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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.

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 et al., 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

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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).

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 (3–4 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; Gill and Ghaemi, 2008; Asiello and Baeumner, 2011; 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 TwistAmp™ 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 (Lutz et al., 2010; Shen et al., 2011; Amer et al., 2013; Euler et al., 2013; Ahmed et al., 2014; Jaroenram and Owens, 2014; Lillis et al., 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 (Wetzel et al., 1992; Nolasco et al., 1993; James, 1999). 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 32 RRV infected different rose varieties (confirmed using RRV specific diagnostic primers RRV-F and RRV-R) collected during 2013–2015 from different states in the U.S.