Journal of Virological Methods 266 (2019) 25–29

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of real-time RT-PCR assays for two viruses infecting pome fruit

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ARTICLE INFO

Keywords:
ASGV
AGCaV
real-time RT-qPCR
Malus

ABSTRACT

Apple stem grooving virus (ASGV) and *Apple green crinkle-associated virus* (AGCaV) negatively impact production, maintenance, and distribution of apples and other *Malus* species world-wide. Due to the increasing diversity of isolates found by high-throughput sequencing, we have developed real-time RT-qPCR assays for these two viruses. Primers and probes were designed against alignments of representative extant sequences from around the world, and reaction conditions optimized for sensitivity and specificity. Assays were validated against a panel of virus isolates, and compared to extant endpoint RT-PCR and ELISA assays. The new real-time RT-qPCR assays showed greater detection sensitivity than extant assays and were able to detect their target viruses from different host tissues.

1. Introduction

Viruses cause significant losses to pome fruit world-wide, either by modification of the host vascular system, as in the case of *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV), by damaging or deforming the fruit, as with *Apple green crinkle-associated virus* (AGCaV), or more indirectly by stunting or reducing the yield of infected plants (Martelli et al., 2007; Hadidi and Barba, 2011; James et al., 2013). Our ability to control these pathogens, and thus the diseases they cause, relies on our ability to detect them early in the infection cycle, ideally before symptom expression, so that the afflicted trees can be removed. Similarly, rapid and sensitive screening of germplasm and planting stocks is essential to preventing many of these viruses from being introduced into an orchard, state, or country. Recognizing this need, here we developed real-time RT-qPCR assays for the detection of ASGV and AGCaV, two viruses that are common in commercial apple plantings and nursery stock in the Pacific Northwest, USA.

The first, ASGV, is a member of the genus *Capillovirus*, possessing a single-stranded RNA genome of 6500 nt (Yoshikawa et al., 1993). ASGV infects many woody tree species, including members of the *Malus, Pyrus, Prunus, Actinidia*, and *Citrus* genera. In *Malus* and *Pyrus* it causes, as the name would suggest, long depressions in the wood of afflicted plants in susceptible species. Necrosis of the graft union and attendant poor growth or decline is observed in some cases. ASGV has also been reported to cause topworking disease and decline in combination with other apple infecting viruses (Massart et al., 2011).

The second, AGCaV, is a more recently reported virus (James et al., 2013) that has been associated with apple green crinkle. This long-standing disease, first reported in Japan and New Zealand during the 1930s, causes deformation, cracking, and red-brown discoloration on the fruit of susceptible cultivars; in some cases tree decline may also occur (Chamberlain et al., 1974; James et al., 2013). The virus itself is member of the genus *Foveavirus*, with a single-stranded RNA genome of 9266 nt and a genome organization similar to that of ASPV. Two strains of this virus have been described, one associated with apple green crinkle, while the second was found in quince (*Cydonia oblonga*) trees expressing quince fruit deformation or quince sooty ringspot disease symptoms (Morelli et al., 2017).

Both of these viruses are found in all apple producing regions of the world (James et al., 2013; Massart et al., 2011) and infect a wide range of apple and other pome fruit cultivars. As neither has a known insect vector, the primary means of spread is through the propagation of infected planting material. As such detection is paramount for disease control. At present, endpoint RT-PCR methods exist for both pathogens (James, 1999; James et al., 2013; Menzel et al., 2003), as do ELISA assays for ASGV. Yet the real-time RT-PCR platform offers greater speed and sensitivity than extant methods. Therefore in this study we developed RT-qPCR assays for our two chosen viruses, and compared them to extant methods.

2. Methods

2.1. Sample collection and extraction

All samples were obtained from the collection maintained at the

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https://doi.org/10.1016/j.jviromet.2018.12.008
Received 9 August 2018; Received in revised form 29 October 2018; Accepted 17 December 2018
Available online 14 January 2019

Table 1
Primers and probes developed in this study for the amplification of *Apple stem grooving virus* (ASGV) and *Apple green crinkle-associated virus* (AGCaV) by RT-qPCR and for generating the RNA transcripts.

Assay	Primer Name	Orientation	Sequence (5'-3')	Binding Site
ASGV RT-qPCR	ASGV-RT-F	+	AGGTCCCTCTCAGCAAGAATCG	5604-55625
	ASGV-RT-R	–	CCGGGTCTATGTGGGTCTTC	5700-5719
	ASGV-RT-P	+	6FAM-TTTGGAGAACATGAGTTTGGAAGACGTGC-BHQ1	5631-5659
AGCaV RT-qPCR	AGCaV-1-F	+	TCCCACCACCTCATATCAATCAC	898-920
	AGCaV-1-R	–	GAGGATTGAGGCCTTTCATAGAAAC	973-997
	AGCaV-1-P	+	6FAM-AGGCCATAGGCCAGAAAATGCGA-BHQ1	930-951
ASGV RNA Standard	ASGV-T-T7-F	+	TAATACGACTCACTATAGGGGTTAGAAGGTCACGCTCAAT	5534-5553
	ASGV-T-R	–	GTCTTATCAGATGACCCAAAGA	5842-5863
AGCaV RNA Standard	AGCaV-T-T7-F	+	TAATACGACTCACTATAGGGACCAGTGTCTGGGTCATATCTT	807-828
	AGCaV-T-R	–	GGCAAACAACTTTCAACTTTCC	1004-1025

Washington State University research and extension center in Prosser, WA. Combined leaf blade, midrib, and woody tissue samples were collected from each source plant, and total RNA extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with the addition of 2.5% w/v PVP-40, 0.1% v/v sodium metabisulfite, and 0.2% v/v Antifoam A to the extraction buffer.

2.2. RT-qPCR primer design

Primers and TaqMan probes were designed using the Biosearch Real-Time Design online software (LGC Biosearch Technologies, Novato, CA) using standard parameters against an alignment of representative complete genome sequences obtained from the NCBI database for each virus (ASGV: KX988001, KR185346, KX686111, KX668488, LT160740, KU198289, LC143387, LC084659, HE978837, KJ579253, KF434636, JQ308181, KU605672, KR106996, JX080201, D14995, AB004063, and AY596172; AGCaV: KT835289, and HE963831). *In silico* analysis of the primer and probe properties, including Tm, hetero- and homodimer formation was performed with Oligo Analyzer online software (IDT Technologies, Coralville, IA). Primers designed for this study are shown in Table 1.

2.3. RT-qPCR reaction optimization

The reaction conditions for each of the primer and probe sets designed for the target viruses were optimized on a BioRad CFX96 real-time thermocycler (BioRad Laboratories, Hercules, CA) using Invitrogen SuperScript™ III Platinum™ One-Step qRT-PCR reagents (Thermo-Fisher Scientific, Waltham, MA) against known positive, negative, and no-template controls; positive and negative samples were confirmed for ASGV and AGCaV using published endpoint PCR assays (Menzel et al., 2003; James et al., 2013). The following reaction conditions were examined: annealing temperature (58 °C–70 °C), primer concentration (300 nM–600 nM), probe concentration (50 nM - 200 nM), and $MgSO_4$ concentration (3 mM–6 mM). Effects on Cq and reaction efficiency were assessed for the different reaction conditions.

2.4. Reaction specificity and sensitivity

Optimized assays were validated against 1) a panel of representative samples known or suspected, from high-throughput sequencing results (data not shown) to contain one or both of these viruses, from the U.S and other countries, 2) the related non-target virus species ASPV and Cherry virus A (CVA), and 3) virus-free representatives of the common host species *Malus pumila* and *Pyrus communis* to ensure reaction specificity.

To determine reaction sensitivity, synthetic RNA transcripts of the ASGV and AGCaV target regions were generated. The transcription templates were amplified by one-step RT-PCR using virus-specific primers (Table 1), extracted using a GenElute PCR extraction kit (Sigma-Aldrich, St. Louis, MO), and then quantified on a Qubit 4 fluorometer (Thermo-Fisher). RNA transcripts were synthesized using a MAXIscript SP6/T7 kit (Thermo-Fisher), as per the manufacturer's instructions. RNA concentration was measured as above and the copy number of each was calculated based on expected transcript length. A dilution series from 10^{10} through 10^{1} copies/µl was created for each of the transcripts, in a total RNA extract from a virus-free apple plant to better reflect the inhibitory secondary metabolites and compounds found in a real virus-infected sample. Each sample and dilution series point was tested in triplicate to account for technical error. Reaction efficiency was calculated using LinRegPCR Ver. 2017.0.

Samples were also collected from different tissue types, including leaves, buds, canes, and roots, from plants infected with either ASGV, AGCaV, or both to evaluate the effect of tissue type on virus recovery and detection using these assays. Samples were tested as described above.

2.5. Comparison to extant assays

Finally, the two real-time RT-qPCR assays developed in this study were compared to extant endpoint RT-PCR assays currently in use, Menzel et al. (2003) and James et al. (2013), for ASGV and AGCaV respectively, and in the case of ASGV, an ELISA assay (Agdia Inc., Elkhart, IN) performed as per the manufacturer's instructions. All assays were tested against the panel of ASGV and AGCaV positive and negative samples described above.

3. Results & discussion

3.1. Reaction optimization

During the optimization of the ASGV assay we found that while an annealing/extension temperature of 64 °C was optimal for high titer samples, it reduced reaction sensitivity and failed to amplify low-titer samples, therefore, a temperature of 58 °C was selected. Adjusting primer and/or probe concentration produced no significant effect on the assay, so the assay was further optimized with a concentration of 400 nM each of the forward and reverse primers, and 100 nM of the FAM-labelled probe. Finally, higher Mg^{2+} concentrations were observed to be inhibitory to the reaction, increasing Ct values with each additional 1 mM. Increased Mg^{2+} also caused non-target amplification, therefore the concentration was left at 3 mM, the default for the reagents used. The optimized conditions for the ASGV assay were as follows: 400 nM of each of the forward and reverse primers, 100 nM of FAM-labelled probe, 3 mM $MgSO_4$, 0.4 µl Superscript III / Platinum Taq enzyme mix, and 2 µl of total RNA in a final reaction volume of 20 µl. One-step thermocycling conditions were 50 °C for 15 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 10 s, and 58 °C for 45 s.

Optimization of the AGCaV assay was similar, with no noticeable effects observed with different primer and/or probe concentrations, and a general trend of decreasing sensitivity with an increase in annealing/extension temperature. Increasing Mg^{2+} concentration did, however,

Table 2
Comparison of the RT-qPCR assays designed in this study to extant endpoint RT-PCR and ELISA assays for the detection of *Apple stem grooving virus* (ASGV) and *Apple green crinkle-associated virus* (AGCaV). +, virus detected; -, virus not detected.

Type	Species / Cultivar/Sample		Source Country	ASGV			AGCaV	
				Cq	RT-PCR	ELISA	Cq	RT-PCR
Virus Positives	Malus pumila	1	USA	26.4	+	–	–	–
		2	USA	30.3	+	–	–	–
		3	USA	29.1	+	–	–	–
		4	USA	34.2	+	–	–	–
		5	USA	33.5	+	–	–	–
		6	USA	36.4	+	–	–	–
		7	Australia	32.6	+	–	–	–
		8	Australia	31.2	+	–	–	–
		9	Australia	30.1	+	–	–	–
		10	Australia	28.3	+	–	–	–
		11	Japan	29.3	+	–	–	–
		12	France	30.4	+	–	–	–
		13	USA	31.4	–	–	–	–
		14	USA	28.5	–	–	–	–
		15	USA	34.8	–	–	–	–
		16	USA	–	–	–	35.4	–
		17	USA	–	–	–	26.4	+
		18	Australia	–	–	–	29.5	–
		19	Australia	–	–	–	28.2	+
		20	USA	25.5	+	–	30.6	–
		21	USA	28.1	+	–	32.9	–
		22	USA	32.9	+	–	29.2	–
		23	USA	25.3	+	–	36.8	+
		24	Australia	27.1	+	–	27.5	–
		25	South Africa	27.8	+	+	30.2	–
Virus Negatives	*M. pumila cv. Fuji*		USA	–	N/A	N/A	–	N/A
	M. pumila cv. Red Delicious		USA	–	N/A	N/A	–	N/A
	M. pumila cv. Ellis Bitter		USA	–	N/A	N/A	–	N/A
	M. pumila cv. M9		USA	–	N/A	N/A	–	N/A
	Pyrus pyrifolia cv. Bartlett		USA	–	N/A	N/A	–	N/A
	P. pyrifolia cv. Bosc		USA	–	N/A	N/A	–	N/A
	Cydonia oblonga cv. Smyrna		USA	–	N/A	N/A	–	N/A
	C. oblonga cv. Cookes Jumbo		USA	–	N/A	N/A	–	N/A
	Prunus avium cv. Bing		USA	–	N/A	N/A	–	N/A
Non-Target Viruses	ACLSV +		USA	–	N/A	N/A	–	N/A
	ASPV +		USA	–	N/A	N/A	–	N/A
	CVA +		USA	–	N/A	N/A	–	N/A

increase reaction sensitivity over the base concentration. The final optimized reaction conditions for the AGCaV assay were: 400 nM of each of the forward and reverse primers, 100 nM of FAM-labelled probe, 6 mM $MgSO_4$, 0.4 µl Superscript III / Platinum Taq enzyme mix, and 2 µl of total RNA in a final reaction volume of 20 µl. One-step thermocycling conditions were 50 °C for 15 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 10 s, and 58 °C for 45 s.

3.2. Specificity and sensitivity

Neither the AGSV or AGCaV assays produced any detectable amplification from virus-free extractions from representative host species and cultivars, including fruit bearing apple, pear, and quince cultivars, from cider apple or rootstock varieties, or from cherry (*Prunus avium*) (Table 2). Similarly, no amplification was detected from non-target virus species, including viruses from the same genera, such as ASPV, or *Cherry virus A* (CVA), or to ACLSV, commonly found to co-infect trees with ASGV or AGCaV (Table 2).

Using an RNA transcript of known concentration diluted in virus-free total RNA extract from apple, the two assays were found to detect as few as 10^1 copies per reaction, at an average Cq of approximately 37 cycles (Fig. 1). Using the dilution series for each assay, reaction efficiencies were calculated to be 121.0% for ASGV and 119.3% for AGCaV respectively (Fig. 1). These results indicate that there is potential inhibition of the reaction from the host plant extract or presence of the reverse transcriptase (Suslov and Steindler, 2005).

Finally, an examination of detection of these viruses from different tissue types revealed something interesting. While ASGV was readily detectable from leaf, stem, root, and bud tissues, with minor variation in titer, AGCaV was only found in leaf, stem, and root tissues from singly and doubly infected plants, yet not in buds (Table 3). This was observed to occur in two separate plants, and in at least one case, cannot be attributed to unsuccessful RNA extraction, for ASGV was successfully amplified from the same sample extract (Table 3). This may suggest that AGCaV has a different tissue tropism than ASGV but, as this was based on single biological samples, further research is required before this can be concluded.

3.3. Comparison to extant assays

Both RT-qPCR assays designed in this study showed significantly greater detection capability than the respective endpoint RT-PCR assays (Menzel et al., 2003; James et al., 2013) when screened against a panel of suspected ASGV and/or AGCaV positive samples from five different countries around the world. For example, the ASGV real-time assay detected 21 ASGV positives from the samples examined, whilst only 18 were detected by endpoint RT-PCR. The difference cannot be solely attributed to virus titer in these plants, for endpoint RT-PCR readily detected other isolates with comparable Cq values (Table 2). Even more notably, ELISA detected only 1 of the 21 ASGV positives, suggesting it is not comparable to either type of PCR in terms of specificity or sensitivity.

The AGCaV real-time RT-qPCR assay detected 10 AGCaV positives from the panel tested, compared to only 3 detected by endpoint RT-

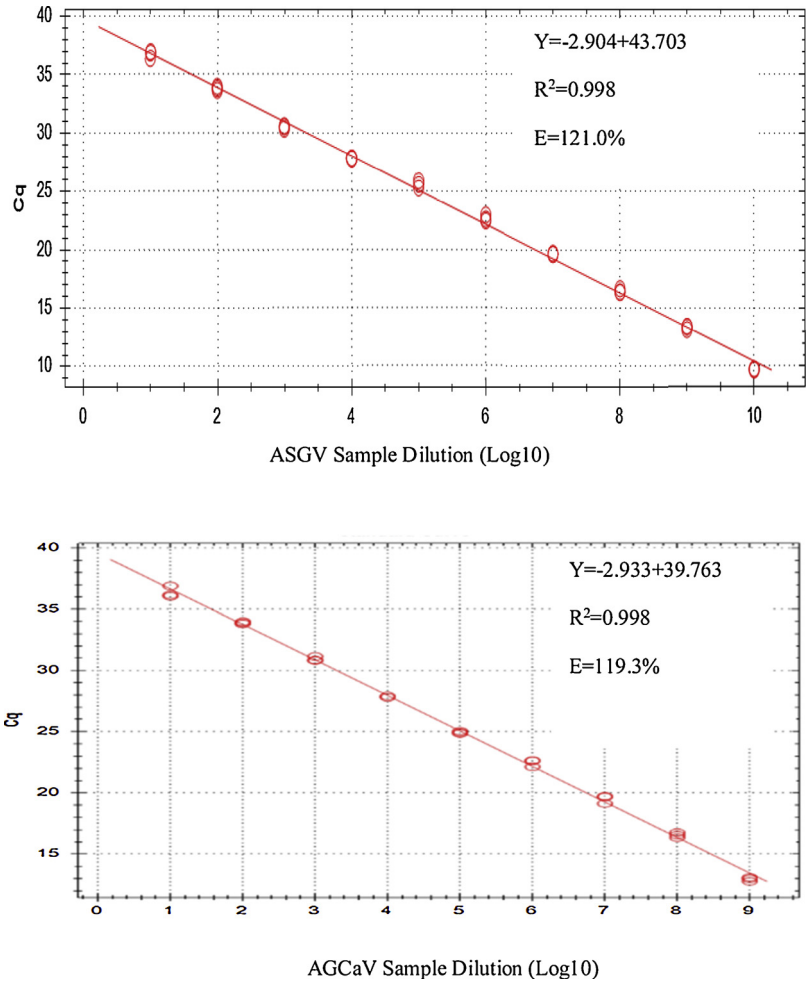

Fig. 1. Standard curves generated from the serial dilution (10^{10} through 10^{1} copies per reaction) of an artificial RNA standard for *Apple stem grooving virus* (ASGV, top) and *Apple green crinkle-associated virus* (AGCaV, bottom). RNA standards were diluted in virus-free *M. pumila* extract. Reactions were performed technical replicates of three.

Table 3
Detection of *Apple stem grooving virus* (ASGV) and *Apple green crinkle-associated virus* (AGCaV) in different tissues from either single- or double-infected apple samples. -, not detected.

Viruses Present	Tissue Type	ASGV Cq	AGCaV Cq
ASGV	Leaves	23.8	–
	Buds	28.4	–
	Stem	22.1	–
	Root	21.3	–
AGCaV	Leaves	–	27.2
	Buds	–	–
	Stem	–	30.6
	Root	–	22.9
ASGV + AGCaV	Leaves	24.9	32.5
	Buds	25.1	–
	Stem	22.5	23.4
	Root	22.7	31.2

PCR. Given the significant variation in Cq values from the positives (Table 2), these data suggest that the endpoint AGCaV assay may not be reliable for the detection of the range of sequence variation present in the global AGCaV population.

3.4. Summary

Sensitive, accurate detection methods are critical for detection of viruses for biosecurity, control, or eradication schemes, as well as for the production of virus-tested propagative material. Real time RT-qPCR is a platform that satisfies both of these conditions, while also reducing turnaround time and eliminating the need for post-assay steps such as gel electrophoresis. In this study we developed one-step RT-qPCR assays for ASGV and AGCaV, two viruses that are commonly found in pome fruit around the world, that have the potential to affect tree vigor, as well as fruit yield and quality. These assays were both capable of detecting low titers of the targeted viruses from a range of tissues. Furthermore, both developed assays showed significantly greater detection range than the extant endpoint assays, and ELISA assay in the case of ASGV.

Acknowledgements

The authors wish to acknowledge the assistance of T. Vasile in performing the ELISA tests, and A. Wright for critical reading of the manuscript. This work was supported in part by funding from the U.S. Department of Agriculture Animal Plant Health Inspection Service as part of the National Clean Plant Network cooperative agreement. All plants propagated in this study were maintained in a USDA-APHIS inspected quarantine facility under Permit PCIP-15-01326.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:https://doi.org/10.1016/j.jviromet.2018.12.008.

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