A Duplex Real-Time RT-PCR System with an Internal Control Offers Sensitive and Reliable Broad-Spectrum Detection of *Squash mosaic virus* Variants

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Abstract

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Squash mosaic virus (SqMV), a seedborne virus, belongs to the genus *Comovirus* in the subfamily *Comovirinae* of the family *Secoviridae*. SqMV has a bipartite single-stranded RNA genome (RNA1 and RNA2) encapsidated separately with two capsid proteins. With the recent identification of a third genotype in SqMV, a greater genetic diversity with only 88 to 89% sequence identity among them are recognized. With the existence of genetic diversity, a previously developed quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) failed to detect isolates in this new genotype. Therefore, it was necessary to create a new qRT-PCR that would react with all SqMV isolates in three

different genotypes. From a multiple sequence alignment of the available SqMV sequences in GenBank, a conserved sequence segment in the 3' untranslated region of RNA2 was identified for primer and probe design. A new qRT-PCR was developed, which provided broad-spectrum reactions to SqMV isolates, including those from the newly recognized third genotype. To improve the reliability in determining the sample quality and result interpretation, an internal amplification control with an endogenous gene sequence (18S ribosomal RNA) was successfully incorporated to develop a duplex qRT-PCR system that was useful for seed health test.

Squash mosaic virus (SqMV), in the genus *Comovirus*, subfamily *Comovirinae* of the family *Secoviridae* (Sanfaço et al. 2012), is a well-recognized virus. With the increasing international seed trade activity, this seedborne virus may be introduced to a region or a field if the seed is not thoroughly tested. SqMV infection could result in a disease epidemic on cucurbit crops, including cucumber, melon, squash, pumpkin, and watermelon. Since its first discovery in California in 1934 (Kendrick 1934), SqMV has continually been reported in other U.S. states, including California (Alvarez and Campbell 1978; Grogan et al. 1959; Haudenshield and Palukaitis 1998; Ling et al. 2011; Milne et al. 1969), Arizona (Haudenshield and Palukaitis 1998; Nelson and Knuhtsen 1973a,b), Florida (Hiebert and Purcifull 1981), New York (Hu et al. 1993), and Illinois (Jossey and Babadoost 2008). Its occurrence has also been reported in a number of other countries, including Japan (Han et al. 2002; Yoshida et al. 1980), Australia (Courtts and Jones 2005), China (Hu et al. 2009), and Spain (Li et al. 2015).

SqMV genome consists of bipartite single-stranded RNA (RNA1 and RNA2) that are encapsidated separately with two capsid proteins. Both RNA molecules contain a genome-linked viral protein (VPg) and a poly (A) tail. VPg functions as a 5' mRNA cap in translation initiation and, when uridylylated, as a primer for RNA synthesis by the virus-encoded RNA-dependent RNA polymerase (RdRp). RNA1 is translated into a single polyprotein that is processed into five functional proteins, including 32K protease cofactor, 58K helicase, VPg, 24K protease, and 87K RdRp. RNA2 is translated into two large overlapping polyproteins (52K replication protein from the larger polyprotein and 42K movement protein from the smaller polyprotein), followed by a larger coat protein and a smaller coat protein.

Studies on the genetic diversity of SqMV isolates initially divided them into two serotypes (Knuhtsen and Nelson 1968) and six biotypes

(Nelson and Knuhtsen 1973b). However, a recent genome sequence analysis showed the presence of a greater genetic diversity (Li et al. 2015). At least three genotypes, with only 88 to 89% nucleotide sequence identity among them, have been recognized (Haudenshield and Palukaitis 1998; Li et al. 2015). Presence of such broad genetic diversity and possible serological specificity among various genotypes poses a greater challenge to reliably detect SqMV isolates in seed and plant health tests. SqMV is a seedborne and seed-transmitted virus (Alvarez and Campbell 1978; Grogan et al. 1959; Milne et al. 1969). The virus is transmitted in the field to other cucurbit plants by beetles (Freitag 1956). In addition, its ease of mechanical transmission could result in a disease outbreak from cultivation activities. Seed transmission rates, ranging from 1% in commercial seed lots (Grogan et al. 1959; Milne et al. 1969) to an average of 10.6% in 62 experimental cantaloupe seed lots (Alvarez and Campbell 1978), have been reported. A typical seed health test for SqMV detection was conducted using enzyme-linked immunosorbent assay (ELISA) (http://www.worldseed.org/cms/medias/file/TradeIssues/PhytosanitaryMatters/SeedHealthTesting/ISHI-Veg/Cucurbits_SqMV_CGMMV_MNSV_010811_2012.pdf). Due to the understanding of greater genetic diversity (Haudenshield and Palukaitis 1998; Li et al. 2015) and possible serological specificity (Knuhtsen and Nelson 1968) among SqMV isolates in different genotypes, a more sensitive and reliable molecular-based assay is urgently needed for seed health tests to meet the demand for increasing global seed trade activities on cucurbit seed.

To improve the efficiency in the seed health test, Ling and colleagues (2011) initially developed a TaqMan real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR, herein after designated as SqMV_2011) which was capable of detecting all SqMV isolates in both genotypes that were recognized at that time. Surprisingly, this qRT-PCR could not detect isolates in the recently recognized third genotype, RZ-SqMV (Li et al. 2015). The complete genome sequence of this novel genotype was recently determined using the small RNA deep sequencing and assembly technology (Kreuze et al. 2009; Li et al. 2012, 2015). The RNA1 and RNA2 of RZ-SqMV had 5,858 and 3,370 nucleotides (nt), respectively. BLASTn sequence analysis of RZ-SqMV in the National Center for Biotechnology Information GenBank database showed only 87 to 88% nucleotide sequence identity in RNA1 and 84 to 86% in RNA2 to SqMV isolates in two other genotypes (Li et al. 2015).

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Despite the presence of such strong sequence diversity among the three SqMV genotypes, several conserved sequence regions identified from a multisequence alignment could be suitable for primer and probe design. In this article, we report on the development of a new qRT-PCR (SqMV_2015) useful for a seed health assay to allow for a reliable and sensitive detection of diverse isolates of SqMV in all known genotypes.

Materials and Methods

Virus sources. In 2010, a new SqMV isolate (designated as RZ-SqMV), representing a third genotype, was collected from a squash field in Spain for evaluation of squash germplasm materials (Li et al. 2015). Preliminary data showed that qRT-PCR (SqMV_2011 system), developed by Ling et al. (2011), even though it reacted to isolates in two known genotypes of SqMV at that time, failed to react to this new isolate. A virus culture of RZ-SqMV was obtained and established on melon plants in a greenhouse in Charleston, SC through mechanical inoculation. Another 14 SqMV-infected materials (leaf or seed samples) used for validation of the established qRT-PCR were supplied by collaborative seed companies, including nine seed samples from Peru, three leaf samples from Spain, and two samples from the Netherlands. A commercial melon seed lot ('Honeydew Green Flesh') from the United States, previously determined to contain an SqMV isolate (CA-SqMV) in genotype 1 (Ling et al. 2011), was also used for comparative analysis. Genotyping of those SqMV isolates in genotypes 1 and 2 was determined based on partial sequence information obtained from amplicons in two genomic regions using primers specific to the reference isolate M96148, targeting 440 to 968 or 1,619 to 2,077 nt, respectively. Although a majority of the tested isolates (11 of 14) were from genotype 1, the isolate 13896.12 was a genotype 2. Two other isolates (13896.11 and 13896.14) from Spain were previously determined to be genotype 3 (Li et al. 2015).

RNA sample preparation. Total plant RNA was purified using a TRIzol reagent (Life Technologies). For RNA isolation from leaves, 0.2 to 0.5 g of leaf tissue in a Bioreba tissue extraction bag was processed in 1.5 ml of TRIzol reagent using a Homex homogenizer (Bioreba). For RNA isolation from seed, 0.25 g of seed was first crushed with a hammer, then processed in 3 ml of TRIzol reagent into slurry using Homex homogenizer. The resulting suspension was transferred into a 2-ml microtube and spun at $12,000 \times g$ for 10 min. Approximately 1 ml of supernatant was transferred into a new tube with 0.4 ml of chloroform, which was shaken vigorously by hand for 15 s and left at room temperature for 2 to 15 min. After centrifugation at $12,000 \times g$ for 15 min at 4°C , 600 μl of supernatant was transferred to a new tube, which was mixed with 1 ml of isopropanol and left at room temperature for 10 min. The mixture was centrifuged at $12,000 \times g$ for 10 min at 4°C . After pouring off the supernatant, the pellet was washed once with 70% ethanol, air dried for 5 to 10 min, and redissolved in 200 μl of RNase-free water. RNA concentration was measured using a NanoDrop spectrophotometer 2000 (Thermo Scientific) and serial dilutions were made with RNase-free water.

Primer and TaqMan probe design. To improve reliability in using qRT-PCR for sensitive detection of all genotypes of SqMV, a

conserved sequence in the 3' untranslated region (UTR; nucleotides 3,273 to 3,354) was identified from a multiple sequence alignment using six available RNA2 sequences, including RZ-SqMV. A new TaqMan probe (nucleotides 3,294 to 3,321: FAM-TCT TTCCAGTTTCGCTTTAGAAGCCTCT-TAMRA) and corresponding forward primer (nucleotides 3,275 to 3,293: 5'-TCTG GGT TTTGTTGTGGCT-3') and reverse primer (nucleotides 3,354 to 3,333: 5'-GCATCAAAGAAAACAAGCTCTT-3') were designed and synthesized by Integrated DNA Technologies (Fig. 1). In order to evaluate the quality of prepared RNA samples useful for real-time RT-PCR, an internal control with a plant endogenous sequence, 18S ribosomal RNA (rRNA), was included in a duplex reaction. The 18S rRNA-specific TaqMan probe for internal control was labeled with a different fluorescent dye (CY5), with their sequences (449F: 5'-GTGACGGAGAATTAGGGTTCGA-3'; 498R: 5'-CTGCCTTCCTTGGATGTGGTA-3'; and 475 P: CY5-CCG GAGAGGGAGCCTGAGAAACGG-BHQ-2) derived from a previous publication (Osman et al. 2007). For comparative analysis, the old qRT-PCR (SqMV_2011 system) developed by Ling and colleagues (2011) was also included with its primers and TaqMan probe described in detail there.

Real-time RT-PCR. Each qRT-PCR assay was prepared using Takara's One-Step Ex Taq qRT-PCR kit in a reduced reaction volume of 10 μl . Each simplex reaction consisted of 5 μl of $2\times$ master reaction buffer, 0.25 μl of Ex Taq HS mix (5 U/ μl), 0.25 μl of RTase mix (5 U/ μl), 0.25 μl of forward primer (20 μM), 0.25 μl of reverse primer (20 μM), 0.25 μl of TaqMan probe (10 μM), 0.19 μl of ROX reference dye (1 $\times$), and 0.5 μl of RNA preparation. For a duplex reaction, in addition to the above reaction components, 0.25 μl of the forward primer (20 μM), 0.25 μl of the reverse primer (20 μM), and 0.25 μl of the TaqMan probe (10 μM) for the 18S rRNA were also included in the reaction mixture. Real-time RT-PCR was carried out on a Stratagene Mx3000P detection system in a thermal cycling profile, with an initial RT cycle at 50°C for 30 min and denaturation at 95°C for 2 min, then 40 cycles of 95°C for 10 s and 55°C for 30 s. In the duplex reaction, both fluorescent signals (FAM and CY5) were measured simultaneously. In addition, for each experiment, both a non-template and a healthy sample were also used as negative controls.

Seed health assay. To determine the sensitivity and reliability of the new qRT-PCR (hereinafter referred to as SqMV_2015) for a seed health test, a commercial seed sample previously determined by ELISA to be infected by SqMV was used. With total RNA preparations extracted from 5, 10, and 100 seeds, along with two health control samples of 5 hybrid cantaloupe melon seeds and 2 pumpkin seeds, a duplex qRT-PCR, including an internal control 18S rRNA, was carried out accordingly. Each reaction was repeated at least three times.

Results

Comparative evaluation of two qRT-PCR systems. To determine relative sensitivity of the new qRT-PCR system (SqMV_2015), both RZ-SqMV (genotype 3) and CA-SqMV (genotype 1) were used as templates. Total plant RNA preparation in an RZ-SqMV-infected sample was adjusted to 100 ng/ μl and then serially diluted with RNase-free water in 10-fold increments up to 1 pg/ μl (Table 1).

Fig. 1. Selection of a conserved sequence in the 3' untranslated region to design TaqMan probe and primers in a new quantitative real-time reverse-transcription polymerase chain reaction (SqMV_2015) with broad-spectrum reaction to *Squash mosaic virus* (SqMV) isolates in all three genotypes. A multiple sequence alignment was generated to identify well-conserved sequences in the 3' untranslated region of RNA-2 from six isolates belonging to genotype 1 (M96148, AB054689, and AF059533), genotype 2 (AF059532 and EU421060), and genotype 3 (KP223324).

Table 1. Comparative sensitivity between the new quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (SqMV_2015) and the old qRT-PCR (SqMV_2011) in detecting *Squash mosaic virus* (SqMV) isolates from two genotypes of SqMV^a

Sample dilution ^b	Experiment 1		Experiment 2		Experiment 3	
	SqMV_2015	SqMV_2011	SqMV_2015	SqMV_2011	SqMV_2015	SqMV_2011
RZ-SqMV RNA (genotype 3)						
1:100	21.83	No Ct	22.49	No Ct	22.04	No Ct
1:1,000	25.80	No Ct	24.92	No Ct	25.52	No Ct
1:10,000	29.78	No Ct	28.59	No Ct	29.21	No Ct
1:100,000	33.74	No Ct	32.72	No Ct	32.43	No Ct
1:1,000,000	37.20	No Ct	36.60	No Ct	35.65	No Ct
1:10,000,000	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
CA-SqMV RNA (genotype 1)						
1:1	11.94	15.21	13.53	15.80	13.86	15.56
1:10	16.28	17.87	15.69	18.21	16.04	18.60
1:100	19.12	21.43	18.71	20.83	19.02	21.53
1:1,000	22.72	24.80	22.81	24.55	22.73	24.57
1:10,000	28.60	29.99	32.32	33.22	33.92	35.57
1:100,000	32.38	35.51	34.94	36.88	No Ct	No Ct
1:1,000,000	36.79	37.87	No Ct	No Ct	No Ct	No Ct
1:10,000,000	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1:100,000,000	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Nontemplate (H ₂ O)	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct

^a Ct = cycle threshold.

^b Dilution 1:100 was equivalent to the purified plant RNA preparation at 100 ng/μl.

Table 2. Development of a sensitive and reliable duplex quantitative reverse-transcription polymerase chain reaction system (SqMV_2015) for a seed health assay to detect *Squash mosaic virus* (SqMV), with the 18S ribosomal RNA (rRNA) as an internal control^a

Preparations ^b	Experiment 1 (Ct)		Experiment 2 (Ct)		Experiment 3 (Ct)		Means Ct	
	CY5/18S rRNA	FAM/SqMV	CY5/18S rRNA	FAM/SqMV	CY5/18S rRNA	FAM/SqMV	CY5/18S rRNA	FAM/SqMV
5 seeds	10.85	13.92	11.12	13.44	11.60	14.56	11.19	13.97
10 seeds	10.43	11.99	11.27	12.89	11.13	13.47	10.94	12.78
100 seeds	11.61	16.54	11.76	15.24	11.87	16.08	11.75	15.95
CK1	10.12	No Ct	10.02	No Ct	9.77	No Ct	9.97	No Ct
CK2	11.04	No Ct	11.39	No Ct	10.74	No Ct	11.05	No Ct
CK+	17.47	21.62	17.26	20.49	17.40	20.74	17.38	20.95

^a Ct = cycle threshold.

^b CK1 = RNA preparations prepared from healthy hybrid cantaloupe melon seed, CK2 = RNA preparations prepared from healthy pumpkin seed, and CK+ = RNA preparation prepared from RZ-SqMV (genotype 3)-infected melon tissue. RNA template concentration was 100 ng/μl for all samples.

Table 3. Comparative analysis between the new quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (SqMV_2015) and the old qRT-PCR (SqMV_2011) for detection of *Squash mosaic virus* (SqMV)-infected seed and tissue samples

Isolate designation	Genotype ^a	Country of origin	Crop species	Tissue type	Year of collection	qRT-PCR (SqMV_2015)	qRT-PCR (SqMV_2011)
13896.1	1	Peru	Melon	Seed	2011	19.72	21.26
13896.2	1	Peru	Melon	Seed	2011	18.53	18.89
13896.3	1	Peru	Melon	Seed	2011	25.37	27.50
13896.4	1	Peru	Melon	Seed	2011	26.24	28.28
13896.5	1	Peru	Melon	Seed	2011	23.88	24.57
13896.6	1	Peru	Melon	Seed	2011	24.89	25.15
13896.7	1	Peru	Melon	Seed	2010	16.34	19.70
13896.8	1	Peru	Melon	Seed	2010	18.90	22.00
13896.9	1	Netherlands	Rootstock	Seed	2012	17.04	17.60
13896.10	1	Netherlands	Rootstock	Leaf	2012	14.63	15.62
13896.11	3	Spain	Melon	Leaf	2010	13.02	No Ct
13896.12	2	Spain	Melon	Leaf	2012	13.86	14.77
13896.13	1	Peru	Melon	Seed	2010	12.96	15.35
13896.14	3	Spain	Squash	Leaf	2010	22.83	No Ct
Nontemplate	-	-	-	-	-	No Ct	No Ct

^a Genotype of these isolates was determined based on partial sequence information obtained on PCR products amplified in two sequence regions using primers specific to M96148 targeting 440 to 968 or 1,619 to 2,077 nucleotides. Phylogenetic relationship analysis was carried out using the unweighted pair-group method with arithmetic means, Jukes-Cantor nucleotide distance measure, and a bootstrap of 100 replicates. Representative isolates for each genotype—genotype 1 (GenBank accession numbers M96148, AB054689, and AF059533), genotype 2 (AF059532 and EU421060), and genotype 3 (KP223324)—were used for alignments to determine their respective genotypes for the test isolates.

On the other hand, the CA-SqMV was a cDNA product generated from 320 ng of total RNA in a 20- μ l reaction volume. This cDNA product was diluted in 10-fold increments with RNase-free water up to 1×10^{-8} (Table 1). An aliquot of 0.5 μ l of each dilution was added to each qRT-PCR. These comparative experiments were repeated three times (Table 1). The results showed that the SqMV_2015 qRT-PCR system was able to detect both genotypes at 1×10^{-4} to 1×10^{-6} dilutions (Table 1). However, the old SqMV_2011 qRT-PCR system (Ling et al. 2011) could only detect genotype 1 (CA-SqMV) isolates but not genotype 3 (RZ-SqMV) isolates (Table 1). Remarkably, the SqMV_2015 qRT-PCR system generated stronger fluorescent signals with approximately 1 to 3 cycle threshold (Ct) values less (more sensitive) than that of the SqMV_2011 qRT-PCR system using the same preparation for detection of SqMV in CA-SqMV preparations (Table 1).

Development of a duplex qRT-PCR system with an internal 18S rRNA control to improve sample integrity and detection reliability. RNA integrity is a pivotal factor for detection reliability. Poor quality of RNA preparation often resulted in high Ct value and even no Ct value. To enhance the detection reliability, 18S rRNA-specific probe and primers were included in each reaction. One preparation from five healthy hybrid cantaloupe melon seeds and another from two healthy pumpkin seeds were also included as negative controls. Consistent strong Ct values (10.94 to 11.75) generated in the duplex qRT-PCR system (Table 2) from 18S rRNA internal controls in healthy samples and in virus-infected seed preparations (5, 10, and 100 seeds) suggested that the quality of RNA preparations was suitable for qRT-PCR. Thus, such low Ct values obtained from those test samples indicated that those seed samples did, indeed, carry SqMV. Therefore, those other samples with nondetectable levels of readings (no Ct) could be unambiguously called SqMV free (Table 2).

Application of the SqMV_2015 qRT-PCR for seed and plant health tests. In this study, the new TaqMan RT-PCR (SqMV_2015) developed was capable of detecting SqMV isolates in diverse genotypes. Application of this sensitive and reliable SqMV detection system should help the seed industry to make a science-based decision on an SqMV-contaminated seed lot or an infected crop. Therefore, any potential introduction of a contaminated seed lot due to a likely poor result from possible lower sensitivity using ELISA could be minimized using this highly sensitive and reliable virus detection technology. The duplex detection system with an internal control should also offer additional advantages to evaluate the quality of RNA preparation as well as the reliability of the test results.

To determine whether the newly developed SqMV_2015 qRT-PCR system could offer a broad-spectrum reaction to various SqMV isolates collected around the world, seed and leaf samples from Peru, Spain, and the Netherlands that represent three different SqMV genotypes based on partial sequencing were included for testing. Results showed that the SqMV_2015 qRT-PCR system was capable of detecting all SqMV isolates in three genotypes (Table 3). On the other hand, the SqMV_2011 qRT-PCR system was not able to detect two SqMV samples (13896.11 and 13896.14) belonging to genotype 3 (Table 3). Confirmation of these isolates as genotype 3 of SqMV was determined using genome sequencing, as previously reported (Li et al. 2015).

Discussion

In the present study, a sensitive and broad-spectrum qRT-PCR system (SqMV_2015) was developed to allow for a sensitive detection of all genotypes of SqMV, including the newly recognized genotype 3 (Li et al. 2015). Such improvement in SqMV detection was necessary due to the understanding of a possible serological specificity (Knuhtsen and Nelson 1968) as well as the recognition of a greater genetic diversity (Li et al. 2015). With the existence of greater genetic diversity of SqMV, the previously developed qRT-PCR system (SqMV_2011) that recognized isolates from genotypes 1 and 2 (Ling et al. 2011) would need improvement to allow for detection of all isolates, including those from genotype 3. In the development of this new SqMV_2015 qRT-PCR detection system, a very well-conserved sequence region identified in the 3' UTR of RNA2 was used. On the other hand, the old SqMV_2011

qRT-PCR detection system was developed based on a relatively conserved sequence region selected in the coding region of RNA2 for a small coat protein (Ling et al. 2011). Apparently, sequences in the 3' UTR were more conserved among various isolates of SqMV in different genotypes than those in the coding region. This may explain why the newly developed qRT-PCR (SqMV_2015) was more sensitive and reliable in detecting diverse isolates of SqMV. In addition, by incorporating an internal amplification control of 18S rRNA (Osman et al. 2007), the duplex qRT-PCR system could be reliably used in seed and plant health assays. Such a duplex system not only would allow assessment of the sample RNA preparation quality but also an accurate interpretation of the results.

Understanding and characterizing the genetic diversity of a virus is important in developing a sensitive and reliable PCR-based detection method. For SqMV, the SqMV_2011 qRT-PCR system was developed based on two known genotypes at that time and was capable of detecting both genotypes in seed and plant health tests (Ling et al. 2011). With recent identification and sequencing of RZ-SqMV, a third genotype was recognized (Li et al. 2015). The newly developed SqMV_2015 qRT-PCR system was created based on the current knowledge of the SqMV genetic diversity in three genotypes. As more SqMV isolates from the world are collected and sequenced, it is possible that other genotypes could be identified. If that is the case, then additional improvements to qRT-PCR might be necessary to detect those emerging isolates with greater confidence.

A reliable disease diagnosis is dependent on accurate and sensitive virus detection. With technology improvement and more affordable real-time PCR equipment and reagents, qRT-PCR has been broadly adapted for plant virus detection. With the existence of genetic diversity, selection of conserved sequence regions that could be suitable for primer and probe design is important. Improvement to the qRT-PCR detection technology should be based on the well-characterized virus genetic diversity and validation of the technology on field-collected samples. In addition, the design strategy in using a duplex qRT-PCR with an internal amplification control may have general implications for improving the reliability of qRT-PCR on virus detection in routine disease diagnosis.

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