A field based detection method for *Rose rosette virus* using isothermal probe-based Reverse transcription-recombinase polymerase amplification assay

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ABSTRACT

Rose rosette disease, caused by *Rose rosette virus* (RRV; genus *Emaravirus*) is a major threat to the rose industry in the U.S. The only strategy currently available for disease management is early detection and eradication of the infected plants, thereby limiting its potential spread. Current RT-PCR based diagnostic methods for RRV are time consuming and are inconsistent in detecting the virus from symptomatic plants. Real-time RT-qPCR assay is highly sensitive for detection of RRV, but it is expensive and requires well-equipped laboratories. Both the RT-PCR and RT-qPCR cannot be used in a field-based testing for RRV. Hence a novel probe based, isothermal reverse transcription-recombinase polymerase amplification (RT-exoRPA) assay, using primer/probe designed based on the nucleocapsid gene of the RRV has been developed. The assay is highly specific and did not give a positive reaction to other viruses infecting roses belonging to both inclusive and exclusive genus. Dilution assays using the *in vitro* transcript showed that the primer/probe set is highly sensitive, with a detection limit of 1 fg/μl. In addition, a rapid technique for the extraction of viral RNA (< 5 min) has been standardized from RRV infected tissue sources, using PBS-T buffer (pH 7.4), which facilitates the virus adsorption onto the PCR tubes at 4°C for 2 min, followed by denaturation to release the RNA. RT-exoRPA analysis of the infected plants using the primer/probe indicated that the virus could be detected from leaves, stems, petals, pollen, primary roots and secondary roots. In addition, the assay was efficiently used in the diagnosis of RRV from different rose varieties, collected from different states in the U.S. The entire process, including the extraction can be completed in 25 min, with less sophisticated equipments. The developed assay can be used with high efficiency in large scale field testing for rapid detection of RRV in commercial nurseries and landscapes.

Keywords: Emaravirus; Isothermal; Recombinase polymerase assay; *Rose rosette virus*.

1. Introduction

Roses are one of the most important ornamental flowering shrubs grown worldwide. In the United States, the 2014 total wholesale production of roses accounts for 204 million U.S. Dollars (United States Department of Agriculture, 2015). To date, several virus species belonging to at least eight different genera have been reported to infect roses (Milleza et al., 2013); and some of these can have a significant impact on rose production. Of the many viruses identified, *Rose rosette virus* (RRV, genus *Emaravirus*) (Laney et al., 2011) is described to be the most economically important one. RRV has a multipartite (7 genomic

RNA segments), single stranded negative-sense RNA genome (-ssRNA) (Laney et al., 2011; Di Bello et al., 2015; Babu et al., 2016a), and is associated with the most devastating rose rosette disease, which causes huge economic losses to home gardeners and the rose nursery and landscape industries (Stanley, 2013).

RRV is transmitted by the eriophyid mite species *Phyllocoptes fructiphilus* (Amrine, 1988, Laney et al., 2011); and causes varying symptoms on infected roses, including lateral shoot growth, excessive thorniness, witches' broom, mosaic, and red pigmentation, and eventually leads to the death of the plant in one to two years (Amrine, 2002; Babu et al., 2014, 2015). The only management option for the control of RRV is timely identification and eradication of the infected plants, thereby preventing or limiting its potential spread. This in turn requires a highly reliable, rapid, specific and sensitive detection assay for routine sampling of plants exhibiting RRV symptoms. The most commonly used diagnostic tool for RRV is end-point reverse transcription-polymerase chain reaction (RT-PCR) assay using specific primers designed based on the RNA1 genomic RNA segment of RRV (Laney et al., 2011). However, these primers are found to be less sensitive and inconsistent in detecting the virus even from symptomatic tissues (Babu et al., 2016b). Recently a SYBR green and TaqMan Real-time RT-PCR assay using a single primer-probe, designed based on the RNA3 genomic RNA segment (Dobhal et al., 2016) and a TaqMan RT-qPCR assay based on multiple gene targets (Babu et al., 2016b) were developed for the detection of RRV.

Even though end-point RT-PCR/PCR and RT-qPCR are very useful for the detection and diagnosis of plant virus infection (Mackay et al., 2002), due to their high sensitivity and specificity (Osman and Rowhani, 2008; Dai et al., 2012; Arif and Ochoa-Corona, 2013; Tang et al., 2014), their impact on certification and indexing programs is still limited in practice because of the expensive equipment needed, and time needed for conducting the assay (3 to 4 h). With the advancement in molecular diagnosis, several isothermal nucleic acid amplification techniques including but not limited to, strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), and rolling-circle amplification (RCA) methods (Andras et al., 2001; Asiello and Baeumner, 2011; Gill and Ghaemi, 2008; Kim and Easley, 2011) have been developed. Among the techniques, RPA is a relatively novel DNA amplification and detection technology (Piepenburg et al., 2006), which utilizes an enzymatic mixture of polymerase and DNA recombination proteins. The phage-derived recombinase enzyme binds to the single stranded oligonucleotide primers, which then

efficiently scans double stranded DNA (dsDNA) template to identify homologous sequences and further facilitate the annealing of the primers to the template. The *Staphylococcus aureus*-derived DNA polymerase then displaces the dsDNA strands, and elongates the primer, resulting in an exponential amplification of the target in less than 20 min (Shibata et al., 1979; Yonesaki et al., 1985; Formosa and Alberts, 1986; West, 2003; Piepenburg et al., 2006; Krejci et al., 2012). RPA can be run at a constant low temperature of 37–42°C (Hoff, 2006; Piepenburg et al., 2006; Euler et al., 2012a, b), thereby avoiding the need for sophisticated thermal cyclers. RPA products can be detected by agarose gel electrophoresis (Piepenburg et al., 2006), in real-time using TwistAmpTM exo probes (TwistDx, Cambridge, UK) (Euler et al., 2012a; Boyle et al., 2013; Euler et al., 2013) or by a lateral flow dipstick assay (MileniaBiotec, Giessen, Germany). Application of RPA in the diagnosis of viruses, bacteria and fungi have been previously reported (Lillis et al., 2014; Lutz et al., 2010; Shen et al., 2011; Amer et al., 2013; Euler et al., 2013; Ahmed et al., 2014; Jaroenram and Owens, 2014; Mekuria et al., 2014; Silva et al., 2014; Zhang et al., 2014; Miles et al., 2015; Londoño et al., 2016). For RRV, a gel-based RT-RPA assay using primer sets designed based on multiple gene targets have been developed (Babu et al., 2017).

This novel isothermal amplification technique has wide capabilities in field based detection, but requires a simple and rapid technique for the extraction of nucleic acids. At present, several manual methods as well as commercial kits are available for the extraction of RNA. However most of these kits are expensive and time consuming (30 min); and can be carried out only in a sterile lab conditions. As an alternative to tedious and time consuming RNA extraction techniques, several methods involving capturing of viral particles and subsequent release of RNA have been previously reported. This includes direct binding (DB)-RT-PCR (Rowhani et al., 1995) and Immuno-capture (IC)-RT-PCR (James, 1999; Wetzel et al., 1992; Nolasco et al., 1993). However, these two methods are time consuming and the latter requires use of an antibody for the virus capture.

The objectives of this study were: 1) to develop a probe based reverse transcription-recombinase polymerase amplification (RT-exoRPA) assay for the RRV; 2) to evaluate the specificity of the RT-exoRPA primers/probe set against other common rose infecting viruses belonging to the exclusive and inclusive genus; 3) to determine the sensitivity of the RT-exoRPA primers/probe in detecting the virus; 4) to develop a rapid viral RNA extraction procedure for RRV (< 5 min) from different tissue sources; and 5) to evaluate the efficiency

of the developed primers/probe in detecting RRV from different tissue sources of infected rose plants, as well as from several rose varieties collected from different states in the U.S.

2. Materials and methods

2.1. Sample collection

Eight pink double Knock Out[®] rose plants (*Rosa Radtkopink*) with characteristic symptoms of Rose rosette disease were collected from a rose nursery in Florida in 2013 (**Table 1**). Two non-symptomatic pink double Knock Out[®] rose plants were collected from another nursery in Florida with no reports of RRV. These plants were maintained in a quarantine facility at the North Florida Research and Education Center, University of Florida. Four RRV infected rose plants (OK-1, OK-2, OK-3 and OK-4), and one non-symptomatic rose plant (OK-5) were also collected from landscape at Stillwater, Oklahoma. These plant tissues were stored in -20°C at Henry Bellmon Research Center, Oklahoma State University. In addition, 15 RRV infected and one non-infected double Knock Out[®] rose plant samples, and ten RRV infected different rose varieties (confirmed using RRV specific diagnostic primers RRV-F and RRV-R) collected during 2013 to 2015 from different states in the U.S. were used in this study. Moreover, 16 different rose cultivar samples showing typical symptoms of RRV were collected from Tulsa, Oklahoma during 2016. All these samples were stored at the Plant Disease and Insect Diagnostic Lab (PDIDL) at Oklahoma State University. The details of the samples are indicated in **Table 2**.

2.2. RPA primer and probe design

The primers and probe sequences for the RT-exoRPA assay was selected by determining suitable conserved regions of RRV genomic RNA segment 3. All the available RRV RNA3 segments (24 sequences) from the National Center for Biotechnology Information database (NCBI) were aligned and highly conserved regions were identified using BioEdit version 7.0.5.3 (Hall, 1999). The conserved regions were further used for designing the primers and probe using the PrimerQuest software (Integrated DNA Technologies, Coralville, IA) (<http://www.idtdna.com/Primerquest/Home/Index>) (**Table 3**). The primers and exo-probe were designed based on the criteria suggested in the TwistAmp[™] reaction kit manual. The thermodynamics, internal structures, and self-dimer formation of the primers were examined *in silico* using mFOLD (Zuker, 2003) and Autodimer software (Vallone and Butler, 2004). The specificities of the primers and probe were assessed *in silico* using Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/blast/>), with Blastn. The

primers were synthesised by IDT (Integrated DNA Technologies, Coralville, IA) and the exo-probe by the LGC Biosearch Technologies (Petaluma, CA).

2.3. RT-RPA assay and transcript preparation

Preliminary RT-RPA assays to assess the primers were carried out using TwistAmp® Basic RT kit (TwistDX, Cambridge, UK). The RT-RPA reaction was performed in a 50 µl volume containing 1 µg total RNA of an RRV infected rose plant, 29.5 µl of 1x rehydration buffer, 0.24 µM of each primer. The master mix was distributed into each 0.2 ml reaction tube containing a dried enzyme pellet, and 14 mM magnesium acetate was pipetted into the tube lids. The lids were closed, and the tubes were vortexed briefly and centrifuged. The tubes were then incubated at 42°C in a heating block for 20 min. Negative controls including non-template control (NTC) and total RNA from a healthy rose plant were included in the assay. The amplified product was analyzed on 1% agarose gel electrophoresis, purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia, CA), and cloned into pCR2.1-TOPO TA vector (Invitrogen, Life Technologies, Grand Island, NY), following the manufacturers' protocol. Inserts from the isolated plasmids were sequenced using the M13F and M13R primers, and the sequences were compared with those in the NCBI database using Blastn. RNA transcripts from the cloned plasmids were generated using the MAXIscript *in vitro* transcription kit (Ambion, Austin, TX) as per the manufacturer's protocol, and using T7 RNA polymerase. The developed transcripts were quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE).

2.4. RT-exoRPA assay

RT-exoRPA assay was performed using RT-exoRPA primers and probe, using TwistAmp® exo RT kit (TwistDX, Cambridge, UK). The RT-exoRPA reaction was performed in a 50 µl volume containing 1 µg total RNA of an RRV infected rose plant, 29.5 µl of 1x rehydration buffer, 0.24 µM of each primer, and 0.12 µM of exo-probe. The master mix was distributed into each 0.2 ml reaction tube containing a dried enzyme pellet, and 14 mM Magnesium acetate was pipetted into the tube lids. The lids were closed, and the tubes were vortexed briefly and centrifuged. The tubes were then incubated at 40 cycles of, 42°C for 30s in Applied Biosystems 7500 Fast Instrument (Invitrogen, Applied Biosystems), and the fluorescence was recorded. Negative controls including NTC and total RNA from a healthy rose plant were included in the assay.

2.5. Specificity and sensitivity assay

The specificity of the designed primers and probe were tested using RT-exoRPA, against other common rose infecting viruses, both exclusive and inclusive (**Table 4**). These lyophilized reference viruses were obtained from Agdia, Inc (Agdia, Elkhart, IN), and RT-PCR confirmed using virus specific primers listed in **Table 4**. A positive control including the specific RNA transcript (1 ng/μl) as well as the total RNA from the RRV infected plant samples, and the negative control including RNA from healthy rose plant as well as the NTC, were included in the RT-exoRPA specificity assay. For the sensitivity assay, a series of dilutions of the RNA transcript ranging from 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg were prepared in Diethyl pyrocarbonate (DEPC) treated water and subjected to RT-exoRPA analysis, along with the RNA from healthy rose plant and NTC. The assay was repeated once more, and the fluorescence detected using Applied Biosystems 7500 Fast Instrument. For comparison, the diluted transcripts (10 pg to 1 fg) were also subjected to end-point RT-PCR analysis using the RPA primers. In addition, sensitivity assay was performed on viral RNA obtained from RRV infected leaf tissues, using the Direct antigen-capture method as described in section 2.6. The viral RNA was subjected to a series of dilutions ranging from 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg in DEPC-treated water, and subjected to RT-exoRPA analysis, along with RNA from healthy rose plant and NTC. The analysis was replicated and the fluorescence recorded using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, California).

2.6. Sample preparation: Direct antigen-capture method

RRV infected leaves (100 mg) were ground in 1 ml of the phosphate-buffered saline pH 7.4 (PBS) solution containing 0.05% Tween-20 (1X PBS-T) buffer. Fifty microliters of the resulting crude sap was placed into a 0.5 ml Polypropylene PCR tube (TempAssure PCR tubes, USA scientific, FL), without trapping any air bubbles. The tubes were then incubated in ice for 2 min. Following the incubation, the sap was completely removed, and the tubes were washed twice with 50 μl of 1X PBS-T buffer. Thirty microlitres of DEPC-water containing RNasin (100 U/ml) was added to the PCR tube, followed by immediate denaturation at 95°C for 1 min, and cooled on ice for 1 min. The resulting solution was then subsequently used in the RT-exoRPA analysis. The schematic representation of the direct antigen-capture method for the extraction of viral RNA is shown in **Fig. 1**. For optimization of the direct antigen capture method, different incubation time (15 s, 30 s, 1.5 min, 2 min, 3 min, 5 min and 10 min) of the crude sap in the PCR tube at 4°C was also evaluated. The

concentration of elutes were quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE).

2.7. Screening of field samples

The eight pink double Knock Out[®] rose plants along with the two non symptomatic plants from Florida were subjected to RT-exoRPA analysis. A total of 12 samples per plant, including 2 each of leafs, stems, petals, anthers (pollen), primary roots and secondary roots, were used in the analysis. Among the samples, all the leaves, petals and anthers were ground in a micro-centrifuge tube using a micropestle (<50 mg/500 µl 1X PBS-T buffer), while the stem, primary and secondary roots were ground using a mortar and pestle. All the sap incubations were performed in ice for 2 min. RT-exoRPA fluorescence for the Florida samples were analyzed using the Applied Biosystems 7500 Fast Instrument. Five samples collected from the landscapes in Stillwater, Oklahoma were subjected to RT-exoRPA analysis, and included 4 samples per plant (leaf, stem, primary and secondary roots). Anthers from one RRV infected sample were tested. The fluorescence for the Oklahoma samples was analyzed using the Rotor-Gene 1.7.87 (Corbett Research, Australia). To further validate the assay, previously collected rose samples (2013-2015) including 15 RRV infected and one non-infected double Knock Out[®] rose plant samples, as well as RRV infected thirty two different rose varieties (**Table 2**), were subjected to RT-exoRPA analysis. In addition, 16 different rose cultivar samples showing typical symptoms of RRV, collected from Tulsa, Oklahoma (2016), were also tested using the RT-exoRPA analysis. Positive control (1 ng/µl of specific RNA transcript) and negative controls including total RNA from healthy rose plant as well as the NTC were included in the analysis.

3. Results

3.1. *In silico* analysis of the primers and probe

One set of RPA primers, based on the genomic RNA segment RNA3 (nucleocapsid) was designed for the RT-exoRPA analysis of the RRV (**Table 3**). The primers were designed based on the nucleotide position 1126-1393 from the genomic RNA segment RNA3. *In silico* analysis of the primer set indicated 97 to 100% identity (Query coverage 100%) with that of RRV RNA3. The RPA-exoprobe was designed based on the nucleotide position 1197-1247 of the genomic RNA segment RNA3. *In silico* analysis of the probe sequences indicated 100% identity to the RRV genomic RNA segment RNA3 with 100% query coverage and

with an E-value of $5e-17$. There were no matches detected with other viruses, including those listed in the exclusivity and inclusivity panel; as well as with bacteria.

3.2. RT-RPA analysis

RT-RPA analysis of the RRV infected samples using the primer set produced an amplification product of 267 bp (data not shown). Sequence analysis of these cloned amplicon indicated 99% and 100% identity to genomic RNA segment RNA3 of several RRV isolates in the NCBI database. No amplification was obtained with the healthy rose plant or with the NTC.

3.3. Specificity assay

RT-exoRPA analysis on the thirteen other viruses infecting roses, belonging to the exclusive and inclusive panel did not produce any cross amplification (data not shown). The positive control (1 ng/ μ L RNA transcript as well as the total RNA from the RRV infected plant sample) produced a positive reaction, while the RNA from the healthy rose plant as well as the NTC did not produce any reaction.

3.4. Sensitivity assay

The RT-exoRPA analysis indicated that the primer/probe set could detect the diluted transcripts up to a concentration of 1 fg/ μ L (**Fig. 2a**). Similar trends were observed on repeating the experiment (data not shown). End-point RT-PCR analysis using RPA primers on diluted transcripts also produced a positive reaction up to a concentration of 1fg/ μ L (**Fig. 3**), confirming previous results (Babu et al., 2017). Sensitivity assay using virus RNA prepared by Direct antigen-capture method also showed that the primer/probe could detect the virus up to a concentration of 1 fg/ μ L (**Fig. 2b**)

3.5. Direct-Antigen Capture analysis

Crude sap of infected leaves ground in 1x PBS-T was used to determine the optimum conditions, such as time of incubation, for the efficient recovery of the viral nucleic acid. Different time incubations of the crude sap at 4°C indicated that the capture of viral particles to the PCR tube can be attained from 15 s to 3:00 min (**Fig. 4**). Analysis of the crude sap incubation period for 5 min and 10 min did not yield any positive reaction on RT-exoRPA analysis. Similar trends were observed on repeating the experiment (data not shown). Concentration analysis of the RNA elutes from different incubation times indicated a lower concentration of the RNA (3.51 ng/ μ L) at 15 s, followed by a gradual increase in the concentration with the increase in incubation time. The concentrations obtained from different time of incubations are 7.38 ng/ μ L (30 s), 11.03 ng/ μ L (1 min), 12.86 ng/ μ L (1:30

min), and 14.59 ng/μl (2:00 min). The highest concentration obtained was 16.14 ng/μl at 3:00 min. There was decrease in the concentration of the RNA at time intervals of 5 and 10 min (0.21 and 0.13 ng/μl respectively).

3.6. Screening of field samples

RT-exoRPA analysis tested positive with majority of the Florida samples, irrespective of the tissue sources; with the exception of FL-2 and FL-6 (**Table 1**). A representative figure showing the detection of RRV from different tissue sources of two plant samples are shown in **Fig. 5**. In case of FL-2 (83% positive detection), the primer/probe failed to detect the virus from both the primary roots collected. In sample FL-6 (75% positive detection), the assay failed to detect the virus from one sample each of stem, petal and secondary root. Among the three RRV infected rose samples collected from Stillwater, Oklahoma, RT-exoRPA analysis detected the virus from all the samples, while the non-symptomatic RRV suspected plant OK-5, did not produce any positive reaction with any of the tissue sources, indicating the absence of the virus in these samples (**Fig. 6**). RT-exoRPA analysis of the Knock Out[®] series of rose plants collected from different states in the U.S., showed positive detection of RRV from all the 15 infected samples, while the non-infected sample from Edgefield, South Carolina (201302247) tested negative (**Table 2**). Among the 32 different rose varieties collected from 2013 to 2015 from different states in the U.S., RT-exoRPA analysis showed positive detection of RRV from majority of the rose cultivars (75% positive detection). Analysis of the different rose varieties collected during 2016 from Tulsa, Oklahoma, also produced a positive reaction with all the samples tested (**Table 2**). The positive control (1 ng/μl RNA transcript) produced a positive reaction in RT-exoRPA analysis, while the RNA from the healthy rose plant as well as the NTC did not produce any reaction.

4. Discussion

This study describes the development of a probe based isothermal RT-exoRPA assay for the rapid, sensitive, reliable and specific detection of RRV, which possesses a major threat to the rose industry. The potential impact of the Rose rosette disease on rose industries in U.S. could be extremely fatal, and hence the development of a diagnostic tool which will facilitate its timely identification in labs with minimum equipment requirement is critical.

Preliminary *in silico* characterization of the primers and the probe sequence, as well as the amplicon sequences obtained from the end-point RT-RPA assay indicated 100% identity to the RRV genomic RNA segments RNA2. The primers/probe did not produce any

nonspecific cross reactivity (false positive) on RT-exoRPA analysis against twelve other rose infecting viruses (exclusive) and one inclusive virus, or RNA from healthy control rose plants, suggesting the high specificity of the designed primer/probe.

The developed RT-exoRPA assay is highly sensitive with a detection limit of 1 fg/ μ L. This was comparable to the sensitivity produced by the RPA primers in an end-point RT-PCR analysis. Such a comparable similarity between the RT-exoRPA and PCR/RT-PCR based techniques has also been previously reported in case of other pathogens including *Francisella tularensis* (Euler et al., 2012b), bio-threat agents including *Yersinia pestis*, *Bacillus anthracis*, variola virus, *Rift Valley fever virus*, *Ebola virus*, *Sudan virus*, and *Marburg virus* (Euler et al., 2013), *Dengue virus* (Teoh et al., 2015) and *White spot syndrome virus* (Xia et al., 2014). In addition, a comparative analysis indicated that the RT-exoRPA assay had a sensitivity limit equivalent with the previously developed RT-qPCR assay (1 fg/ μ L) for RRV (Babu et al., 2016b). Even though a comparable sensitivity exists between these methods, the expertise and time required to perform a RPA assay is considerably reduced (20 min) as compared to RT-PCR (3 to 4 h) and RT-qPCR (1 to 1.5 h).

The developed direct-antigen capture method is a rapid and efficient technique to obtain template viral RNA for molecular diagnosis, and can be completed in less than 5 min. Several rapid and simple extraction techniques for the plant viruses including direct binding (DB)-RT-PCR (Rowhani et al., 1995) are previously reported. However, this method is time consuming. Another method called as tube capture (TC)-RT-PCR or Immuno-capture (IC)-RT-PCR (James, 1999) has been reported, in which the viral particles are captured on to the PCR tubes using specific antibodies, either monoclonal or polyclonal. Production of viral specific antibodies is expensive, laborious and time consuming. This poses a constraint on the utilization of IC-RT-PCR as a source of molecular diagnostic tool. The direct antigen capture method used in this study differs from the IC-RT-PCR technique in avoiding the usage of antibody for the capture of the viral particles. Indeed, the virus particles are adsorbed on to the PCR tube by PBS-T buffer, followed by the denaturation at 95°C for 1 min to release the viral RNA. The capturing method described in this study also resembles that involved in indirect-ELISA or Direct-antigen coated ELISA (Koenig and Paul, 1982), in which the conventional carbonate-bicarbonate coating buffer is used to trap the virus particles on to the ELISA plate. Our attempts to use the coating buffer to capture the RRV virus and use subsequently in RT-exoRPA analysis did not produce any positive result (data not shown).

This could potentially be due to the trace amounts of buffer left over in the PCR tube, and subsequent buffer incompatibility with the RT-exoRPA analysis.

Another important factor for the efficient extraction technique is the time of incubation of the plant sap at 4°C. Our studies indicated that, even at lower incubation times (15 s, 30 s, 1 min and 1:30 min), efficient trapping of RRV particles occur, but is slightly inconsistent with the different tissue sources (data not shown). The optimum time of incubation was thus set at 2 min, which was highly efficient in trapping, and consistently detecting the virus in our assay. Higher incubation time of 5 and 10 min produced negative effect on the virus adsorption on to the PCR tube, and could potentially be due to the degradation of the viral particles. This is also reflected in the decrease in the RNA concentration at incubation times of 5 and 10 min, as compared to the lower incubation times. In addition, incubation of sap at 4°C for 5 or 10 min increases the viscous nature of the sap, which further affects the downstream cleaning step. The left-over contaminants could also be the potential reasons for the negative reaction on RT-exoRPA analysis. The extraction technique produced an RNA concentration ranging from 3.51 to 16.14 ng/μl, at different time of incubation ranging from 15 s to 3 min. This low concentration of the RNA in the elute, could be attributed to the low virus titer in 50 μl of the sap used for the adsorption. However, our study shows that this concentration of RNAs was sufficient to be detected by the RT-exoRPA analysis, indicating the high sensitivity of the developed technique.

RNA isolation from infected rose plant tissue is often complicated due to the abundance of phenolic compounds, carbohydrates, pigments, and other unknown compounds. These compounds irreversibly interact with the nucleic acid and proteins leading to oxidation and degradation, reducing the quality of RNA and subsequent analysis (Hu et al., 2002; Salzman et al., 1999). Our attempts to use the diluted plant sap for the RT-exoRPA assay did not produce any significant results (data not shown), indicating huge amount of inhibitors in the rose tissues. The major advantage of the direct-antigen capture method is that it is possible to completely remove the host tissue and subsequent inhibitors, leaving behind only the virus particles in the tube. Washing using the PBS-T buffer after the sap incubation, makes the sample free of debris.

RT-exoRPA analysis of the collected samples using different tissue sources indicated a positive detection of the virus, suggesting the robustness of the assay. The failure to detect the virus in certain tissue sources could be due to the inhibitory sap present during the extraction technique, which potentially affected the further analysis. Positive detection of the

virus from different tissue sources provides a major advantage in selecting the source of tissue for indexing purposes. RNA extraction from primary and secondary root as well as from the stem tissue using the Qiagen RNeasy Plant mini kit did not yield high quality RNA, for its subsequent analysis in endpoint-RT-PCR using previously published RRV primers (Laney et al., 2011) (data not shown). This could potentially be due to the high amount of inhibitory compounds present in these host tissue, interfering with the extraction of high quality RNA. Even though the virus is described to be systemic in nature; there hasn't been any scientific evidence of its occurrence in the roots and the stem tissues. With the developed RT-exoRPA analysis coupled with the rapid and high pure extraction technique, the virus could be easily detected in these tissues. Even though roses are vegetatively propagated, the discovery of the RRV from pollen (anther) tissue in RRV infected roses, using the RT-exoRPA analysis, suggests that the virus could potentially be sexually transmitted, but lacks scientific evidence at this point. This paper describes the first report on the detection of these viruses from pollen tissues. Even though RRV can be easily detected from different tissue sources (stem, petals, pollen and roots), from a diagnostic perspective, the best source of samples are fresh young leaves. Unlike the Florida samples, which were maintained in a quarantine green house in controlled environmental conditions, the Oklahoma samples collected in these studies were growing in the landscapes at a temperature below the freezing point. Collection of tissue sources for the RRV testing was tedious in these plants, due to the extreme tissue conditions caused by the cold weather. Detection of the RRV from the primary and secondary roots of these plants suggests that the viruses over-winter these extreme cold conditions by surviving in the roots. This finding suggests that the plants surviving in extreme cold conditions could be efficiently diagnosed for RRV by using the root as the tissue source, even in the absence of other tissue sources including the leaves, green stem, petals etc. The ability of the primer/probe in detecting the RRV from rose samples irrespective of their location and varieties indicates the high efficiency and broad range applicability of the developed assay. However, some of the rose varieties collected during 2013 to 2015, showed negative reaction with RT-exoRPA assay, as compared to the positive reaction with the end-point RT-PCR analysis using the RRV specific primers RRV-F and RRV-R (**Table 2**). Failure of the primer/probe in detecting the RRV from these samples could potentially be due to the RNA degradation or inhibitors in samples stored in -20°C for long term (1 to 3 years). Moreover, many of the samples were in fair-poor condition at the time of submission to PDIDL. Samples collected in 2016 were tested after a brief period of

storage at 4°C, and showed 100% detection of RRV from these symptomatic or suspected samples. Even though, samples stored in -80°C may test positive for RRV; it is always good or better to relay on fresh tissue sources.

This study is the first report on the use of a novel probe based isothermal amplification technique, RT-exoRPA for the detection of RRV. The developed assay is highly specific, sensitive, and rapid (25 min including the RNA extraction). Even though the cost per sample for the RT-exoRPA analysis is approximately \$6, which is equivalent to the cost per sample for the end-point RT-PCR/RT-qPCR, the time required for performing the RT-exoRPA analysis is much less, and can even be operated using a simple fluorescent reader. In our study, the fluorescence recordings have been performed using Applied Biosystems 7500 Fast Instrument or Rotor-Gene 1.7.87, due to the non-availability of a portable fluorescent reader. However, the assay can also be easily operated using a simple fluorescent reader. Coupling the rapid extraction technique (< 5min) for viral RNA without any sophisticated equipments, and RT-exoRPA analysis (20 min) using a simple fluorescent reader, the developed technique can be well suited for field based detection. This would provide an added advantage for performing on-site testing of suspected rose plants in the field itself. This would facilitate the commercial growers and nursery personnel to perform the on-site testing of the suspected plants; from RNA extraction to RT-exoRPA analysis in a short period of time, without the need to transfer them to a lab for testing. The developed RT-exoRPA assay is expected to directly support commercial nurseries and diagnostic laboratories, for the rapid detection and confirmation of the virus, which will facilitate the timely eradication measures to be initiated, thereby minimizing the risk associated with spread of the RRV.

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Table 1. RT-exoRPA analysis of *Rose rosette virus* (RRV) from different tissue sources of infected Pink Double Knock Out[®] roses from Florida.

Sample Name	RT-exoRPA											
	Tissue sources											
	Leaf		Stem		Petal		Anther/Pollen		Primary root		Secondary root	
	L1	L2	S1	S2	P1	P2	An1	An2	R1a	R1b	R2a	R2b
FL-1 ^a	+	+	+	+	+	+	+	+	+	+	+	+
FL-2 ^a	+	+	+	+	+	+	+	+	-	-	+	+
FL-3 ^a	+	+	+	+	+	+	+	+	+	+	+	+
FL-4 ^a	+	+	+	+	+	+	+	+	+	+	+	+
FL-5 ^a	+	+	+	+	+	+	+	+	+	+	+	+
FL-6 ^a	+	+	-	+	-	+	+	+	+	+	-	+
FL-7 ^a	+	+	+	+	+	+	+	+	+	+	+	+
FL-8 ^a	+	+	+	+	+	+	+	+	+	+	+	+
FL-9	-	-	-	-	-	-	-	-	-	-	-	-
(Healthy rose plant)												
FL-10	-	-	-	-	-	-	-	-	-	-	-	-
(Healthy rose plant)												

^a Infected Pink Double Knock Out[®] roses confirmed positive for *Rose rosette virus* using end-point RT-PCR primers RRV-F and RRV-R (Laney et al., 2011)

Table 2. RT-exoRPA analysis of *Rose rosette virus* (RRV) from Knock Out[®] roses and other rose varieties collected from different states in the U.S.

Sample Number	Varieties	Date of collection	County	State	RT-PCR RRV-F /R ^a	RT-exoRPA
Knock Out[®] varieties						
201300218	Double Knock Out [®]	04-11-2013	Tulsa	Oklahoma	+	+
201300219	Double Knock Out [®]	04-11-2013	Tulsa	Oklahoma	+	+
201300222	Knock Out [®]	04-11-2013	Tulsa	Oklahoma	+	+
201301118	Knock Out [®]	05-20-2013	Oklahoma	Oklahoma	+	+
201302391	Knock Out [®]	11-08-2013	Oklahoma	Oklahoma	+	+
201302247	Double Knock Out [®]	10-16-2013	Edgefield	South Carolina	-	-
201301691	Double Knock Out [®]	07-07-2015	Augusta	Virginia	+	+
201301102	Knock Out [®]	05-10-2013	Wake	North Carolina	+	+
201301104	Knock Out [®]	05-10-2013	Wake	North Carolina	+	+
201301105	Knock Out [®]	05-10-2013	Wake	North Carolina	+	+
201301719	Knock Out [®]	07-29-2013	Fayette	Kentucky	+	+
201301754	Knock Out [®]	08-05-2013	Fayette	Kentucky	+	+
201301755	Knock Out [®]	08-05-2013	Fayette	Kentucky	+	+
201301645	Knock Out [®]	06-24-2014	Belknap	New Hampshire	+	+
201302248	Knock Out [®]	09-22-2014	Allegan	Michigan	+	+
201301528	Knock Out [®]	06-19-2015	Montgomery	Virginia	+	+
Rose varieties (2013 -2015)						
201301940	Benjamin Britten	08/29/2013	Tulsa	Oklahoma	Not tested	+
201302232	Chris Everett	10/14/2013	Wagoner	Oklahoma	+	+
201401827	Easter Basket	07/15/2014	Tulsa	Oklahoma	+	+
201501592	Livin Easy	06/26/2015	Cleveland	Oklahoma	+	+
201401820	Multiflora	07/11/2014	Columbia	New York	+	-
201302234	Old Blush	10/14/2013	Orange	North Carolina	+	-
201301541	Pink Home Run	07/01/2013	Tulsa	Oklahoma	+	+

201301166	Rosy Red Robin Hood	05/28/2013	Tippecanoe	Indiana	+	+
201401962	Rugosa rose	07/31/2014	Montgomery	Virginia	+	+
201301713	Watercolors	07/26/2013	Tulsa	Oklahoma	+	+
201300956	Unknown	30-04-2013	Boone	Indiana	+	-
201301191	Unknown	30-05-2013	Boone	Indiana	+	+
201301277	Unknown	13-06-2013	Tulsa	Oklahoma	+	-
201301490	Unknown	10-07-2013	Montgomery	Virginia	+	-
201301704	Unknown	23-07-2013	Middlesex	New Jersey	+	+
201400532	Unknown	30-05-2014	Ingham	Michigan	+	-
201401610	Unknown	24-06-2014	Montgomery	Virginia	+	+
201401961	Unknown	31-07-2014	Montgomery	Virginia	+	+
201402097	Unknown	22-08-2014	Suffolk	New York	+	+
201402119	Unknown	27-08-2014	Ingham	New York	+	+
201402121	Unknown	27-08-2014	Allen	Indiana	+	+
201402397	Unknown	09-10-2014	New Castle	Delaware	+	+
201402455	Unknown	24-10-2014	Lee	Alabama	+	+
201501138	Unknown	28-04-2015	Tippecanoe	Indiana	+	-
201501140	Unknown	28-04-2015	Laurens	Georgia	+	+
201501434	Unknown	05-06-2015	Gwinnett	Georgia	+	+
201501562	Unknown	24-06-2015	Elbert	Georgia	+	+
201501593	Unknown	26-06-2015	McClain	Oklahoma	+	+
201501951	Unknown	03-08-2015	Washtenaw	Michigan	+	+
201501974	Unknown	06-08-2015	Tippecanoe	Indiana	+	-
201502196	Unknown	31-08-2015	Ingham	Michigan	+	+
201502497	Unknown	12-10-2015	Washtenaw	Michigan	+	+
Rose varieties (2016)						
201601644	Elina	04/19/2016	Tulsa	Oklahoma	+	+
201601694	Elle	04/19/2016	Tulsa	Oklahoma	+	+
201601695	Glowing Peace	04/19/2016	Tulsa	Oklahoma	+	+
201601696	Grenada	04/19/2016	Tulsa	Oklahoma	+	+
201601697	Gypsy	04/19/2016	Tulsa	Oklahoma	+	+

201601700	Palace Eze	04/19/2016	Tulsa	Oklahoma	+	+
201601701	Pretty Lady	04/19/2016	Tulsa	Oklahoma	+	+
201601702	Renaissance Clair	04/19/2016	Tulsa	Oklahoma	+	+
201601703	Renaissance Naomi	04/19/2016	Tulsa	Oklahoma	+	+
201601704	Renaissance Natalie	04/19/2016	Tulsa	Oklahoma	+	+
201601705	Scarborough Faire	04/19/2016	Tulsa	Oklahoma	+	+
201601706	Town and Country Cottage Rift	04/19/2016	Tulsa	Oklahoma	+	+
201601707	Town and Country Luray	04/19/2016	Tulsa	Oklahoma	+	+
201601708	Uetersen Climbing rose	04/19/2016	Tulsa	Oklahoma	+	+
Positive control ^b						+
FL-9 (Healthy rose)	Pink Double Knock Out [®]	-	Gadsden	Florida	-	-
FL-10 (Healthy rose)	Pink Double Knock Out [®]	-	Gadsden	Florida	-	-
NTC ^c					-	-

^a End-point RT-PCR analysis of the samples using conventional *Rose rosette virus* specific diagnostic primers RRV-F and RRV-R (Laney et al., 2011). The analysis was performed at the time of sample collection

^b Positive control includes viral RNA transcripts produced from cloned amplicons, obtained using primers RPA-267F and RPA-267R

^c Non-template control (NTC)

Table 3. Details of primers and probe used in the RT-exoRPA analysis of *Rose rosette virus*

Genomic RNA segment	Primers/probe		Sequence (5'-3')	bp	Tm (°C)	GC %	Target region	Amplicon size (bp)
RNA3	Primers	RPA-267F	TGAAGCTGCTCCTTGATTTCAGGGACCTA	30	71	50	1126-1156	267
		RPA-267R	AAGCACATCCAACACTCTTGCAGCCGATAC	30	71	50	1363-1393	
	Probe	RPA-267 exoP2	GTAAGGTGCTAGACTAAAATTGTTGGGACTT [FAM-dT] G [A-THF] A [BHQ-dT] CTCTGAAGTAAAAGG ^a	51	63.8	37.3	1197-1247	

^a FAM = Fluorescein; THF = Tetrahydrofuran; BHQ = Black Hole Quencher

Table 4. Exclusive and inclusive list of other common rose infecting viruses used in the specificity assay for the *Rose rosette virus* RT-exoRPA primers and probe

Virus panel	Genus	Virus Species	Acronym	Virus Source	Reference for primers used ^a
Exclusive	<i>Ilarvirus</i>	<i>Prunus necrotic ringspot virus</i>	PNRSV	Agdia Inc.	Mackenzie et al., 1997
		<i>Apple mosaic virus</i>	ApMV	Agdia Inc.	Menzel et al., 2002
		<i>Tobacco streak virus</i>	TSV	Agdia Inc.	Pappu et al., 2008
	<i>Nepovirus</i>	<i>Arabis mosaic virus</i>	ArMV	Agdia Inc.	Mackenzie et al., 1997
		<i>Tobacco ringspot virus</i>	TRSV	Agdia Inc.	Maliogka et al., 2004
		<i>Tomato ringspot virus</i>	ToRSV	Agdia Inc.	Maliogka et al., 2004
	<i>Tospovirus</i>	<i>Tomato spotted wilt virus</i>	TSWV	Agdia Inc.	Chu et al., 2001
		<i>Impatiens necrotic spot virus</i>	INSV	Agdia Inc.	Chu et al., 2001
	<i>Alfamovirus</i>	<i>Alfalfa mosaic virus</i>	AMV	Agdia Inc.	Xu and Nie 2006
	<i>Tobamovirus</i>	<i>Tobacco mosaic virus</i>	TMV	Agdia Inc.	Kumar et al., 2011
	<i>Cucumovirus</i>	<i>Cucumber mosaic virus 1</i>	CMV-1	Agdia Inc.	Chen et al., 2011
		<i>Cucumber mosaic virus 2</i>	CMV-2	Agdia Inc.	Chen et al., 2011
Inclusive	<i>Emaravirus</i>	<i>Maize red stripe virus</i> or <i>wheat mosaic virus</i>	WMoV	Agdia Inc.	Elbeaino et al., 2013

^a Details of primers used for the end-point RT-PCR confirmation of the specific viruses belonging to the exclusive and inclusive genus, are mentioned in the listed references.

Figure legends

Fig. 1. Schematic representation of the direct antigen-capture method used for the extraction of *Rose rosette virus* RNA for subsequent use in RT-exoRPA analysis.

Fig. 2. RT-exoRPA based sensitivity analysis of the primers/probe on a) diluted transcripts and b) diluted viral RNA prepared using direct antigen-capture method.

Fig. 3. Sensitivity assay on the diluted transcripts using end-point reverse transcription-polymerase chain reaction using RPA primers RPA-267F and RPA-267R. M = 100 bp DNA ladder.

Fig. 4. RT-exoRPA analysis of the *Rose rosette virus* RNA obtained from different incubation times of the sap at 4°C, in the direct antigen-capture method. + ve ctrl = 1 ng/ul of viral RNA transcript

Fig. 5. RT-exoRPA analysis of two Pink Double Knock Out[®] rose samples from Florida using *Rose rosette virus* specific primers/probe, showing the detection of RRV from different tissue sources. A) FL-1; B) FL-3. L1, L2: leaf samples; S1, S2: stem samples; P1, P2: petals; An1, An2: anthers/pollen; R1a, R1b: primary root samples; R2a, R2b: secondary root samples; +ve = Positive control (1 ng/ul of viral RNA transcript)

Fig. 6. RT-exoRPA analysis of the field samples from Oklahoma using the *Rose rosette virus* specific primers/probe. Sample details: OK-1, OK-2, OK-3 and OK-4. L = leaf samples; POL = anthers/pollen; S = stem; PR = primary roots; SR = secondary roots.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.