**Detection of *Strawberry necrotic shock virus* using conventional and TaqMan®
quantitative RT-PCR**

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Abstract

Graft-indexing of an advanced selection from the University of Florida strawberry breeding program produced virus-like symptoms on *Fragaria vesca*. However; RT-PCR testing of the material did not detect the presence of any of 16 strawberry virus species or members of virus groups for which strawberries are routinely indexed. Large scale sequencing of the material revealed the presence of an isolate of Strawberry necrotic shock virus. The nucleotide sequence of this isolate from Florida shows a significant number of base changes in the annealing sites of the primers compared to the primers currently in use for the detection of SNSV thereby explaining the most the probable reason for the inability to detect the virus in the original screening. RT-PCR and Taqman® qPCR assays were developed based on conserved virus sequences identified in this isolate from Florida and other sequences for SNSV currently present in GenBank. The two assays were applied successfully on multiple samples collected from several areas across the United States as well as isolates from around the world. Comparison between the RT-PCR and the qPCR assays revealed that the qPCR assay is at least 100 times more sensitive than conventional PCR.

Keywords: *Strawberry necrotic shock virus*, virus detection, Taqman qPCR

28 Introduction

29 Strawberry (*Fragaria* spp.) is infected by multiple virus and virus-like agents (Tzanetakis and
30 Martin, 2013; Xiang et al., 2015). One of those agents is the ilarvirus *Strawberry necrotic*
31 *shock virus* (SNSV), first characterized from strawberry in 1956 (Frazier et al., 1962). The
32 virus name was derived from the transient necrotic reaction induced when infected tissue is
33 grafted onto *F. vesca* var. *semperflorens* 'Alpine'. SNSV does not cause visible symptoms on
34 modern strawberry cultivars when present as single infection (Martin and Tzanetakis, 2006)
35 but may act synergistically and causes decline association with other viruses that infect
36 *Fragaria* and *Rubus* (Martin and Tzanetakis, 2013; Martin et al., 2013).

37 SNSV belongs to subgroup 1 of the genus *Ilarvirus*, family *Bromoviridae* (Tzanetakis
38 et al., 2004). Ilarviruses have a single-stranded, positive-sense, tripartite RNA genome. RNA
39 1 is translated to a polyprotein with methyltransferase and helicase domains. RNA 2
40 encodes the RNA dependent RNA polymerase and a putative suppressor of RNA silencing
41 (Shimura et al., 2013) in members of Ilarvirus subgroups 1 and 2. RNA 3 encodes the
42 movement and coat proteins (MP and CP respectively), the latter being expressed via a
43 subgenomic RNA, RNA 4 (King et al., 2012).

44 Ilarviruses are distributed unevenly in plant tissues and develop relatively low titres
45 that may fluctuate during the growing season (Uyemoto et al., 1992; Pallas et al., 2012).
46 These traits makes detection a challenging undertaking and there has been a need for
47 sensitive tests to ensure reliable detection and indexing, a premise satisfied in many cases
48 by PCR and its variants (Barker et al., 1993; Mekuria et al., 2003; Dang et al., 2009).
49 Oligonucleotide primers CPbegF/CPendR, designed to study the population structure of
50 SNSV (Tzanetakis et al., 2004), have also been used for routine detection of the virus. These

primers were designed in the untranslated regions flanking the CP gene, in which mutations are more likely to occur compared to coding regions that are under selective pressure.

The present study was initiated when a University of Florida strawberry breeding selection (FL) failed to pass graft indexing (Converse, 1987) and showed virus-like symptoms. None of 16 viruses and virus groups reported in strawberry, namely *Arabis mosaic virus*, *Beet pseudo- yellow virus*, *Cucumber mosaic virus*, *Fragaria chiloensis latent virus*, *Prunus necrotic ring spot virus*, *Raspberry ring spot virus*, *Strawberry crinkle virus*, *Strawberry latent ring spot virus*, *Strawberry mild yellow edge virus*, *Strawberry mottle virus*, *Strawberry necrotic shock virus*, *Strawberry pallidosis associated virus*, *Strawberry vein banding virus*, *Tobacco ring spot virus*, *Tomato ring spot virus* and genus *Tospovirus* could be detected by the RT-PCR assays currently used for indexing strawberry germplasm (California Seed and Plant Lab Inc, Elverta, California).

Material was subjected to large scale (or next generation) sequencing, revealing the presence of SNSV. This necessitated the development of new, more sensitive, method(s) for SNSV to insure detection. This communication reports the characterization of the FL isolate of SNSV and development of conventional and TaqMan® qPCR assays based on newly identified highly conserved sequences.

Materials and Methods

Amplification and sequencing of SNSV FL RNA 3

Double stranded RNA (dsRNA) enriched nucleic acids were extracted from the leaf samples and were subjected to 454 pyrosequencing as described by Ho et al. (2015). Specific primers were designed from the 5' and 3' termini of RNA 3 (Table 1) of the SNSV type isolate

(GenBank accession NC_008706). The full length RNA of the FL isolate was amplified from cDNA produced for the pyrosequencing and LA *Taq* DNA polymerase kit (Clontech Laboratories, Inc., CA). The PCR mixture contained LA Buffer II (proprietary; 25mM Mg²⁺ plus), 0.4 mM dNTPs, 400 nm each of the primers and 0.5 unit of LA *Taq* polymerase. The molecule was amplified using initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 20s, annealing at 52°C for 20s and extension at 72°C for 2 min 30s terminating with a 5 min extension at 72°C. The product was purified using the GeneJET PCR purification kit (Thermo Fisher Scientific, MA), cloned into PCR2.1 TOPO vector (Life Technologies, CA) and introduced in to *Escherichia coli* competent cells by electroporation. Three recombinant plasmids were sequenced using SNSV CPR1 (5'-AGCGGCATCCAACTTGTCAA-3'), M13 forward and M13 reverse primers and assembled using CAP3 (Huang and Madan, 1999) to obtain the consensus sequence of the molecule.

Nucleic acid extraction and reverse transcription

Total nucleic acids were extracted from leaf tissues of 177 samples collected from nurseries and commercial fields in Arkansas, California, Missouri and the National Clonal Germplasm Repository in Corvallis, Oregon essentially according to the method of Poudel et al. (2013). Reverse transcription (RT) was conducted using Maxima RT buffer, 2U/μl volume Maxima reverse transcriptase, 0.25U/μl volume RiboLockTM RNase inhibitor (all three from Thermo Scientific), total nucleic acids at 10% volume, 6 ng random primers, 0.4 mM dNTPs. Random primers were chosen over a SNSV-specific primer with the notion that the reaction could be used not only for the detection of SNSV but all *Rubus* or strawberry viruses as is the common practice in laboratories working with berry viruses. The mixture was incubated first

at 50°C for 75 min followed by 5 min at 85°C for enzyme inactivation. The cDNA products were stored at -20°C until used.

Assay design and standardization

RNAs 1 and 2 of subgroup 1 ilarviruses are highly conserved; showing about 70% nt identity between species (Tzanetakis et al., 2010). Yet, the CP gene presents a better target because of its abundance, being present in RNAs 3 and 4, an important factor given the low titer of ilarviruses (Uyemoto et al., 1992; Pallas et al., 2012). In addition, there is more information on the CP sequence variability compared any other gene coded by the virus.

All available (21) SNSV CP sequences (Table 2, last accessed May 20th 2016) were aligned using MUSCLE (Edgar, 2004). Several primer sets for the conventional PCRs were designed to have 100% nucleotide identity to all isolates. After preliminary trials with different primer combinations and PCR conditions (data not shown), conditions were optimized for a reaction that amplify both a 372 base region of the CP gene using primers SNSVuniF/R (Table 1) and a 721 base region of the NADH dehydrogenase ND-2 subunit transcript (Tzanetakis et al., 2007), included in the reaction to ensure the integrity of the extracted nucleic acids. The reaction mixture (25 µl) contained equal amount of virus and internal control primers (400 nM each), 10X *Taq* buffer (proprietary), 400 µM dNTPs, 0.5U of *Taq* DNA polymerase (Genscript, NJ) and RNase/DNase-free water. The PCR reactions run on either a Mastercycler GX2 (Eppendorf) or a C1000 thermocycler (Biorad Laboratories) initiated with 2 min denaturation at 94°C followed by 40 cycles of denaturation at 94°C for 20s, annealing at 58°C for 20s and extension at 72°C for 30s. All PCR products were sequenced to confirm the identity of the products.

RealTime PCR tool (Integrated DNA Technologies - IDT) was used to investigate the suitability of CP regions for Taqman[®] qPCR oligo/probe design before sequence alignment. Oligo physical properties were checked using OligoAnalyzer tool (version 3.1, IDT). Several qPCR oligo/probe sets were synthesized with a fluorophore group (FAM) at the 5' end, a quencher group (3' Iowa Black FQ) at the 3' end, and an additional quencher (ZEN) at the 10th nucleotide from the 5' end to reduce background emission noise. Working concentrations of oligo/probe were set at 500 and 250 nM, respectively. Routine quantitative PCR was performed with cDNA corresponding to 0.4ng/μl of total RNA using the 2x Universal Master Mix (proprietary; Thermo Scientific). Reactions were run on either a CFX96 qPCR thermocycler (BioRad Laboratories) or an ABI 7500 Fast machine (ThermoFisher) with a program comprising of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

Quality and sensitivity

A region of RNA 3 was amplified using primers 1276F/1911R (Table 1 and

Name	Call	Ct value
Sample 1	Presence	17.8
Sample 2	Presence	20.9
Sample 3	Presence	20.5
Sample 4	Presence	19.9
Sample 5	Presence	20.4
Sample 6	Presence	20.2
Sample 7	Presence	21.9
Sample 8	Presence	22.4
Sample 9	Presence	21.4
Sample 10	Presence	21.6
Sample 11	Presence	21.6
Sample 12	Presence	20.5
Sample 13	Presence	19.3
Sample 14	Presence	19.2
Sample 15	Presence	20.8
Sample 16	Presence	22.2
Sample 17	Presence	21.3
Sample 18	Presence	18.9
Healthy	Absent	N/A
RTwater	Absent	N/A
qPCRwater	Absent	N/A

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156 Figure 1A) and the product was cloned and sequenced as described above. The 10 pmol of
157 SpeI-linearized recombinant plasmid template was *in vitro* transcribed with T7 primer
158 according to the manufacturer's instruction (Thermo Scientific). The reaction was
159 terminated using 1:20 vol/vol of 0.5 M EDTA, ethanol-precipitated, and digested with 1U of
160 RNase-free DNase I (Thermo Scientific) for 1 h at 37 °C. The transcribed RNA was purified,
161 quantified and stored at -80°C until use.

162 Fifty (50) ng of RNA was used for RT as described above using a 0.4 mM primer
163 1933R (Table 1). Ten-fold serial dilutions of the cDNA corresponding to 0.4 ng-0.04 fg/μl
164 RNA were prepared. Standard curves were constructed from the cDNA dilutions as
165 described earlier (Poudel et al., 2014). To eliminate variability due to reagents/enzymes, the
166 same cDNA dilutions were used in the comparison of assays, in which conventional PCRs

reactions were set-up using the 2X Taqman® mastermix, primers SNSVuniF/R and RNase/DNase-free water applying the conventional PCR program described above.

Results

Analysis of the large scale sequencing-derived data (Ho et al., 2015) revealed that the FL selection was infected by an isolate of SNSV (GenBank accession No KU053514). The FL isolate RNA 3 is 2249 nucleotide (nt) and shared 93% nt identities to the type isolate (GenBank accession NC_008706) and 99% identity to isolate SNSV-840 from Australia (Sharman et al., 2011). Sequence alignments confirmed several mismatches in the CPbegF/CPendR priming sites, one in CPbegF and four in CPendR, the putative reason behind the false negative in the original RT-PCR screening.

Primer set SNSVuniF/R (Table 1) shows 100% identity to all available SNSV sequences and was successfully used in the amplification of 55 isolates from both *Rubus* and strawberry which included isolates AY363229- AY363235 and AY363241 (Table 2). The SNSV products were preferentially amplified compared to the internal control in infected material (Fig. 1). In SNSV-free samples, the internal control product was efficiently amplified.

Among the several PCR oligos/probe sets designed and tested for qPCR, assay SNSV1667 (Table 1) was selected for routine detection based on Cq values consistency, intensity and noise of fluorescence signals (Fig. 1). Standard curve was constructed with efficiency of 91.5%, correlation coefficient=0.985, and y-intercepts=43.010 (Figure 2).

The comparison of conventional and qPCR assays using a 10-fold serial dilutions of cDNA corresponding to the RNA concentration from 0.4ng-0.04 fg/μl confirmed significantly

higher sensitivity for the qPCR assay. The conventional PCR provided visible amplicons at a 4pg/ μ l, whereas the qPCR assay could detect signal up to a 40fg/ μ l (Figure 3).

Discussion

All major berry crops are expanding to new areas and are exposed to new pathogens and/or new pathogen combinations (Martin et al., 2013; Tzanetakis and Martin 2013). In general terms there is significant knowledge on the effects of single virus infection in berry crops (Jelkmann et al., 1990; Posthuma et al., 2000; Thompson and Jelkmann, 2003). The situation with SNSV is more complex. Since the early 1970s SNSV was hypothesized to be a strain of *Tobacco streak virus* (TSV), primarily based on immunology (Jones, 1975; Johnson et al., 1984; Stace Smith et al., 1987). In the period between the assignment of the SNSV as a strain of TSV and the re-establishment as a stand-alone species (Tzanetakis et al., 2004), there have been several publications on 'TSV' infecting berry crops (Converse, 1972; 1979; Finn and Martin, 1996). Whether those refer to TSV or SNSV is unknown, limiting our knowledge of the effect of the individual viruses on the crops. Even if the effect of SNSV in single infections is minimal, there is only speculation on its effects in mixed infections. This is of immense importance in berry crops as more often than not, mixed infections have devastating effects on plant health. An example of a berry virus complex is that of *Blackberry yellow vein associated virus* and *Blackberry virus Y* both of which are asymptomatic in single infections but could cause blackberry death when they co-infect plants (Susaimuthu et al., 2008). All those facts make reliable detection of a virus that is emerging in both strawberry and *Rubus* (Li and Yang, 2011; Sharman et al., 2011; Tzanetakis, 2012; Silva-Rosales et al., 2013; Khadgi, 2015) a significant tool for disease control.

The primer set developed previously for the study of SNSV population structure was also used for detection. As the FL isolate was not detected by this primer set, we developed two sensitive assays based on more conserved areas of the SNSV genome, providing better confidence in the results. Whereas the efficiency of the qPCR assays allows for an assessment of the quality of the template this is not the case for conventional PCR. For this reason, the SNSV conventional RT-PCR assay is combined with an internal control, the NADH dehydrogenase ND-2 subunit which provides extra assurance on the quality of the nucleic acids. Amplification of a 721 base pair NADH product indicates good RNA quality appropriate for the assay (Fig. 1). The absence of the 721 bp band but presence of a ~1300 bp product (depending on the plant species) indicates that the RNA is degraded and the primers amplified the genomic copy of the gene, including an intron. If there are no visible bands then there is either, human error, significant nuclease contamination or inhibitors that do not allow for optimal amplification of the template. In SNSV-infected samples, the conventional PCR reaction preferentially amplified virus specific products (Fig. 1). Hence, this protocol provides answers not only the presence of the virus but also the integrity of the assay.

The optimum length for a Taqman[®] qPCR assay is about 150 nt long, and with a standard curve with efficiency close to 100%, a correlation coefficient of more than 0.99, slopes close to -3.32, and y-intercepts close to 40 (Anonymous, 2016a). Due to the diversity in the SNSV isolates deposited in GenBank, the qPCR product is 295 nt long and the primers include three degenerate positions making it less than optimal for absolute quantification of the virus, which, in any case, was not one of the expected outputs of the research presented here but has proven to work optimally on field-collected material (Fig. 1). The protocol has

the advantage of higher detection sensitivity, 100 times more sensitive to the detection range of conventional PCR, and requires shorter preparation time. Still both assays provide excellent detection and are recommended as new standard diagnostic tests for SNSV, especially for high quality propagation material as those entering certification programs (G1) and need to be tested for all *Rubus* and strawberry viruses of concern (Anonymous, 2016b).

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337 **Tables**

338 Table 1. List of primers used for the amplification and detection of *Strawberry necrotic*
 339 *shock virus* (SNSV). F: Forward, R: Reverse

Primer name	Primer sequences (5'-3')
RNA3-5'F	GTATTCTCTGAGCATAATACCACTT
RNA3-3'R	GCATCTCCTTAGGGAGGCATCATTAA
1933R	ACTCWTWGGWGGCATCARCG
1276F	CCATCCAACGCCATGTCTGT
1911R	ATTMRGTCGTGATATGGCGA
SNSVuniF	AACAACTCCAATGGTTGCCCAACT
SNSVuniR	ACCAAATGTCCCATCGGACACGGCA
	Probe: TTGCCGTGTCCGATGGGACATTTGGT
SNSV1667	F: CCGAAATCAATGAYGACACGAAGA
	R: ATTMRGTCGTGATATGGCGA

340 Table 2. *Strawberry necrotic shock virus* isolates used for the designing of primers and
 341 probes for conventional and qPCR detection assays.

No.	Genbank Accession	Host	Country
1	JF781586	<i>Fragaria x ananassa</i>	Australia
2	JF781587	<i>Fragaria vesca</i>	Australia
3	JF781588	<i>Fragaria vesca</i>	Australia
4	HQ830017	<i>Fragaria x ananassa</i> <i>Fragaria x ananassa</i> cv.	China
5	AY363229	'Ooishi-shikinari' (NCGR 1291)	Japan
6	AY363230	<i>Fragaria x ananassa</i> cv. 'Koro' (NCGR 1268)	Japan
7	AY363231	<i>Fragaria x ananassa</i> cv. 'Sokusei' (NCGR 1294)	Japan
8	JQ629415	<i>Fragaria x ananassa</i>	Irapuato, Mexico
9	AY363228	<i>Fragaria x ananassa</i>	Maryland, USA
10	AY363232	<i>Fragaria x ananassa</i> cv. 'US-70' (NCGR 1189)	Mississippi, USA
11	AY363233	<i>Fragaria x ananassa</i> cv. 'US-159' (NCGR 1191)	Mississippi, USA
12	AY363234	<i>Fragaria x ananassa</i> cv. 'Melcher 86-17' (NCGR 1196)	Louisiana, USA
13	AY363235	<i>Fragaria x ananassa</i> cv. 'Sweet Charlie' (NCGR 1314)	Florida, USA
14	AY363236	<i>Fragaria x ananassa</i> (NCGR 9034)	California, USA
15	AY363237	<i>Fragaria x ananassa</i> (NCGR 9078)	North Carolina, USA
16	AY363238	<i>Rubus ursinus</i> cv. 'Dyke' (NCGR 139)	Pacific northwest, USA
17	AY363239	<i>Rubus</i> hybrid cv 'New Logan' (NCGR 9012)	Pacific northwest, USA
18	AY363240	<i>Rubus ursinus</i> (NCGR 9025)	Pacific northwest, USA
19	AY363241	<i>Rubus occidentalis</i> cv. 'Munger' (NCGR 9003)	Pacific northwest, USA
20	AY363242	<i>Rubus idaeus</i>	Pacific northwest, USA
21	KU053514	<i>Fragaria x ananassa</i>	Florida, USA

Figures

(A)

(B)

Name	Call	Ct value
Sample 1	Presence	17.8
Sample 2	Presence	20.9
Sample 3	Presence	20.5
Sample 4	Presence	19.9
Sample 5	Presence	20.4
Sample 6	Presence	20.2
Sample 7	Presence	21.9
Sample 8	Presence	22.4
Sample 9	Presence	21.4
Sample 10	Presence	21.6
Sample 11	Presence	21.6
Sample 12	Presence	20.5
Sample 13	Presence	19.3
Sample 14	Presence	19.2
Sample 15	Presence	20.8
Sample 16	Presence	22.2
Sample 17	Presence	21.3
Sample 18	Presence	18.9
Healthy	Absent	N/A
RTwater	Absent	N/A
qPCRwater	Absent	N/A

Figure 1. (A) Positions of primers/probe developed for the conventional and qPCR detections in conserved regions of *Strawberry necrotic shock virus* (SNSV) RNA 3. The base line represent the RNA 3 region selected for the development of PCR and qPCR primers and probe. Lines represent the regions amplified in conventional (SNSVuniF/R and CPbegF/CPendR) and qPCR assays (SNSV1667) whereas arrows indicate the primer annealing sites. (B) Top: Agarose gel electrophoresis of conventional PCR for SNSV detection including virus specific and internal control primers multiplexed; Bottom: RT-qPCR assay for SNSV detection of field material (C): Agarose gel electrophoresis of RT-PCR amplification of the internal control, NADH dehydrogenase ND-2 subunit gene, using different templates. Lane 1: 1Kb DNA marker; lane 2: SNSV-free, high quality nucleic acid; lane 3: Degraded RNA; lane 4: water control, lane 5: Impure RNA with inhibitors; lane 6: SNSV-infected, high quality nucleic acid.

Figure 2. Standard curves for the *Strawberry necrotic shock virus* TaqMan® quantitative PCR assay. Cross marks represent cDNA dilutions. E = efficiency; R^2 = correlation coefficient; y-int = y-intercepts.

A.

B.

Figure 3. Comparison of detection sensitivity of conventional PCR and qPCR assays of *Strawberry necrotic shock virus* (SNSV). The cDNA obtained from *in vitro* transcribed RNA was serially diluted 10-fold with the corresponding concentration of RNA ranging from 0.4 ng-0.04 fg/ μ l and used for both conventional and qPCR assays. Agarose gel electrophoresis of the amplified products from conventional PCR assay detecting SNSV up to 4pg virus RNA/ μ l (A) and qPCR amplification curves showing detection of SNSV up to 40fg virus RNA/ μ l (B).