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12-2015

Understanding the Causal Agent of Rose rosette disease

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Recommended Citation

Understanding the Causal Agent of Rose rosette disease.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

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University of Maryland
Bachelor of Science in Plant Science 2012

December 2015
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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ABSTRACT

A number viruses are known to infect roses, ranging from those in the genera *Nepovirus*, and *Ilarvirus*, which have been reported since the inception of rose virology, to recently discovered viruses in the genera *Carmovirus*, *Closterovirus*, *Emaravirus*, *Luteovirus*, *Rosadnavirus*, and *Potyvirus*. Of the viral diseases in rose, arguably the most damaging is Rose rosette (RRD), which is associated with the *Emaravirus*, *Rose rosette virus* (RRV). The objective of this thesis is to fill in the gaps in knowledge on the epidemiological aspects of RRD and RRV. There has been significant progress in the epidemiology of the RRD agent prior to the discovery of *Rose rosette virus* (RRV). The elusive agent was known to be graft transmissible, vectored by the eriophyid mite, *Phyllocoptes fructiphilus* in an uncharacterized manner, and associated with virus-like double membrane-bound bodies. RRV, the putative casual agent, was detected in all plants with RRD symptoms. However, this correlation does not prove causation of the disease. Given the complex symptomology observed the question of whether RRV causes RRD solely or as part of a virus complex, as is the case of numerous disorders of perennial plants, once thought to be caused by a single virus, was still unclear. Resistance is an important first line of defense when managing any disease, and here we identified potential sources resistance for producers, rosarians, and breeders. To date few viruses, believed to be transmitted by eriophyid viruses have been conclusively demonstrated to do so. The mode of transmission is elucidated for an even smaller subset of those viruses. In this study Koch's postulates were fulfilled for RRV; additional RRV genome segments were discovered; *Phyllocoptes fructiphillus* was verified as a vector of RRV; resistant rose varieties were identified; and the acquisition and inoculation access periods (AAP and IAP respectively) for RRV were determined.

ACKNOWLEDGMENTS

DEDICATION

      To my parents, Dennis and Maria and who always fed my interest in science. To my grandmother Marlene Carnovale who taught me how to propagate plants, and let me work in her kitchen (lab). To my grandfather Bruno Carnovale, for reminding me to go after what you want and not doing so is worse than failing. To my little sister, Aubrey for always being there, and just because. To my family, friends, and mentors. For that one white hair I discovered the other day, it has just begun.

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CITATIONS:

Di Bello, P. L., Ho, T., & Tzanetakis, I. E. (2015). The evolution of emaraviruses is becoming more complex: seven segments identified in the causal agent of Rose rosette disease. Virus research, 210, 241-244.

Di Bello, P. L., Thekke-Veetil, T., Druciarek, T., & Tzanetakis, I. E. (2015). Transmission attributes and resistance to rose rosette virus. Virus research, (Submitted).

CHAPTER 1

Introduction

Abstract

A number viruses are known to affect rose, ranging from those in the genera *Nepovirus*, and *Ilarvirus*, which have been reported in rose since the inception of rose virology, to recently discovered viruses in the genera *Carmovirus*, *Closterovirus*, *Emaravirus*, *Luteovirus*, *Rosadnavirus*, and *Potyvirus*. Of the viral disease in rose, arguably the most damaging is Rose rosette (RRD), which is associated with the *Emaravirus*, *Rose rosette virus* (RRV). The objective of this thesis is to fill in the gaps in knowledge on the epidemiological aspects of RRD. Koch's postulates were fulfilled for RRV; additional RRV genome segments were identified; *Phyllocoptes fructiphillus* was verified as a vector of RRV; resistant rose varieties were identified; and the acquisition and inoculation access periods (AAP and IAP respectively) for RRV were determined.

Virus diseases and virus-like diseases of Rose

Rose mosaic

Rose mosaic is a complex disease with worldwide distribution which manifests an array of symptoms including mosaic, line and "oak leaf" patterns, ringspots, mottling, and yellow netting (Fig 1) (Horst et al., 2007). Symptoms may be confined to a few leaves and are thus easily overlooked (Horst et al., 2007). Symptoms are associated with single or mixed infections of *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV), *Arabis mosaic virus* (ArMV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) (Cammack, 1966; Halliwell and Milbrath, 1962; Horst et al., 2007; McDaniel et al., 1971). When ELISA positive ApMV, ArMV or PNRSV scion were grafted to material negative to those viruses, chlorotic patches, puckering, and distortion developed in the case of ApMV, vein-banding or vein netting in the case of ArMV and ringspots and line patterns in the case of PNRSV; symptoms were more dramatic when plants where infected by two of the viruses (Wong et al., 1988)(THOMAS, 1981). Infections with TRSV or ToRSV are associated with wavy line patters, ringspots, and blotches (Halliwell and Milbrath, 1962; McDaniel et al., 1971). However these results should be considered with caution as it is unknown whether scions or rootstock where infected with additional viruses, and thus the symptomology observed could have been the result of mixed virus infections.

Rose ring pattern & Rose flower break

Rose ring pattern was first reported on 'Queen Elizabeth' in 1957 (Hunter, 1966). The disease causes flower variegation (Fig 2) which is vaguely reminiscent of flower break in tulips. Other symptoms include fine ringspots, line patterns, leaf distortion and mottling. In 'Queen

Elizabeth' flowers are malformed and crimpled at the margins (Hunter, 1966) and flower yield could be reduced by 45-50% (Ahmed et al., 2004). The disease has been found in Egypt (Ahmed et al., 2004), New Zealand (Milleza et al., 2013) and, the United Kingdom (Hunter, 1966). The disease agent was successfully graft transmitted to and caused color break in *Rosa multiflora* (Hunter, 1966) whereas mechanical transmission was successful to *N. tabacum* (Ahmed et al., 2004). The nature of the causal agent is still elusive.

Rose spring dwarf

Rose spring dwarf disease is characterized by balling or rosetting of newly emerging growth in the spring (Salem et al., 2008). New growth is curved, distorted, and leaf veins develop yellow vein banding (Salem et al., 2005). Symptoms tend to alleviate in mature leaves whereas canes develop a zig-zag pattern (Salem et al., 2008). Rose spring dwarf associated virus (RSDaV) was discovered by index grafting to multiflora rose, wherein disease symptoms developed; still it is largely asymptomatic in a number of rose cultivars (Salem et al., 2008). The disease is cosmopolitan but given the rather recent discovery of the virus its presence has only been confirmed in the Americas (Rivera and Engel, 2010; Salem et al., 2008). RSDaV, belongs to the genus *Luteovirus* (Salem et al., 2008) which includes viruses that are phloem limited and transmitted in a persistent circulative manner (Salem et al., 2008). RSDaV is vectored by at least two aphid species, *Metapolophium dirhodum* and *Rhodobium* porosum, is able to cause the synonymous disease to presumed healthy roses; alas the virosome of those "healthy" roses remains unknown (Salem et al., 2008). *M. dirhodum* transmitted RSDaV to *N. benjthamiana*, and *R. porosum* to barley and oat, suggesting that RSDaV may have additional natural hosts; in all cases alternative host infection remained asymptomatic (Salem et al., 2008).

Rose rosette disease

Rose rosette disease (RRD) is manifested by an array of symptoms including excessive thorniness, mosaic, mottling, reddening on young shoots, malformed flowers, and an overall decline in vigor leading to early death (Fig 3) (Laney et al., 2011). In the 1940s this disease was reported as witches' broom and mosaic on roses in North America (Conners, 1940). The causal agent of the disease was shown to be transmitted by the eriophyid mite *Phyllocoptes fructiphillus* (Allington et al., 1968), and be associated with double membrane bound bodies (Gergerich and Kim, 1983). Still the etiology of the disease remains unclear. A new emaravirus, the presumed causal agent, was detected in all RRD roses tested (Laney et al., 2011).

Viruses known to infect rose

Nepoviruses in Rose

Nepoviruses are positive sense bipartite RNA virus belonging to the family *Secoviridae* (King et al., 2012). RNA 1 is 7,200 to 8,400 nt long and encodes the replication-associated proteins; RNA 2 is 3,700 to 7,300 nt long and encodes a polyprotein with structural and movement protein domains. RNAs have a virus-linked protein (Vpg) attached to the 5' termini and are polyadenylated at the 3' termini (King et al., 2012). Nepoviruses generally have weak silencing suppressors and may benefit from co-infection with viruses with stronger silencing counterparts (Siddiqui et al., 2011). Nepoviruses are transmitted by nematodes in the genus *Xiphinema* (Brown, 1986; McGuire and others, 1964; Schmidt et al., 1963a; Teliz et al., 1966; Trudgill et al., 1983), with the exception of *Blackcurrant reversion virus*, which is transmitted by eriophyid mites (Jacob, 1976)**.** Nepoviruses are also readily seed and pollen transmissible (Martin et al., 2012b; McGuire and others, 1964; Mellor and Stace-Smith, 1963; Murant, 1970a).

Arabis mosaic virus

Arabis mosaic virus (ArMV) is a subgroup A *Nepovirus,* (Digiaro et al., 2007). ArMV was first described in the 1940s, and has subsequently been detected in over 100 plant species (Bos, 1971). ArMV is associated with rose mosaic (Wong et al., 1988); (Smith and Markham, 1944). The virus is transmitted by *X. diversicaudatum* (Trudgill *et al.*, 1983*;* (Brown, 1986), and *X. coxi* (Schmidt et al., 1963b). Both the adult and larvae of *X. diversicaudatum* can transmit ArMV after feeding on an infected plant for one day (Jha and Posnette, 1961). *X. diversicaudatum* retains ArMV for over 100 days in the absence of a host plant (Taylor and Thomas, 1968). ArMV is transmitted by pollen, and seed in at least 15 different species in 12 families (Murant, 1970b). Roses co-infected with ArMV and PNRSV develop chlorotic vein-banding symptoms (Wong et al., 1988)(THOMAS, 1981).

Tomato rinsport virus

Tomato ringspot virus (ToRSV) is a subgroup C nepovirus (Digiaro et al., 2007). and transmitted by the dagger nematode *Xiphinema americanum*, with both the larval and adult stages able to vector the virus after an hour feeding on infected material (Teliz et al., 1966). Seed transmissibility and pollen transmission has been shown in a number of crops (Mellor and Stace-Smith, 1963); (Braun et al., 1973); (Scarborough et al., 1977). Infections by ToRSV in rose is associated with wavy line patters, ringspots, and blotches in the leaves (Halliwell and Milbrath, 1962).

Tobacco ringspot virus

Tobacco ringspot virus (TRSV) is a subgroup A nepovirus (Digiaro et al., 2007), which was isolated from wild blackberries growing in North Carolina in 1965 (Rush and Gooding Jr, 1970); (Wei and Clover, 2008). TRSV is seed and pollen transmitted, and can be transmitted by the American dagger nematode *Xiphinema americanum* (Martin et al., 2012b; McGuire and others, 1964). TRSV infection in rose is associated with wavy line patters, ringspots, and blotches (McDaniel et al., 1971)**.**

Ilarviruses in Rose

Ilarviruses are positive sense tripartite RNA virus, in the family *Bromoviridae*. RNA 1 is 3.4-kb and codes for a single protein with methylransferase and helicase motifs (Poudel et al., 2014). RNA 2 is 2.8-kb and codes for two protein, a RNA-dependent RNA polymerase, and the 2b gene, which in other members of *Bromoviridae* plays a role in RNA silencing (Shimura et al., 2013; Siddiqui et al., 2011). RNA 3 2.3-kb codes for the viral movement and coat proteins (Bachman et al., 1994). Ilarviruses are seed, and pollen transmissible with thrips playing a role in pollen movement and transmission (Greber et al., 1991; Megahed et al., 1967); (Cameron and Thompson, 1985).

Blackberry chlorotic ringspot virus

Blackberry chlorotic ringspot virus (BCRV) is a subgroup 1 Ilarvirus which was simultaneously isolated in the United Kingdom and the United States of America in 2006 (Jones et al., 2006a; Tzanetakis et al., 2007). BCRV has been found in roses affected with RRD, but does not correlate well with the disease (Poudel et al., 2014). BCRV infects apple, blackberry, raspberry, and rose (Poudel et al., 2013b; Tzanetakis et al., 2007). The virus is 58% seed

transmissible in *R. multiflora*. Isolates of BCRV are likely pollen transmissible between blackberry, raspberry, and rose as isolates do not diverge between host (Poudel *et al.*, 2013; (Poudel and Tzanetakis, 2013).

Tobacco streak virus

Tobacco streak virus (TSV) is a subgroup 1 ilarvirus (Jones et al., 2006a), originally discovered in the 1930 in tobacco, with a host range of over 150 plant species (Fulton, 1948; Johnson et al., 1936). TSV is both seed and pollen transmissible, with thrips possibly playing a role in transmission as they move pollen between plants (Klose et al., 1996; Sdoodee and Teakle, 1988). A TSV-like virus was first reported in *Rosa setigera* in 1970, which presented with chlorosis, irregular patterns, as well as vein clearing, and leaf distortion (Fulton, 1970). A small survey in Oregon found 4 out of 17 roses infected with TSV (Converse and Bartlett, 1979). However these two reports should be taken with caution as ilarviruses antisera is known to cross react among species (Jones et al., 2006b; Tzanetakis et al., 2004).

Prunus necrotic ringspot virus

Prunus necrotic ringspot virus (PNRSV) is a subgroup 3 ilarvirus known to infect hops, rose, and several other rosaceous species (Fulton, 1970b; Hammond, 2003). In rose PNRSV induces ringspots and line patterns; symptomology becomes more severe when co-infected with *Apple mosaic virus* (Wong et al., 1988)(THOMAS, 1981). There are multiple PNRSV serotypes with at least three in rose; being distant from those found in *Prunus* (Moury et al., 2001).

Apple mosaic virus

Apple mosaic virus (ApMV) is a subgroup 3 ilarvirus (Mink, 1992) which has a host range of over 100 different species, within 19 different families (Fulton, 1972). Chlorotic

patches, puckering, and leaf distortion are associated with infection of rose with ApMV (Thomas, 1981; Wong et al., 1988). Molecular characterization of the coat protein of rose, and apple isolates, revealed 100% identity between some isolates (Valasevich et al., 2014).

Other viruses infecting rose

Strawberry latent ringspot virus

Strawberry latent ringspot virus (SLRSV) is an unclassified member of the *Secoviridae*. Originally SLRSV was discovered infecting strawberry, raspberry, cherry, plum, black currant, and elder (Lister, 1964). SLRSV has a large host range of over 125 species of both monocot and dicots, including many rosaceous species (Murant, 1974). In roses infected with SLRSV symptoms are associated with yellow fleck, leathery appearance of leaves, stunting of leaves and shoots (Horst et al., 2007). Symptoms do not always develop right away nor do all varieties express symptoms (Horst et al., 2007). SLRSV is transmitted by the nematodes *Xiphinema cox* (Putz et al., 1970)*, and *X. diversicaudatum* (Lister, 1964). Both adults and larvae vector SLRSV, with retention being up to 84 days in the absence of a host (Taylor and Thomas, 1968). SLRSV is also transmitted by pollen and seed, with seed transmission in various species exceeding 70% (Lister, 1964; Murant, 1974; Tang et al., 2012; Taylor and Thomas, 1968). Mixed infections with ArMV or PNRSV increase symptom severity (Horst et al., 2007).

Rosa rugosa leaf distortion virus

Five *Rosa rugosa* varieties demonstrated stunting, leaf distortion, and circular lines in new growth (Lockhart et al., 2011). TEM revealed spherical virion of 30-32 nm, encapsidating a ~4.2kb ssRNA genome (Lockhart et al., 2008). Further characterization lead to the discovery of the first member of the family *Tombusviridae* to infect rose, Rosa rugosa leaf distortion virus

(RrLDV) (Lockhart et al., 2008). RrLDV was graft transmitted to healthy *Rosa rugosa* and symptoms typical of the disease developed (Mollov et al., 2013b). Phylogenetically, the RrLDV is related to Pelargonium line pattern virus and Pelargonium chlorotic ring pattern virus, two unclassified viruses that group within the genus *Pelarspovirus* (Scheets et al., 2015).

Rose yellow leaf virus

Rose yellow leaf disease was noted in 'Fiesta' and 'Softee', and is characterized by blotchy yellow mosaics, leaf yellowing, and premature senescence (Lockhart et al., 2011). Spherical 30-32 nm virions encapsidating a ~4.2kb ssRNA genome was present in infected roses (Lockhart et al., 2011). Virion purification followed by cloning and sequencing, revealed a new member of the family *Tombusviridae*, Rose yellow leaf virus (RYLV), (Mollov et al., 2014). RYLV along RrLDV belong to the Pelargonium line pattern/chlorotic ring pattern virus group (Mollov et al., 2014). Interestingly, when transmitted to the cultivar 'Ballerina' rings and lines occurred on the canes, which is notable of rose streak disease (Lockhart et al., 2011), an indication that the virus may also be associated with rose streak.

Rose yellow mosaic virus

Rose yellow mosaic is characterized by yellow mosaic, premature leaf senescence, and stem necrotic lesions (Lockhart, 2011). Symptomatic plants were found infected with filamentous virus, a novel member of the *Potyviridae*, Rose yellow mosaic virus (RoYMV) (Mollov et al., 2013a). RoYMV was found in nine different rose cultivars between New York, and Minnesota (Mollov et al., 2013a). Compared with rose mosaic disease associated with ApMV or PNRSV, where symptoms are expressed early in the season; roses infected with RoYMV remain symptomatic through the season (Mollov et al., 2013a). RoYMV is a distinct

member of the family *Potyviridae*, unassigned to a genus, sharing less that 35% sequence similarity with other members (Mollov et al., 2013a). While the HC-Pro and coat protein of RoYMV have highest identity with potyviruses, a genus transmitted by aphids, a hallmark protein associated with transmission, HC-Pro, does not contain any of the motifs associated with aphid transmission (Mollov et al., 2013a). The virus instead contains a eriophyid mite motif in its HC-Pro and full polyprotein clusters with other mite transmitted viruses, suggesting eriophyids as possible vectors (Mollov et al., 2013a). It may also be that the aphid motifs were lost after continuous virus transmission through vegetative propagation (Mollov et al., 2013a).

Rose yellow vein virus

Rose yellow vein disease (Fig 3) is characterized by vein-yellowing in rose cultivars and leaf distortion in *R. rugosa.* (Mollov et al., 2012). Transmission electron microscopy revealed 48-50 nm spherical particles in affected plants (Mollov et al., 2012). Particle purification followed by cloning led to the discovery of a new virus, Rose yellow vein virus (RYVV), genus *Rosadnavirus* in the family *Caulimoviridae*, a group of circular dsDNA viruses (Geering, 2014; Mollov et al., 2013c). RYVV has a unique genome organization compared to that of other species of *Caulimoviridae*, and shares 22-38% similarity to the genome of this family (Mollov et al., 2013c). RYVV was graft transmitted from 'Dr. Merkeley', to healthy 'George Vancouver' and systemic vein-yellowing developed six to seven months later. The virus has been detected in Maryland, Minnesota, New York, and New Zealand (Mollov personal communication, Mollov et al., 2012; Perez-Egusquiza et al., 2012).

Wild rose leaf rosette virus

Wild rose leaf rosette disease (WRLRD), reminiscent of herbicide damage was described on wild roses in China (He et al., 2014). Large scale sequencing revealed the presence of *Apple stem grooving virus* (ASGV), *Blackberry chlorotic ringspot virus* (BCRV) and *Prunus necrotic ringspot virus* (PNRSV) and a previously uncharacterized *Closterovirus* dubbed rose leaf rosette-associated virus (RLRaV). RLRaV shares significant identity with the closterovirus, *Citrus tristeza virus* (CTV) (He et al., 2014). Twenty samples with WRLRD were tested for ASGV, BCRV, PNRSV, and RLRaV. A total of twelve tested positive for RLRaV with seven being positive only for RLRaV among the viruses tested (He et al., 2014). The authors conclude that this is evidence that RLRaV causes WRLRD, but this is unlikely given the poor correlation. While the symptoms are not classical of RRD, the rose genotype plays a role in symptom expression and it is intriguing that the authors did not test for RRV, a virus perfectly correlated with RRD. While a new virus was discovered, additional epidemiology work, namely vector transmission to eliminate the possibility of an abiotic nature for the disease needs to be conducted.

Rose rosette virus

Rose rosette virus is an emaraviruses, negative strand multipartite RNA viruses with the following five recognized members: *European moountain ash ringspot-associated virus* (EMARAV), *Fig mosaic virus* (FMV), *Pigeon pea sterility mosaic virus*, *Raspberry leaf blotch virus* and *Rose rosette virus* (RRV) ("ICTV Master Species List," 2014). Additionally three other recently discovered viruses phylogenetically fit within the genus Wheat mosaic virus (WMV), Redbud yellow ringspot virus (RYRSV) and Blackberry leaf mottle associated virus (BLMaV) (Hassan et al., 2011; Laney, 2010; McGavin et al., 2012). The virus has four RNAs, consistent

with the genomes of the two other emaraviruses sequenced at the time; EMARaV and FMV. All three viruses possessed protein homology with the exception being EMARaV p4. Based on homology with other bunyaviruses, RNA 1 encodes the RNA-dependent-RNA polymerase, RNA 2 codes for the glycoprotein precursor and RNA 3 for the nucleocapsid. RNA 4 has the movement protein of the virus. The genome organization of emaraviruses fluctuates with FMV and RLBV having 6 and 5 RNAs respectively.

All of the diseases associated with emaraviruses including PPSMV have been shown or speculated to be transmitted by eriophyid mites (Mielke-Ehret and Muehlbach, 2012). Similarly the causal agent of RRD has been shown to be transmitted by the mite *P. fructiphilius* but it has not been demonstrate whether RRV is vectored by the mite (Amrine et al., 1988). Successful transmission of RRV using *P. fructiphilius* to a virus-free background and subsequent development of typical symptoms of RRD would provide evidence that RRV is the causal agent of the disease.

Disease Causation

Koch's postulates is a well-known procedure for determining the causal agent of a disease. The four steps/postulates: 1. the microorganism must be present in all cases of the disease; 2.the pathogen can be isolated from the diseased host and grown in pure culture; 3. the pathogen from the pure culture must cause the disease when inoculated into a healthy, susceptible host; and 4. the pathogen must be re-isolated from the new host and shown to be the same as the originally inoculated pathogen (Falkow, 2004). For plant viruses the isolation and grown in pure culture step are replaced by infectious clones, virion isolation, and or mechanical inoculation to local lesion host. These options are not viable for a number of viruses.

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Since its advent next generation sequencing (NGS) methods have been increasing used to identity new viruses (Barba et al., 2014). Additionally the use of NGS has been proposed for the fulfillment of Koch's postulates in humans where the last step is unethical.

<u>**Research Goals**</u>

Since other methods to fulfill Koch's postulates were unavailable for RRV we elected to use a molecular approach. *P. fructiphillus* was proven to be the vector of RRV by collecting mites from roses which tested positive for RRV with typical symptoms of the disease, and moving them to virus-free roses as determined after NGS. The classic symptoms of RRD developed and the plant tested positive for RRV. Nucleic acids of this RRD/ RRV infected plant was examined by next generation sequencing. Reads in this run were found to match low percentage with blastx to emaraviruses with extra RNAs. These reads were further characterized and determined to be three additional segments of the RRV genome. Twenty-one different rose cultivars were infested with mites which had fed on RRV infected material to screen for resistance, with at least one of each cultivar grafted with a RRV infected scion for those varieties in which transmission did not occur. *P. fructiphillus* was reared on RRV infected material and allowed to feed on RRV-free for various periods to determine the virus IAP. To establish the AAP, mites reared on RRV-free roses were allowed to feed on RRV-infected leaves for different time periods.

The epidemiological data of the virus and disease presented here, namely; *P. fructiphillus* transmission of RRV; resistance to *P. fructiphillus* and RRV; AAP; IAP; fill in important knowledge gaps that will assist in the management of RRV and RRD. Additionally the approach to fulfill Koch's is practical and useful in cases where viral disease causation is not yet determined.

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Figure 1: Rose mosaic symptoms on ornamental rose presented as "oak leaf" patterns.

Figure 2: Flower variegation, likely caused by Rose flower break on ornamental rose.

Figure 3: Overly redden young shoots, leaf distortion, and witches'-broom formation on Knock-Out rose infected with *Rose rosette virus*.

Figure 4: Vein-yellowing and leaf distortion in a rose infected with Rose yellow vein virus, courtesy of D. Mollov.

CHAPTER 2

The evolution of emaraviruses is becoming more complex: seven segments identified in the

causal agent of Rose rosette disease

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The evolution of emaraviruses is becoming more complex: seven segments identified in the causal agent of Rose rosette disease

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Abstract

There are few plant diseases more devastating than rose rosette, a disorder that leads to total loss for the rose industry and rosarians alike. There has been circumstantial evidence that *Rose rosette virus* (RRV) is the causal agent of the disease. Notwithstanding, there are several diseases of woody plants that were once thought to be caused by a single virus but are now proven to be caused by virus complexes. In this study we established that RRV can solely cause rose rosette and determined that the virus genome comprises of at least seven RNAs. The implications of these discoveries in the genetic makeup and evolution of emaraviruses are discussed.

Abstract: 109 words, Main body: 2428 words

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First described in North America as witches' broom and mosaic (Conners, 1941), rose rosette disease (RRD) is the most destructive disorder of rose in North America. Symptoms include mosaic and mottle, flower and leaf malformation, excessive thorniness, increase in lateral shoot formation, young shoots reddening, and overall decline in vigor, leading to plant death. Rose rosette virus (RRV), an emaravirus, has been detected in all plants with RRD symptoms (Laney et al., 2011) but given the complex disease symptomology, it is still unclear whether RRV causes RRD solely or as part of a complex, similar to numerous other diseases of perennial plants once thought to be caused by a single virus (Martin & Tzanetakis, 2006; Martin *et al.*, 2013; Uyemoto & Scott, 1992). Fulfillment of Koch's postulates is necessary to establish causality of a disease (Falkow, 2004). For plant viruses, infectious clones and serial passage to local lesion hosts have routinely substituted "isolation" and "growth in pure culture". These options are not viable for a number of viruses because of the nature of their particles or their hosts which are not amendable to classical purification, or where the construction of an infectious clone is not yet feasible as is the case of emaraviruses. For this reason, new approaches involving next generation sequencing (NGS) were implemented to   assess the role of RRV in RRD development.

RRV was reported as a quadra-segmented virus as is the type member of the genus, European mountain ash ringspot associated virus (EMARaV; Mielke and Muehlbach, 2007). Still other members or tentative members of the genus, including fig mosaic virus (FMV; (Ishikawa et al., 2012), pigeonpea sterility mosaic virus (PPSMV(Elbeaino et al., 2014), raspberry leaf blotch virus (RLBV; McGavin et al., 2012), and wheat mosaic virus (WMoV; Tatineni *et al.*, 2014) have additional RNAs. In this study we examine the hypothesis that the RRV genome includes additional segments as its genetic relatives.

Roses with typical RRD symptoms were screened for *Phyllocoptes fructiphilus* mites,

vector of the RRD disease agent, and tested for RRV (Laney et al., 2011). Fifteen to fifty mites

were transferred from RRD/RRV-infected material to each of 35 asymptomatic and RRV-free

roses. Every 30 days post mite infestation, roses were tested for RRV using reverse transcription

(RT)-PCR as previously described (Laney et al., 2011). A plant that developed RRD symptoms

was chosen for further analysis. Total nucleic acids were extracted essentially as described

(Poudel et al., 2013), using two grams of leaf tissue collected from several areas of the plant to

eliminate sampling variability. The material was digested with DNase I (D4263, 25 units, Sigma-

Aldrich) in the presence of RNase inhibitor (Ribolock[TM], 6 units, Thermo Fisher) before

phenol:chloroform extraction. The ribosomal RNA was eliminated using the Ribo-Zero™

Magnetic Kit (Plant Leaf) (Epicentre) following the manufacturer's recommendation. This

material was used in two separate NGS preparations using the approach described in (Laney et

al., 2011) or by utilizing oligonucleotide primer PDAP213 in both the RT and PCR steps (Table

S1). PDAP213 anneals to a conserved 13 nucleotide region of both the 5' and 3' untranslated

regions of all emaraviruses identified to date, allowing for the simultaneous amplification of all

emaravirus RNAs. The RT reaction was incubated at 64°C before the addition of the Maxima

Reverse Transcriptase (Thermo Fisher) and cDNA was synthesized at the same temperature to

avoid mispriming. The PCR reactions consisted of 3 min at 94°C, followed by 40 cycles of 30

sec at 94°C, 30 sec at 64°C, 30 sec at 68°C, and a final step of 10 min at 68°C using LA Taq

(Takara). The extension time of 30 sec under the same PCR conditions allowed for amplification

of targets exceeding 5 Kb (Tzanetakis, personal observation) but not allowing for efficient

amplification of the 7 Kb RRV RNA 1. The PCR product was purified as described before

(Laney et al., 2011).

Although there is evidence that DNA virus transcripts can be detected in the RNA fraction of the nucleic acid extraction (Laney et al., 2012), their presence was assessed in the RRD-infected material using an aliquot taken prior to DNase digestion step. Rolling circle amplification (RCA) was performed using the illustra TempliPhi 100 Amplification Kit (GE) according to manufacturer's recommendations. To ensure that reactions were not affected by plant inhibitors, a control RCA was performed on RRD-extracted nucleic acids spiked with a pUC-type plasmid.

Degenerate oligo-primed (DOP) RT-PCR and PDAP213-generated products were sequenced in two separate reactions using the 454 GS Junior system (Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK). To identify sequences of viral origin, 454 output data was analyzed automatically using VirFind (Ho and Tzanetakis, 2014) with Newbler (Roche) as the *de novo* assembler, and further manually checked by comparing against GenBank sequences using NCBI Blastx (Altschul et al., 1997) applying the BLOSUM45 matrix.

Sequences of putative novel RRV RNAs were PCR amplified from PDAP213-primed cDNA with primers designed using Geneious 6.0 (Biomatters, Table S1). Amplicons were cloned and sequenced as previously described (Laney et al., 2011), with sequences assembled for at least 3x coverage. Thirteen RRV isolates (Arkansas: 4; Missouri: 4; Oklahoma: 1; mite transmission trials: 4) and an equal amount of RRV negative-free samples were used to establish the RRV nature of the newly identified segments.

ORFs were predicted using NCBI ORF Finder (Wheeler et al., 2003) and analyzed using Blastp BLOSUM45 (Altschul et al., 1997). Sequences were aligned and amino acid identity and homology were calculated using MUSCLE (Edgar, 2004) with the following emaravirus

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sequences; EMARaV p4 (YP003104766), FMV p5 (BAM13841), FMV p6 (BAM13855), PPSMV p5 (CCW28369), PPSMV p6 (CCW28370), RLBV p5 (CBZ42028), WMoV p5 (AIK23036), WMoV p6 (AIK23037), WMoV p7 (AIK23038) and WMoV p8 (AIK23039). Protein conserved domains were determined using NCBI Conserved Domain Search (Marchler-Bauer et al., 2011), whereas protein secondary structures were predicted using PSIPred (Buchan et al., 2013). Protein-protein interactions were analyzed with COTH (Mukherjee and Zhang, 2011), glycosylation sites using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), transmembrane helices using TMHMM 2.0 (Krogh et al., 2001), signal peptides and cleavage sites using SignalP 4.1 (Bendtsen et al., 2004), and subcellular localization using TargetP (Emanuelsson et al., 2000). The RNA binding properties of the proteins were analyzed using BindN at 85% specificity (Wang and Brown, 2006).

The DOP-RT-PCR run generated 191,540 raw reads, with a mean length of 404nt (40-1,196). VirFind assembled them to 18,623 contigs and singletons, and detected 541 unique hits to the four previously identified RRV RNAs. The VirFind Blastx step identified 142 sequences similar to emaravirus orthologs (FMV p5:62; FMV p6:67; EMARaV p4:13). The PDAP213 run was multiplexed with two other samples and after barcode extraction 63,327 raw reads were identified with a mean length of 436nt (51-1,200). They were assembled into 13,995 contigs and singletons. VirFind Blastn detected 836 unique hits to RRV RNAs 2 and 4. The Blastx step identified 15 hits to RRV and other emaravirus orthologs. Both approaches failed to identify any other virus-like sequences whereas RCA products were only generated in the plasmid control, verifying the absence of any other viruses in the sequenced material. The plant tested positive for RRV developed typical RRD symptoms (Fig. 1) verifying that RRV is transmitted by *P. fructiphilus* the sole causal agent of the disease.

Sequences resembling emaravirus ORFs were used to design primers for confirmation of the nature of the RNAs. The three novel segments were detected in four plants used in the RRD causality study; the plant used in NGS and three additional plants that developed RRD symptoms and tested positive for the virus, as well as isolates from different geographic areas. No segment was present in the RRV-free material providing concrete evidence that they are part of the virus genome.

RRV RNA 5 is 1665 nt long and contains a single ORF between CTA_{1604} - CAU_{201} coding for a putative protein p5 with a molecular mass of 55 kDa (Table 2). The predicted size is similar to that of FMV p5 59/57 kDa (depending on the isolate), PPSMV p5 55 kDa, RLBV p5 56 kDa, WMoV p5 56 kDa, and WMoV p6 58 kDa. RRV p5 is predicted to bind RNA (Wang and Brown, 2006) and contain one O- (Ser_{18}) and four N-glycosylation sites ($Asn_{65, 149, 434, 464}$). The p5 protein is predicted to form homodimers as well as interact with RRV p2, and p7 (Mukherjee and Zhang, 2011). These interactions were also predicted for the FMV p5 ortholog and FMV p2, p5 and p6. Secondary structural comparisons between RRV p5 and other emaravirus p5s (FMV, PPSMV, RLBV, WMoV), and WMoV p6, identified a highly conserved region starting approximately at 130 aa from the N' termini of the proteins ((Buchan et al., 2013); Fig. S1). Global alignment of the protein as seen in Table 1 revealed significant sequence identity and homology between orthologs.

RNA 6 is 1,402 nt long and possibly codes for two proteins. The smaller ORF, encodes a 7.4kDa protein, p6a (UUA_{712} – CAU_{524}). The protein is predicted to have two transmembrane domains and be involved in the secretory pathway (Krogh *et al.*, 2001; (Emanuelsson et al., 2000) although such an ORF was not observed in other emaraviruses. The 27.1 kDa p6b (UUA_{1334} – CAU_{633}), shares 30% identity and 51% homology with FMV p6, and18% identity

and 32% homology with EMARaV p4 (Edgar, 2004). RRV p6b exhibits similar secondary structure to its EMARaV and FMV ortholog (Buchan *et al.*, 2013, Fig. S2). This protein is also predicted to be involved in the secretory pathway, interacts with RRV proteins p2, and p5, and includes RNA binding motifs (Emanuelsson et al., 2000; Mukherjee and Zhang, 2011; Wang and Brown, 2006). Similar protein properties were also observed in the FMV p6.

RNA 7 is 1649 nt long with a single ORF (UUA_{1588} - CAU_{191}), encoding a putative protein of 54.1 kDa (p7). RRV p7 shares aa sequence and N-terminal secondary structure identity with all emaravirus p5 and WMoV p6 ((Edgar, 2004); Fig. S1). Similar to its orthologs, p7 is predicted to form homodimers as well as interact with glycoproteins and RRV p5 (Mukherjee and Zhang, 2011). A potential *O*-glycosylation site (Thr_{317}) was found along with three *N*-linked glycosylation sites ($Asn_{87, 282, 434}$).

These findings indicate that RRV and emaraviruses in general are more complex than originally thought. Understanding of the causal agent and its biology is prerequisite for any attempts for disease control. This communication proves evidence beyond doubt that RRV is transmitted by *P. fructiphilus* and causes RRD as there are no other disease agents identified in material used in the analysis; rejecting the hypothesis that the disease is the result of the synergism of multiple viruses.

Three additional RRV RNAs have been discovered. Several segments are of similar size; RNAs 3 and 4 differ by three bases whereas RNAs 5 and 7 differ by 16 bases. This fact, coupled with the lack of similarities of the putative proteins with those found in the databases; the potential difference in protein expression patterns, translated as low reads in sequencing runs may have led to the additional RNAs been undetected before this study. Newer, more robust sequencing methodologies coupled with novel bioinformatics analysis provide longer contigs

which is easier to manipulate and analyze. This has been the case of RRV where the original NGS data, retrieved after Illumina sequencing, did not include RNA 3-7, unlike the runs presented in this study.

In the VirFind dataset, both RRV p5 and p7 appeared as blastx hits to FMV p5. When these contigs were mapped against their ortholog, it was clear that the sequences were distinct and belong to different segments. Because of the discovery of those orthologs we tested the hypothesis of additional emaraviruses infecting rose using the *Emaravirus* degenerate primers targeting polymerase Motifs A-C (Elbeaino et al., 2013). The single amplicon was cloned and multiple clones sequenced. The sequences obtained belonged to RRV RNA 1 (over 99% identity to NC_015298); providing another line of evidence that the analyzed material was only infected by RRV.

RRV along WMoV have two FMV p5-like orthologs (p5 and p6 for WMoV). RRV p5 and RRV p7 are highly homologous, 73%, compared with 35%-55% to emaravirus orthologs, and WMoV p5/p6 which share 42% homology. This ponders the question as to how two gene copies arose in two different emaraviruses; are they products of duplication that led to the diversification of two paralogs or reassortment between strains or emaravirus species.

The protein similarity of the novel RRV proteins to their orthlogs is primarily at the structural level. Phylogenetic analysis based on the polymerase places RRV and FMV in a single clade distinct from other members of the group (Elbeaino et al., 2014). This topology can explain the loss of primary sequence homology when comparing all available p5/p7 orthologs.

The discovery of RRV p6 and its similarity to EMARaV p4, along with the EMARaV p4 lack of homology to other emaravirus movement proteins (Yu et al., 2013), suggest that other

emaraviruses may also have a similar protein and that the putative EMARaV movement protein is yet to be discovered. RRV RNA 6 is predicted to have two ORFs, inside an otherwise large untranslated region. The expression of p6a can only be speculated since there is no information on the protein expression strategy of emaraviruses; still the presence of transmembrane domains in the protein indicates that its expression is probable and not a bioinformatics artifact. Of the newly discovered RNAs and putative proteins, no functional domains or conversed regions were identified outside the genus, yet homology, and secondary structure including transmembrane domains suggest a shared function.

The obvious question is: why there are no WMoV p7/p8 orthologs identified in RRV? The data in this study combines one Illumina and two 454 reactions analyzed using low e value ($e=10^{-2}$) with the most powerful tool for virus detection and discovery known to date. Additional efforts to discover additional RRV RNA segments, if any, included increasing VirFind Blastn/Blastx e-values, and performed further Blastx search against a database containing only emaravirus amino acid sequences. Still, no additional segments were discovered.

Emaraviruses identified to date have different number of segments, an indication of genome plasticity; they need a core of proteins involved in replication, protection and virion assembly and movement whereas they may acquire additional segments-proteins either via reassortment or duplication to facilitate other functions in a similar fashion to closteroviruses where protein numbers and functions vary greatly between members of the family, genera and even groups within a genus (Dolja et al., 2006; Tatineni 2008; Tatineni et al. 2011) with the auxiliary proteins possibly involved in pathogenicity and/or host range. As emaravirus knowledge is expanding we can determine whether this hypothesis stands.

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Table 1. Amino acid identities and similarity among emaravirus orthologs. Percent amino acid conservation: identity (italics); similarity (bold) using MUSCLE.

	FMV p5	PPSMV p5	RLBV p5	RRV p5	RRV p7	WMoV p5
PPSMV p5	*31*	-	-	-	-	-
	53	**-**	**-**	**-**	**-**	**-**
RLBV p5	*16*	*13*	-	-	-	-
	36	**33**	**-**	**-**	**-**	**-**
RRV p5	*36*	*38*	*17*	-	-	-
	58	**56**	**37**	**-**	**-**	**-**
RRV p7	*35*	*35*	*16*	*53*	-	-
	55	**54**	**35**	**73**	**-**	**-**
WMoV p5	16	17	18	17	17	-
	35	**35**	**38**	**35**	**36**	**-**
WMoV p6	13	13	18	14	14	20
	30	**31**	**39**	**33**	**34**	**42**

Fig. 3. Rose rosette symptoms on the plant used in mite transmission studies and genome sequencing.

Fig. 4. Genome organization of *Rose rosette virus* RNAs 5–7 and co-ordinates of the predicted proteins.
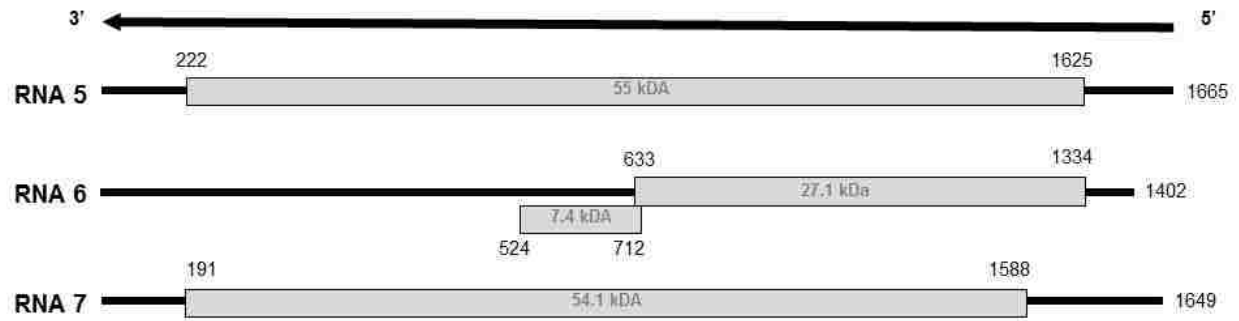
Suppl Table 2. List of oligonucleotide primers used in the study

Primer name	Sequence
PDAP213	GGCGACCCGCTCCGGTACCCTAGTAGTGAACTCC
RRVp5detF	TTTATGTATGGTGAGTTCTTCCC
RRVp5detR	TGTTCTAATAGCTGAAACTTTGGC
RRVp6detF	TGCCTGAAAGCATCCATCAT
RRVp6detR	CCATGACTGATGAAGCACTAGC
RRVp7detF	TTTAAGGTGAAATACTTCGC
RRVp7detR	GAGAGCCAGATTATTCGC

Suppl. Fig. 1 Secondary structural comparisons between emaravirus p5 orthologs identified a highly conserved region in the N' termini of the proteins
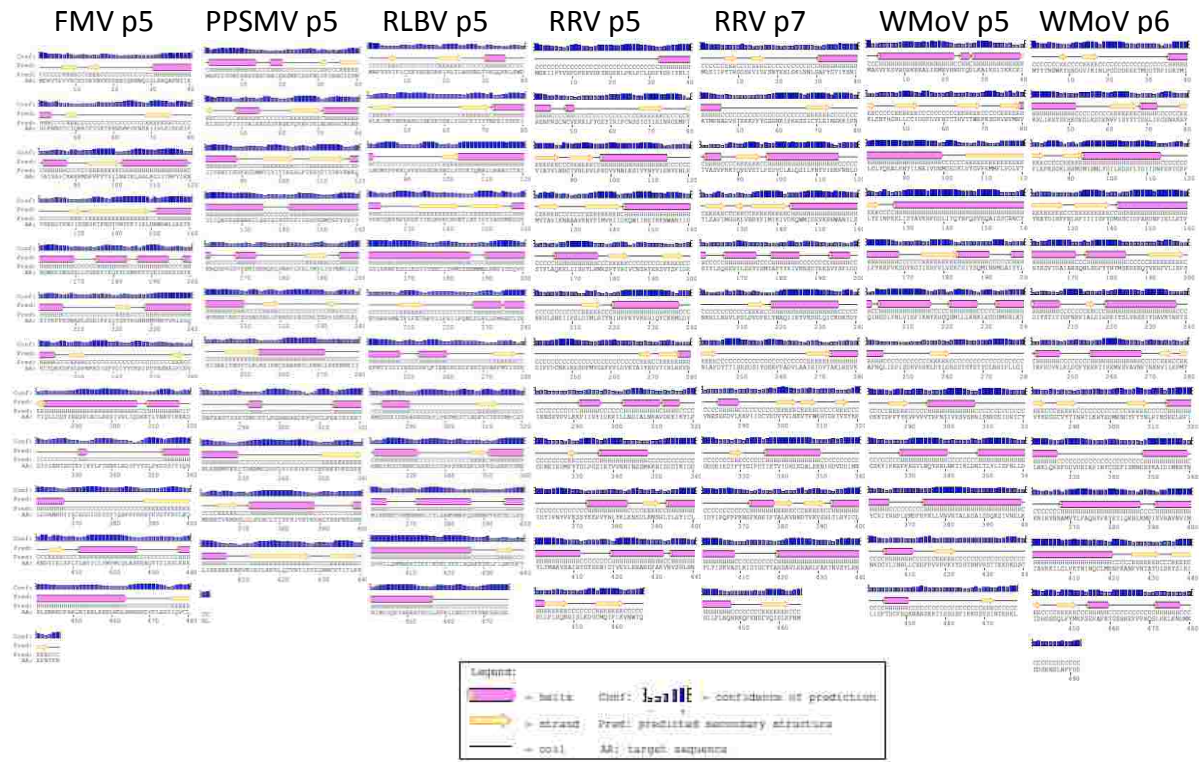
Suppl. Fig. 2. Secondary structural comparison between RRV p6 & FMV p6 and EMARaV p4.

| RRV p6 | FMV p6 | EMARaV p4 |
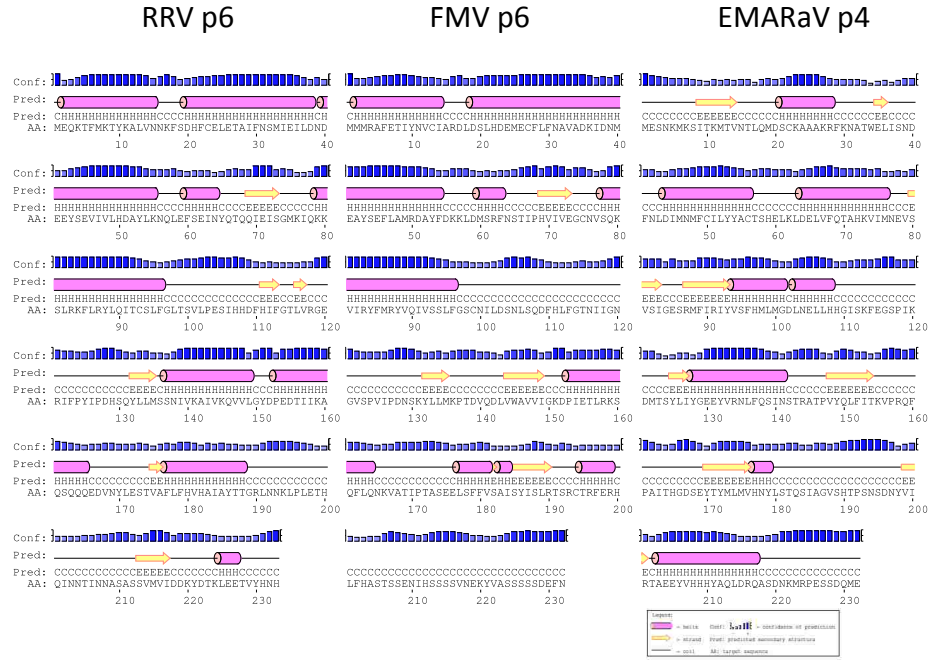
CHAPTER 3

Transmission attributes and resistance to rose rosette virus

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Transmission attributes and resistance to rose rosette virus

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Abstract

Rosette caused by the rose rosette virus (RRV) is the most devastating malady of rose in the United States. The virus is vectored by the eriophyid mite *Phyllocoptes fructiphilus* in an uncharacterized manner. There are different hypotheses on virus transmission and movement in the plant. This is because RRV was only recently identified and all assumptions were based on visual observations of material that may or may have not been infected by the virus. This study addresses several basic and applied aspects of virus and disease epidemiology. A new detection protocol based on all the genetic information available for RRV was developed and used. RRV was confirmed to move systemically in rose. Twenty rose genotypes were screened by mite inoculation and/or grafting and one was identified with resistance to the virus. The acquisition and inoculation access periods were also studied revealing a slow acquisition but rather rapid transmission time frame.

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1. Introduction

Since the 1940s Rose rosette disease (RRD) has plagued roses across North America (Conners, 1941). Early manifestations of RRD include reddened and swollen veins, leaf mosaic and mottling. Newly emerging shoots remain red longer when compared to healthy shoots, have an over-abundance of thorns and tend to bunch together, forming witches'-brooms with malformed flowers (Fig. 1). Infected roses begin to lose their esthetic appeal; experience an overall decline in vigor resulting in plant death 3 to 5 years after the initial infection.

Rose rosette virus (RRV) has been found in perfect association with the disease (Laney et al., 2011); confirmed to be transmitted by *Phyllocoptes fructiphilus* and be the causal agent of the disease (Di Bello et al., 2015). RRV belongs to the multipartite, negative strand RNA virus genus *Emaravirus* which currently includes five members: European mountain ash ringspot-associated virus (EMARaV), fig mosaic virus (FMV), pigeon pea sterility mosaic virus, raspberry leaf blotch virus and RRV (ICTV Master Species List, 2014). Four other recently characterized viruses; wheat mosaic virus (WMoV), redbud yellow ringspot virus (RYRSV), blackberry leaf mottle associated virus (BLMaV), and pigeon pea sterility mosaic virus II (PPSMVII) are also likely members of the genus (Elbeaino et al., 2009; 2014; 2015; Hassan et al., 2011; Laney, 2010; Tatineni et al., 2014). Partial emaravirus-like sequence has also been reported from *Arctium tomentosum* and *Cordyline fruticosa* (Bi et al., 2012; Melzer et al., 2014).

All recognized and tentative emaraviruses have been shown or are hypothesized to be transmitted by eriophyid mites (Caglayan et al., 2012; Kulkarni et al., 2002; Mielke-Ehret and Muehlbach, 2012). Little is known on how emaraviruses interact with their vectors including the mode of transmission. In the case of EMARaV, viral mRNA was detected in *Eriophyes pyri* suggesting that the virus replicates in the mite (Mielke-Ehret et al., 2010; Walia and Falk, 2012).

Acquisition and inoculation access periods (IAP and AAP respectively) have been accessed for PPSMV (Kulkarni et al., 2002) using immunological detection. In view of the recent discovery of PPSMVII it is not clear whether those results refer to PPSMV, PPSMVII or the combination of the two. If such a scenario is true it may be that the results are skewed because of possible interaction between the viruses that affect transmission times.

The basis of any epidemiological study is the development of sensitive, universal protocols able to detect the majority if not all the variants in a virus population. Here we describe such an assay, an RT-PCR, based on all available data on the population structure of the virus. Using this protocol we were able to screen a wide spectrum of rose varieties commonly grown in the United States. We verified that RRV moves systemically in plants and transmission experiments demonstrated that RRV is transmissible with *Phyllocoptes fructiphilus* with AAP and IAP of five days and one hour, respectively.

2. Materials and methods

2.1 Detection

RRV RNA3 sequences from 23 isolates available in GenBank were aligned (accession numbers HQ891892-HQ891913 and HQ871944) and oligonucleotide primers were designed in a region with 100% nucleotide identity among isolates. PCR conditions were optimized to amplify a 271 base region of RRV in infected plants or a 721 base fragment of the mRNA of NADH dehydrogenase ND-2 subunit in RRV-free material (Tzanetakis et al., 2007). Total nucleic acids were extracted from infected and RRV-free samples, reverse transcribed and amplified essentially as described in Poudel et al. (2013) with modifications incorporated for the optimum amplification of both virus and internal control products. The PCR program consisted of initial

denaturation for 2 minutes at 94°C followed by 40 cycles of denaturation at 94°C for 20s, annealing at 60°C for 20s and extension at 72°C for 30s. The reaction mixture contained equal concentration of virus-specific (F 5'-GCACATCCAACACTCTTGCAGC-3' and R 5'-CTTATTTGAAGCTGCTCCTTGATTTCC-3') and internal control primers (5' GGACTCCTGACGTATACGAAGGATC 3' and R 5' AGTAGATGCTATCACACATAC AAT 3') (400 nM).

2.2 . Systemic movement

There is a constant debate in the rose community whether RRV moves systemically or symptoms in different areas of the canopy are due to multiple mite infection sites. Movement was examined by testing for virus presence in the roots in addition to grafting experiments, part of the resistance screening presented below. Total nucleic acids were extracted as previously described with an additional washing step (Poudel et al. 2013) to minimize the presence of inhibitors that may be present in root tissue, and tested for the virus. The absence of inhibitors was determined by amplification of the mitochondrion cytochrome oxidase subunit I mRNA by RT-PCR (Papayiannis et al., 2011). Virus-specific amplicons were sequenced to confirm results.

2.3. Resistance screening

All roses used in the resistance and transmission studies described below were inspected for mites and verified to be RRV-free prior to initiation of the studies. Twenty rose varieties were screened for virus and mite resistance (Table 1). Roses were maintained in an insect-proof greenhouse and watered, fertilized, and pruned as needed. At least two weeks prior to infestation

plants were defoliated to force out new growth. From each field RRV-infected rose used for infestations an average of 50 mites were collected and slide-mounted in modified Berlese medium for phase contrast microscopic examination (Amrine and Manson 1996) and were all taxonomically identified as *P. fructiphilus* (Keifer, 1940). To separate between mite and virus resistance, screening was done in a serial mode: three to eight plants from each variety were inoculated with mites and one or two plants was grafted with a RRV-infected scion. A rose variety that developed symptoms and tested positive in either mite or graft transmission was not further evaluated. Viruliferous mites were collected from RRV-infected roses (as assessed by RT-PCR amplification) with typical RRD symptoms and 50 were transferred to each respective rose using an eyelash tool (de Lillo et al., 2010) to at least three plants/variety (Gispert et al., 1998). The infestation site was marked with a paperclip to monitor symptom development. Virus infection was assessed three months post inoculation using the protocol described above.

Plants were periodically checked under a stereoscope for mite presence. In varieties where no living mites were observed, additional experiments were performed to confirm the ability of *P. fructiphilus* to establish colonies. Fifty adult mites were transferred to leaves of those putative resistant varieties in modified Munger cells (Druciarek et al., 2014) and kept in growth chambers from 25°C to 30°C on a 16h photoperiod. The presence of mite eggs or immature stages was evaluated seven and fourteen days respectively after the transfer.

For varieties where no symptoms were observed and RRV was not detected, follow-up experiments were performed to determine whether the absence of infection was either due to resistance to the virus and/or mites. Twenty plants per variety were infested with 25 mites reared on infected material, whereas an additional 10 plants per variety were grafted with scions of a

RRV-infected Knock Out® rose. The presence of the virus was determined using RT-PCR monthly for up to three months post mite infestation or grafting.

2.4. *Transmission*

One-inch 'Julia Child' rose plants, maintained in growth chambers at 25°C to 30°C on a 16 h photoperiod were used to determine the AAP. Non-viruliferous mite colonies were established by eyelash transfer from RT-PCR RRV-free field roses to RRV-free 'Julia Child' roses. Colony plants were regularly tested for the virus and reared under the above described temperature and light conditions. Mites were transferred to modified Munger cells with RRV-infected 'Julia Child' leaves, and allowed to feed for 1, 6, 12 and 24h or 5 days. Following the respective acquisition time, 25 mites were transferred to a plant node marked with a permanent marker to each of five roses/time point and the experiment was repeated three or four times. Mites were eliminated 14 days after transfer using Worry Free® (0.3% Pyrethrins and 3% Piperonyl butoxide) and Sevin® (22.5% Carbaryl), around the infested nodes and moved to the green house. Still, experiments for each time point were kept in separate, isolated, areas to prevent mite movement in case the chemical control failed to eliminate all individuals. Five additional plants received 25 mites each, directly collected from RRV-free stock colonies to insure the integrity of the experiment. Roses were monitored for symptoms and tested for RRV three months post transmission.

'Julia Child' plants were also used to determine the IAP with plants maintained in growth chambers under the conditions described above. Viruliferous mites were collected from stock colonies in growth chambers under similar conditions or collected directly from infected

roses used in the AAP experiments. Symptomless and RRV-free roses, as determined after RRV-specific RT-PCR, were infested with 25 mites each at a node marked with a permanent marker. To determine the IAP, mites from RRV-infected leaves were transferred to RRV-free roses and allowed to feed for 1, 6 and 12 hours and 1, 5, or 14 days. Mites were eliminated as described above. Each trial consisted of five roses and was repeated four times. Seven plants, used as controls were infested with 25 mites from RRV-positive material and vectors were eliminated a month after inoculation.

3. **Results**

3.1. Detection

 A universal detection protocol based on the population structure of the virus available to date was developed. Although the previous test worked well, it was based on the sequence of a single isolate and the possibility of escapes, especially in early infections, in the absence of visual symptoms, could not be ignored. In addition, this protocol provides the advantage of validating the test and the quality of the extracted material by preferentially amplifying the virus product in infected plants or a plant mRNA in RRV-free material (Fig. 2). We compared more than 40 RRV samples using the Laney et al (2011) and the new test and the latter gave at least as good if not brighter amplicons than the previous assay (data not shown).

3.2. Systemic movement

A total of 20 RRD symptomatic and RRV-positive 'Julia Child' plants were tested for virus movement to the root. All samples amplified the internal control indicating the integrity of RT-PCR and 50% of the roots tested positive for the virus as determined by sequencing all amplicons.

3.3. Resistance screening

Sixteen out of the twenty varieties infested with viruliferous mites developed symptoms akin with RRD (Table 1) including leaf distortion, vein yellowing, mosaic, and mottling (Fig. 3), and test positive for the virus. 'Marmalade Skies' was the only genotype that did not develop excessive thorniness. Active mites were only found on 'All Ablaze Cl', 'Julia Child', and 'Pink Double Knock-out', but mites laid eggs and nymphs developed in all other genotypes when they fed on detached leaves in munger cages. In the initial screening described, 'Bonica', 'Home Run', and 'Stormy Weather', did not develop symptoms or tested positive for the virus when infested with mites or grafted. When additional plants where infected with viruliferous mites, 'Bonica' and 'Home Run' proved susceptible and typical rosette symptoms developed. 'Stormy Weather' did not sustain virus replication after having tested 35 plants between mite and graft transmission (Table 1, supplemental data).

3.4.   Transmission

In the AAP trial a single rose tested positive for RRV when mites were allowed to feed for five days prior to transferring to a healthy rose (Supplemental material). Mites successfully

transmitted RRV and typical symptoms developed in all IAP time points tested other than six hours (Table 2). Infection ranged from 5% to 60% with infection of control plants, where mites were allowed to feed for 30 days, being 100%. Symptoms developed in about a month under greenhouse conditions, and were more prominent and developed sooner when mites were allowed to feed for 14 days compared to all other time points.

4. **Discussion**

We developed a universal RRV detection protocol which allowed for the simultaneous assessment of the quality of the extracted nucleic acids. This confers better confidence to the results, especially when working with asymptomatic plants, a potential issue when moving propagation material to areas where the virus and disease are not endemic.

It is more often than not that rosarians only remove symptomatic tissue with the premise that RRV is not systemic and disease could be eliminated by pruning. We used a 'proof-of-concept' approach using grafting, part of our resistance screening, and testing root tissue where mites are unable to reach and feed on. Based on these results management recommendations based on pruning of symptomatic areas are rather inefficient and should be avoided to minimize the persistence of the virus after overwintering in the root system.

Resistance to any pathogen has many attributes; genotype, virus, environment, vector biology, and time. 'Stormy Weather' appears to be resistant under greenhouse conditions which were manipulated for optimal mite survival and symptom development. Independent of these results, genotypes need to be tested under field conditions and complex virus population

structures. If resistance stands under field conditions then the molecular mechanisms behind the phenotype need to be determined as this material provides the basis for control of RRD.

The study on PPSMV transmission were done using 1-20 mites/plant with transmission ranging from 40-100% (Kulkarni et al., 2002). Using 10 mites/plant the agent was transmitted at 100% efficiency after 24h AAP while needing 2h for IAP. In the present study we used a significant higher number of mites/plant and extended the AAP and IAP range to gain additional confidence on the results obtained. Mites require a feeding time of 5 days before becoming viruliferous, an indication that the RRV probably needs to infect and propagate in the mite as suggested in the EMARaV/ *Eriophyes pyri* complex (Mielke-Ehret et al., 2010) whereas mites could re-transmit after feeding for less than an hour on RRV-free material. Further work with a larger set of genotypes needs to be conducted to determine the source or resistance among the parents of the resistant/tolerant varieties. We aim to continue research with progeny from viruliferous females in order to determine whether transovarial transmission plays a role in the epidemiology of RRV.

Acknowledgements

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Table 1. List of varieties screened for resistance to rose rosette virus by *Phyllocoptes fructiphilus* infestation and grafting.

| | No. infected/ no. infested | |
Variety	Mite transmission	Grafting
All Ablaze Cl	2/5	1/1
Bellind's Dream	1/5	-
Blaze improved Cl	0/5	2/2
Bonica	1/25	1/11
Carefree Spirit	0/7	1/1
Double Knock-Out	1/8	1/1
Easy Does it	1/3	1/1
Francis Melliand	0/5	1/1
Home Run	2/30	0/10
Iceberg	1/7	-
Julia Child	2/5	-
Knock Out	1/6	-
Marmalade Skies	3/7	1/1
Pink Double Knock Out	1/6	-
Pink Knock Out	1/6	-
Queen Elizabeth	1/6	-
Stormy Weather	0/25	0/10
Sunshine Daydream	1/5	-
Veterans Honor	1/5	-
Yabba Dabba Doo	1/3	-

Table 2. Rose rosette virus inoculation access periods (IAP) tested using *Phyllocoptes fructiphilus*.

IAP	infected/infested				
	Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Total**
1 hour	1/5	0/5	0/5	0/5	1/20 (5%)
6 hours	0/5	0/5	0/5	0/5	0/20 (0%)
12 hours	1/5	1/5	0/5	0/5	2/20 (10%)
24 hours	1/5	2/5	2/5	1/5	6/20 (30%)
5 days	2/5	0/5	3/5	5/5	10/20 (50%)
14 days	1/5	1/5	5/5	5/5	12/20 (60%)

Supplemental Table 1. Rose rosette virus acquisition access period (AAP) experiments using *Phyllocoptes fructiphilus*.

AAP	infected/ infested				
	Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Total**
1 hour	0/5	0/5	0/5	-	0/15 (0%)
6 hours	0/5	0/5	0/5	-	0/15 (0%)
12 hours	0/5	0/5	0/5	-	0/15 (0%)
24 hours	0/5	0/5	0/5	0/5	0/20 (0%)
3 days	0/5	0/5	0/5	0/5	0/20 (0%)
5 days	1/5	0/5	0/5	0/5	1/20 (5%)

Supplemental Table 2. List of varieties additionally screened for resistance by *Phyllocoptes fructiphilus* infestation and grafting.

	infected/ infested						Grafting
	Mite transmission						
Variety	Initial	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Total	
Bonica	0/5	0/5	0/5	1/5	0/5	1/25	1/11
Home Run	0/10	0/5	2/5	0/5	0/5	2/30	0/10
Stormy Weather	0/5	0/5	0/5	0/5	0/5	0/25	0/10

Figure 1: Knock Out® rose infected with Rose rosette virus displaying typical rosette symptoms. Younger shoots cluster into a witches'-brooms.

Figure 2: Rose rosette virus (RRV) detection including primers targeting the NADH dehydrogenase ND-2 subunit gene. Lanes 1: 100 bp ladder; lanes 2-7: RRV-infected material; lanes 8-9: RRV-free samples; lane 10: Water control.
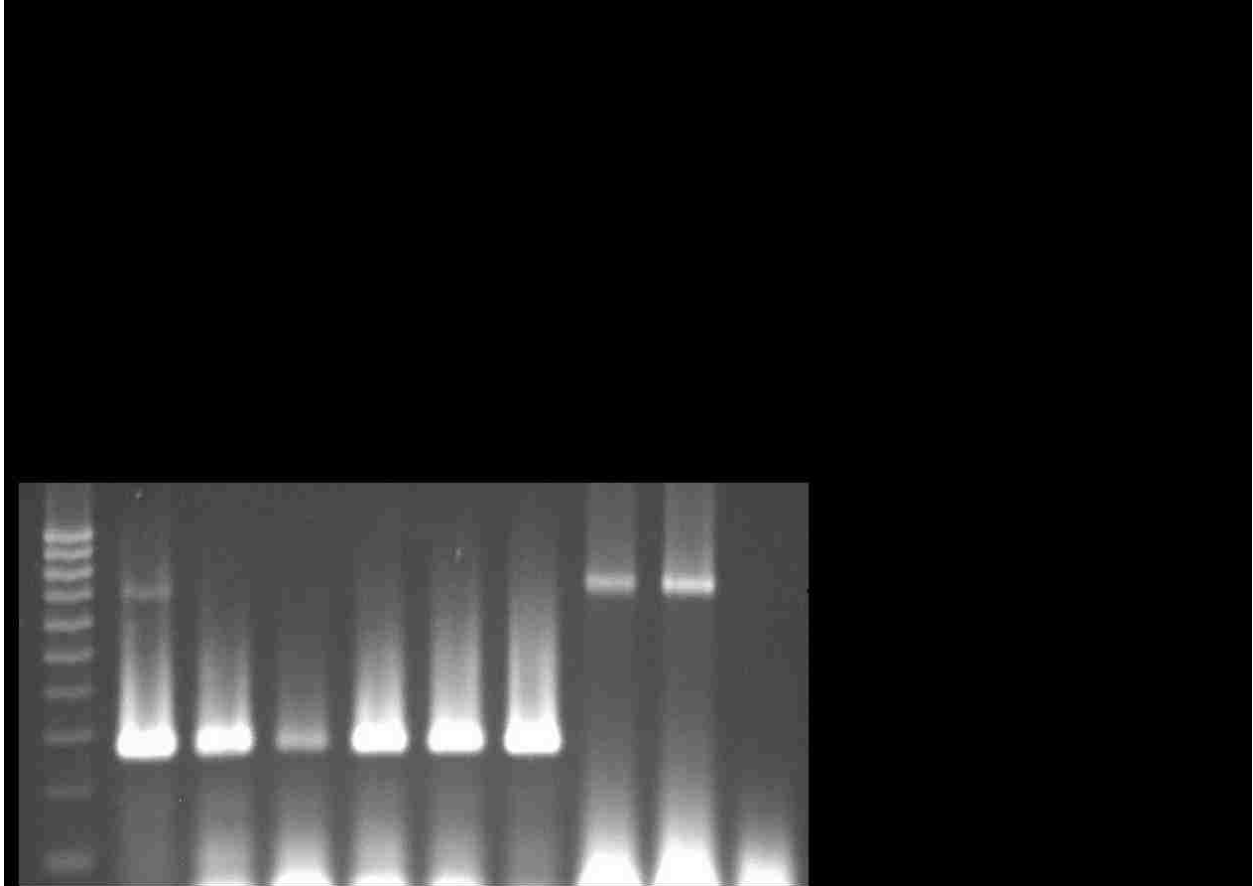
Figure 3: Rose rosette disease symptom progression after mite transmission. A. Twenty four (24) days post Infestation: enations and leaf distortion; B. Ninety four (94) days PI: red and distorted newly emerged shoots.

CHAPTER 4

Conclusion

Abstract

Work presented in this Thesis sought to determine the causal agent and study the epidemiology of the RRD agent. There has been significant progress on the biology of the RRD agent prior to the discovery of *Rose rosette virus* (RRV). The elusive agent was known to be graft transmissible, vectored by the eriophyid mite, *Phyllocoptes fructiphilus*, and associated with virus-like double membrane-bound bodies (Allington et al., 1968; Amire et al., 1988; Gergerich and Kim, 1983). RRV, the putative casual agent, was detected in all plants with RRD symptoms (Laney et al., 2011). However, this correlation does not prove causation of the disease. Given the complex symptomology observed the question of whether RRV causes RRD solely or as part of a virus complex, as is the case of numerous disorders of perennial plants, once thought to be caused by a single virus, was still unclear (Martin et al., 2012; Martin and Tzanetakis, 2006; Poudel et al., 2013; Uyemoto, 1992). Resistance is an important first line of defense when managing any disease, and here we identified five potential sources resistance for producers, rosarians and breeders. To date few viruses, once assumed to be transmitted by eriophyid viruses have been conclusively demonstrated to do so. The mode of transmission is elucidated for an even smaller subset of those viruses. Here we provide such information for the causal agenst of the most important rose disease in North America

Significance:

A.

Correlation between a pathogen and a disease is one of the important aspects in the route to determine causality, yet it is not proof. The same is true for vector transmission, a bottleneck that point to the nature of the disease agent. Koch's postulates still remain the standard to prove

beyond a reasonable doubt that a pathogen causes a disease (Falkow, 2004). Koch's postulates have remained rather elusive for viruses which are not culturable outside of their host. For plant viruses in particular the development of infectious clones or the use of serial passage to local lesion hosts is commonly considered equivalent to "isolating the agent" and "growing the agent in pure culture". Yet, infectious clones or the use of serial passage are still a hurdle for many plant viruses especially those with unstable particles, or where infectious clones have not yet been developed. For this reason new approaches involving next generation sequencing (NGS) have been implemented to identify or confirm disease causality (Barba *et al.*, 2014; Falkow, 2004; Fredericks & Relman, 1996; Mokili *et al.*, 2012). Such an approach was employed in the case of the RRV/RRD.

Utilizing this molecular approach to Koch's postulates we showed the RRV was the only virus present in a rose which developed typical RRD symptoms, after it was exposed to *P. fructiphillus*, the RRD agent vector, which were collected from a RRV/RRD infected rose. Thus, it was demonstrated that RRV is the causal agent of RRD and that RRV, is vectored by eriophyid mites. Both of these notions had been assumed since (Laney et al., 2011), but never proven. The approach to Koch's postulates can be readily adopted for other diseases

Additional RRV genome segments were discovered. The function of the putative encoded proteins could not be inferred with bioinformatics prediction tools but it was determined that they share homology to proteins coded by other emaraviruses. The similarities between two of the segments may indicate a reassortment or duplication event, but when comparing with other emaraviruses this same event is present in the distantly related Wheat mosaic virus, making this a rather primordial event, and perhaps pivotal to the existence of the genus as we know it today. This information adds to the rich complexity of a genus in which phylogenic relations between

the viruses cannot be predicted based on the relatedness of the host or the vector, making its evolution rather unusual.

B:

The work presented in this Thesis addresses significant gaps in knowledge in the epidemiology of RRV; Resistance to *P. fructiphillus* and RRV; virus acquisition and inoculation access periods, important aspects of any disease control strategies.

By infesting different genotypes with mites that fed on infected material and grafting RRV-infected scions five varieties which may possess various degrees of resistance to mite transmission of RRV or the virus itself were identified. Resistance has similarly been identified in two other emaravirus-associated diseases, pigeon pea sterility mosaic and wheat mosaic (Kulkarni et al., 2003; Marcon et al., 1997; Reddy et al., 1993). Those results will provide important background to producers, rosarians and rose breeders alike in the quest to control RRD as it spreads across much of the US. Systemic movement of RRV to the roots is a significant fact to consider when making management recommendations; e.g. pruning out symptomatic areas, an ineffective measure for virus elimination.

AAP and IAP for RRV and *P. fructiphillus* were determined using 'Julia Child'. *P. fructiphillus* become viruliferous after feeding for five days, and then efficiently vectors RRV to a new rose after feeding for a day. *Aceria ficus*, which trasmits FMV and the casual agent of fig mosaic acquires the disease agent within a few minutes and then vectors after an IAP of 16 hours (Caglayan et al., 2012; Elbeaino et al., 2009; Proeseler, 1972). *A. cajani,* the vector of the disease agent of pigeon pea sterility disease, becomes viruliferous after 15 minutes of feeding on infected material, and then transmits after two hours of feeding (Elbeaino et al., 2014; Kulkarni et al., 2002).

Future Work

Through this work a number of question and potential avenues for additional research have evolved. Additional work is needed to establish whether the presented varieties are field resistant. If the results presented here also stand in a field setting then this germplasm may be used for the development of a rose population that will assist in the development of molecular markers or even genes that confer resistance to the virus.

Different geographic population of *A. tosichella*, the wheat curl eriophyid mite transmit the Wheat mosaic virus at different efficiencies (Seifers et al., 2002). Given the spread of RRV across the US it is important to initiate a similar study for RRV and *P. fructiphillus* so as to determine whether there are mite and/or virus populations that transmit with different efficiencies.

(Proeseler, 1972) reported the fig mosaic disease agent was retained through the eriophyid's molt, such work should be conducted with RRV. Progeny from viruliferous females should be assessed for the virus, to determine whether transovarial transmission is a significant factor in RRV epidemiology. RRV retention by the vector is another important aspect of virus epidemiological knowledge that needs to be addressed.

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