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

Shefali Dobhal[a,b,1], Jennifer D. Olson[b], Mohammad Arif[a,b,1], Johnny A. Garcia Suarez[a,b,c], Francisco M. Ochoa-Corona[a,b,*]

[a] National Institute for Microbial Forensics & Food and Agricultural Biosecurity, Stillwater, OK, USA
[b] Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, USA
[c] Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Ecuador

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A B S T R A C T

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

1. Introduction

Roses (*Rosa* spp.) belong to the family Rosaceae and are among the most popular flowering shrubs in the United States with a total wholesale value of $194 million (Paret, 2014). Roses are cherished for their desirable aesthetics, landscaping and industrial products. In recent years, Rose rosette disease (RRD) has become a devastating disease of roses causing economic losses to ornamental nurseries in different parts of the United States including the state of Oklahoma where RRD is commonly observed on both nursery and landscape roses. In 2012, RRD caused severe losses to the Tulsa Municipal Rose Garden where thousands of rose plants were eradicated (Stanley, 2013). RRD is a viral disease of commonly cultivated roses and the wild rose *Rosa multiflora*. It was proposed to be caused by a newly characterized *Rose rosette virus* (RRV), a ssRNA virus in the genus *Emaravirus* (Laney et al., 2011). RRD was first observed in 1940 in Manitoba, Canada (Conners, 1941). RRD is reported to be widespread in Kansas, Oklahoma, Missouri and Arkansas, and has become widespread in north, south-central and southeast parts of the United States (Crowe, 1983; Christine, 2006; Epstein and Hill, 1995; Laney et al., 2011; Windham et al., 2014a,b). RRD has become a serious problem in these states and a threat to the rose industry and landscape roses. RRD is transmitted by grafting and the eriophyid mite *Phyllocoptes fructiphilus* (Amrine et al., 1988; Laney et al., 2011). The mites do not fly but can passively move long distances by air currents and can spread to nearby roses infecting rose plantings (Epstein and Hill, 1999; Windham et al., 2014a).

The symptoms of the disease are highly variable depending on the rose cultivar, and stage of development of the plant. Symptoms may vary within the same cultivar at the same or different locations (Epstein and Hill, 1995, 1999; Windham et al., 2014a,b). RRD symptoms include rapid elongation of lateral shoots: reddening of leaves and shoots, masses of shoot proliferation (witches broom), excessive thorns, malformation, reduced flowering, and deformed buds and flowers (Amrine et al., 1988; Epstein and Hill, 1995) (Supplementary Fig. 1). RRD infected plants become severely disfigured in one to three years after infection and are removed due to their

* Corresponding author at: Oklahoma State University, 127 Noble Research Center, Stillwater, OK 74078, USA.
E-mail address: ochoaco@okstate.edu (F.M. Ochoa-Corona).
[1] Present address: Department of Plant Pathology, Kansas State University, Manhattan, KS 66506, USA.

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unpleasant appearance. Diagnosis of RRD in early stages is difficult and often confusing due to symptoms resembling herbicide damage or caused by other plant viruses and/or pest problems. Similar to other viral diseases, there is no chemical control for RRD. Once a rose plant becomes infected, complete removal of plants is recommended as the plants are likely to harbor the vector eriophyid mites. Reliable early detection of the disease is needed to prevent the spread of infection to the healthy plants.

Laney et al. (2011) reported an endpoint RT-PCR primer sets for RRV detection. However, the primers were used by other groups which had difficulty in detecting all RRV infected samples (J. Olson; unpublished). Therefore, a reliable, accurate, sensitive and validated assay for detection of RRV is required. Moreover, rose tissues are rich in PCR inhibitors and frequently produced false negatives; therefore, incorporation of amplification facilitators (like BSA and PVP) in the reaction mix would be helpful to develop robust and validated protocol. Nucleic acid based methods are rapid, reliable, have high specificity and discriminatory abilities and are more sensitive than immunological methods (Dai et al., 2012; Arif et al., 2012, 2013, 2014a; Ouyang et al., 2013). Reverse transcription-polymerase chain reaction (RT-PCR) is considered a sensitive and rapid method for detection of RNA viruses (Arif et al., 2014b; Dobhal et al., 2014a). The sensitivity of reverse transcription PCR (RT-PCR) depends importantly on the thermodynamics of the oligonucleotide primers (Arif and Ochoa-Corona, 2013). High sensitivity allows high capability for detection of minute quantities of virus particles, leading to early detection of virus diseases (Arif et al., 2014b). Other DNA amplification assays such as real time or quantitative PCR (qPCR) offer increased sensitivity, accuracy and speed compared to end-point PCR for detection of target genes (Arif et al., 2014b, 2015). SYBR Green and TaqMan qPCR are the two most popular formats of qPCR and are based on two different chemistries (Bustin, 2005). SYBR Green assays use a fluorescent dye that intercalates with all double-stranded DNA, including non-specific products that may be synthesized during the reaction, whereas, TaqMan assays use a DNA probe that fluoresces only after the probe is cleaved during the amplification of a specific product (Tomlinson et al., 2005). TaqMan assays offer highly specific detection and analysis of the amplified products (Obrepalska-Steplowska et al., 2008). However, PCR probe-based analyses are expensive, and often require considerable information about the targeted gene and may be complex (VanGuilder et al., 2008). In contrast, SYBR Green assays are cost effective and relatively simple to use (VanGuilder et al., 2008). High Resolution Melting (HRM) analyses coupled to SYBR Green PCR exploits the SYBR-Green dye that intercalates only in double-stranded DNA and measures a decrease of fluorescence once the dsDNA start melting. The decreasing fluorescence values are plotted against the temperature generating a melting curve unique and characteristic of the amplified fragment that is characteristic of the targeted species or even strains of the studied viral pathogen, facilitating discrimination from artifacts created by non-specific products (Varga and James, 2005; Reed et al., 2007; Winder et al., 2011). Both SYBR Green qPCR and HRM curve analyses can be performed in a single tube. This method allows detection and discrimination of the target simultaneously (Varga and James, 2005), and has the advantage of monitoring the non-specific amplification.

This research reports the design of a single pair of diagnostic primer and demonstrates its application in three RT-PCR chemistries: endpoint RT-PCR, TaqMan RT-qPCR, and SYBR Green RT-qPCR assays. The SYBR Green RT-qPCR can also be combined with HRM curve analyses for the detection of RRV. Moreover, a multi-target artificial positive control to include a probe target was also constructed for routine application in diagnostics. The application of a single primer set with three different chemistries offer flexibility to operators in a diagnostic network to adopt any of the three methods depending on the available resources in the laboratory. The three developed methods will also assist epidemiological studies, breeding, biosecurity, and management of the disease.

2. Materials and methods

2.1. Source of viruses and infected plant material

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

2.2. Primer and probe design

Twenty three sequences of RRV nucleocapsid gene p3 located in the RNA3 region were retrieved from NCBI GenBank database. The selected GenBank accessions were: HQ871944 and HQ891892–HQ891913 (Laney et al., 2011), aligned using CLUSTALX2 (Larkin et al., 2007) and examined for conserved regions. The last date NCBI GenBank database accessed was 17th of September, 2013. A primer pair RRV2F (Forward: 5′-TGCTATAAGTCTCATTGGAAGAGAAA-3′) and RRV2R (Reverse: 5′-CCTATAGCTTCATCATTCCTCTTTG-3′), and the RRV probe-2 (5′-TGCTAGAGACATTGGTACAACAAGCAA-3′) were designed from a consensus sequence generated after aligning the targeted gene to amplify a fragment of 104 bp. The web interface application Primer3 (Rozen and Skaletsky, 2000) was used for primer design. The thermodynamics, internal structures, and self-dimer formation of the primers were examined *in-silico* using mFold (Zuker, 2003) following parameters described by Arif and Ochoa-Corona (2013). The preliminary specificity of the primer set was assessed *in-silico* using BLASTn (Altschul et al., 1990). The primers and probe 5′-6-carboxyfluorescein (FAM)-3′ were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA).

2.3. Artificial positive control (APC)

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