A rapid assay for detection of *Rose rosette virus* using reverse transcription recombinase polymerase amplification using multiple gene targets

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Abstract.

Rose rosette disease caused by *Rose rosette virus* (RRV; genus *Emaravirus*) is the most economically relevant disease of Knock Out[®] series roses in the U.S. As there are no effective chemical control options for the disease, the most critical disease management strategies include the use of clean plants for propagation and early detection and destroyal of infected plants. The most common detection method for RRV is end-point reverse transcription-polymerase chain reaction (RT-PCR). However, this method is time consuming and can yield inconsistent results. Another method is real-time PCR (RT-qPCR) which is highly sensitive. However, the method is limited to diagnostic labs with the equipment and expertizes; and is time consuming. Thus a critical limitation for early detection of RRV from rose samples is the lack of an easy to use and rapid lab assay. To address this limitation, isothermal reverse transcription-recombinase polymerase amplification (RT-RPA) assay based on multiple gene targets for specific detection of RRV was developed. The assay is highly specific and did not cross react with other common rose infecting viruses belonging to the inclusive and exclusive genus. Dilution assays using the *in vitro* transcripts showed that the designed primer sets (RPA-267, RPA-131, and RPA-321) are highly sensitive in consistently detecting RRV with a detection limit of 1 fg. Testing of the infected plants using the primer sets indicated that the virus could be detected from leaves, stems and petals of roses. The primer pair RPA-267 produced 100% positive detection of the virus from infected leaf tissues, while primer set RPA-131 produced 100% detection from stems and petals. The primer set RPA-321 produced 83%, 87.5% and 75% positive detection from leaves, petals and stem tissues, respectively. The assay can be completed in less than 20 min as compared to the end-point RT-PCR assay (3-4 h) and RT-qPCR (1.5 h). The RT-RPA assay is reliable, rapid, highly sensitive, and can be easily used in diagnostic laboratories for detection of RRV with no need for any special equipment.

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

1. Introduction

Roses are one of the most important ornamental flowering shrubs grown worldwide. In the United States, the 2014 total sales of shrub roses accounts for 204 million U.S. Dollars (United States Department of Agriculture, 2015). Among the diseases of roses, the rose rosette disease has become the most devastating, causing huge economic losses to nurseries,

landscapes and gardeners (Stanley, 2013). The causal agent of Rose rosette disease was associated to be a single stranded negative sense RNA virus called *Rose rosette virus* (RRV, genus *Emaravirus*) (Laney et al., 2011). The virus is transmitted by an eriophyid mite species *Phyllocoptes fructiphilus* (Amrine, 1988, Laney et al., 2011). It can also be transmitted by grafting. The virus has multipartite genomes and is reported to contain seven genomic RNA segments - RNA1 (RNA-dependent RNA polymerase, RdRp), RNA2 (glycoprotein), RNA3 (nucleocapsid), RNA4 (movement protein), and RNA5, RNA6 and RNA7 of unknown function (Laney et al., 2011; Di Bello et al., 2015).

The only effective strategy currently available for disease management is timely identification and eradication of the infected plants, which will facilitate in limiting the spread of infection to healthy plants. This can only be achieved with a highly reliable, rapid, specific and sensitive detection assay for RRV. The only available 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). Even though RT-PCR and PCR are widely used in virus detection (Mackay et al., 2002) because of their sensitivity and specificity (Dai et al., 2012; Arif and Ochoa-Corona, 2013), their utilization in the field of plant virus detection is limited due to the need for expensive thermal cyclers, time consuming (4-5 h) and requires skilled personnel. A possible alternative to these constraints are isothermal nucleic acid amplification techniques which are carried out at a single temperature in a shorter period of time.

Recombinase polymerase amplification (RPA) is a novel isothermal DNA amplification and detection technology (Piepenburg et al., 2006), which utilizes an enzymatic mixture of polymerase and DNA recombination proteins. The phage-derived recombinase binds to the single stranded oligonucleotide primers to form a stable helical filament, which is highly efficient at scanning double stranded DNA (dsDNA) template to identify homologous sequences in targeted DNA sequences. The recombinase proteins then facilitate the strand-inversion and the formation of a D-loop structure where the primers are introduced at the cognate site of the template, and the *Staphylococcus aureus*-derived DNA polymerase (Sau DNA polymerase) possessing strand displacement activity elongates the primer, resulting in an exponential amplification of the target (West, 2003; Krejci et al., 2012) in less than 30 min (Shibata et al., 1979; Yonesaki et al., 1985; Formosa and Alberts, 1986; Piepenburg et al., 2006). The advantage of this method is that the reaction runs at a constant low temperature of about (37–42°C) (Hoff, 2006; Piepenburg et al., 2006; Euler et al., 2012 a,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., 2012; Boyle et al., 2013; Euler et al., 2013) or simply by a lateral flow dipstick assay (MileniaBiotec, Giessen, Germany). Application of RPA in the amplification of nucleic acid from bacteria and/or viruses have been well documented (Lutz et al., 2010; Shen et al., 2011; Amer et al., 2013; Euler et al., 2013; Ahmed et al., 2014; Jaroenram et al., 2014; Mekuria et al., 2014; Silva et al., 2014; Zhang et al., 2014).

Development of molecular diagnostic tools requires the design of primers, highly specific to the pathogen of interest. However mutations, genetic drift and selection pressure produces a variety of sequence variants that can be difficult to target effectively. Such problems are frequently encountered in the case of RNA viruses (Jenkins et al., 2002), due to the error prone RNA polymerase enzyme. Failure to discriminate between closely related target molecules results in false positives, which poses a major bias to the diagnosis. Unlike other diagnostic tools, in RPA, an additional factor affecting the specificity includes the homology search by recombinase enzymes. Previous studies have shown that mismatches in the primer sequences could drastically produce a negative effect on the RPA assay (Daher et al., 2015). At present, there is little information about the genetic diversity of RRV. However considering the fact that the rate of mutation is high in RNA viruses (Drake and Holland, 1999; Garcí'a-Arenal et al., 2001; Elena and Sanjuán, 2005), an efficient strategy to overcome this problem would be to develop primers targeting multiple regions of the genomic RNAs, and use them in subsequent confirmation of the virus. Detection using multiple primer sets could be advantageous in detecting the virus, if one of them failed to detect due to potential presence of genetically variable strains.

The objectives of this study were: 1) to develop reverse transcription-recombinase polymerase amplification (RT-RPA) assay based on primers designed from multiple gene targets; 2) to evaluate the specificity of the RT-RPA primer sets against other common rose infecting viruses belonging to the exclusive and inclusive genus; 3) to determine the sensitivity of the RT-RPA primers in detecting the virus; and 4) to evaluate the efficiency of the developed primers in detecting RRV from different tissue sources of infected roses.

2. Materials and methods

2.1. Sample collection and RNA extraction

Eight pink double Knock Out[®] rose plants (*Rosa Radtkopink*) with characteristic symptoms of RRV were collected from a rose nursery in Florida. Two non symptomatic pink double Knock Out[®] rose plants were collected from another nursery in Florida with no history of RRV. Total RNA was extracted from leaf tissues (100 mg) of the collected samples using the Qiagen RNeasy plant mini kit (Qiagen Inc., Valencia, CA).

2.2. RPA primer design

The primer sequences were selected by determining suitable conserved regions of RRV genomic RNA segments 2 and 3. All the available RRV RNA2 (2 sequences) and RNA3 (24 sequences) segments 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 using the PrimerQuest software (Integrated DNA Technologies, Coralville, IA) (<http://www.idtdna.com/Primerquest/Home/Index>) (**Table 1**). The primers 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 specificity of the primers were assessed *in silico* using Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/blast/>), with Blastn. All the primer sets were synthesised by IDT (Integrated DNA Technologies, Coralville, IA).

2.3 RT-RPA assay and transcript preparation

Preliminary RT-RPA assay to assess the primer sets 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. The amplified product was then either subjected to protein denaturation by heating at 65°C for 10 min, or subjected to high speed centrifugation for 3-5 minutes to pellet the protein. Negative controls including non template water (NTC) and total RNA from a healthy rose plant were included in each round of RT-RPA assay. The amplified products were analyzed on 1% agarose gel

electrophoresis, purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia, CA), and cloned into the 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 databases 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. Specificity assay

The specificity of the three RT-RPA primers were tested against other common rose infecting viruses, both exclusive and inclusive (**Table 2**). 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 healthy rose plant as well as the non-template control, was included in each reaction. These reference viruses were also tested using end-point RT-PCR using individual RPA primer sets.

2.5. Sensitivity assays: Transcript dilution assay

A series of dilution of the individual RNA transcripts ranging from 10 pg, 1 pg, 100 fg, 10 fg and 1 fg were prepared in Diethyl pyrocarbonate (DEPC) treated water and subjected to RT-RPA analysis using the individual primer sets. For comparison of the sensitivity of the RT-RPA, diluted transcripts were also subjected to end-point RT-PCR analysis using the individual primer sets.

2.6. Screening of field samples

The collected eight pink double Knock Out[®] rose plants along with the two non symptomatic plants were subjected to RT-RPA analysis using the three primer sets, as mentioned above. Five samples per plant (3 leaf, 1 stem, 1 flower petal) were used in the analysis. Positive control (1 ng/μL of specific RNA transcript) and negative controls including total RNA from healthy rose plant as well as the non-template control were included in each reaction.

3. Results

3.1. In silico analysis of the primers and probes

Three sets of RPA primers, one of them based on the genomic RNA segment RNA2 (glycoprotein) and two of them based on genomic RNA segment RNA3 (nucleocapsid) were

designed for the RT-RPA analysis of the RRV (**Table 1**). Among the primer sets designed, RPA-131 was designed based on the nucleotide position 1273-1404, from the genomic RNA segment RNA2. The primer sets RPA-267 and RPA-321 were designed based on the nucleotide position 1126-1393 and 505-826 respectively from the genomic RNA segment RNA3. *In silico* analysis of the RPA-131 primer sequences using BLASTn indicated 100% identity (Query coverage 100%) with that of RRV RNA2, while the primer sets RPA-267 and RPA-321 indicated 97-100% identity (Query coverage 100%) with that of RRV RNA3. There were no matches detected with other viruses or bacteria, including those listed in the exclusivity and inclusivity panel.

3.2. RT-RPA analysis

RT-RPA analysis of the RRV infected samples using the primer set RPA-131, RPA-267 and RPA-321 produced amplification products of 131 bp, 267 bp and 321 bp, respectively (**Fig. 1**). Sequence analysis of these cloned amplicons indicated 100% identity to that of the corresponding RRV genomic RNA segments. No amplification was obtained with RNA from healthy rose plants or with the non-template control (NTC).

3.3. Specificity assay

End-point RT-PCR analysis using all the three primer sets on the thirteen other viruses infecting roses, belonging to the exclusive and inclusive panel did not produce any reaction (data not shown). RT-RPA analysis using the three primer sets did not produce any cross-amplification with this panel of viruses. 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 in both the end-point RT-PCR and RT-RPA analysis, while the RNA from the healthy rose plant as well as the NTC did not produce any reaction.

3.4. Sensitivity assay

Each of the RPA primer sets detected as little as 1 fg of the transcripts in RT-RPA analysis (**Fig. 2**). End-point RT-PCR analysis using the RPA primers on diluted transcripts also produced a positive reaction up to a concentration of 1fg (**Fig. 3**).

3.5. Screening of field samples

RT-RPA analysis of the field samples indicated positive reaction with majority of the samples (**Fig. 4**) (**Table 3**). Among the leaf samples (n=24) (3 each from eight plants) tested, RPA-131 failed to detect the virus from one sample (96%) and RPA-321 from four samples (83%); while the primer set RPA-267 detected the virus from all the 24 samples tested. Among the flower petals analysed, primer sets RPA-267 and RPA-321, detected the virus in

all the samples with the exception of one sample. RPA-131 primer detected the virus from all the 8 infected flower petals. In case of stem, primer RPA-131 produced 100% positive virus detection from all the eight infected samples, while the primer RPA-267 and RPA-321 produced only 75% positive detection. The positive control (1 ng/ μ L specific RNA transcript) produced a positive reaction in RT-RPA 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 gel-based RT-RPA assay using three specific primer sets, for rapid, sensitive, reliable and specific detection of RRV, which possesses a major threat to the rose industry.

Application of RPA in the diagnosis of pathogens, have been previously reported (Euler et al., 2013; Lutz et al., 2010; Shen et al., 2011; Ahmed et al., 2014; Jaroenram et al., 2014; Mekuria et al., 2014; Silva et al., 2014; Zhang et al., 2014), but are based on single gene targets. The efficiency of the RPA assay depends on the primer-template homology search by the recombinase enzyme. Previous studies have showed that mismatches in the primer sequences reduced the efficiency of the RPA amplification. Patil et al., 2011 reported that at a rate of 12% mismatches in the primer sequences, the efficiency of the recombinase enzyme in homology search as well as rate of strand-exchange was reduced by 50%; which in turn decreases the yield of the RPA assay. Such type of primer-template mismatches are more likely to occur as a result of sequence variants arising from mutation, selection or genetic drift. Hence to overcome these issues, development of a primer based on the highly conserved regions of the pathogen is needed. However looking at the criteria for the synthesis of RPA primers including the 30-35 bp length, GC content and secondary structure, this is not always possible. An efficient strategy to overcome this problem would be to develop primers targeting multiple regions of the pathogen, which would facilitate the detection by either of the primers, if one of them failed to detect due to potential presence of genetically variable strains.

Preliminary *in silico* characterization of the three sets of primer sequences, as well as the amplicon sequences obtained from the RT-RPA assay using specific primers, indicated 100% identity to the RRV genomic RNA segments. In order to assess the specificity, testing of the primers was performed against other viruses infecting roses. All the three primer sets did not produce any nonspecific cross reactivity (false positive) when tested against other twelve rose

infecting viruses (exclusive) and one inclusive virus, or RNA from healthy control rose plants. This suggests that the developed assay is highly specific.

The developed RT-RPA assay using all the three primer sets is highly sensitive with a detection limit of 1 fg/ μ L of the RRV transcripts. This was comparable to the sensitivity produced by the RPA primers in an end-point RT-PCR analysis. Such a comparable similarity between the RPA and PCR/RT-PCR based techniques has been also previously reported in case of other pathogens including *Francisella tularensis* (Euler et al., 2012), *dengue virus* (Teoh et al., 2015) and *White spot syndrome virus* (Xia et al., 2014). Even though comparable sensitivity exists between the two methods, the time and expertise required to perform a RPA assay is considerably reduced, as compared to RT-PCR. The RT-RPA assay developed in this study could be completed in 15-20 min; while the end-point RT-PCR generally requires about 3-4 hours. In addition to the shorter time, the requirement of a constant temperature of 42°C provides RT-RPA advantages over RT-PCR, which normally requires varying cycling temperatures under stringent conditions. This could be easily achieved with a simple heating device, as compared to the costly thermal cyclers used in PCR/RT-PCR analysis.

A major limitation in using the gel-based RT-RPA assay is the presence of protein mixture in the reaction tube, which could pose a constraint on the proper migration of the amplicons in gel electrophoresis. An alternative strategy to overcome these protein traces is to perform purification of the RT-RPA product, before loading on to the gel. However in our study, we have performed an additional centrifugation of the RT-RPA product for 3 min at 13,000 rpm, to sediment the protein components, making the RT-RPA product clear for gel based testing. In our studies, sample testing has been performed using total RNA extract from the RRV infected tissues. Our attempts to use the diluted plant sap for the RT-RPA assay did not produce any significant results (data not shown). This could potentially be due to the presence of huge amount of inhibitors in infected rose plant tissues, including phenolic compounds, carbohydrates, pigments, and other unknown compounds.

All the infected pink double Knock Out[®] rose plants tested positive for RRV using RT-RPA analysis with all the primer sets. Multiple sampling of individual plant including leaves, petals and stem confirm reproducibility of the assay. The assay indicated high reproducibility and proved to be highly efficient in detecting the RRV from infected. Positive detection of the virus from different tissue sources provides a major advantage in selecting the source of tissue for detection purposes. The RT-RPA primers can facilitate the detection of the virus in

the stem, which could be utilized in the studies of virus transmission and movement within the plant. Among the primer sets, RPA-267 produced 100% RRV detection from leaf tissues, while primer set RPA-131 showed 100% detection from stems and petals. The variability in the detection of the virus using the different primer sets could potentially be due to the inhibitory compounds. Hence utilization of multiple diagnostic primers proves to be a better strategy for detection and confirmation of the virus.

This study is the first report on the use of a novel isothermal amplification technique, RT-RPA for the detection of RRV infecting roses using multiple gene targets. The RT-RPA assay described in this study is faster and sensitive compared to the currently available RT-PCR based diagnostic assay. This RT-RPA assay is expected to directly support commercial nurseries and diagnostic laboratories, for the rapid detection and confirmation of the virus.

Acknowledgments

Florida Nursery Growers and Landscape Association, Dean for Research, Institute of Food and Agriculture Sciences, University of Florida and USDA-SCRI grant (2014-51181-22644) funded this research. We also appreciate the help provided by Fanny Iriarte, Laura Ritchie, Barron Riddle and Jim Aldrich, Biological scientists at NFREC, Quincy in managing the plants in the quarantine facility. We also thank all the commercial nurseries in Florida supporting our project including Tri-B Nursery, Simpson Nurseries, May Nursery and Hackney Nursery.

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Table 1. Details of primers used in the RT-RPA analysis of *Rose rosette virus*

Genomic RNA segment	Primers	Sequence (5'-3')	bp	Tm (°C)	GC %	Target region	Amplicon size (bp)
RNA2	RPA-131F	GATGTACATGCACCACAGACAGTTGCAGTAG	31	69	48.4	1273-1304	131
	RPA-131R	GATGGAGCCGTTGAATGCTTAGCAGATCTCA	31	71	48.4	1373-1404	
RNA3	RPA-267F	TGAAGCTGCTCCTTGATTTCAGGGACCTA	30	71	50	1126-1156	267
	RPA-267R	AAGCACATCCAACACTCTTGCAGCCGATAC	30	71	50	1363-1393	
RNA3	RPA-321F	CCTCTATCAGCAGCTAAAGCAGGAGCAAAG	30	69	50	505-535	321
	RPA-321R	GTATGAGCTCTATCCAGCTGAAGTGTTGGC	30	69	50	796-826	

Table 2. Exclusive and inclusive list of other rose infecting viruses used in the specificity assay using *Rose rosette virus* specific RT-RPA primers

Virus panel	Genus	Virus Species	Acronym	Virus Source
Exclusive	<i>Illarvirus</i>	<i>Prunus necrotic ringspot virus</i>	PNRSV	Agdia Inc.
		<i>Apple mosaic virus</i>	ApMV	Agdia Inc.
		<i>Tobacco streak virus</i>	TSV	Agdia Inc.
	<i>Nepovirus</i>	<i>Arabid mosaic virus</i>	ArMV	Agdia Inc.
		<i>Tobacco ringspot virus</i>	TRSV	Agdia Inc.
		<i>Tomato ringspot virus</i>	ToRSV	Agdia Inc.
	<i>Tospovirus</i>	<i>Tomato spotted wilt virus</i>	TSWV	Agdia Inc.
		<i>Impatiens necrotic spot virus</i>	INSV	Agdia Inc.
	<i>Alfavirus</i>	<i>Alfalfa mosaic virus</i>	AMV	Agdia Inc.
	<i>Tobamovirus</i>	<i>Tobacco mosaic virus</i>	TMV	Agdia Inc.
	<i>Cucumovirus</i>	<i>Cucumber mosaic virus 1</i>	CMV-1	Agdia Inc.
		<i>Cucumber mosaic virus 2</i>	CMV-2	Agdia Inc.
Inclusive	<i>Emaravirus</i>	<i>Maize red stripe virus or wheat mosaic virus</i>	WMoV	Agdia Inc.

Table 3. RT-RPA analysis of *Rose rosette virus* infected pink double Knock Out® roses, showing differential compatibility of three set of primers on different rose tissues

Sample ID	Tissue source	Lane ^a	Primer		
			RPA-131	RPA-267	RPA-321
UF-RRV_1	Leaf	1	+	+	-
	Leaf	2	+	+	-
	Leaf	3	+	+	+
	Petal	4	+	+	-
	Stem	5	+	+	+
UF-RRV_2	Leaf	6	+	+	+
	Leaf	7	+	+	+
	Leaf	8	+	+	+
	Petal	9	+	-	-
	Stem	10	+	+	+
UF-RRV_3	Leaf	11	+	+	+
	Leaf	12	+	+	+
	Leaf	13	+	+	+
	Petal	14	+	+	+
	Stem	15	+	+	+
UF-RRV_4	Leaf	16	+	+	+
	Leaf	17	+	+	+
	Leaf	18	+	+	+
	Petal	19	+	+	+
	Stem	20	+	+	-
UF-RRV_5	Leaf	21	+	+	+
	Leaf	22	+	+	+
	Leaf	23	+	+	+
	Petal	24	+	+	+
	Stem	25	+	-	+
UF-RRV_6	Leaf	26	+	+	-
	Leaf	27	+	+	+
	Leaf	28	+	+	+
	Petal	29	+	+	+
	Stem	30	+	+	+
UF-RRV_7	Leaf	31	+	+	+
	Leaf	32	+	+	+
	Leaf	33	+	+	-
	Petal	34	+	+	+
	Stem	35	+	-	-
UF-RRV_8	Leaf	36	-	+	+
	Leaf	37	+	+	+
	Leaf	38	+	+	+
	Petal	39	+	+	+
	Stem	40	+	+	+
Healthy Rose	Leaf	41	-	-	-
NTC ^b	-	N	-	-	-

Table 4. RT-RPA analysis of *Rose rosette virus* (RRV) from Knock Out[®] roses collected from different states in the U.S.

Sample Number	Cultivar	Date of collection	County	State	RT-PCR RRV-F /R ^b	RT-RPA		
						Primer set		
						RPA-131	RPA-267	RPA-321
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 City	Oklahoma	+	+	+	+
201302391	Knock Out [®]	11-08-2013	Oklahoma City	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	+	+	+	+
Positive control ^a					-	+	+	+
Healthy rose 1	Pink Double Knock Out [®]	-	Gadsden	Florida	-	-	-	-
Healthy rose 2	Pink Double Knock Out [®]	-	Gadsden	Florida	-	-	-	-
NTC ^c					-	-	-	-

^a Positive control includes specific transcripts produced using respective primers.

^b End-point RT-PCR analysis of the samples using conventional RRV specific diagnostic primers RRV-F and RRV-R (Laney et al., 2011).

^c Non-template control (NTC).

^a Lane indicates the well position of the sample analyzed using specific primers in agarose gel electrophoresis (Figure 4)

^b Non template control

Figure legends

Figure 1. End-point reverse transcription recombinase polymerase amplification assay of *rose rosette virus* infected leaf tissues using specific primers. Lane 1: RPA-131; Lane 2: RPA-267 and Lane 3: RPA-321. Lane 4: Non template control (NTC); M = 1 Kb DNA ladder.

Figure 2. Sensitivity assay using end-point reverse transcription recombinase polymerase amplification, on the diluted transcripts using *rose rosette virus* specific primers RPA-131, RPA-267 and RPA-321. M = 100 bp DNA ladder.

Figure 3. Sensitivity assays using end-point RT-PCR on the diluted transcripts using RPA primers, RPA-131, RPA-267 and RPA-321. M = 100 bp DNA ladder.

Figure 4. End-point RT-RPA analysis of the field samples using the *Rose rosette virus* specific primers, RPA-131, RPA-267 and RPA-321. Lane 1-5: UF-RRV_1; Lane 6-10: UF-RRV_2; Lane 11-15: UF-RRV_3; Lane 16-20: UF-RRV_4; Lane 21-25: UF-RRV_5; Lane 26-30: UF-RRV_6; Lane 31-35: UF-RRV_7; Lane 36-40: UF-RRV_8; Lane 41: Healthy rose leaf tissue; Lane P = Positive control (RRV specific transcript 1ng/μL); Lane N = Non template control. M = 100 bp DNA ladder. The details of the tissue source for each plant are described in table 3.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.