High throughput sequencing and RT-qPCR assay reveal the presence of rose cryptic virus-1 in the United Kingdom

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Abstract

Rose cryptic virus-1 (RoCV1) also known as *Rosa multiflora* cryptic virus is a partitivirus affecting roses, one of the most important ornamental crops worldwide. RoCV1 has previously been reported in the US, Canada and New Zealand, and has now been identified in the United Kingdom for the first time. Using High Throughput Sequencing (HTS) RoCV1 sequences were found in two samples collected in 2007 and 2012. This discovery led to the development of a RT-qPCR (TaqMan) assay for the detection of this virus. As part of a rose virus survey in the UK, 251 samples were analysed using the newly developed RoCV1 RT-qPCR test, following ELISA analysis for other common rose viruses. The results of the RT-qPCR test were confirmed using published conventional PCR primers and Sanger sequencing of amplified products. Results suggest that RoCV1 could have been infecting roses in the UK since at least 2007, with a large number of recently collected samples (43%) found to be infected. Cryptoviruses are not thought to cause direct economic losses in their plant host, although it is not clear what impact they might have in mixed infections.

Keywords RoCV1 · TaqMan · HTS · *Rosa* · Diagnostic methods

Roses are one of the most important ornamental crops worldwide, they are grown for their desirable aesthetics and scent for gardens and landscaping, and their use in industrial products (Dobhal et al. 2016). The garden industry contributes £9 billion to the UK economy every year. The UK's Department for Environment, Food & Rural Affairs (Defra) valued ornamental plant production at £1.1 billion in 2015, with an estimated

disease loss of £630 million within UK ornamental plant production of which £40 million could be attributed specifically to viral diseases (Department for Environment 2016; Little 2016).

A number of viruses have been reported affecting roses in the UK including arabis mosaic virus (ArMV), prunus necrotic ringspot virus (PNRSV) and strawberry latent ringspot virus (SLRSV) (Thomas 1984). Rose mosaic disease, one of the most common diseases in roses, is caused by single or mixed infections of these viruses, and others such as apple mosaic virus (ApMV). Symptoms include chlorotic line patterns, ring spots, mottles in leaves, yellow net and yellow mosaic. Infected plants are less vigorous and more likely to die during winter (Horst et al. 1983).

Rose cryptic virus-1 (RoCV1) also known as *Rosa multiflora* cryptic virus (Martin and Tzanetakis 2008) is a partitivirus, related to other species in the genus *Alphacryptovirus*, but not yet classified within this genus (King et al. 2012).

Partitivirus genomes are generally composed of two monocistronic dsRNA segments of 1.4–3.0 kbp, which are encapsidated in isometric particles about 30–40 nm in diameter. The larger RNA encodes a viral RNA-dependent RNA polymerase (RdRp), and the smaller one encodes a coat

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protein (CP) (King et al. 2012). However, RoCV1 has been shown to contain a third dsRNA segment (Sabanadzovic and Abou Ghanem-Sabanadzovic 2008), the origin of which is not clear, though several hypotheses have been published: (i) it may represent a co-infection by two different partitiviruses, (ii) the virus may possess two different versions of dsRNA2, or (iii) the third dsRNA element may represent a satellite virus (Nibert et al. 2014).

Cryptoviruses are reported to be widespread in nature and have escaped detection for many years because most of them cause no visible symptoms or, in a few situations, very mild symptoms (Hull 2014). RoCV1 has been found in asymptomatic plants as well as plants with mottling, leaf spots and necrosis (Milleza et al. 2013), as well as in mixed infections with rose spring dwarf-associated virus (Salem et al. 2008) or rose rosette emaravirus (Martin and Tzanetakis 2008).

In common with other partitiviruses there are no known natural vectors for RoCV1, it cannot move from cell-to-cell but can be transmitted by pollen and seed (Boccardo et al. 1987).

It was first reported in the US and it is thought to be widespread there, being reported from several locations (Martin and Tzanetakis 2008; Sabanadzovic and Abou Ghanem-Sabanadzovic 2008; Lockhart et al. 2011). Subsequently it has been reported in New Zealand, being the most prevalent virus infecting roses (Milleza et al. 2013), and in Canada (James et al. 2015). This is the first report describing the detection of RoCV1 in rose in the United Kingdom.

Rose samples were submitted to the plant clinic at Fera Science Ltd. (York, UK) in 2007 (LS11S16) and 2012 (LS13S9), suspected of having a viral infection based on symptoms: distortion on new growth, and wrinkled and streaked leaves. ELISA analysis was performed for a range of viruses using commercially available kits following the manufacturer's instructions, as follows: arabis mosaic virus (ArMV; Leibniz Institute DSMZ GmbH, Germany); prunus necrotic ringspot virus (PNRSV; Loewe Biochemica GmbH, Germany); apple mosaic virus (ApMV; Loewe Biochemica GmbH, Germany); strawberry latent ringspot virus (SLRSV; BIOREBA AG, Switzerland); and impatiens necrotic spot virus (INSV; BIOREBA AG, Switzerland). In each case a negative control corresponding to the same host plant species as the test sample was used. The result was considered positive when the optical density (OD) value at 405 nm after 1 h for a given sample was greater than 3× the mean OD value of the corresponding negative control. Each sample was tested in duplicate. Sample LS11S16 gave a positive result for ApMV and sample LS13S9 gave a positive result for SLRSV.

Subsequently, High Throughput Sequencing (HTS) using nucleic acid extracted from samples LS11S16 and LS13S9 was performed. Ribosome depleted indexed sequencing

libraries were prepared from extracted RNA using the ScriptSeq complete plant leaf kit (Illumina, USA) which were then sequenced on an Illumina MiSeq instrument using a V3, 2 × 300 cycle run kit (Illumina, USA). Sequence reads were trimmed for quality using Sickle (Najoshi 2011), assembled using Trinity (Grabherr et al. 2011) and then compared to the GenBank nr and nt databases using BLAST+ (Camacho et al. 2009). Reads of viral origin were inspected using MEGAN community edition (Huson et al. 2016). Sequences of RoCV1 were detected in both samples: for LS11S16, the total number of reads was 654,746 and 1893 mapped to RoCV1 (GenBank Accession Nos. MK075821; MK075822; MK075823; MK075824; MK075825). For LS13S9, the total number of reads was 317,946 and 62 mapped to RoCV1 (MK075826; MK075827; MK075828).

As part of a survey of rose viruses in the UK, 251 leaf samples were collected in autumn 2017 and spring/early summer 2018 from roses in the Royal Horticultural Society gardens at Harlow Carr (Harrogate, North Yorkshire, UK) and Wisley (Woking, Surrey, UK). In addition, samples of rose leaves were taken from a nursery in the Midlands, a public garden near London, the Royal National Rose Society Garden (St Albans, UK) and from samples submitted to the plant clinic at Fera Science Ltd. (York, UK).

ELISA analysis was performed for the same viruses as described above, and also tested for the following: alfalfa mosaic virus (AMV; BIOREBA AG, Switzerland); raspberry ringspot virus (RpRSV; Leibniz Institute DSMZ GmbH, Germany); cucumber mosaic virus (CMV; Agdia Inc., US); tomato spotted wilt virus (TSWV; Leibniz Institute DSMZ GmbH, Germany); and tobacco ringspot virus (TRSV; Agdia Inc., US).

ELISA results showed that six samples were infected with SLRSV (2.4%); ten samples were infected with ArMV (4%); and three samples with both viruses (1.2%).

To further investigate the incidence of RoCV1 in the UK, primers and a probe for the specific detection of RoCV1 by RT-qPCR (TaqMan) were designed (RoCV1–2-Fw-5'-TGATCGACCAAAGTTGCAACC-3'/RoCV1–2-Rv-5'-GAAGATAAGACAATGCAGTCA CTTTCTT-3'/RoCV1–2-Pe-5'-FAM-ATTCGGACTGAATTTGCTA-MGBNFQ-3') using Primer Express 3.0.1 (Applied Biosystems) in regions conserved within the RoCV1 genome (dsRNA1 segment) and divergent from other species. Sequences used for assay design included those generated using HTS, and sequences obtained from GenBank® of target and non-target viruses (NC_010346; KM598758.1; JX492318.1; EU413666.1; EU350962.1; EU024675.1; DQ093961.2).

Samples collected for the survey were analysed using the new assay. Nucleic acid was extracted from the leaf samples using a magnetic bead-based extraction method and the

KingFisher® mL platform (Thermo Scientific). Nucleic acid samples were also tested using an RT-qPCR simplex assay designed to the plant COX (Weller et al. 2000) and subsequently modified (Hughes et al. 2006). RT-qPCR was performed on previously extracted RNA in 96 well plates on either an ABI Prism 7500 or Viia7 (Applied Biosystems). Reactions contained iTaq One-step (2x), iScript™ reverse transcriptase (Bio Rad), 300 nM of each primer (RoCV1–2-Fw and RoCV1–2-Rv), 100 nM of probe (RoCV1–2-Pe) and 1 µl of extracted RNA (concentration as extracted) in a final reaction volume of 20 µl. The cycling conditions used were: 10 min at 50 °C, 2 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Negative controls where water replaced template were included in all runs and all samples were tested in duplicate. Results were scored positive if a C_T value <40 was recorded.

Previously published primers for the dsRNA2 segment (Sabanadzovic and Abou Ghanem-Sabanadzovic 2008) were used to confirm the results obtained by RT-qPCR. A two-step RT-PCR was performed on a single sample, 7B3, which tested positive for RoCV1 using RT-qPCR. Generation of cDNA was performed using SuperScript™ II Reverse Transcriptase (Thermo Scientific) following the manufacturer's instructions. cDNA (10% by volume) was added in a total volume of 30 µl, containing dNTP mix, 5x Phusion® HF Buffer, Thermo Scientific® Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) and primers (300 nM). Cycling was done using a C1000™ Thermal Cycler (Bio-Rad) as follows: 30 min at 40 °C, 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, a final extension step of 72 °C for 10 min was included.

Using agarose gel electrophoresis, a PCR product of the expected size (610 bp) was obtained and PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen, Germany), following the manufacturer's instructions before being sequenced (Eurofins Genomics, Germany) using both PCR primers (GenBank Accession No. MK075829).

The resulting sequence was compared to published nucleic acid data (99% identity; GenBank Accession Nos. KM598759.1; EU024677.1; EU350963.1; EU413667.1) and amino acid data (89–99% identity; ABY60413.1; ABV89764.1; YP_001686787.1), confirming that the sample 7B3 was infected with RoCV1.

The remaining samples were then tested for RoCV1 using the RT-qPCR, the results showed 43% (75 samples of 251, including 7B3) were positive (C_T between 17.4 and 38.4). Of these, two samples (0.8%) were found to be co-infected with RoCV1 and SLRSV, eight (3.2%) with RoCV1 and ArMV and two (0.8%) with all three viruses. All the samples tested positive with the plant COX assay previously, confirming the success of the nucleic acid extraction.

Following conventional PCR, products from three RoCV1 positive samples (4B3, 13A3, 16B1) were sent for Sanger sequencing (GenBank Accession No. MK075830; MK075831; MK075832). Comparison of the nucleic acid sequences with published data confirmed the presence of RoCV1 with a 95%–100% identity.

Furthermore, eight samples (11C, 16A1, 19A3, 21A1, 69A2, 74A2, 89 and 99) with high C_t values for RT-qPCR were analysed by conventional PCR. For three of these samples PCR gave a band of the expected size (610 bp) while the remaining gave negative results. To investigate if these samples were false-positive results following RT-qPCR or false negatives following testing using conventional PCR, the DNA amplified using the RT-qPCR from the eight samples were cloned using a pGEM®-T Easy Vector System (Promega), following the manufacturer's recommended protocols. Ligation of the insert was performed, bacterial cultures grown overnight after transformation, clones containing inserts were identified, and plasmid DNA was purified. Following sequencing of the plasmid inserts, comparisons were made with published sequence for RoCV1. The results showed that the sequence of all inserts have a high sequence identity (80–100%) to GenBank accessions KM598758.1; JX492318.1; EU413666.1; EU024675.1; AFQ36917.1; YP_001686786.1 and ABV89762.1. The results led us to conclude that the samples were infected, giving positive results with the RT-qPCR and negative results with the conventional RT-PCR most likely due to lower sensitivity.

ELISA analysis of the samples in the UK rose survey demonstrated the presence of SLRSV and ArMV, both previously described in the UK (Thomas 1980). Whilst the symptoms were frequently as described in the literature, SLRSV-infected plants (Fig. 1a) and plants with mixed infections (SLRSV + ArMV) display a chlorotic mosaic whilst ArMV-infected plants have yellow vein clearing symptoms (Fig. 1b). Nevertheless, not all the samples followed this pattern, some samples infected with ArMV had chlorotic mosaic symptoms typical of SLRSV infections. Further analysis is required to confirm these results.

Based on the provenance of samples, these results suggest that RoCV1 has been in the UK at least since 2007. Following the genus demarcation criteria from the International Committee on Taxonomy of Viruses (Vainio et al. 2018), < 24% amino acid sequence identity within the RdRP gene indicates a new species. Analysis of the sequence of samples LS11S16 and LS13S9, show that the amino acid sequences for RNA1 (RdRP protein) share between 98.85–100% identity with published sequences.

RoCV1 coat protein nucleic acid sequences (dsRNA2) from this work (GenBank Accession No. MK075822; MK075824; MK075827; MK075829; MK075830;

Fig. 1 Rose leaves showing rose mosaic disease symptoms, infected with (a) SLRSV and RoCV1 (b) ArMV and RoCV1 and (c) an asymptomatic rose stem infected with RoCV1

MK075831; MK075832) showed little difference ($<0.5\%$) between isolates. For samples LS11S16 and LS13S9 dsRNA 3 was detected (MK075823; MK075825; MK075828). Comparison of the nucleic acid sequence showed a 99% of identity with published sequences.

RoCV1 has been reported in the US (Martin and Tzanetakis 2008; Sabanadzovic and Abou Ghanem-Sabanadzovic 2008; Lockhart et al. 2011), Canada (James et al. 2015), and New Zealand where it was the most prevalent virus in roses with a 48% incidence (Milleza et al. 2013). By using HTS we have shown the presence of RoCV1 in the UK for the first time, follow on testing using RT-qPCR (TaqMan) has shown that a large percentage (43%) of the samples tested were infected with RoCV1. There is no known natural vector for RoCV1; it is assumed to be pollen and seed transmitted like other cryptoviruses. Therefore, it is probable that RoCV1 has spread between the US, New Zealand, Canada and the UK during commercial trade of planting material.

In New Zealand, infected roses were associated with mottling, leaf spots and necrosis and also one with flower break but it was also found in asymptomatic samples (Milleza et al. 2013). In this study, several samples were asymptomatic, others had vein banding, mottling or leaf distortion symptoms. It is possible that these symptoms could be caused by another, as yet unidentified virus (or pathogen), by herbicide damage or adverse cultural conditions.

A sample with symptoms similar to those caused by rose rosette virus was positive for RoCV1; further studies will be undertaken to investigate if those symptoms were due to mixed infections with other, as yet undescribed viruses. We also found roses with symptoms of rose mosaic disease which were infected with RoCV1, where ELISA analysis indicated that they were co-infected with ArMV, SLRSV or all three viruses. It is unknown whether the symptoms caused by ArMV or SLRSV were impacted by co-infection with RoCV1, although we found samples with mosaic symptoms which were not infected with RoCV1. Cryptoviruses are not thought to cause direct economic losses in their plant host,

although it is not clear what impact they have in mixed infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. It is original and has not been submitted or published elsewhere.

Informed consent Informed consent was obtained from all individual participants included in the study. All the authors have seen and approved the manuscript, and all have taken a valid role through either study design, data generation or manuscript preparation.

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