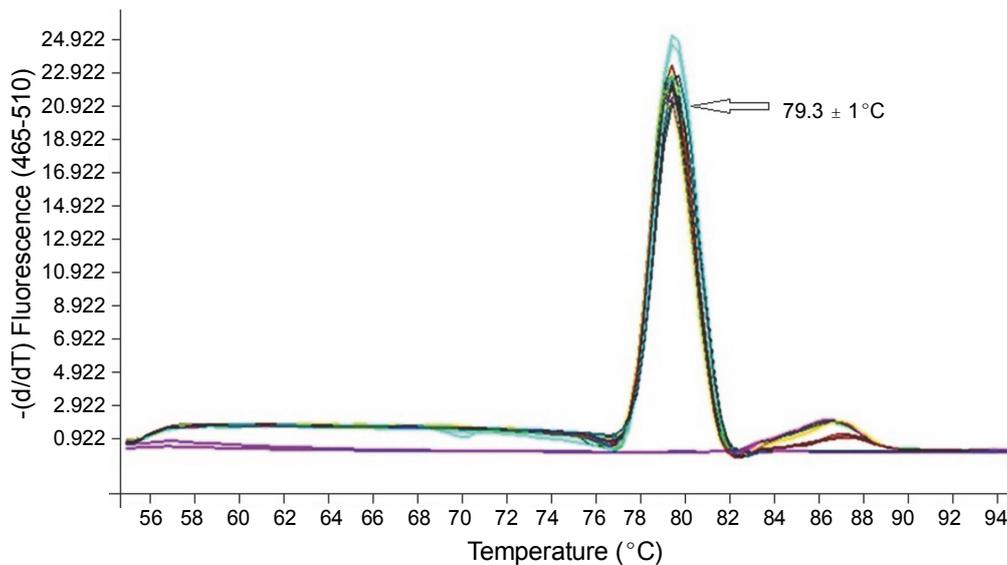
Fig. 2. Melting curve analysis of RT-qPCR assay for ASBVd detection of symptomatic sample. The positive sample shows the specific peaks at 79.3 ± 1 °C (lines green, red, gray, yellow, brown and turquoise), negative samples (water and RNA from ASBVd-free plant) do not show peak (violet lines). The X-axis shows the melting temperature and the Y-axis derivate intensity of fluorescence -(d/dT) from SYBR Green dye in the melting temperature process
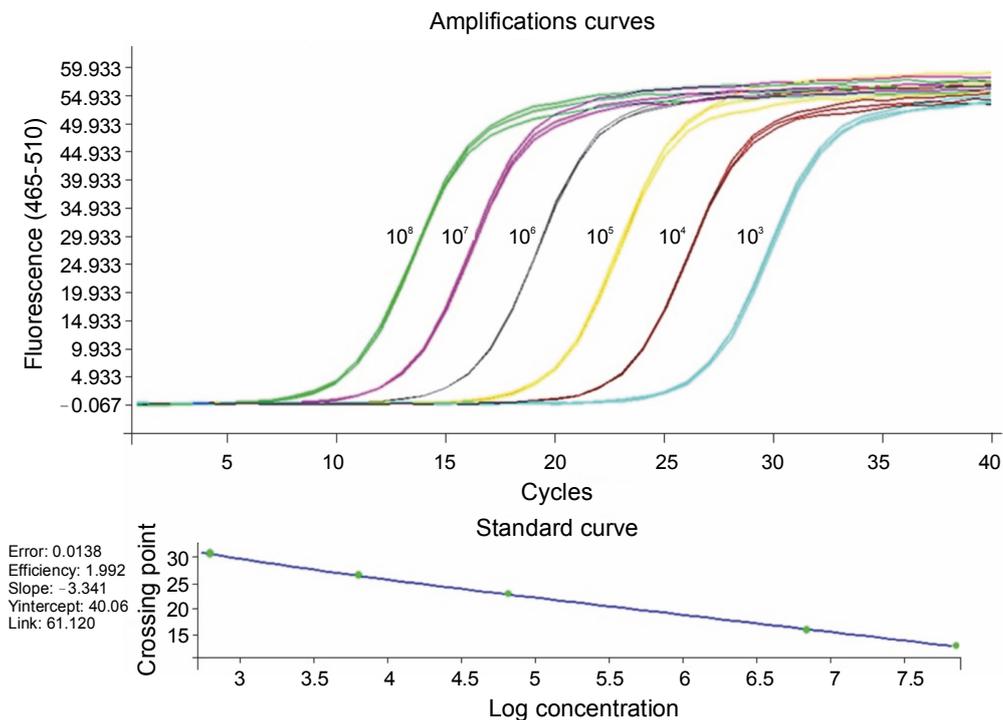
Fig. 3. Standard curve of RT-qPCR assay for ASBVd detection and quantification; upper: the assay amplification kinetics performed accurately enough to quantify ASBVd levels in different samples of avocado crops (slope = – 3.341 and efficiency = 1.992); the lines green, pink, gray, yellow, brown and turquoise represents standard serial dilutions from 6.37E + 08 to 6.37E + 03 copies/μl; the X-axis shows the amplification cycles and the Y-axis the intensity of fluorescence from SYBR Green dye used into RT-qPCR assay; bottom: linear regression of serial dilution for ASBVd standard, parameters in the left are the error produce in the triplicate of the sample; the efficiency of amplification which one if it is close to 2 theoretically duplicate the product amplified; slope of the linear regression which one if it is close to – 3.332 theoretically means that the dilutions have a good performance; graphics in the right shows X-axis is the log of concentration of plasmid and the Y-axis is the crossing point from each standard dilution

Table 2. Limit of Detection (LOD) comparison between RT-PCR and RT-qPCR for ASBVd detection using serial dilution of 2 quantified samples (A and B) with different genome viroid loads (A: $3.41E + 02$ copies/µl and B: $1.78E + 05$ copies/µl of viroid genome); the results show that the RT-qPCR was more sensitive than RT-PCR

| Dilution	Sample A			Sample B		
	Cp[a]	RT-qPCR[b] concentration (copies/µl)	RT-PCR[c] result	Cp[a]	RT-qPCR[b] concentration (copies/µl)	RT-PCR[c] result
10^0	30.15	$3.41E + 02$	negative	21.12	$1.78E + 05$	positive
10^{-1}	33.02	$4.68E + 01$	negative	24.55	$1.65E + 04$	positive
10^{-2}	35.42	$8.83E + 00$	negative	28.02	$1.50E + 03$	positive
10^{-3}	0	0	negative	31.98	$9.59E + 01$	negative
10^{-4}	0	0	negative	34.84	$1.32E + 01$	negative
10^{-5}	0	0	negative	0	0	negative

a – crossing point: the crossing point is the cycle at which fluorescence achieves a defined threshold (it corresponds to the cycle at which a statistically significant increase in fluorescence is first detected); b – Reverse Transcription Real Time Polymerase Chain Reaction; c – Reverse Transcription Polymerase Chain Reaction
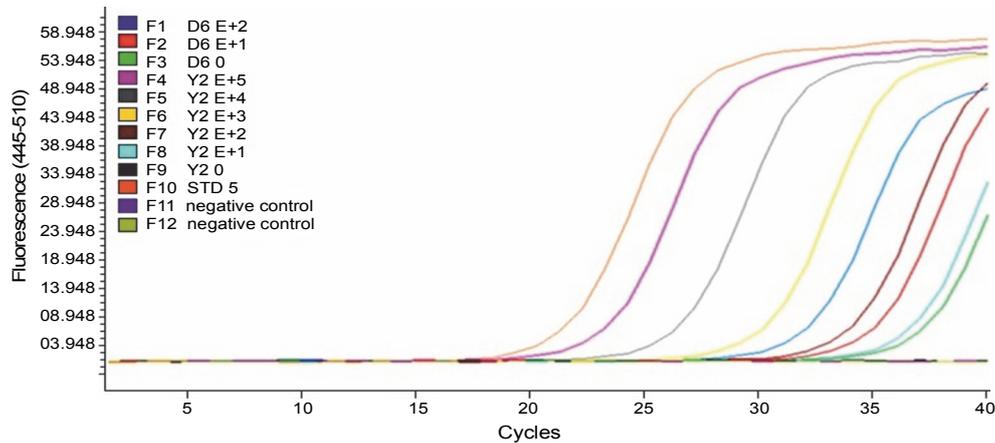

Fig. 4A. Amplification of serial dilution of two samples of ASBVd to determine the lower detection limit. Kinetic amplification of serial dilution of higher (B: $1.78E + 05$ copies/µl) and lower sample (A: $3.41E + 02$ copies/µl)
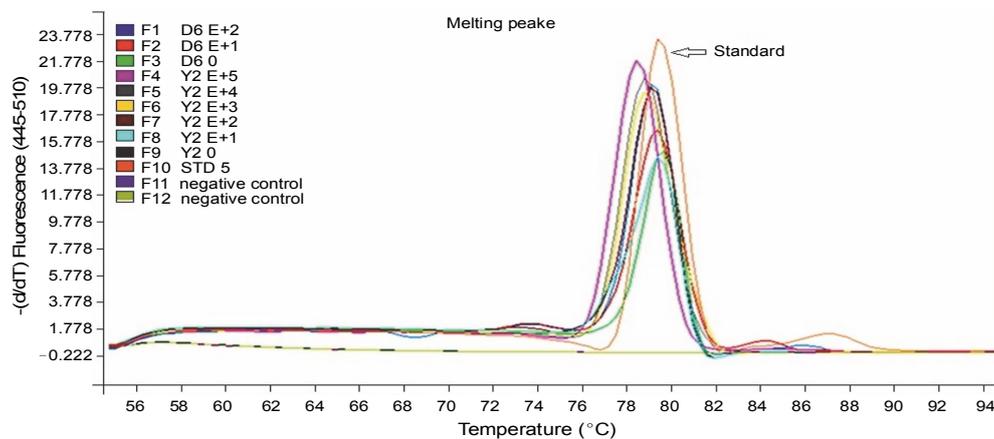
Fig. 4B. Melting curve analysis shows a specific product for ASBVd between 78.46 and 79.52 °C possibly due to a small internal change in the sequence; blue line – sample A: $3.41E + 02$ copies/µl; red line – sample A: $3.41E + 01$ copies/µl; green line – sample A: 3.41 copies/µl; pink line – sample B: $1.78E + 05$ copies/µl; gray line – sample B: $1.78E + 04$ copies/µl; yellow line – sample B: $1.78E + 03$ copies/µl; brown line – sample B: $1.78E + 02$ copies/µl; turquoise line – sample B: $1.78E + 01$ copies/µl; dark green line – sample B: 1.78 copies/µl; orange line – standard $6.27E + 05$; violet and medium green lines – negatives control (water and RNA from ASBVd-free plant, respectively)
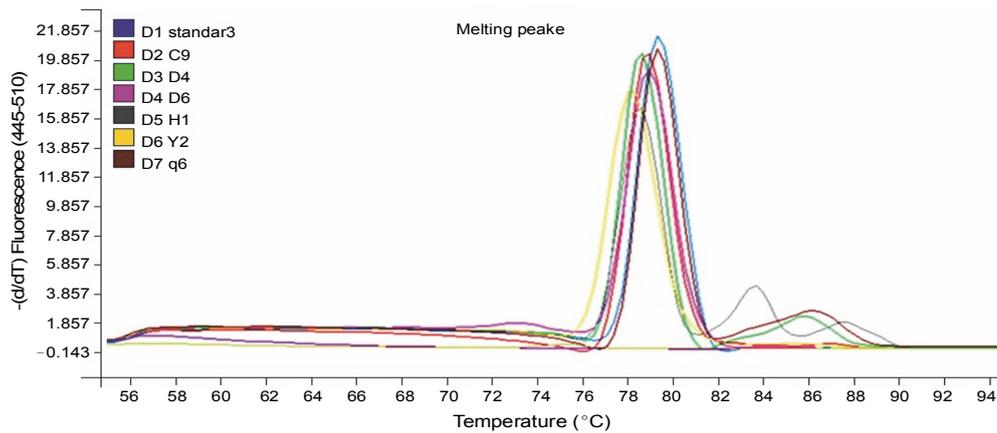
Fig. 5A. Amplification by RT-qPCR of 380 avocado trees (leaves samples) for ASBVd detection; kinetic amplification of some positive samples
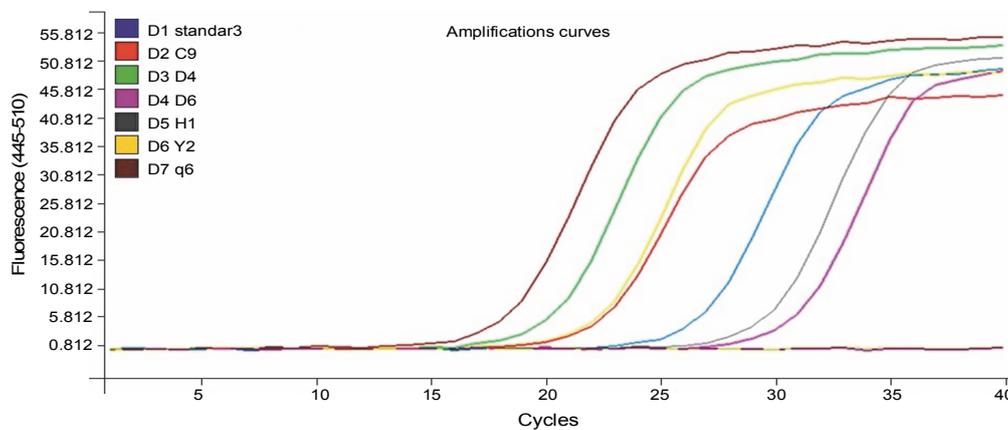
Fig. 5B. Melting curve analysis showing the specific product for ASBVd between 78.33°C and 79.46°C may be due to a small internal change in the sequence; blue line: standard 6.37 E + 03 copies/µl; red line: sample C9; green line: sample D4; pink line: sample D6; gray line: sample H1; yellow line: sample Y2 and brown line: sample q6; violet and medium green lines: negatives control (water and RNA from ASBVd-free plant, respectively)

Results

Detection of ASBVd by RT-PCR and RT-qPCR

Detections of ASBVd from symptomatic avocado leaves by RT-PCR and RT-qPCR were carried out using primers previously developed by Schnell and coworkers, (1997) and primers developed in the current study (Table 1). Four symptomless avocado leaves tested positive for ASBVd in RT-qPCR reaction and were further confirmed by sequencing.

The RT-qPCR primers designed from a full circular viroid genome (249 bp) were able to detect viroid samples better than RT-PCR, avoiding false negatives. The specific amplifications of the target RNA of ASBVd (Fig. 1A) and β-actin (Fig. 1B) were confirmed by agarose gel electrophoresis. The melting curve analysis

showed the absence of nonspecific peaks (Fig. 2), and a specific peak (TM) of positive samples (79.3 ± 1°C) was determined.

Detection limit and the standard curve

The linear range of quantification of the RT-qPCR assay for ASBVd was determined by using 10-fold serial dilutions of the standard (range from 6.37 E + 03 to 6.37 E + 08 copies/µl). The standard curve (Fig. 3.) covered a linear range of 5 orders of magnitude. The slope (−3.341) and the correlation coefficient (R2 = 0.986) of the standard curve showed that this assay could be used to quantify the target RNA in infected avocado plants.

From serial dilutions of 2 samples (A: 3.41 E + 02 copies/µl; B: 1.78 E + 05 copies/µl), the RT-qPCR assay was able to detect the amplification in 1E-024 and 1E-04

Table 3. The results of positive amplification for ASBVd by RT-qPCR in avocado leaves. The melting curve analysis showed a melting temperature (TM) in a range between 78.33°C and 79.46°C (the difference in the TM was probably due to a small nucleotide change in the viroid sequence)

Positive samples	Cp[a]	Viroid concentration (copies/μl)	TM[b]
D4	19.92	4.52 E + 5	78.77
D6	30.58	6.40 E + 2	79.13
C9	21.93	1.13 E + 5	79.1
q6	18.06	1.63 E + 6	79.46
H1	29.47	1.12 E + 3	78.68
Y2	21.93	1.13 E + 5	78.33

a – crossing point; b – melting temperature: the melting temperature is defined as the temperature at which 50% of the DNA helices are dissociated and the fluorescence decays

dilutions (samples A and B, respectively), while the RT-PCR assay detected the viroid in the 1E-02 dilution (sample B) only. The results showed that the detection sensitivity of RT-qPCR assay was about 100 times higher than the RT-PCR (Table 2) and it also detected the lowest viroid titer. This RT-qPCR assay enabled us to detect as few as 8.8 copies/μl of the ASBVd in avocado total RNA extracts. The amplification kinetics (Fig. 4A) and the melting curve (Fig. 4B) confirmed the specific amplification of the ASBVd.

Detection of ASBVd from avocado leaves

Detection of ASBVd by the RT-qPCR assay was carried out using the total RNA isolated from 380 avocado leaves. Six samples tested positive for ASBVd (Fig. 5A and Table 3), the melting curve analysis of the positive samples showed a Tm between 78.33°C and 79.46°C (Fig. 5B). The difference in the melting temperature was probably due to a small change in the nucleotide sequences of viroids.

Discussion

The need for sensitive, rapid, and reliable methods of detection of pathogens including microbes, viruses, and viroids is critical for the health of many plants (Candresse et al., 1998; Saponari et al., 2013). In this study, RT-qPCR detected the avocado sunblotch viroid in infected samples with viroid levels that has been too low to be detected for routine methods (conventional RT-PCR, Nucleic acid spot hybridization-NASH, etc.). These results highlight a higher sensitivity and specificity of RT-qPCR, which makes the detection and quantification of ASBVd possible already in early infections of avocado trees. The 100-fold increase in the sensitivity gained by using RT-qPCR is likely to increase the effectiveness of certification programs that require the initial vegetative material of avocado to be free from ASBVd.

A careful selection of primers has proven to be the most effective approach to increase the sensitivity of the method, compared with RT-PCR (Bustin et al., 2009; Zhang et al., 2013a; Zhang et al., 2013b). When using primers reported by Schnell and coworkers (1997) for RT-qPCR, 4 of the 10 positive samples detected by RT-PCR were reported as negative by the RT-qPCR and further sequencing. The RT-qPCR method allowed obtaining more accurate results and detect lower virus titer (8.8 copies/μl), when compared to conventional RT-PCR.

Changes in single nucleotides in viroid genome sequences can result in altered symptoms in avocado trees (Schnell et al., 2001). By sequencing, we determined that changes in nucleotide sequences are also responsible for the differences in melting temperatures observed in RT-qPCR (C to T in position 202 and G to A in position 207). Our findings were similar to the results reported by Schnell and coworkers (2001) and Cambrón (2011), and variations require further studies. Based on the sensitivity, specificity, accuracy, and repeatability of this RT-qPCR assay, it is suggested that its employment for routine diagnostics in breeding programs might decrease the viroid incidence in avocado crops and its further accumulation in plants.

Acknowledgments

Ethics statement

This study was approved by the Research and Development Committee of Camposol S.A. The assay did not involve endangered or protected species and hence no specific permissions were required.

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