Rose Rosette Disease: Recent Advances on Molecular Diagnostic Tools

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Abstract. *Rose rosette emaravirus* (RRV, genus *Emaravirus*), the causal agent of rose rosette disease, is the topmost pathogen of concern for the rose industry in the United States. The only strategy available for disease management is early identification and eradication of the infected plants. Highly reliable, specific, and sensitive detection assays are thus required to test and confirm the presence of RRV in suspected plant samples. RRV is only a recently characterized virus and hence limits the diagnostic tools available for its early detection. With a U.S. Department of Agriculture (USDA) Specialty Crop Research Initiative (SCRI) project sponsorship, several diagnostic tools including end-point reverse transcription-polymerase chain reaction (RT-PCR) and RT-qPCR assays targeting single and multiple genes targets were developed for routine diagnostics. This review introduces an overall view of the different diagnostic tools developed, which are reliable, highly sensitive, and can be easily implemented for detection and identification in laboratories providing diagnostic services and confirmation of RRV-infected samples.

ROSE ROSETTE DISEASE

Roses are one of the most important ornamental flowering shrubs grown worldwide. In the United States, the total sales of shrub rose's value was 204 million U.S. dollars in 2014 (U.S. Department of Agriculture, 2015). Among the diseases of roses, rose rosette disease has become the most devastating, causing economic losses to nurseries, landscapes, and gardeners (Stanley, 2013). The disease affects many rose species and cultivars, and has become widespread in the north-central, south-central, and southeast regions. Also, the disease has been identified on cultivated roses in the midwest, portions of the northeast, and a few western states in the United States.

Rose rosette disease is associated with RRV (genus *Emaravirus*), a single-stranded negative-sense RNA virus (Laney et al., 2011). The virus is transmitted by the eriophyid mite species *Phyllocoptes fructiphilus* (Amrine et al., 1988; Laney et al., 2011) and by grafting (Amrine et al., 1988). The mites do not fly but passively move long distances via air currents, spreading to nearby roses and infecting rose plantings with RRV (Epstein

and Hill, 1999; Windham et al., 2014a). *Rose rosette emaravirus* has a multipartite genome enclosed by a double membrane-bound body of 120–150 nm in diameter (Gergerich and Kim, 1983; Gergerich et al., 1983). The RRV virion is reported to contain seven genomic RNA segments, some of which encode known functions. For example, RNA1, RNA2, RNA3, and RNA4 encode an RNA-dependent RNA polymerase (RdRp), a glycoprotein, a nucleocapsid, and a movement protein, respectively, whereas RNA5, RNA6, and RNA7 have unknown function (Babu et al., 2016a; Di Bello et al., 2015; Laney et al., 2011).

SYMPTOMS

The symptoms of rose rosette disease are highly variable and depend on the rose cultivar, growth stage, and environmental conditions (Windham et al., 2014a, 2014b). Some of the symptoms include excessive thorniness, witches' broom, red pigmentation which does not disappear as the plant matures, and excessive lateral shoot growth. The disease ultimately leads to the death of the plant in 1–2 years (Amrine, 2002). Diagnosis of rose rosette disease in early stages could be misleading because of its resemblance to symptoms caused by herbicide damage or other plant viruses. The only effective strategy for disease management is early identification and eradication of the infected plants, thereby limiting the spread of RRV. Early identification requires a highly reliable, specific, and sensitive detection assay for either detection or confirmation of the rose rosette disease.

Rose rosette emaravirus is a recently characterized virus and little is known about its diversity and biology. Currently, neither

enzyme-linked immunosorbent assay nor a rapid lateral flow immunoassay is commercially available for testing RRV. A multidisciplinary team of scientists united by the USDA SCRI project entitled *Combating rose rosette disease: Short and long term approaches* is developing molecular and serological diagnostic tools for rapid and sensitive detection of RRV. This short review describes some of the molecular diagnostic tools currently developed, which include end-point RT-PCR and TaqMan RT-qPCR.

AVAILABLE DIAGNOSTIC TOOLS

The availability of the sequence information of four genomic RNA segments—RNA1 (RdRp), RNA2 (glycoprotein), RNA3 (nucleocapsid), and RNA4 (movement protein)—facilitated the development of the first molecular diagnostic method based on end-point RT-PCR. This method used specific primers designed based on the RNA1 genomic segment of RRV (Laney et al., 2011). The primer sequences were RRV F 5'-CAGAATGA-ACCATAGATGTC-3' and RRV R 5'-ATGGTCTGCTCGAGATT-3'. This method requires the following cycling conditions: reverse transcription at 48 °C for 30 min; initial denaturation at 94 °C for 2 min; followed by 30 cycles of 94 °C for 30 s, annealing at 53 °C for 10 s, and extension at 72 °C for 30 s; and a final extension for 10 min at 72 °C. This initial primer set was used for testing symptomatic rose tissues of 84 cultivated and *Rosa multiflora* roses collected from nine states, along with 30 asymptomatic plants (20 from Arkansas and five each from Missouri and Tennessee). RRV was detected in all symptomatic roses but not in the asymptomatic plants. These primers are still in use in

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a number of plant diagnostic clinics for routine testing of RRV. The same primer set was later found to be less sensitive and often inconsistent in the detection of the virus from symptomatic tissues (Babu et al., 2016b; J. Olson, personal communication).

DEVELOPMENT OF REAL-TIME RT-PCR DIAGNOSTIC TOOLS

Nucleic acid-based methods including end-point polymerase chain reaction (PCR) and RT-PCR are widely used in virus detection (Mackay et al., 2002); however, the sensitivity of these techniques may be questionable in cases when low virus titer is combined with the presence of inhibitors, which is the case for rose tissue infected by RRV. The development of quantitative real-time PCR has revolutionized plant virus detection (Balaji et al., 2003; Boonham et al., 2002; Drosten et al., 2002; Eun et al., 2000; Jarosova and Kundu, 2010; Korimbocus et al., 2002; Lunello et al., 2004; Mortimer-Jones et al., 2009; Mumford et al., 2000; Roberts et al., 2000; Schoen et al., 1996) because of its increased sensitivity (100–1000-fold more sensitive than end-point PCR/RT-PCR), accuracy, and high throughput capability (Mackay et al., 2002). Seeking for more consistent assays, we hypothesized that it was possible to develop a RT-qPCR assay adjustable to different reagent formats targeting different genomic regions of the virus with increased specificity and sensitivity.

The RRV multidisciplinary team developed a diagnostic tool for the detection of RRV that consists of a single pair of diagnostic primers, which can be used with three different RT-PCR chemistries: end-point RT-PCR, TaqMan RT-qPCR, and SYBR green-based RT-qPCR which can be coupled with high-resolution melting analysis (Dobhal et al., 2016). The implementation of an assay consisting of a single primer set to serve three different chemistries brings flexibility to diagnosticians furnished with different equipment capabilities in a diagnostic network. Further improvement contributed after publication of Dobhal et al. (2016) includes the substitution of SYBR green by

LCGreen using same reported stepwise denaturation increments conditions for improved accuracy. The primers were designed after the analysis of the nucleocapsid gene RNA3 of the RRV. The sequences of primers, probe, and their characteristics are listed in Table 1. Moreover, a synthetic, clonable, and noninfectious multitarget artificial positive control (APC) (plasmid) containing forward and reverse complement sequences of the designed primers and probe for RRV, along with the primer sequences of five other viruses of interest in ornamental crops including *Cucumber mosaic virus*, *Hosta virus X*, *Tobacco mosaic virus*, *Tomato spotted wilt orthotospovirus*, and *Impatiens necrotic spot orthotospovirus*, was engineered for routine application in detection and diagnostic assays. The construct was designed to be used as a source of positive control for the testing of RRV (amplifies a 125 bp product) and other five viruses (Fig. 1). The cycling conditions for the primers and probe are as follows:

- Two-step end-point RT-PCR: Reverse transcription at 48 °C for 30 min; cDNA amplification 94 °C for 3 min; followed by 38 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 3 min.
- TaqMan RT-qPCR assay: Reverse transcription at 48 °C for 30 min; followed by two initial holds at 50 and 95 °C, 2 min each; followed by 40 cycles: 95 °C for 20 s and 58 °C for 45 s.
- SYBR green assay coupled with high-resolution melting (HRM) analysis: Reverse transcription 48 °C for 30 min; cDNA amplification at 50 °C and 95 °C 2 min each, followed by 40 cycles at 95 °C for 20 s and 54 °C for 45 s.

After amplification, high-resolution melting analysis was performed as previously described using 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 the first step and a 2 s stop for each step afterward (Dobhal et al., 2016). Specificity analysis of the primers and probe with other near-neighbor viruses and viruses commonly infecting roses including *Apple mosaic virus*,

Arabis mosaic virus, *Groundnut ringspot orthotospovirus*, *High plains wheat mosaic virus* (formerly *High plains virus*), *Impatiens necrotic spot orthotospovirus*, *Maize stripe tenuivirus*, *Prunus necrotic ringspot virus*, *Tobacco mosaic virus*, *Tomato chlorotic spot virus*, *Tomato spotted wilt orthotospovirus*, and *Tomato ringspot virus* did not amplify any product, indicating the specificity of the primers and probe to RRV. Further sensitivity analysis of the diluted APC plasmids from 1 ng to 1 fg demonstrated that the primers are highly sensitive to a detection limit of 1 fg/μL for all the three RT-PCR chemistries. Total RNA extracted using RNeasy Plant Mini kit from 30 symptomatic and asymptomatic rose samples from eight different states in the United States were tested with end-point RT-PCR and TaqMan RT-qPCR. The assays tested positive with all collected samples and were able to detect RRV in asymptomatic plant samples 3 months before the onset of visual symptoms. The plants were field-monitored samples from Edmond, OK. These particular plants were initially healthy looking and tested RRV positive until dying. We speculate that the rose plants get hardly damaged because of both RRV and winter.

Even though RRV is reported to have little genetic variability within the four reported genomic RNA segments RNA1, RNA2, RNA3, and RNA4 (Laney et al., 2011), additional studies with isolates from different geographic locations need to be conducted. In general, RNA viruses are considered to have a high mutational rate (Jenkins et al., 2002), and hence, they may acquire synonymous or silent nucleotide mutations within primer and probe target sequences, allowing them to potentially evade detection and resulting in false negatives. To overcome these issues, TaqMan RT-qPCR assays based on multiple gene targets were developed for the detection of RRV (Babu et al., 2016b). The use of multiple gene targets offers additional confirmatory testing for operators in diagnostic networks. The sequence of the primers and probes and their characteristics is listed in Table 1. The TaqMan RT-qPCR was performed with the following thermal program:

Table 1. Details of primers and probes used for the detection of *rose rosette emaravirus* (RRV) for use in end-point reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR assays.

Genomic RNA segment	Primers and probes	Sequence (5'–3')	bp	Temp (°C)	GC (%)	Target region	Amplicon size (bp)	Reference
RNA 3	RRV2F	TGCTATAAGTCTCATTGGAAGAGAAA	26	59.8	34.6	559–663	104	Dobhal et al., 2016
	RRV2R	CCTATAGCTTCATCATTCCTCTTTG	25	59.6	40			
	RRVP	TGCTAGAGACATTGGTACAACAAGCAA	27	65.1	40.7			
RNA2	RRV_2-1For	CCACAGACAGTTGCAGTAGTT	21	62	47.6	1,285–1,402	117	Babu et al., 2016b
	RRV_2-1Probe	ACAGCTGAAGCCATCATGAACCTT	24	67	46			
	RRV_2-1Rev	TGGAGCCGTTGAATGCTTAG	20	62	50			
RNA2	RRV_2-2For	CCATTGCAGGTTGTTGCATT	20	62	45	145–245	100	
	RRV_2-2Probe	TGAACAAGGGTGGACCATTCACACA	24	68	50			
	RRV_2-2Rev	TTGGCTCTACCCCTTTCTTTCC	21	62	47.6			
RNA3	RRV_3-2For	ACACTCTTGACGCTGATACTG	21	62	47.6	65–182	117	
	RRV_3-2Probe	AGCTTCGGGTCTCTCAAGTTGACAA	24	68	50			
	RRV_3-2Rev	CTGGGTCCAATTCTGAACCTCTC	22	62	50			
RNA3	RRV_3-5For	CTGATACTGTTATCATCGGAGCTG	24	62	45.8	77–171	94	
	RRV_3-5Probe	AGCTTCGGGTCTCTCAAGTTGACAA	24	68	50			
	RRV_3-5Rev	TCTGAACTCTCAGGCTTCACTA	22	62	45.5

Fig. 1. The artificial positive control, synthetically constructed to be made by a tandem of forward and reverse complement primer sequences to include probes from *rose rosette emaravirus* (yellow) and other five viruses, ligated into a multiple cloning site of pUC57. Note that a probe sequence is included to allow quantitative polymerase chain reaction (PCR). Each PCR product has a unique sequence for each target virus which is useful for PCR product verification and quality control. (reprinted with permission; Dobhal et al., 2016).

50 °C for 15 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s. The primer sets for the three additional RRV gene targets were highly specific, when tested against other viruses that frequently coinfect rose plants (*Alfalfa mosaic virus*, *Apple mosaic virus*, *Arabidopsis mosaic virus*, *Cucumber mosaic virus 1*, *Cucumber mosaic virus 2*, *Impatiens necrotic spot orthotospovirus*, *Prunus necrotic ringspot virus*, *Tobacco mosaic virus*, *Tobacco ringspot virus*, *Tobacco streak virus*, *Tomato ringspot virus*, and *Tomato spotted wilt orthotospovirus*) including two taxonomically (genus) related viruses (*Maize red stripe tenuivirus* or *Wheat mosaic virus*). Sensitivity analysis of these primers using viral RNA transcripts indicated that all four primer/probe sets were able to detect the virus up to 1 fg/μL. All primers and probes were assessed three times over a period of 1 month using infected RRV samples (confirmed by RT-PCR) confirming their high reproducibility as compared with the inconsistencies reported with previously existing assays. All four primer/probe sets were tested against the total RNA of 15 RRV-infected Double Knock Out roses and one asymptomatic plant, collected during 2013–15 (stored in –80 °C) from different states in the United States, and tested positive for 13 symptomatic plant samples (87%). The asymptomatic plant tested negative for RRV. Failure of the primer/probe sets in detecting the RRV from two samples has been attributed to the degraded RNA or inhibitors in samples stored in –80 °C for long term.

For molecular diagnostics of RRV, the best source of samples was fresh young

leaves. Even though samples stored in –80 °C may test positive for RRV, it is always good to rely on fresh tissue sources. A comparative analysis of the various diagnostic tools available for RRV is presented in Table 2. The reviewed molecular diagnostic tools can be efficiently implemented by diagnostic laboratories for rapid detection and identification of RRV to support commercial nurseries, landscapers, and home owners in early stages of virus identification, facilitating and speeding the implementation of timely eradication measures, which will minimize the risk associated with the spread of the RRV to healthy roses. Diagnostic research regarding RRV and *Emaravirus* is not completed. The RRV multidisciplinary team actively focuses on developing a genus broad detection and diagnostic tool for *Emaravirus* and explores sensitive color visualization assays using self-quenched primers (SqPs) and sample processing methods to circumvent the presence of putative PCR inhibitors in rose tissues.

FORTHCOMING RRV DIAGNOSTICS

Thermophilic helicase-dependent amplification (tHDA) is an isothermal nucleic acid amplification technique that does not require a temperature cycling. A comparison of primers designed with the same thermodynamic criteria but different GC content in their targeted amplification products is in progress to assess fluorescence using SqPs reacting in tHDA standard temperature and chemistry. Model viruses tested are RRV, *High plains wheat mosaic emaravirus*, and *Hosta virus X*. The products amplified by

quantitative thermophilic helicase dependent amplification (qtHDA) with SqPs are visualized in 2% agarose gel electrophoresis. Detection limits using plasmid DNA carrying the target sequences for RRV and *High plains wheat mosaic virus* are 0.0001 ng. This study explored primer design criteria for tHDA with SqP assays exploring alternative sensitive detection. The use of SqPs may reduce the cost of qPCR and qtHDA by half and still maintain good sensitivity. tHDA with SqP also bring new possibilities for field deployment primer design in biosecurity and microbial forensics (Molina Cárdenas et al., 2016).

Loop-mediated amplification of DNA (LAMP) is another isothermal amplification method that combines specificity, sensitivity, and easy points of care implementation because it allows visual detection. Alignments of RRV P3 (RNA3) and P4 (RNA 4) gene sequences allowed LAMP primer design for broad detection of reported isolates. Optimal isothermal amplification was obtained with Bst 2.0 Warm-Start DNA polymerase and Optigene master mix. The detections were 1 pg/μL and 1 fg/μL, respectively, using plasmid carrying the targeted sequence. Products were also visualized by electrophoresis. The visual detection limit of plasmid in colorimetric reactions using hydroxynaphthol blue (120 μM) without bovine serum albumin (BSA) and polyvinylpyrrolidone (PVP) was 0.01 ng/μL. No cross-reactions with cDNA from 10 frequently rose coinfecting or related viruses were detected. The method has potential application in biosecurity, microbial forensics, and nursery virus-free monitoring of germplasm (Salazar Aguirre et al., 2016).

Table 2. Comparative features of the currently available molecular diagnostic tools for *rose rosette emaravirus* (RRV).

Method	Primers/probes	Sensitivity	Time (h)	Cost per reaction (\$)	Remarks	Reference
End-point RT-PCR	RRV F and RRV R	Not tested	4–5	5–6	Used in diagnostic clinics for routine diagnostics. Tested positive with 84 samples collected from different states. Inconsistent results with symptomatic leaves.	Laney et al., 2011
End-point RT-PCR	RRV2F and RRV2R	1 fg/μL	3–4	5–6	Consistently detected RRV from 30 samples collected from different states.	Dobhal et al., 2016
TaqMan RT-qPCR	RRV2F and RRV2R and RRV2 probe	1 fg/μL	1–1.5	4–5	Consistently detected RRV from 30 samples collected from different states.	
SYBR RT-qPCR + HRM	RRV2F and RRV2R	1 fg/μL	1–2	4–5	Further testing required as only eight samples of 30 were tested.	
TaqMan RT-qPCR	RRV_2-1 F/R and probe	1 fg/μL	1–1.5	4–5	Consistently detected RRV from 13 samples of 15 collected from different states from 2013–15. Failure of detection could potentially be due to degraded RNA from long-term storage.	Babu et al., 2016b
	RRV_2-2 F/R and probe	1 fg/μL	1–1.5	4–5		
	RRV_3-2 F/R and probe	1 fg/μL	1–1.5	4–5		
	RRV_3-5 F/R and probe	1 fg/μL	1–1.5	4–5		

RT-PCR = reverse transcription-polymerase chain reaction; RT-qPCR = quantitative reverse transcription-polymerase chain reaction.

The genus *Emaravirus* has six confirmed species and two unclassified species, attributed to variety of symptoms. Seeking rapid detection and an effective screening of existing and new emaraviruses for detection/diagnostics, taxonomic confirmation, plant virus discovery and biosecurity applications, sequences of RdRp (RNA 1) of *European mountain ash ringspot-associated emaravirus*, *Fig mosaic emaravirus*, *High plains wheat mosaic emaravirus*, *Pigeonpea sterility mosaic emaravirus*, *Pigeonpea sterility mosaic emaravirus 2*, *Raspberry leaf blotch emaravirus*, *Redbud yellow ringspot-associated emaravirus*, and RRV were studied and genus broad-specific detection primers designed. The sensitivity of this RT-PCR was found to be 100 fg/reaction. The multiple *Emaravirus* detection was confirmed with rose samples showing symptoms caused by *European mountain ash ringspot-associated emaravirus*, *Fig mosaic emaravirus*, *High plains wheat mosaic emaravirus*, and RRV. Other emaraviruses, *Pigeonpea sterility mosaic emaravirus*, *Pigeonpea sterility mosaic emaravirus 2*, *Raspberry leaf blotch emaravirus*, and *Redbud yellow ringspot-associated emaravirus*, were detected in silico using Primer-Blast. This method will address the need for sensitive molecular detection and discovery of novel emaraviruses.

This review described current and forthcoming different diagnostic tools developed for RRV, which have been developed seeking extended reliability, high sensitivity, and easy implementation for detection and identification in laboratories providing diagnostic services and confirmation of RRV-infected samples (Olmedo-Velarde et al., 2016).

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