Real-time RT-PCR for detection of *Raspberry bushy dwarf virus*, *Raspberry leaf mottle virus* and characterizing synergistic interactions in mixed infections☆

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ABSTRACT

Two TaqMan-based real-time One-Step RT-PCR assays were developed for the rapid and efficient detection of *Raspberry bushy dwarf virus* (RBDV) and *Raspberry leaf mottle virus* (RLMV), two of the most common raspberry viruses in North America and Europe. The primers and probes were designed from conserved fragments of the polymerase region of each virus and were effective for the detection of different isolates tested in this study. The RBDV assay amplified a 94 bp amplicon and was able to detect as few as 30 viral copies. Whereas the RLMV assay amplified a 180 bp amplicon and detected as few as 300 viral copies from plant and aphid RNA extracts. Both assays were significantly more sensitive than their corresponding conventional RT-PCR methods. The sensitivity of the RLMV assay was also tested on single aphids after a fixed acquisition access period (AAP). In addition, the assays revealed a novel synergistic interaction between the two viruses, where the concentration of RBDV was enhanced ~400-fold when it occurred in combination with RLMV compared to its concentration in single infections. The significance of this finding and the importance of the development of real-time RT-PCR assays for the detection of RBDV and RLMV are discussed.

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1. Introduction

Real-time PCR is increasingly being used for detection and quantitation of plant viruses and has become the method of choice for studies on synergistic interactions between viruses because of its high sensitivity and reproducibility (Lopez et al., 2006; Osman et al., 2007; Wintermantel et al., 2008; Shiller et al., 2010; Tatineni et al., 2010; Harper et al., 2011). Other advantages of this technique include shorter detection times (especially for RNA viruses that require a reverse transcription step), improved specificity, closed-tube procedures that reduce contamination and high throughputs.

Raspberry bushy dwarf virus (RBDV) and *Raspberry leaf mottle virus* (RLMV) are two of the most common raspberry viruses in the Pacific Northwest (PNW) (Oregon and Washington, U.S. and British Columbia, Canada) and Europe (Martin et al., in press). RBDV, a pollen-borne idaeovirus, has long been cited as the causal agent of crumbly fruit disease in some raspberry and blackberry cultivars including 'Meeker' and 'Marion', respectively (Strik and Martin, 2003). RLMV is a member of the genus *Closterovirus* (fam. *Closteroviridae*) (Tzanetakis et al., 2007a; McGavin and MacFarlane, 2010) and is present at high incidence in commercial 'Meeker' fields in Washington State (Quito-Avila and Martin, unpublished data). RLMV is transmitted by the large raspberry aphid *Amphorophora agathonica* in a semi-persistent manner and has been implicated as a component in viral complexes responsible for important raspberry diseases such as raspberry mosaic, raspberry decline (Murant, 1974; Converse et al., 1987; Cadman, 1951, 1952; Jones and Murant, 1975; Halgren et al., 2007; Jones et al., 2002), and raspberry crumbly fruit (Quito-Avila et al., 2009).

Because of the high error-prone nature of RNA polymerases and the frequent recombination events between diverged sequences, RNA viruses are known for being genetically diverse even at the species level. This is particularly true for viruses with large genomes such as closteroviruses, which have been reported to be more diverse towards the 3′ end of the genome (Karasev, 2000; Ayllon et al., 2006; Sentandreu et al., 2006). At least two isolates have been reported for RLMV in North America and Europe where high variability at the amino acid and nucleotide levels were found in sequences belonging to the coat protein (CP) and the heat shock protein 70 homolog (HSP70 h) (Tzanetakis et al., 2007a; McGavin and MacFarlane, 2010). Likewise, several RBDV isolates have been reported from different hosts including red raspberry, blackberry, *Rubus multibracteatus* from China, and, recently, grapevine (Ellis

et al., 2005; Chamberlain et al., 2003; Mavric et al., 2009; Viršček et al., 2010; Valasevich et al., 2011).

In this study, two TaqMan-based One-Step-Real-Time RT-PCR assays for the rapid detection and quantification of RLMV and RBDV were developed. The primers and probes were designed from conserved genomic regions to detect multiple isolates of each virus. In addition, based on the results of a recent survey conducted on raspberry fields in northern Washington, where RLMV is present at high incidence and the severity of RBDV produced crumbly fruit is more prevalent, the assays were used to evaluate the titer of each virus in single and mixed infections. Virus complexes, involving at least two different viruses, are rather common in nature and often result in the induction of more severe symptoms compared to symptoms produced by either of the viruses in single infections. This phenomenon is usually caused by synergistic interactions that result in increases in vector transmission efficiencies, cell-to-cell movement and increases in virus replication resulting in higher virus titers (Kassanis, 1963; Pruss et al., 1997; Vance, 1991, 1999; Voinnet et al., 1999; Karyeija et al., 2000; Wintermantel et al., 2008; Tatineni et al., 2010). In *Rubus*, Blackberry yellow vein disease (BYVD) is probably one of the best examples, described thus far, of disorders caused by virus complexes. The disease, initially thought to be caused by the crinivirus *Blackberry yellow vein associated virus* (BYVaV), was found to be the result of an interaction involving at least two viruses: BYVaV and the potyvirus *Blackberry virus Y* (BVY). The viruses do not cause symptoms when present alone, whereas in mixed infections, the titer of BVY was increased several fold, the titer of BYVaV was decreased approximately 500-fold and dramatic vein yellowing and line patterns developed (Susaimuthu et al., 2008).

The real-time assays reported in this paper were validated by detecting each virus from singly infected plants and 50 mixed-infected plants obtained from commercial raspberry fields all of the cultivar 'Meeker', and RLMV from single aphids after a short virus acquisition period.

2. Materials and methods

2.1. Virus isolates and plant material

Five isolates of RLMV were used in this study. Three of them: RLMV-GC-8-102, RLMV-GC-8-153 and RLMV-WSU-991 had been used previously for sequencing analysis and virus variability studies (Tzanetakis et al., 2007a). Two new variants (RLMV-8 W and RLMV-F-APh) obtained from commercial raspberry fields in Washington State were also included. RBDV isolates used for sequence alignments and primer design included RBDV-1860, RBDV-Fav-15, RBDV-Fav-33 and RBDV-Rub-30 obtained from the *Rubus* virus collection at the USDA Agricultural Research Service (USDA-ARS), in Corvallis, Oregon; and RBDV-9S obtained from commercial fields in northern Washington in 2009. The virus isolates were maintained in one of the virus collection greenhouses at the USDA-ARS. This is an aphid-and-pollen-free facility so that no cross-infections of different isolates can occur.

More than 400 raspberry plants from 20 different commercial fields in Washington and Oregon were sampled in 2010 and tested for RLMV and RBDV as part of a separate study. Fifty of these samples, from plants doubly-infected with RBDV and RLMV were selected for validating each real-time assay developed in this study.

2.2. RNA extraction and conventional RT-PCR

Total RNA was extracted and precipitated in isopropanol as described (Halgren et al., 2007), followed by resuspension in 500 μl of wash buffer and 25 μl of glass milk (15 μl for single aphids)

(Rott and Jelkmann, 2001). The glass milk was pelleted at $1400 \times g$, washed with and 500 μl of wash buffer and dried in a speed vac at room temperature. The RNA was resuspended in 150 μl of TE. The procedure was the same for plants and aphids, except that the amount of extraction buffer used for single aphids was 350 μl instead of 1 ml used for leaves. In order to compare the sensitivity of TaqMan assays relative to conventional RT-PCR, reverse transcription reactions, using plant and aphid RNA, were performed using random primers as described (Halgren et al., 2007). Two and one half microliters of the RT product were used as template for the PCR in a final volume of 25 μl. The reaction was carried out according to the polymerase manufacturer's instructions (TaKaRa Bio, Shiga, Japan). In addition, conventional RT-PCR was performed using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) using the same real-time primers but no probe to account for differences in detection sensitivity attributed to the buffer/enzyme system. To assess the RNA quality and effectiveness of the RT reaction from aphid RNA, the universal Actin primers Act-2F (5′-ATGGTCGGYATGGGNCAGAAGGAC-3′) and Act8-R (5′-GATTCCATACCCAGGAAGGADGG-3′) (Staley et al., 2010) were used to amplify a 683 bp transcript region. The detection primers used routinely in our laboratory for detection of RBDV and RLMV (Tzanetakis et al., 2007a) were employed for sensitivity comparison purposes. The PCR program for amplification of the targets consisted of initial denaturation for 4 min at 94 °C followed by 40 cycles with denaturation for 40 s at 94 °C, annealing for 25 s at 55 °C for RBDV and RLMV, and 52 °C for Actin, and extension for 40 s at 72 °C, with a final 7-min extension step at 72 °C.

2.3. Primers and TaqMan probes

A polymerase region of about 800 bases for RLMV and 600 bases for RBDV was amplified by PCR from each of the five isolates included in this study. Five individual PCR-products per isolate were cloned into the StrataClone PCR Cloning Vector pSC-A-amp/kan (Agilent Technologies, La Jolla, CA, USA) and subsequently sequenced in an ABI 3730XL DNA analyzer by Macrogen (Seoul, Korea). The sequences corresponding to each virus were aligned by ClustalW (Thompson et al., 1994) and used to design primer/probe sets from conserved regions. Sequences used in the alignments can be retrieved from the GenBank under accession numbers JN651152–JN651156 for RBDV and JN651157–JN651161 for RLMV.

Three different TaqMan assays were designed and tested for each virus. Sets showing the lowest *Ct* values (*Ct value*: the cycle at which the amplification/luminescence reaches a pre-established threshold) were selected for further detection and quantification purposes (data not shown). The sequence of the primer/probe sets that were tested and selected are listed in Table 1. In addition, a TaqMan assay for amplification of the highly conserved *ndhB* plant gene was designed. This gene has been shown to be constitutively expressed under different environmental and pathological conditions in *Rubus* and other species (Thompson et al., 2003; Tzanetakis et al., 2007b), hence, it was used to normalize the RNA template in samples containing single infections (RLMV or RBDV) and mixed infections (RBDV plus RLMV). All TaqMan probes were manufactured by IDT (Integrated DNA Technologies Inc., IA, USA) using double-quenched probes (5′FAM/ZEN/3′IBFQ). The AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) was used to perform the reverse transcription and PCR in a single tube. The optimal reaction consisted of 10 μl of 2× RT-PCR buffer, 1 μl of primer/probe mix (5 μM primers and 2.5 μM probe), 3 μl of RNA sample (150 ng total), 0.8 μl of the 25× RT-PCR Enzyme-Mix, and nuclease-free water to a final volume of 20 μl. Each sample was done in triplicate using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following

Table 1
List of primers and probes tested for detection of *Raspberry bushy dwarf virus* (RBDV), *Raspberry leaf mottle virus* (RLMV) and the endogenous control (*ndhB*). Bolded denote the sets with the highest sensitivity and amplification efficiencies.

Assay	Forward	Reverse	Probe	Ampl. size (bp)
RBDV/1st	TGGAGAATGTGGGCAATGAG	CCAATTCTATCGACCTCAGCTC	5′-/56-FAM/CGGAAAACC/ZEN/GGAAACACCCGAAG/3IABkFQ/-3′	145
RBDV/2nd	GGAAGGTGTCTGACTCTATGA	CCAAATTTATGGTGACAATAG	5′-/56-FAM/ATGAGTTGC/ZEN/CGTCACGTATGCCG/3IABkFQ/-3′	124
RBDV/3rd	**TGGGAGATCCAATGTTCATAGT**	**CATCAGACTCTCAGTCATCGT**	**5′-/56-FAM/ACGATGAGT/ZEN/ATGTCGTTCATTGTCCCT/3IABkFQ/-3′**	**94**
RLMV/1st	TCTCCAGTCCCAAACTCTTTG	CATCCCTTTTCAGCAACCAC	5′-/56-FAM/CGTCAGTCC/ZEN/GTTATCTCCATCTCCGA/3IABkFQ/-3′	144
RLMV/2nd	GATATTGCTGTTTCCGCTGTG	TTGGTGAAAGGACTCTGAAGG	5′-/56-FAM/AGGAACGTA/ZEN/GGGCTCTGACTTAGGC/3IABkFQ/-3′	149
RLMV3rd	**TACTCCAGTCCCAAACTCTTCG**	**ATCGAGGTCGGTCATGAGAG**	**5′-/56-FAM/AACTTCGGC/ZEN/CAACTTCTCGGC/3IABkFQ/-3′**	**180**
ndhB	AAGCAAAAGTTCCTAGATTCATGG	TTGCGTATTCGTCCATAGGTC	TGCTTGCATATCCACCATTTGAGTCTCC	132

parameters: 15 min at 45 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 50 s at 60 °C.

2.4. Sensitivity and efficiency assays

A standard curve (threshold values [*Ct*] versus number of target copies) was generated to determine the efficiency and detection limit of each assay. The cDNA used to generate *in vitro* transcripts was obtained by RT-PCR using RNA extracts from each virus. The primer locations for the amplification of each template were the same as the ones used for sequence alignments, except that the forward primer included a T7-promoter sequence at the 5′ end.

RNA transcripts were synthesized directly from purified PCR-products using T7-RNA polymerase (New England Biolabs, Bedford, MA) for 2 h at 37 °C. The DNA template was then removed by adding Turbo DNase (Ambion, Foster City, CA, USA) incubated at 37 °C for 2 h. RNA transcripts were then purified using phenol–chloroform and quantified using a ND-1000 Spectrophotometer® (NanoDrop Technologies,Wilmington, USA). The absence of the DNA template in transcript preparations was later confirmed by RT-PCR without reverse transcriptase.

The concentration of the transcript solution was determined by the formula: μg of RNA × 10^6 pg/1 μg × 1 pmol/340 pg × 1/number of nucleotides of transcript. The Avogadro's constant was then applied to the concentration value to obtain the number of RNA copies.

A 10-factor serial dilution was prepared for each virus to obtain concentrations of 3×10^{12}–3×10^1 targets resuspended in RNA extracts from virus-free raspberry plants or virus-free aphids (the latter used for the RLMV assay only).

2.5. Normalization and relative quantitation of virus titer in single and mixed infections

Linear regression and efficiency analyses were performed by the StepOnePlus software from Applied Biosystems. The efficiency (*E*), defined as the amount of target used as template at each amplification cycle, was calculated by the software according to the formula: $E = 10^{[-1/m]} - 1$; where *E* is the percent efficiency and *m* the slope of the curve.

Because it has been reported that duplex real time PCR tends to obscure true results when testing virus interactions (Tatineni et al., 2010), all the assays were performed independently, as singleplex, using the same chemistries (see Section 2.3) for all the probes used in this study.

A comparative ΔΔ*Ct* method was applied to compare the titer of RBDV and RLMV when present in mixed infections versus single infections. This method is based on differences in *Ct* values of the target between treated and untreated samples, each of which is normalized to the respective *Ct* value obtained from the amplification of a housekeeping gene. Since the efficiencies of each assay may vary, an additional step, which normalizes the efficiencies of

the target and the housekeeping gene, is in order (Pfaffl, 2001). This calculation is summarized in the formula:

$$\text{Fold change} = \frac{(E_{target})^{\Delta Ct\ target}}{(E_{ndhB})^{\Delta Ct\ ndhB}}$$

where *E* is the PCR efficiency of the assay and Δ*Ct* the difference of *Ct* values between two treatments. Each biological replicate was done in triplicate.

2.6. Comparison of assays with conventional detection methods

While ELISA is the method of choice for large scale detection of RBDV, conventional RT-PCR has proven more sensitive and specific and is also used in routine diagnosis of the virus (Kokko et al., 1996). Tzanetakis et al. (2007a) developed a set of degenerate primers for the detection of RLMV by conventional RT-PCR. The primers were derived from the coat protein region and have been successfully used to detect multiple isolates of the virus. In order to evaluate, in a practical manner, the sensitivity of these conventional methods compared to the TaqMan assays, 50 mg of each of five different leaves co-infected with RBDV and RLMV were used for RNA extraction. Ten-fold serial dilutions of the RNA, from 150 ng/μl to 150×10^{-9} ng/μl, were prepared. 3 μl of each RNA dilution were used as template for conventional RT-PCR and TaqMan real-time PCR.

In addition, the RLMV assay was used to evaluate the sensitivity and efficiency at detecting the virus from single aphids. Batches of 25 raspberry aphids (*A. agathonica*) were starved for 12 h and then allowed to feed on RLMV infected plants for an acquisition access period (AAP) of 12 h or 18 h. The virus acquisition experiments were carried out in growth chambers at 16 h light and a constant temperature of 24 °C. Once their AAP was finished, the aphids were collected individually and frozen at −80 °C for subsequent use. Three separate experiments were conducted for each AAP (75 aphids total per AAP).

3. Results

3.1. Sensitivity and specificity of TaqMan assays

The TaqMan assay for RLMV was able to detect as few as 300 (3×10^2) target copies diluted in RNA extracts derived from plants or aphids. The *Ct* values obtained from the standard curves ranging from 25 for the highest dilution (3×10^2) to 6 for the lowest dilution (3×10^8) (Fig. 1). There was no significant difference between the standard curves obtained from the two separate experiments i.e. from aphid extracts and plant extracts (data not shown), indicating that the primers can be reliably used to detect RLMV from aphids and plants with the same sensitivity.

The TaqMan assay designed to detect RBDV showed to be more sensitive than the one for RLMV as it was able to detect as few as 30 copies of the target diluted in plant extracts. The *Ct* values obtained
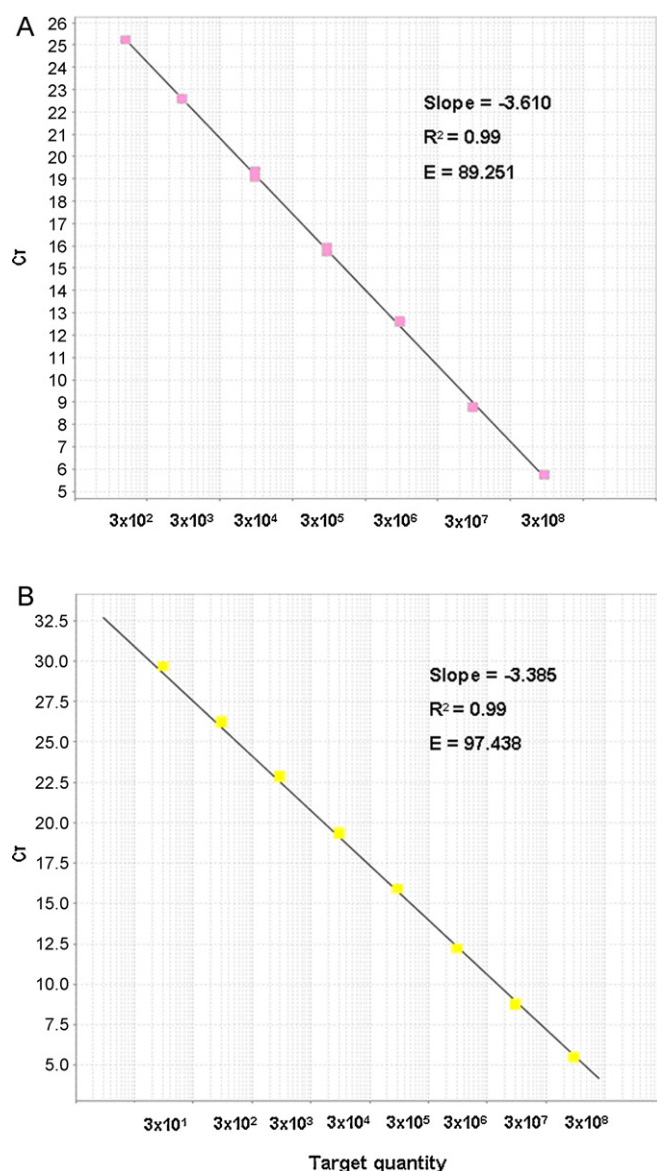
Fig. 1. Standard curves showing the quantification ranges for RLMV (A) and RBDV (B). The slopes, correlation coefficients (R^2) and efficiencies (E) of each assay are shown.

from both assays ranging from 30 ± 0.6 for the most diluted samples, to 6 ± 0.4 for the least diluted samples (3×10^{12}) (Fig. 1). The assays were able to detect all the virus variants maintained in the USDA-ARS *Rubus* virus collection. The efficiencies at which each isolate was detected were statistically the same to one another (data not shown). Fifty different samples obtained from commercial fields (known to have both viruses) were tested by each assay. All samples were positive for each virus with Ct values ranging from 22 to 28 (data not shown). Neither assay produced any amplification signal when virus-free raspberry plants ('Meeker' or 'Munger') and other hosts such as grapes, were tested as negative controls, indicating the specificity of each assay.

3.2. Comparison to conventional RT-PCR

The sensitivity of each assay was significantly higher than its conventional counterpart. While RT-PCR detected RBDV from samples containing an input of up to 450×10^{-4} ng of RNA, the TaqMan assay detected the virus from samples with 450×10^{-7} ng of input RNA. Conventional RT-PCR was able to detect RLMV only in the first 2 dilutions (450 and 45 ng of RNA input); whereas the TaqMan assay amplified samples with 100 times less RNA (450×10^{-3}) (Fig. 2). In addition, the RLMV assay detected the virus in 50 out of the 75 single aphids (67%) that were allowed a 12 h AAP and 60 out of 75 (80%) aphids after an 18 h AAP. Conventional RT-PCR from the same aphid RNA extracts failed to detect the virus at either AAP. The extrapolation of these amplifications to the standard curve revealed a virus load per aphid between 350 and 600 copies for both AAPs (data not shown). Conventional RT-PCR performed with the AgPath-ID[TM] One-Step RT-PCR system without the probe (see Section 2) showed similar sensitivity results to the conventional two-step RT-PCR system (Fig. 2). This finding suggests that the Taq-Man probe component is the determinant factor for the increased sensitivity in the reaction.

3.3. Virus titer in single and mixed infections

Thirty-eight of the 50 leaves sampled from mixed-infected plants collected in the field showed a consistent pattern in the amplification of each virus. RBDV amplification showed an average Ct of 26 when present in single infections and 15 in mixed infections (Fig. 3). The normalized Ct values revealed a ~400-fold increase in titer of RBDV when present in association with RLMV compared to the RBDV titer in single infections. The RLMV titer was neither increased nor reduced when present in combination with RBDV compared to single infections (data not shown). Semi-quantitative RT-PCR, using the same RNA input, was performed from single and mixed infections in order to validate the previous result. Fig. 3 shows the intensity of the amplicons after 25 cycles visualized in an ethidium bromide gel under UV light. Enhanced signals were also observed when RBDV was detected by ELISA in mixed infections compared to single infections (data not shown).

4. Discussion

The Pacific Northwest is a leading producer of red raspberries. 'Meeker' is the most widely planted cultivar in this region and accounts for over 80% of the total raspberry acreage. More than 40 viruses have been reported to infect *Rubus* spp. in different parts of the world (Martin et al., in press; Martin and Tzanetakis, 2005; Quito-Avila et al., 2011). Mixed infections with some of these viruses may result in synergistic interactions leading to more severe symptoms (Jones, 1980, 1982) and widely fluctuating virus titers (Susaimuthu et al., 2008). Therefore, being able to detect these pathogens with highly sensitive and reliable methods is important when it comes to diagnostics and certification programs.

RBDV and RLMV are two of the most common viruses present in raspberry production areas in the PNW. In a recent survey conducted in multiple commercial raspberry fields in northern Washington State, it was found that the incidence of RLMV in five year-old plantings was between 50% and 100% (Quito-Avila and Martin, unpublished data). This virus has been involved in virus complexes responsible for important diseases such as raspberry mosaic (Jones, 1980; Tzanetakis et al., 2007a; McGavin and MacFarlane, 2010) and raspberry crumbly fruit (Quito-Avila et al., 2009). The latter, which has been attributed to RBDV, is by far the most important viral disease in the PNW. Three TaqMan real-time PCR assays, derived from conserved regions of the polymerase gene, were developed for each virus. Assays with the highest amplification efficiencies, consequently highest sensitivities, are highlighted in this communication and recommended for reliable detection of these viruses. Different RLMV isolates reported in the U.S. and Europe have been detected successfully by conventional RT-PCR
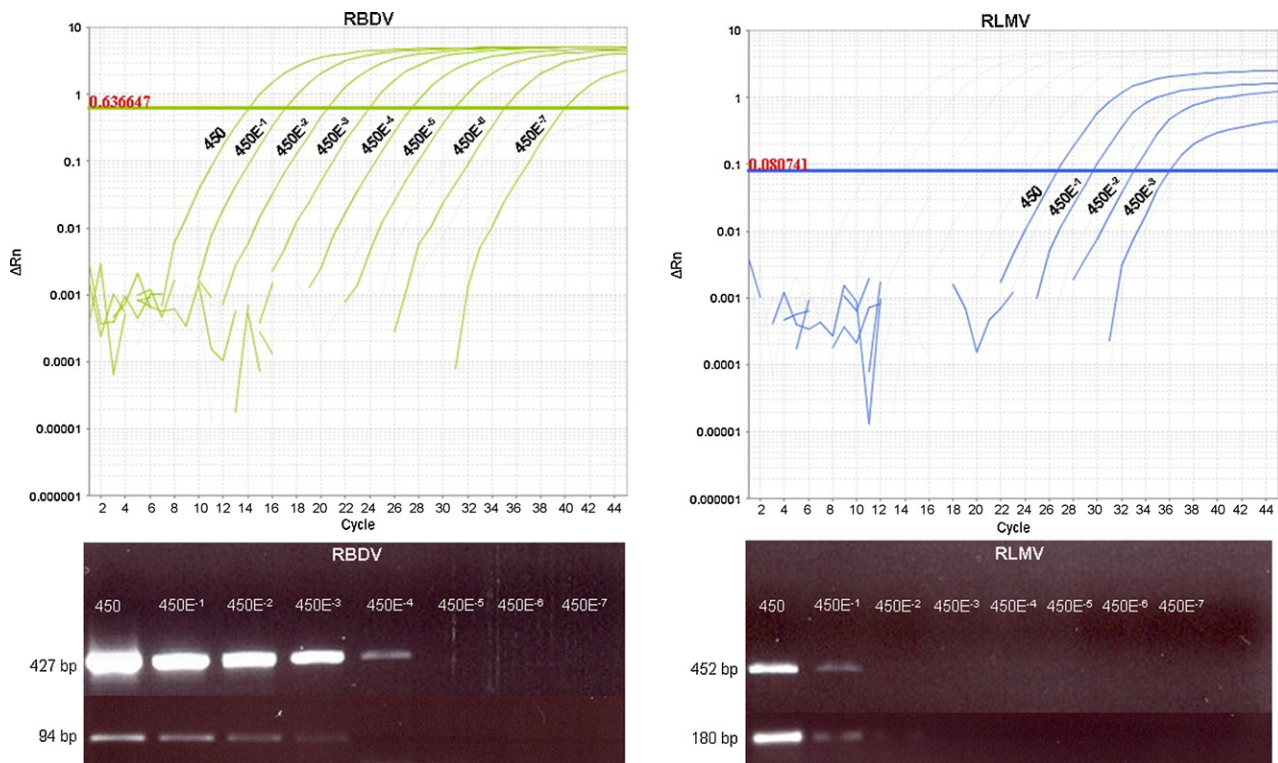
Fig. 2. Sensitivity comparison of TaqMan assays with respect to their corresponding conventional RT-PCR. Left panel: RBDV targets amplified by real-time RT-PCR (amplification curves) and conventional RT-PCR (electrophoretic bands: 427 bp amplified by coat protein primers and 94 bp amplified by the real-time primers without the probe). Right panel: RLMV targets amplified by the real-time RT-PCR assay (amplification curves) and conventional RT-PCR (electrophoretic bands: 452 bp amplified by primers flanking the coat protein homolog and 180 bp amplified by the real-time primers without the probe).

using specific or degenerate primers derived from the coat protein region (Tzanetakis et al., 2007a; McGavin and MacFarlane, 2010). Likewise, RBDV is detected readily by DAS-ELISA (Pūpola et al., 2009) and to some extend by conventional RT-PCR. However, these techniques may not be sensitive enough to detect the virus when present in very low concentrations due to host stress or other physiological processes that may result in reduced virus titers, or in mixed infections where virus:virus interactions may result in reduced titers of one of the viruses in a complex (Susaimuthu et al., 2008; Karyeija et al., 2000). It has been shown, for example, that in some cases, *in vitro* techniques, aimed to eliminate viruses in plants, repress the virus replication to a point below the detection threshold (Fridlund, 1989) resulting in false negatives. Retesting of such plants may take weeks to months given that the plants have to be placed in greenhouses at optimum conditions so that the virus (if present) can increase its concentration to a detectable level. The ability to detect RBDV and RLMV from plants containing the virus in low titers represents a new alternative for early screening in *in vitro* virus elimination programs.

In addition, a new synergistic interaction between RBDV and RLMV was determined using the real-time PCR assays developed in this study. It was shown that the concentration of RBDV (*Ideaovirus*, unassigned family) was enhanced by RLMV (*Closterovirus*, fam. *Closteroviridae*). This is not the first case where distantly related viruses interact in a synergistic fashion. Several cases have been reported where the interaction of two unrelated viruses result in an increase in titer of one or both viruses (Goldberg and Brakke, 1987; Anjos et al., 1992; Vance, 1991; Scheets, 1998; Stenger et al., 2007; Susaimuthu et al., 2008). Recently, two new synergistic interactions between related viruses have also been reported where increases in virus titers were observed (Wintermantel et al., 2008; Tatineni et al., 2010).

The increase in RBDV concentration, when present in combination with RLMV, may be due to the existence of the RLMV-23 kDa protein, putatively involved in RNA silencing suppression (Tzanetakis et al., 2007a). This has been the case for several other virus interactions. For example, the potyvirus *Tobacco etch virus* (TEV) encodes a potent RNA silencing suppressor, namely HC-Pro, which enhances the replication of unrelated viruses such as *Cucumber mosaic virus* (fam. *Bromoviridae*) and *Tobacco mosaic virus* (fam. *Virgaviridae*) (Vance, 1999; Voinnet et al., 1999; Pruss et al., 1997).

The significance of the RBDV-RLMV interaction comes from the fact that raspberry fields with increased severity of crumbly fruit disease have a high incidence of RLMV in addition to RBDV. It is hypothesized that the increase in severity of crumbly fruit is due to the enhancement of the RBDV concentration in plants co-infected with RLMV. Field experiments are underway to validate this hypothesis.

The real-time RT-PCR assay developed for RBDV was 10-fold more sensitive than the one for RLMV. This was evidenced by the detection threshold obtained from serial dilutions using plant or aphid extracts containing known amounts of the target.

This difference may be due to the relatively large amplicon amplified by the RLMV assay, which was 30 nucleotides longer than the usually recommended amplicon size (150 bp). This unusual amplicon size, however, was necessary in order to design the primers and probe from conserved stretches that ensure the efficacy for detection of several RLMV isolates. Nevertheless, the RLMV assay proved to be 100 times more sensitive than conventional RT-PCR carried out even with the same buffer/enzyme system. This was evidenced by detection of the virus from serial RNA dilutions and also from single aphids after 12 or 18 h of virus acquisition.
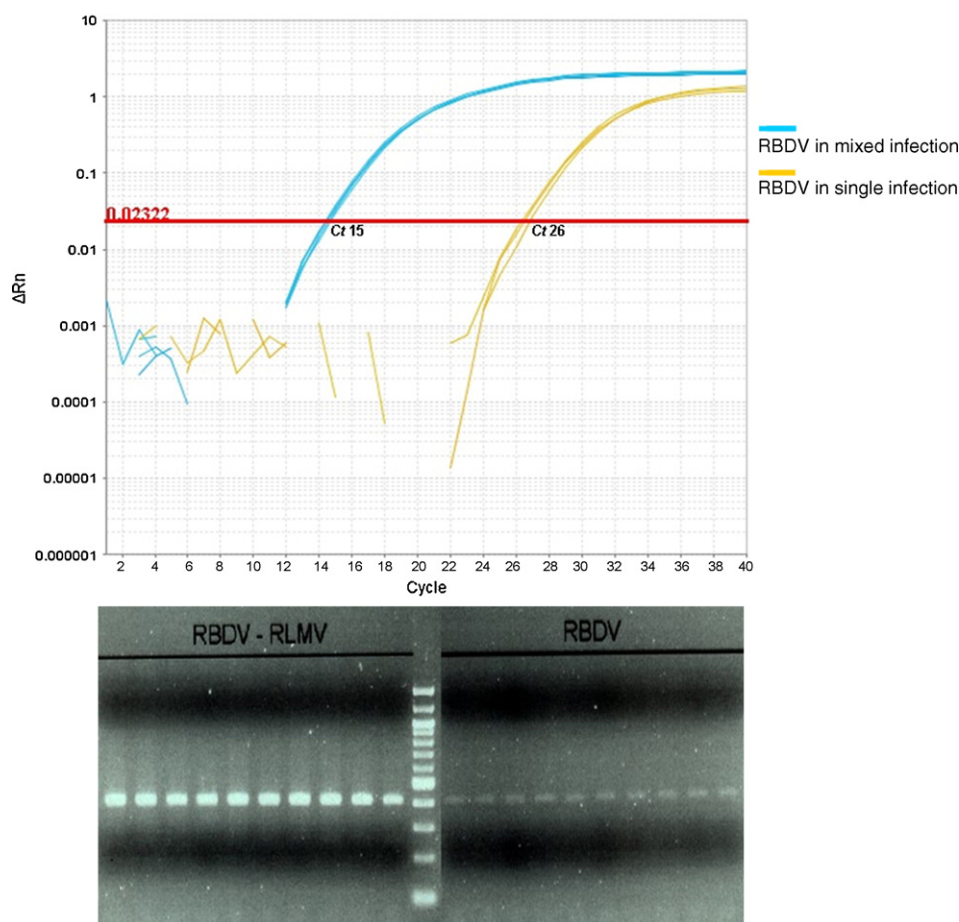
Fig. 3. Amplification of RBDV from plants with single and mixed infections by real-time RT-PCR and conventional RT-PCR. The upper panel shows the curve produced from real-time amplification of RBDV from single and mixed infected plants. The lower panel shows amplicons (427 bp) from conventional RT-PCR stained with ethidium bromide and visualized after 25 cycles of amplification.

The two TaqMan assays developed for the detection of RBDV and RLMV are suitable for several applications, such as analysis of virus replication, virus-vector studies and virus synergistic interactions.

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