A discovery 70 years in the making: characterization of the Rose rosette virus

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Rose rosette was first described in the early 1940s and it has emerged as one of the most devastating diseases of roses. Although it has been 70 years since the disease description, the rosette agent is yet to be characterized. In this communication, we identify and characterize the putative causal agent of the disease, a negative-sense RNA virus and new member of the genus Emaravirus. The virus was detected in 84/84 rose rosette-affected plants collected from the eastern half of the USA, but not in any of 30 symptomless plants tested. The strong correlation between virus and disease is a good indication that the virus, provisionally named Rose rosette virus, is the causal agent of the disease. Diversity studies using two virus proteins, p3 and p4, demonstrated that the virus has low diversity between isolates as they share nucleotide identities ranging from 97 to 99%.
(EMARaV) (GenBank accession nos NC_013105, NC_013106, NC_013107 and NC_013108) and Fig mosaic virus (FMV; FJ769161, AM941711, FM864225 and FM991954). The first and last 13 nt of EMARaV and FMV share more than 90% identity and this property was used to develop primers that would potentially amplify the complete RNAs of the virus. dsRNA was A-tailed by a using Poly(A) Tailing kit (Ambion) according to the manufacturer’s recommendations followed by RT as described previously (Tzanetakis & Martin, 2004). The cDNA was then subjected to PCR by using LA Taq (TakaraBio) and primers designed to match the last 13 bases of the 5’ (5’-AGTAGTGTTCTCC-3’) and 3’ (5’-GGAGTTCACTACT-3’) ends of EMARaV and FMV with an oligoT\_13 extension (Supplementary Table S1). Amplicons were cloned and sequenced (Supplementary Methods) to obtain at least three times genome coverage.

The first and last 13 bases of the virus RNAs were verified using RACE (Supplementary Table S1). Four RT primers, one primer for each RNA species, were chosen upstream of the PCR primers. Total RNA was used as the template for the 5’ RACE RT (Tzanetakis et al., 2007), followed by poly(C)-tailing. The sample was then subjected to PCR using LA Taq (TakaraBio), the virus-specific primers and a primer for the poly(C)-tail (Supplementary Table S1). For the 3’ RACE A-tailed dsRNA was used as a template for the RT-PCR using an oligo d(T) and a specific primer. All amplicons were cloned and sequenced as before. Programs used for bioinformatics analysis can be found in Supplementary Methods.

RNA 1 is 7026 nt long and contains a single ORF between nt 6919–6917 and 91–89 coding for a protein with a molecular mass of 264.8 kDa (Fig. 2). It encodes a putative RNA-dependent RNA-polymerase (RdRp) with signature RdRp motifs from members of the family Bunyaviridae between residues Lys\_1026-Asn\_1304 (Supplementary Fig. S1, available in JGV Online). Motifs A (DXKKWS\_{1106-1111}) and C (SDD\_{1233-1235}) are part of the palm domain of the replicase (Lukashevich et al., 1997; Bruenn, 2003) and are involved in divalent-metal cation binding. Motif B (QGXXXXXSS\_{1192-1200}) is thought to be involved in RNA binding with the conserved Gly\_1193 residue allowing for mobility in the peptide backbone. The Lys residues in motif D (KK\_{1285-1286}) are thought to have catalytic activity as they are reported to be close to the Asp\_1106 residue in motif A through the tertiary structure of the protein. Motif E in emaraviruses (EFLST) is similar to the conserved residues in tospoviruses (EFXSE; Bag et al., 2010), located from residues Glu\_1295 to Thr\_1298 in RRV; however, there is an amino acid substitution from the polar-neutral Thr to the negatively charged Glu. Motif E is believed to be involved in cap-snatching in bunyaviruses as well as having possible endonuclease activity. The genomic RNA of RRV was found to be uncapped, though the mRNA was capped (data not shown).

Fig. 1. RR symptoms on Rosa multiflora. (a) Leaf proliferation at nodes, (b) red pigmentation of stems and leaves, (c) multiple shoots emerging from a single node to form witches’ broom and (d) malformed leaves.
Characterization of the Rose rosette virus

RNA 1 – 7026 nt
RdRp

RNA 2 – 2220 nt
Glycoprotein precursor

RNA 3 – 1544 nt
Nucleocapsid

RNA 4 – 1541 nt
IMP?

(b)

(c)

(d)

RRV  
FMV  
INSV
for cleavage were predicted using SignalP (Bendtsen et al., 2004) between residues Gly20/Thr21 and Ala195/Asp196 though the cleavage site at Gly20/Thr21 was supported by a lower score. Cleavage at Ala195/Asp196 yields two mature glycoproteins, Gp1 of 22.4 kDa and Gp2 of 51.4 kDa. The first 20 aa of Gp1 contain a signal peptide predicted to be secreted to the endoplasmic reticulum. Two transmembrane domains (Sonnhammer et al., 1998) were predicted for Gp1, between residues Val114–Leu136 and Tyr176–Ala193; Gp2 contains one transmembrane domain from Asn391 to Asp413.

The p2 of EMARaV was reported to contain a phlebovirus glycoprotein motif (Mielke & Muehlbach, 2007; Supplementary Fig. S1) and this short motif, GCCXCGXG481–488, was also found in RRV Gp2. The protein has 51 % aa identity (73 % similarity) with FMV and 40 % aa identity (60 % similarity) with the EMARaV orthologues. Because p2 is predicted to have glycosylation sites and transmembrane domains, as well as a glycoprotein motif and a predicted structure similar to other glycoproteins (PHYRE, Supplementary Methods; Kelley & Sternberg, 2009; Fig. 2), we predict that RRV p2 is a glycoprotein precursor.

RNA 3 is 1544 nt long and contains a single ORF (nt 1445–495) for a putative nucleocapsid protein of 35.6 kDa. The predicted size is similar to that of the orthologues from High plains virus (HPV; 32 kDa), Pigeon pea sterility mosaic virus (32 kDa), EMARaV (35.1 kDa) and FMV (35.1 kDa) p3 orthologues. The putative protein shows identity from 26 % aa (47 % similarity) of the putative nucleocapsid of HPV to 60 % aa (74 % similarity) of FMV p3.

RRV p3 is predicted to form homodimers (Mericity; Garian, 2001) and contains stretches of positively charged residues, predicted to bind RNA (BindN at 85 % specificity; Wang & Brown, 2006). The predicted structure (PHYRE; Kelley & Sternberg, 2009) is bowl-shaped with interlocking protrusions and β-sheets, predicted to bind RNA (Fig. 2). This gives credibility to the prediction that the EMARaV, FMV and RRV p3 orthologues are nucleocapsid proteins. No motifs, identified in other viral proteins were found in p3. However, two conserved regions, (NVVSXXKXXXA134–143 and NRLA182–185), reported earlier as possible emaravirus nucleocapsid motifs (Elbeaino et al., 2009) were also found in RRV p3 (Supplementary Fig. S1).

RNA 4 is 1541 nt long and contains a single ORF starting at nt 1458 and terminating at nt 373 (Fig. 2). This ORF encodes a putative 41 kDa protein of undetermined function. The putative protein contains an ATPase motif between Phe78–Asp119 and a dnaK motif between residues Asp305 and Met350 (Supplementary Fig. S1). The protein product shows 59 % aa identity (76 % similarity) to the FMV orthologue. The predicted structure using PHYRE (Kelley & Sternberg, 2009; Fig. 2) shows a horseshoe-shaped monomer that is predicted to form homodimers (Mericity; Garian, 2001). It is possible that p4 also has the peptide binding properties of dnaK-containing heat-shock proteins.
proteins. Although present in an unrelated group of viruses, it has been proven that closteroviral Hsp70h are involved in virus cell–cell movement (Dolja et al., 2006) requiring both the dnaK and ATPase domains for movement (Alzhanova et al., 2001). This suggests that RRV p4 is involved in cell–cell movement in a manner similar to closteroviruses with p4 threading the nucleo-capsid-coated RNAs through the plasmodesmata in an ATP-dependent manner.

The virus RdRp clusters with the RdRps of two members of the newly formed genus Emaravirus (EMARaV and FMV), as supported by absolute bootstrap values (1000/1000 pseudoreplications), providing evidence that RRV is a new member of the genus (Fig. 3).

Koch postulates could not be fulfilled for the virus as there were no local lesion alternative hosts identified and virus purifications were unsuccessful (Laney, 2010). For this reason, symptomatic tissue from 84 cultivated and R. multiflora roses was collected from nine states. Thirty asymptomatic roses were used in this study as negative controls. After total nucleic acid extraction, cDNA was synthesized and subjected to PCR using detection primers. After total nucleic acid extraction, cDNA was synthesized and subjected to PCR using detection primers.

The diversity of RRV p3 and p4 was investigated using 22 isolates from eight different states. The coding regions were obtained using PCR, with the product being directly sequenced. All respective sequences were aligned with CLUSTAL W (Thompson et al., 1994) using the neighbour-joining algorithm, Kimura’s correction and bootstrapping consisting of 1000 pseudoreplicates. Bar: 0.1 change per site.

The virus RdRp clusters with the RdRPs of two members of the family Bunyaviridae (Fig. 3), using the RdRp nucleotide coding region, including species from the genera Emaravirus, Tospovirus, Nairovirus, Orthobunyavirus, Phlebovirus, Hantavirus and Tenuivirus. Citrus psorosis virus (CPsv), the genus Ophiovirus is used as an outgroup. Species include Tomato spotted wilt virus (TSWV), Capsicum chlorosis virus (CaCV), Impatiens necrotic spot virus (INSV), Groundnut bud necrosis virus (GBNV), Tomato zonate spot virus (TZSV), Rice streak virus (RSV), Rice grassy stunt virus (RGSV), Dugbe virus, Nairobi (DUGV), Crimean-Congo hemorrhagic fever virus (CCHFV), Bunyamwera virus (BUNV), Akabane virus (AKAV), La Crosse virus, Orthobunyavirus (LACV), Rift valley fever virus (RVFV), Fig mosaic virus (FMV), European mountain ash ringspot associated virus (EMARaV), RRV (HQ871942) and Hantaan virus (HTNV). Analysis was performed with CLUSTAL W (Thompson et al., 1994) using the neighbour-joining algorithm, Kimura’s correction and bootstrapping consisting of 1000 pseudoreplicates. Bar: 0.1 change per site.

Fig. 3. Phylogram for members of the family Bunyaviridae using the RdRp nucleotide coding region, including species from the genera Emaravirus, Tospovirus, Nairovirus, Orthobunyavirus, Phlebovirus, Hantavirus and Tenuivirus. Citrus psorosis virus (CPsv), the genus Ophiovirus is used as an outgroup. Species include Tomato spotted wilt virus (TSWV), Capsicum chlorosis virus (CaCV), Impatiens necrotic spot virus (INSV), Groundnut bud necrosis virus (GBNV), Tomato zonate spot virus (TZSV), Rice streak virus (RSV), Rice grassy stunt virus (RGSV), Dugbe virus, Nairobi (DUGV), Crimean-Congo hemorrhagic fever virus (CCHFV), Bunyamwera virus (BUNV), Akabane virus (AKAV), La Crosse virus, Orthobunyavirus (LACV), Rift valley fever virus (RVFV), Fig mosaic virus (FMV), European mountain ash ringspot associated virus (EMARaV), RRV (HQ871942) and Hantaan virus (HTNV). Analysis was performed with CLUSTAL W (Thompson et al., 1994) using the neighbour-joining algorithm, Kimura’s correction and bootstrapping consisting of 1000 pseudoreplicates. Bar: 0.1 change per site.

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Ophiovirus; Ophioviridae
Hantavirus
Nairovirus
Orthobunyavirus
Phlebovirus
Tenuivirus
Emaravirus
Tospovirus
results were observed when a diversity survey was performed for EMARaV in Europe. Sequencing the coding region for RNA 3 showed that isolates shared identity between 97 and 99% (Kallinen et al., 2009). Again in this case geographical location did not influence diversity; isolates from Germany and Finland were more conserved than isolates from adjacent trees in Finland.

This communication gives an answer to a question lingering for more than 70 years: what causes RR? The absolute association of a new emaravirus found in 84/84 symptomatic plants, but not in any of the 30 asymptomatic plants, collected from areas more than 1300 km apart, indicates that RRV is most likely the causal agent of the disease. The discovery of the novel virus, provisionally named RRV, and the development of a reliable detection protocol is of great importance to the nursery and the ornamental industries around the world as it is now possible to test for the virus and freely move material within and between countries without the fear of introducing the disease into areas where it is not present.

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