**A simplified strategy for sensitive detection of *Rose rosette virus* compatible
with three RT-PCR chemistries**

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Abstract

Rose rosette disease is a disorder associated with infection by *Rose rosette virus* (RRV), a pathogen of roses that causes devastating effects on most garden cultivated varieties, and the wild invasive rose especially *Rosa multiflora*. Reliable and sensitive detection of this disease in early phases is needed to implement proper control measures. This study assesses a single primer-set based detection method for RRV and demonstrates its application in three different chemistries: Endpoint RT-PCR, TaqMan-quantitative RT-PCR (RT-qPCR) and SYBR Green RT-qPCR with High Resolution Melting analyses. A primer set (RRV2F/2R) was designed from consensus sequences of the nucleocapsid protein gene p3 located in the RNA 3 region of RRV. The specificity of primer set RRV2F/2R was validated *in silico* against published GenBank sequences and *in-vitro* against infected plant samples and an exclusivity panel of near-neighbor and other viruses that commonly infect *Rosa* spp. The developed assay is sensitive with a detection limit of 1 fg from infected plant tissue. Thirty rose samples from 8 different states of the United States were tested using the developed methods. The developed methods are sensitive and reliable, and can be used by diagnostic laboratories for routine testing and disease management decisions.

Keywords: Diagnostics; TaqMan RT-qPCR; SYBR-Green RT-qPCR; High Resolution Melting; HRM; Biosecurity.

1. Introduction

Roses (*Rosa* spp.) belong to the family Rosaceae and are among the most popular flowering shrubs in the United States with a total wholesale value of \$194 million (Paret, 2014). Roses are cherished for their desirable aesthetics, landscaping and industrial products. In recent years, Rose rosette disease (RRD) has become a devastating disease of roses causing economic losses to ornamental nurseries in different parts of the United States including the state of Oklahoma where RRD is commonly observed on both nursery and landscape roses. In 2012, RRD caused severe losses to the Tulsa Municipal Rose Garden where thousands of rose plants were eradicated (Stanley, 2013). RRD is a viral disease of commonly cultivated roses and the wild rose *Rosa multiflora*. It was proposed to be caused by a newly characterized *Rose rosette virus* (RRV), a ssRNA virus in the genus *Emaravirus* (Laney et al., 2011). RRD was first observed in 1940 in Manitoba, Canada (Connors, 1941). RRD is reported to be widespread in Kansas, Oklahoma, Missouri and Arkansas, and has become widespread in north, south-central and southeast parts of the United States (Christine, 2006, Epstein and Hill, 1995, Laney et al., 2011, Windham et al., 2014a and Windham et al., 2014b). RRD has become a serious problem in these states and a threat to the rose industry and landscape roses. RRD is transmitted by grafting and the eriophyid mite *Phyllocoptes fructiphilus* (Amrine, 1988, Laney et al., 2011). The mites do not fly but can passively move long distances by air currents and can spread to nearby roses infecting rose plantings (Epstein and Hill, 1999, Windham et al., 2014a).

The symptoms of the disease are highly variable depending on the rose cultivar, and stage of development of the plant. Symptoms may vary within the same cultivar at the same or different locations (Epstein and Hill, 1995, 1999, Windham et al., 2014a, 2014b). RRD symptoms include rapid elongation of lateral shoots: reddening of leaves and shoots, masses of shoot proliferation (witches broom), excessive thorns, malformation, reduced flowering, and deformed buds and

flowers (Amrine, 2002, Epstein and Hill, 1995) (Supplementary Fig. 1). RRD infected plants become severely disfigured in one to three years after infection and are removed due to their unpleasant appearance. Diagnosis of RRD in early stages is difficult and often confusing due to symptoms resembling herbicide damage or caused by other plant viruses and/or pest problems. Similar to other viral diseases, there is no chemical control for RRD. Once a rose plant becomes infected, complete removal of plants is recommended as the plants are likely to harbor the vector eriophyid mites. Reliable early detection of the disease is needed to prevent the spread of infection to the healthy plants.

Laney et al. (2011) reported an endpoint RT-PCR primer sets for RRV detection. However, the primers were used by other groups which had difficulty in detecting all RRV infected samples (J. Olson; unpublished). Therefore, a reliable, accurate, sensitive and validated assay for detection of RRV is required. Moreover, rose tissues are rich in PCR inhibitors and frequently produced false negatives; therefore, incorporation of amplification facilitators (like BSA and PVP) in the reaction mix would be helpful to develop robust and validated protocol. Nucleic acid based methods are rapid, reliable, have high specificity and discriminatory abilities and are more sensitive than immunological methods (Dai et al., 2012, Arif et al., 2012, 2013b, 2014a and Ouyang et al., 2013). Reverse transcription-polymerase chain reaction (RT-PCR) is considered a sensitive and rapid method for detection of RNA viruses (Arif et al., 2014b). The sensitivity of reverse transcription PCR (RT-PCR) depends importantly on the thermodynamics of the oligonucleotide primers (Arif and Ochoa Corona, 2013). High sensitivity allows high capability for detection of minute quantities of virus particles, leading to early detection of virus diseases (Arif et al., 2014b). Other DNA amplification assays such as real time or quantitative PCR (qPCR) offer increased sensitivity, accuracy and speed compared to end-point PCR for detection

of target genes (Arif et al., 2014b, 2015). SYBR Green and TaqMan qPCR are the two most popular formats of qPCR and are based on two different chemistries (Bustin, 2005). SYBR Green assays use a fluorescent dye that intercalates with all double-stranded DNA, including non-specific products that may be synthesized during the reaction, whereas, TaqMan assays use a DNA probe that fluoresces only after the probe is cleaved during the amplification of a specific product (Tomlinson et al., 2005). TaqMan assays offer highly specific detection and analysis of the amplified products (Obrepalska-Stepłowska et al., 2008). However, PCR probe-based analyses are expensive, and often require considerable information about the targeted gene and may be complex (VanGuilder et al., 2008). In contrast, SYBR Green assays are cost effective and relatively simple to use (VanGuilder et al., 2008). High Resolution Melting (HRM) analyses coupled to SYBR Green PCR exploits the SYBR-Green dye intercalates only in double-stranded DNA and measures a decrease of fluorescence once the dsDNA start melting. The decreasing fluorescence values are plotted against the temperature generating a melting curve unique and characteristic of the amplified fragment that is characteristic of the targeted species or even strains of the studied viral pathogen, facilitating discrimination from artifacts created by non-specific products (Varga and James, 2005, Reed et al., 2007 and Winder et al., 2011). Both SYBR Green qPCR and HRM curve analyses can be performed in a single tube. This method allows detection and discrimination of the target simultaneously (Varga and James, 2005), and has the advantage of monitoring the non-specific amplification.

This research reports the design of a single pair of diagnostic primer and demonstrates its application in three RT-PCR chemistries: endpoint RT-PCR, TaqMan RT-qPCR, and SYBR Green RT-qPCR assays. The SYBR Green RT-qPCR can also be combined with HRM curve analyses for the detection of RRV. Moreover, a multi-target artificial positive control to include a

probe target was also constructed for routine application in diagnostics. The application of a single primer set with three different chemistries offer flexibility to operators in a diagnostic network to adopt any of the three methods depending on the available resources in the laboratory. The three developed methods will also assist epidemiological studies, breeding, biosecurity, and management of the disease.

2. Materials and Methods

2.1. Source of viruses and infected plant material

Frozen plant tissue infected with RRV provided by the Plant Disease and Insect Diagnostic Laboratory (PDIDL), Oklahoma State University, Stillwater, was used as reference positive control. Other eleven virus lyophilized reference controls, including near-neighbor viruses and viruses commonly infecting roses, were used in an exclusivity panel: *High plains virus*, *Maize stripe virus*, *Impatiens necrotic spot virus*, *Tomato spotted wilt virus*, *Groundnut ringspot virus*, *Tomato chlorotic spot virus*, *Apple mosaic virus*, *Arabidopsis mosaic virus*, *Prunus necrotic ringspot virus*, *Tomato ringspot virus*, and *Tobacco mosaic virus* were obtained from Agdia, Inc (Agdia, Elkhart, IN). A healthy *Rosa 'Radrazz'* (Knock Out[®] rose) from PDIDL was used as a source of healthy tissue for negative control. Infected plant material obtained from PDIDL was collected from eight states of the United States: Oklahoma, Indiana, Michigan, California, North Carolina, Virginia, New Jersey and Kentucky (Table 1). Samples consisted mostly of leaves. The majority of samples were symptomatic; however, a few asymptomatic plant samples were also included.

2.2. Primer and probe design

Twenty three sequences of RRV nucleocapsid gene p3 located in the RNA3 region were retrieved from NCBI GenBank database. The selected GenBank accessions were: HQ871944 and HQ891892–HQ891913 (Laney et al., 2011), aligned using CLUSTALX2 (Larkin et al., 2007)

and examined for conserved regions. The last date NCBI GenBank database accessed was 17th of September, 2013. A primer pair RRV2F (Forward: 5'-TGCTATAAGTCTCATTGGAAGAGAAA-3') and RRV2R (Reverse: 5'-CCTATAGCTTCATCATTCTCTTTG-3'), and the RRV probe-2 (5'-TGCTAGAGACATTGGTACAACAAGCAA-3') were designed from a consensus sequence generated after aligning the targeted gene to amplify a fragment of 104 bp. The web interface application Primer3 (Rozen and Skaletsky, 2000) was used for primer design. The thermodynamics, internal structures, and self-dimer formation of the primers were examined *in-silico* using mFold (Zuker, 2003) following parameters described by Arif and Ochoa-Corona (2013). The preliminary specificity of the primer set was assessed *in-silico* using BLASTn (Altschul et al., 1990). The primers and probe 5'-6-carboxyfluorescein (FAM)-3' were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA).

2.3. Artificial positive control (APC)

A synthetic, clonable and non-infectious multi-target artificial positive control (plasmid) was constructed as described by Caasi et al. (2013) for routine application in detection and diagnostics assays. The APC insert consists of a tandem of forward and reverse complement sequences of primers and probe targeting RRV, and amplified a 125 bp PCR amplicon using primer set RRV2F/2R. This APC also included primer sequences for other five major plant viruses used in our laboratory (GenBank accession number KP772215). The multi-target APC was artificially synthesized and ligated into a restriction site of pUC57 vector (GenScript USA Inc, Piscataway, NJ) which allows circularization and results in a clonable gene construct. A graphic description of the APC is shown in Figure 1.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from symptomatic, and asymptomatic rose leaves, reference positive control (RRV infected rose), a healthy *Rosa* 'Radrazz' Knock Out[®] (negative control), and 11 lyophilized reference control viruses. Approximately 100 mg of the leaf tissue for each sample was taken and macerated using a Mini-beadbeater[™] (Biospec Products, Bartlesville, OK,) for 40 s. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. For lyophilized reference control viruses, 450 µL of Qiagen RLT buffer plus β-Mercaptoethanol (as indicated by the manufacturer) was directly added to the lyophilized tissue, mixed, transferred into 2 ml eppendorf tubes, and vortexed for 30 s. The total RNA was subsequently extracted following the manufacturer's instructions. Four microliters of extracted RNA was used to prepare cDNA. First-strand cDNA synthesis was performed using random hexamer primers (5µM) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions and cDNA synthesis was performed at 37 °C for 90 min and used within 24 h. The quality of the RNA was confirmed using the primers for plant internal control mitochondrial *nad5* gene (Menzel et al., 2002).

2.5. Gradient PCR

The optimum annealing temperature (T_a) of the RRV2F/2R primer set was determined performing a gradient PCR with a T_a range of 53.9 to 66 °C. The concentration of the APC DNA (plasmid) used for this assay was 1 ng/reaction. Twenty microliters of the amplified product was visualized in 2% agarose gel electrophoresis in 1X TAE buffer (pH= 8.0). Similarly, the T_a of RRV2F/2R primer set was also tested using cDNA synthesized from a positive RRV infected sample. For this assay 3 µl of cDNA template was added to the PCR mix and tested within a T_a range of 54 to 64 °C.

2.6. Two-step endpoint RT-PCR and specificity assay

Preliminary endpoint RT-PCR was performed with positive RRV infected tissue (positive control) with and without the addition of amplification facilitators (BSA: Bovine serum albumin, and PVP: Polyvinylpyrrolidone; Dobhal et al., 2014b). Endpoint RT-PCR was performed in 20 µl reaction volumes consisting of 10 µl GoTaq Green Master Mix (Promega), 1 µl of each RRV2F and RRV2R (5 µM) primers, 3 µl of cDNA template, 1.6 µl of BSA (50 mg/ml; Ambion, Austin, TX), 2 µl of 10% PVP40 (Sigma-Aldrich, St. Louis, MO) and 1.4 µl nuclease free water (Ambion). Two-step endpoint RT-PCR was performed in a thermal cycler (Biometra, Goettingen, Germany) and the cycling parameters were: initial denaturation of 94 °C for 3 min followed by 38 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 3 min. Positive (APC) and negative (healthy *Rosa* 'Radrazz' Knock Out[®] leaf tissue) controls were included. Twenty microliters of amplified RT-PCR product was electrophoresed in 2% agarose gel in 1X TAE buffer. The amplified products were eluted from agarose gels using Quantum Prep Freeze'N Squeeze Spin Columns (Bio-Rad, Hercules, CA) and directly sequenced at the Oklahoma State University Recombinant DNA/Protein Core Facility using primers RRV2F and RRV2R.

To test the specificity of the primer set RRV2F/2R with RRV, the primer set was tested with cDNA synthesized from reference positive control (infected RRV plant). The cross-reactivity of primer set RRV2F/2R was also tested with 11 reference control viruses in the exclusivity panel. The, healthy Knock Out[®] *Rosa* 'Radrazz' (negative control) was included in each assay. The samples negative for RRV specific RT-PCR were crosschecked for RNA quality using primers specific for plant mitochondrial *nad5* gene (internal control; Menzel et al., 2002). The *nad5* specific RT-PCR was performed by adding amplification facilitators (AFs) such as BSA and PVP into the reaction.

2.7. PCR sensitivity assay using artificial positive control

The detection limit of the primer set RRV2F/2R was determined using a ten-fold serial dilution of plasmid DNA from 1 ng to 1 fg. One microliter of each dilution was used as a template for PCR. PCR was performed in 20 µl of reaction using reaction components and parameters described above. A negative control (non-template control: water) was also included in the assay. Twenty microliter of amplified product was electrophoresed using a 2% agarose gel in 1X TAE buffer.

2.8. Two-step endpoint RT-PCR sensitivity assay with infected plant tissue

A standard curve was prepared with ten-fold serially diluted plasmid DNA (APC) at concentrations of 1 ng to 10 pg using a real-time SYBR Green qPCR. The unknown RRV concentration in the cDNA (prepared from the infected positive plant tissue) was calculated from the APC standard curve as described by Arif et al. (2014b). The quantified cDNA was ten-fold serially diluted from 1 ng to 1 fg. One microliter of each serially diluted cDNA was used as a template in endpoint RT-PCR following the conditions and parameters described above.

2.9. Screening of field samples using two-step endpoint RT-PCR

Thirty symptomatic and asymptomatic rose plant samples received from eight states of the United States were screened using endpoint two-step RT-PCR. Total RNA was individually extracted from each plant sample and a healthy Knock Out[®] *Rosa* 'Radrazz' leaves (negative control); cDNA was synthesized as described above, and 3 µl of cDNA was used for endpoint RT-PCR. Twenty microliters of amplified RT-PCR product was used for electrophoresis. The amplified products were eluted from agarose gels using Quantum Prep Freeze'N Squeeze Spin Columns and directly sequenced as described earlier. The sequences were aligned and compared against sequences available in the GenBank nucleotide database using BLASTn.

2.10. SYBR Green RT-qPCR, HRM analyses and screening of field samples

SYBR Green RT-qPCR and HRM analyses were performed using synthetic APC, a RRV-infected positive control sample, and 11 viruses included in the exclusivity panel. To standardize the method, preliminary experiments were performed with APC plasmid and reference positive control (RRV infected plant tissue) with and without the addition of AFs. SYBR Green RT-qPCR amplification was carried out in 20 μ l reaction mixture containing 10 μ l of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 0.8 μ l (5 μ M) of each RRV2F and RRV2R primers, 2 μ l of template cDNA, 1.6 μ l of BSA (50 mg/ml), 2 μ l of 10% PVP40 and 2.8 μ l of nuclease-free water. Each reaction was performed in three replicates. The cycling parameters included two initial holds at 50 °C and 95 °C for 2 min each, followed by 40 cycles: 95 °C for 20 s and 54 °C for 45 s. The assays were performed in a Rotor-Gene 6000 thermocycler, and data analysis was done using Rotor-Gene 6000 series software 1.7 (Corbett Research, Sydney, Australia) with a manual cycle threshold (*CT*) of 0.1. The cDNA templates were amplified, and subsequently followed by a melting curve HRM protocol: a stepwise denaturation from 65 to 99 °C with 0.2 °C increments at each step, a default of 90 s of pre-melt conditioning on first step and a 2 s stop for each step afterwards was performed. Fluorescence data from melting curves were converted into melting profile charts using the Rotor-Gene 6000 series software 1.7 which plots the negative derivative of fluorescence ($-dF/dT$) vs. temperature (*T*). HRM curve, normalization and temperature shifts were also analyzed. To test the real-time SYBR Green RT-qPCR method with the infected field samples, eight out of the 30 samples were randomly selected and tested using the developed method. Positive and negative controls were included in each assay. The mean T_m was calculated for each sample and the standard deviation (SD) was determined.

2.11. TaqMan RT-qPCR assay and screening of field samples

TaqMan RT-qPCR was performed with the 30 asymptomatic and symptomatic rose samples from eight different states, a reference positive control and 11 viruses in the exclusivity panel. TaqMan RT-qPCR was performed in a 20 µl reaction containing 10 µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.8 µl (5 µM) of each RRV2F and RRV2R primers, 0.8 µl (5 µM) of probe, 1.6 µl of BSA (50 mg/ml), 2 µl of 10% PVP40, 2 µl of template (cDNA in case of infected positive control or negative control or APC), and 2 µl of nuclease-free water. Positive and negative controls were included in each assay and each reaction was performed in three replicates. Cycling parameters included two initial holds at 50 °C and 95 °C - each of 2 min; followed by 40 cycles: 95 °C for 20 s and 58 °C for 45 s. The assays were performed in a Rotor-Gene 6000 thermocycler, and the data analysis was done with a manual *CT* of 0.1 using Rotor-Gene 6000 series software 1.7. The mean of each set of replicates was calculated and the standard deviation was determined.

3. Results

3.1. *In silico* analyses of primers

The primer set RRV2F (T_m 59.8; G+C content 34.6%; 3' complementarity = 2; 26 bp) and RRV2R (T_m 59.6; G+C content 40%; 3' complementarity = 0; 25 bp), and the probe RRV2 (T_m 65.1; G+C content 40.7%; 3' complementarity = 0; 27 bp), were obtained using Primer3 and were found specific for RRV after a pairwise alignment against the available GenBank nucleotide database using BLASTn. The calculated E-value of RRV2F, RRV2R and RRV2 probe were 1e-04, 3e-04 and 3e-05, respectively. BLASTn report of primer set RRV2F/2R showed 100% identity and 100% query coverage with all RRV accessions available in the GenBank nucleotide database. Primer set RRV2F/2R amplifies a conserved segment of 104 bp

from nucleotide 559 to 663 of the RRV nucleocapsid protein gene RNA3 (Accession number: HQ871944.1). The ΔG plot values calculated using mFold were 0.8, 0.6 and 1.0 kcal/mol for RRV2F, RRV2R and RRV2 probe, respectively. The T_m difference between primers RRV2F and RRV2R was 0.2 °C and between the primer set and the probe RRV2 were 5.3 and 5.5, respectively.

3.2. Gradient PCR and two-step endpoint RT-PCR

Primer set RRV2F/2R amplified a diagnostic product from the predicted target of RRV p3 gene within a range of seven annealing temperatures (T_a), from 53.9 (circa 54 °C) to 60 °C. This result indicates primer set RRV2F/2R performed well in a broad range of T_a . The RT-PCR products were obtained within the expected amplification size, of 125 bp from APC and 104 bp from actual RRV (Fig. 2A and B). The primer set did not amplify products from the negative control (*Rosa 'Radrazz'* Knock Out[®]) (Fig. 2B).

3.3. Endpoint PCR and specificity of primers

The RRV infected reference positive control (RRV infected rose tissue) did not amplify the expected product unless PCR AFs BSA and PVP40 were added to the PCR mixture (Supplementary Fig. 2). No cross-amplification was observed with the panel of eleven viruses (exclusivity panel). The cDNA has to be used preferably fresh within 24 h after the synthesis; otherwise a rapid degradation of cDNA templates leading to inconsistencies may be detected. This fact was observed to happen with knowingly positive infected samples.

3.4. Sensitivity assay

The detection limit of primer set RRV2F/2R after a ten-fold serial dilution of APC (plasmid) was 1 fg (Fig. 3A). A SYBR-Green RT-qPCR standard curve is shown in Figure 4A and B.

Using the standard curve, the quantity of virus in the positive infected plant sample was found to be 8 pg which was diluted to 1 pg for starting concentration in endpoint RT-PCR. The detection limit of the RT-PCR to detect RRV from infected plant tissue was 1 fg (equivalent to the detection limit of the plasmid) with an expected amplification of 104 bp. No amplification was observed with the negative plant control (Fig. 3B). The detection limit of endpoint RT-PCR to detect the RRV from the infected host tissue and APC was equivalent.

3.5. Screening of infected field samples with two-step endpoint RT-PCR

All 30 asymptomatic and symptomatic rose samples tested positive (100%) for RRV using two-step endpoint RT-PCR. The expected PCR product of 104 bp was visible, and no amplification was observed with the negative control (healthy Knock Out[®] *Rosa* 'Radrazz') (Fig. 5A and B). Out of 30 samples, seven were randomly selected and sequence using both forward and reverse primers RRV2F and RRV2R. The BLASTn alignment of obtained sequences showed 99% identity with twenty-three p3 RRV sequences available in the GenBank nucleotide database.

3.6. TaqMan RT-qPCR

TaqMan RT-qPCR amplified the diagnostic target in the reference RRV positive control with an average Ct value of 28.76 ± 0.36 (mean $\pm$ SD). Amplification was not detected with the eleven viruses in the exclusivity panel (data not shown). This result confirmed that primer set RRV2F/2R and RRV2 probe were specific for the targeted virus fragment. It was also confirmed that assays carried out with AFs added to the PCR mixture consistently amplified samples that otherwise previously failed to amplify when AFs were not added. All 30 samples that tested positive using endpoint RT PCR also tested positive using TaqMan RT-qPCR. The average Ct values of the thirty field samples varied from 20.26_(0.40) to 36.45_(1.35) (Table 1). A graphic

representation of seven randomly selected samples with the positive and negative control is shown in Figure 6.

3.7. SYBR-Green RT-qPCR and HRM analyses

Positive amplification by SYBR-Green RT-qPCR and HRM of the expected RRV target was only consistently possible after the addition of AFs. The HRM T_m was 75.7 ± 0.03 °C (mean $\pm$ SD, n=3) (data not shown). The SYBR-Green RT-qPCR followed by HRM with AFs allowed amplification of the targeted diagnostic product of RRV reference positive control with a distinct T_m of 75.6 ± 0.1 °C (mean $\pm$ SD, n=6). The melting curve profile of the RRV diagnostic product from APC has a different T_m of 78.85 ± 0.06 °C (mean $\pm$ SD, n=9) as expected. No amplification or HRM curve was obtained with healthy Knock Out[®] *Rosa 'Radrazz'* (negative control) (Fig. 7). Furthermore, no amplification was observed with cDNA from viruses in the exclusivity panel. The eight randomly selected samples out of the 30 asymptomatic and symptomatic samples also tested positive for RRV by SYBR-Green RT-qPCR. The average C_t values of those eight samples are shown in Table 1 and a C_t plot of five samples is shown in Figure 8A. The HRM curve of the five infected plant samples is shown in Figure 8B. The HRM temperatures were 75.7 ± 0.04 °C, 75.7 ± 0.02 °C, 75.7 ± 0.03 °C, 75.7 ± 0.02 °C and 75.7 ± 0.0 °C (mean $\pm$ SD, n=3) for R4, R11, R14, R29 and R30 samples, respectively.

4. Discussion

This study describes and demonstrates the development of a single primer set for sensitive, reliable and highly specific detection of RRV by endpoint RT-PCR, TaqMan RT-qPCR, and SYBR-Green RT-qPCR which can be coupled to HRM analyses.

RRD is a major problem for rose growers in many parts of the United States and is causing dramatic losses in yield and quality of the roses. Since early symptoms of RRD are difficult to

349 diagnose, RRD has spread due to failure in early detection to make timely control measures.
350 Moreover, *Rosa* spp. tissues are rich in inhibitors that cause inconsistent results when large
351 numbers of specimens are routinely tested. These inconsistencies also raised concerns about
352 whether all RRV isolates are being detected. Therefore, a sensitive and reliable diagnostic
353 method considering these two important concerns was needed.

354 In general, PCR based detection methods are more sensitive, reliable and accurate for
355 detection and discrimination of the pathogens than immunological methods, if the primers are
356 designed from signature regions of a targeted gene (Arif et al., 2013 and Arif et al., 2014b). RT-
357 PCR is one of the most popular, efficient, accurate and reliable method for the detection of RNA
358 viruses. A detection endpoint RT-PCR was reported by Laney et al. (2011) by the time RRV
359 genomic organization was reported. This research reports and validates primers designed to
360 perform consistently in endpoint RT-PCR and two RT-qPCR based detection methods cross
361 validated with an exclusivity panel of eleven viruses and demonstrated its application testing
362 field samples. The design and development of this new set of primers focused over the consensus
363 region of the nucleocapsid gene p3 located in the RNA3 of all RRV isolates submitted to the
364 NCBI GenBank to date. The primers were further tested *in silico* for their thermodynamics
365 features following parameters outlined by Arif and Ochoa Corona (2013). The primers did not
366 show complex 3' self-complementary secondary structures. The BLASTn results of primer set
367 RRV2F/2R showed 100% similarity with the p3 gene of RRV and twenty three p3 sequences of
368 RRV in GenBank.

369 RT-PCR requires the extraction of RNA from the plant tissue. RNA isolation from infected
370 rose plant tissue is often complicated due to abundance of phenolic compounds, carbohydrates,
371 pigments, and other unknown compounds. These compounds may play an inhibitory role

interacting irreversibly with nucleic acid and proteins leading to oxidation and degradation reducing the quality of RNA and subsequent analysis (Salzman et al., 1999). It was observed that RRV was not consistently detected from infected rose tissue if AFs were not added. This could have been caused by inhibitors precipitating with viral RNA during the extraction procedure. The addition of BSA and PVP40 as AFs to the PCR mix improved consistently the amplification of 104 bp diagnostic product. The incorporation of AFs in the PCR mix was adapted from a previously reported method by Dobhal et al. (2014b), who demonstrated the incorporation of AFs in PCR mixture were able to relieve the inhibitory effect of inhibitors present in different sample matrices. The incorporation of AFs did not have a negative effect on the specificity of the primers as no cross-reaction was observed with the cDNA of viruses in the exclusivity panel and negative control (healthy tissue of Knock Out[®] *Rosa* 'Radrazz'). Positive amplification was observed only with RRV infected samples, which demonstrated the three methods are specific for detection of RRV. Further sequencing of the amplified product using the primers RRV2F/2R confirmed the RRV identity.

The developed endpoint RT-PCR was further assessed using symptomatic and asymptomatic plant samples from eight different locations in the United States. Endpoint RT-PCR was able to detect the infection in all samples tested. No amplification was observed with cDNA from healthy tissue of Knock Out[®] *Rosa* 'Radrazz' (negative control) showing the RRV primer set RRV2F/2R was not reactive with the host plant. Endpoint RT-PCR was also able to detect RRV in asymptomatic plant samples three months before to the onset of visual symptoms. Samples testing negative for the RRV, tested positive for *nad5* gene used as an internal control confirming these samples were true negatives.

Furthermore, a qPCR standard curve was made with a known APC concentration and the unknown concentration of RRV cDNA synthesized from infected plant tissue RNA was plotted and calculated as described by Arif et al. (2014b). Primer set RRV2F/2R detected RRV at a concentration down to 1 fg in infected plant tissue, similar to the detection limit observed when using APC.

The size of the amplified product and RRV2F/2R thermodynamics allows these set of oligos to be compatible with two real-time RT-qPCR chemistries. In fact, all 30 field samples tested positive to RRV demonstrating primers compatibility with TaqMan RT-qPCR. No contradictory results or cross amplifications were observed after the incorporation of AFs in the Taqman RT-qPCR assays. Primer set RRV2F/2R was also compatible with SYBR Green RT-qPCR. SYBR-Green qPCR chemistry is a less expensive alternative to TaqMan (Arif et al., 2014b).

A HRM curve analyses was also demonstrated to work in conjunction with SYBR-Green RT-qPCR. HRM coupled to RT-qPCR allows simultaneous detection and discrimination of variants within single or multiple PCR products (Winder et al., 2011). As expected the T_m of the field samples ($75.7 \pm 0.04^\circ\text{C}$) were similar to the T_m of RRV reference positive control ($75.6 \pm 0.1^\circ\text{C}$). The T_m of the APC ($78.85 \pm 0.06^\circ\text{C}$) also showed a characteristic melting profile different from positive RRV infected plant tissue, which allows discrimination of positive samples and the APC. The HRM analyses also discriminate false negatives that might had generated by cross-contamination (Winder et al., 2011). No HRM profiles were generated from any of the 11 viruses in the exclusivity panel.

A consistent and reliable single primer pair based detection method compatible with three different RT-PCR chemistries is reported. The selection of the method to be used will depend on the end user's preferences, laboratory capabilities or diagnostic strategy. The three tested

methods are simple, sensitive, reproducible, and have application in detection and discrimination of RRV. RRV detection by RT-PCR can be challenging due to presence of the inhibitory compounds in rose tissue especially when the virus is present in low titer. The use of AFs in the PCR mixture reduces the risk of false negatives. Furthermore, during this study we observed that cDNA synthesized from RNA stored at -80 °C for more than a month may fail to amplify even when AFs were added. Therefore, all assays were performed either with freshly prepared RNA and no older than one week. Best results are obtained when cDNA is used freshly extracted before experimentation and used immediately with any of the reported RT-PCR chemistries. These methods have application in epidemiological studies, biosecurity, as well as rapid screening of the asymptomatic plants in certification and breeding programs.

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No conflict of interest exists.

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Figure Legends

Figure 1: The artificial positive control (APC). Custom synthesized tandem of forward and reverse complement primer sequences ligated into a multiple cloning site of pUC57. Each PCR product produces a unique sequence for each target also useful in product verification and quality control. The APC also contains primer sequences for five other target viruses.

Figure 2: Gradient end point PCR and two steps RT-PCR using primer set RRV2F/2R. Annealing temperature (Ta) ranged from (A) 53.9 to 66 °C using the APC (plasmid DNA), and (B) 54 to 64 °C using cDNA from infected tissue. Lane M, 50 bp DNA ladder; lane N, non-template control (NTC; water).

Figure 3: Sensitivity assays. (A) PCR sensitivity assay using ten-fold serially diluted APC (plasmid DNA) starting from 1 ng to 1 fg. Lane M, 50 bp ladder; lane 1, 1 ng; lane 2, 100 pg; lane 3, 10 pg; lane 4, 1 pg; lane 5, 100 fg; lane 6, 10 fg; lane 7, 1 fg; lane N, non-template control (NTC, water). (B) RT-PCR sensitivity assay using ten-fold serially diluted infected host cDNA from *Rose rosette virus* infected tissue. Lane M, 50 bp ladder; lane 1, 1 pg; lane 2, 100 fg; lane 3, 10 fg; lane 4, 1 fg; lane N, healthy plant control (negative control, healthy Knock Out[®] *Rosa* 'Radrazz').

Figure 4: (A, B) SYBR Green qPCR standard curve/graph generated using ten-fold serial dilution of APC (plasmid DNA) for quantification of unknown cDNA (prepared from infected host tissue). R² is linear correlation coefficient, Y is the slope, and Ex is the reaction efficiency. Each reaction was performed in three replicates. X axis represents the number of cycles, and Y axis is normalized fluorescence.

Figure 5: Screening of Rose samples (30) collected from eight states of the United States using two steps endpoint RT-PCR. (A, B) Lane M, 50 bp ladder; lane P, Positive control (APC), lanes 1-30, Asymptomatic and symptomatic rose samples R1-R30 as indicated in Table 1; lane N, Healthy plant sample (negative control, healthy Knock Out[®] *Rosa* 'Radrazz').

Figure 6: TaqMan RT-qPCR assay using infected field samples. Positive control, Infected *Rose rosette virus* plant; R7, 201301191; R12, 201301166; R9, 2013002284; R13, 201301097; R3, 201301118; R6, 201302232; R5, 201300232 (Table 1); Negative control, Healthy plant sample (healthy Knock Out[®] *Rosa* 'Radrazz'); NTC, Non-template control. Each reaction was performed in three replicates.

Figure 7: HRM analyses showing the melting profile of APC ($T_m = 78.85 \pm 0.06$ °C, mean $\pm$ SD, n=9) and positive RRV infected sample ($T_m = 75.6 \pm 0.1$ °C, mean $\pm$ SD, n=6) amplified using RRV2F/2R primer set (Arrows indicate the T_m). Only the plant sample infected with RRV is amplified.

Figure 8: SYBR-Green RT-qPCR and HRM curve chart of randomly selected infected field samples. (A) SYBR-Green RT-qPCR assay (B) Melt peak chart for cDNA amplified using RRV specific primer set. Positive control, Plasmid; R29, 1; R30, 2; R4, 201301016; R14, 201301704; R11, 201302234; NTC, Non-template control. Each reaction was performed in three replicates. No HRM curves were obtained with the negative control (healthy Knock Out[®] *Rosa* 'Radrazz').

Supplementary Figures Legend

Supplementary Figure 1: Symptoms of Rose rosette disease on landscape roses. (A, B) Infected plant showing reddening of stems, thin elongated and malformed leaves with increased number of prickles, (C, D) Distorted flower buds on rose infected with the RRV often leading to the

585 failure of bud to open, (E) Abnormal red discoloration of leaves and excessive prickles on
586 thickened stem, (F) Rose rosette disease within a bed of asymptomatic plants. Red color and
587 witches broom is noted.

588 Supplementary Figure 2: Endpoint RT-PCR using primer set RRV2F/2R with and without the
589 incorporation of amplification facilitators (AFs) in the PCR mix. Lane M, 50 bp DNA ladder;
590 lane N, non-template control (NTC; water).

Table 1: RT-PCR and RT-qPCR detection of *Rose Rosette virus* (RRV) from symptomatic and asymptomatic *Rosa* spp. collected from different states of the United States

Sample code used in the study	Original sample code	Host*	Location	RT-PCR (RRV)	Average $C_{T(SD)}$ values using TaqMan RT-qPCR	Average $C_{T(SD)}$ values using SYBR-Green RT-qPCR
R1	201301541	Rose (Pink Home Run)	Oklahoma	+	33.16 _(0.09)	35.68 _(0.51)
R2	201301202	Rose	Oklahoma	+	30.58 _(0.48)	28.40 _(1.12)
R3	201301118	Rose	Oklahoma	+	29.38 _(0.19)	x
R4	201301016	Rose (Pink Knock Out [®])	Oklahoma	+	23.84 _(0.54)	23.63 _(0.48)
R5	201300232	Rose	Oklahoma	+	34.13 _(0.09)	x
R6	201302232	Rose (Chris Evert)	Oklahoma	+	31.55 _(0.32)	x
R7	201301191	Rose	Indiana	+	21.94 _(0.14)	x
R8	201301192	Rose	Indiana	+	30.61 _(0.18)	x
R9	201302284	Rose	Michigan	+	27.51 _(0.17)	x
R10	201301814	Rose (Red Velvet)	California	+	32.15 _(0.21)	x
R11	201302234	Rose	North Carolina	+	34.53 _(0.76)	38.15 _(0.33)
R12	201301166	Rose (Rosy-Red Robin Hood)	Indiana	+	24.1 _(1.48)	x
R13	201301097	Rose	Virginia	+	28.91 _(0.25)	x
R14	201301704	Rose	New Jersey	+	26.53 _(0.07)	26.83 _(1.67)
R15	201301815	Rose (South Africa origin)	California	+	36.45 _(1.35)	35.89 _(0.97)
R16	201301105	Rose	North Carolina	+	30.84 _(0.30)	x
R17	201301490	Rose	Virginia	+	27.63 _(0.28)	x
R18	201301719	Rose (Knock Out [®])	Kentucky	+	25.46 _(0.18)	x
R19	201301219	Rose	Michigan	+	28.65 _(0.21)	x
R20	201301104	Rose	North Carolina	+	26.88 _(0.17)	x
R21	201301102	Rose	North Carolina	+	26.82 _(0.26)	x
R22	201301716	Rose	Virginia	+	32.33 _(0.57)	x
R23	201300219	Rose	Oklahoma	+	27.54 _(0.29)	x
R24	201301755	Rose (Knock Out [®])	Kentucky	+	34.49 _(0.61)	x
R25	201301754	Rose (Knock Out [®])	Kentucky	+	36.11 _(0.61)	x
R26	201300222	Rose	Oklahoma	+	33.75 _(0.58)	x
R27	201300220	Rose	Oklahoma	+	31.36 _(0.37)	x
R28	201300218	Rose (Double Knock Out [®])	Oklahoma	+	29.57 _(0.09)	x
R29	1	Rose	Oklahoma	+	20.26 _(0.40)	19.53 _(0.22)
R30	2	Rose	Oklahoma	+	20.43 _(0.19)	19.95 _(1.49)
Positive Reference control	201300956	Rose	Oklahoma	+	23.61 _(0.15)	28.4 _(1.12)
Negative control	NC	Rose (Healthy Knock Out [®])	Oklahoma	-	-	-
Non-template control	NTC	Nuclease free water	Ambion, Austin, TX	-	-	-

C_T values are average of 3 replicates. SD=Standard deviation of 3 replicates. + = samples tested positive. X = samples not tested. - =negative. The assays were performed using cDNA prepared from RNA extracted from the leaf tissue of asymptomatic and symptomatic plant samples (Roses).

*Cultivar was unknown unless otherwise noted in brackets

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8