European Journal of Plant Pathology **110:** 45–52, 2004.

Detection and tentative grouping of *Strawberry crinkle virus* isolates

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Accepted 5 August 2003

Key words: L protein, molecular beacon, NASBA, rhabdovirus, SCV, strawberry

Abstract

A partial sequence of the putative polymerase (L) protein of *Strawberry crinkle virus* (SCV), genus *Cytorhabdovirus*, is described. The virus protein was found to be distantly related to the L protein of the rhabdoviruses *Northern cereal mosaic virus*, *Rice yellow stunt virus* and *Sonchus yellow net virus*. Moreover, a tentative grouping of SCV isolates is described, based on phylogenetic analysis of a region enclosing the GDN-motif within the RNA-dependent RNA polymerase gene. A sequence homology of 98% was found for each tentative group, and heterogeneity of at least 11% was observed between both groups. The tentative grouping did not appear to be related to symptomatology or geographical origin of the isolates. Nevertheless, the use of several reverse-transcription (RT)-PCR primer sets, all directed to different regions within the SCV genome, confirmed the presumptive classification into two groups, namely group I (isolates 1554, KG and Post), and group II (isolates 1553, Hb-A1, 37-1 and 37-2). Additionally, the detection of SCV isolates from herbaceous hosts and strawberry plant material was possible through use of a newly developed gel-based RT-PCR and a gel-free AmpliDet RNA assay. Both methods have the potential to provide rapid, sensitive and specific detection of SCV in *in vitro* propagation material.

Introduction

Strawberry crinkle virus (SCV), genus *Cytorhabdovirus* in the family *Rhabdoviridae*, is a common virus infecting strawberry plants and is responsible for reduction in fruit yield and quality. The natural host range of SCV is restricted to members of the genus *Fragaria.* The virus is transmitted by two aphid genera, namely *Chaetosiphon fragaefolii* and *Chaetosiphon jacobi* (Richardson et al., 1972; Vaughan, 1933). The negative sense genomic organisation resembles that of animal and plant rhabdoviruses, consisting of at least five proteins; glycoprotein (G), nucleocapsid (N) protein, nonstructural (Ns) protein, matrix (M) protein and polymerase (L) protein (Hunter et al., 1990; Schoen and Leone, 1995).

At present, detection of the virus depends on leaf grafting on strawberry indicator plants (Frazier et al., 1987), since serological methods are not sufficient. Recently, polyclonal and monoclonal antibodies were raised against the M, N and Ns proteins of the SCV (Schoen and Leone, 1995). Unfortunately, these antibodies only enabled the serological detection of SCV in herbaceous hosts like *Physalis pubescens*, but not in strawberry plant material. To allow for the development of a molecular detection technique, a partial sequence of the L protein of SCV was elucidated (Posthuma et al., 2002). Although the detection of SCV in *P. pubescens*

was successful, it failed to reliably detect SCV in strawberry indicator plant material.

Recently, a gel-free method for the amplification and detection of RNA, called AmpliDet RNA (Leone et al., 1998), was described based on the nucleic acid sequence-based amplification (NASBA) (Compton, 1991; Kievits et al., 1991) combined with the molecular beacon technology (Tyagi and Kramer, 1996). The method allows the sensitive and robust detection of different RNA targets (messenger RNA, viral RNA, ribosomal RNA) and has been applied to the detection of several plant viruses, such as *Apple stem pitting virus* in woody tissues (Klerks et al., 2001b), *Sugarcane yellow leaf virus* in aphids, and sugarcane (Gonçalves et al., 2002) and potato viruses in potato (Leone et al., 1998; Klerks et al., 2001a; Szemes et al., 2002).

In this paper, a comparison of conserved regions by partial sequence analysis of the putative RNA-dependent RNA polymerase gene (L protein) of different SCV isolates is described. Moreover, a tentative grouping within the collection of SCV isolates is indicated based on alignment of nucleic acid and amino acid sequences. Finally, an easy approach to detection of all SCV isolates in strawberry plants is presented, relying on either a gel-based RT-PCR method or a gel-free AmpliDet RNA method.

Materials and methods

Plant materials and virus isolates

Different SCV isolates originating from The Netherlands (1554, 1553, 37-1, 37-2, R2-7) and Germany (Hb-A1, Hb-B2, KG) were maintained in Strawberry UC-5 indicator plants (Schoen and Leone, 1995). Symptom development was followed for each isolate in at least five strawberry UC-5 indicator plants. Severeness of infection was scored by means of indexing the following symptoms: stunting, local lesions, crinkling and infection rate.

To maintain the virus isolates in a herbaceous host, strawberry leaves were ground in a 1% nicotine solution and used for mechanical inoculation onto *Nicotiana occidentalis* P1. The herbaceous host showed local lesions when infected with SCV. Each week local lesions were excised and were ground for mechanical inoculation onto a new *N. occidentalis* P1 plant. This procedure was repeated until the number of local lesions was sufficient (at least 50 lesions per leaf). Subsequently, 50 lesions were ground in a 1% nicotine solution and used for mechanical inoculation onto *P. pubescens*. This herbaceous host is susceptible to SCV infection and shows local and systemic symptoms when infected. Accordingly, the SCV isolates were further maintained in *P. pubescens* by mechanical inoculation onto uninfected seedlings (Schoen and Leone, 1995).

Infected leaves (5 cm^2 each) from *P. pubescens* and strawberry UC-5 indicator plants were separately ground using the Pollähner press and 1 ml of sample extraction buffer (SEB: 0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_2O$, pH 7.4, 0.05% Tween 20, 2% PVP 44000, 0.2% ovalbumin, 0.5% BSA and 0.05% Na-azide). Subsequently, 100 µl of the suspension was subjected to a commercial nucleic acid extraction kit (Plant RNeasy kit; Westburg) following the manufacturer's instructions. Eight SCV isolates, all maintained in both *P. pubescens* and strawberry UC-5, were used throughout the experiments.

Primers and probes

Reverse transcription (RT)-PCR of the viral RNA was enabled by selecting SCV-specific primers MKC-F (5′ CAT.TGG.TGG.CAG.ACC.CAT.CA 3′) and MKC-R (5′ TTC.AGG.ACC.TAT.TTG.ATG.ACA 3′). Both primers hybridised to a conserved region within the RNA-dependent RNA polymerase-coding gene to generate a specific amplification product of 345 bp. Design of the SCV-specific primers RPC (5′ AAT.TCT.AAT.ACG.ACT.CAC.TAT.AGG.GAG.GAA.TGA.TGG.GTC.TGC.CAC.CAA 3′) and FPC (5′ CCT.ATG.ATC.TTA.TGG.TCA 3′) for amplification of the viral RNA by NASBA (Biomerieux, Boxtel, The Netherlands) was also based on conserved regions within the RNA-dependent RNA polymerase-coding gene of SCV, flanking a sequence of 248 nucleotides. The primer RPC consisted of a 5′ T7-RNA polymerase recognition-site sequence (underscored) and a 3′ target complementary sequence. To enable the real-time detection of NASBA products, an SCV-specific molecular beacon was developed within a conserved region between the primers FPC and RPC. The molecular beacon (5′ GCA.CGT.ATC.TTC.CAG.CCA.CCT.GAT.GAA.CGT.GC 3′) consisted of 5′ and 3′ complementary arms of six nucleotides long (underscored), that enclosed a loop sequence of 20 nucleotides complementary to the specific NASBA product. The arms of

the molecular beacon were designed to form a double stranded structure at 41 °C, which would thereby prevent fluorescence from unhybridised molecular beacons due to fluorescence resonance energy transfer (FRET) (Stryer, 1978). The molecular beacon was labelled with 6-carboxyfluorescein (FAM) and the quencher 4-[4′-dimethylaminophenylazo]-benzoic acid (Dabcyl) at the 5′ and 3′ ends, respectively.

Detection of specific NASBA products was performed by Northern blotting, followed by enhanced chemiluminescent detection (ECL) using a 5′-biotinylated probe Bio-1 (5′ CCA.CCT.TAT.GAC.TCC.TCT.TCT.CT 3′) (methodology performed as described by Leone et al., 1998). The probe Bio-1 was designed to hybridise within the same region as the molecular beacon.

RT-PCR and sample preparation for sequencing

Each RT was performed using 10 µl of sample in 20 µl RT1-mix, consisting of 5 µl of 10× PCR Gold buffer (150 mM Tris–HCl, pH 8.5 and 500 mM KCl), 25 mM $MgCl_2$, 10 units of RNase-inhibitor, 3.5 µl of RNase-free water and 3 µl of reverse primer (5 µM). The sample was incubated for 5 min at 65 °C. Concurrently, 20 µl of RT2-mix (5 µl of dNTP-solution (10 mM of each dNTP diluted in RNase-free water), 10 units of RNase inhibitor, 12.5 units of MmuLV-reverse transcriptase and 14.25 µl of RNase-free water) was added to each reaction and incubated at 37 °C for 60 min and 95 °C for 5 min. The RT-products were stored at −20 °C prior to future use. PCR was performed by adding 5 µl of RT-product to a PCR mix (5 µl of 10× PCR Gold buffer, 8 µl of 25 mM $MgCl_2$, 5 µl of dNTP solution (10 mM of each dNTP diluted in RNase-free water), 1 µl of each primer (5 µM), 2.5 units of AmpliTaq Gold DNA polymerase and 24.5 µl RNase-free water). Each PCR reaction was heated at 95 °C for 10 min to activate the AmpliTaq Gold DNA polymerase before amplification. The PCR regiment for each PCR reaction consisted of 39 cycles of 15 s at 95 °C, 30 s at 58 °C and 60 s at 72 °C (the annealing temperature ranged between 50 ° and 60 °C in case the primer sets specifically designed for sequencing were used). After PCR the samples were stored at −20 °C prior to further analysis.

PCR products were analysed on a 1% pronarose gel (pre-stained with 0.5 µg ethidium bromide per millilitre), and run at 100 V for 60 min in buffer containing 40 mM Tris-borate and 1 mM EDTA, pH 8.0 (1xTAE). A 123 bp DNA marker was used to determine the product size. Prior to sequencing, total RNA was extracted from leaves of *P. pubescens*, each infected with an SCV isolate. The leaves were ground in liquid nitrogen and subsequently 100 mg of tissue was added to 350 µl of RLT-buffer (RNeasy) prior to RNeasy extraction.

Each RT-PCR primer was designed based on preliminary viral genomic sequence data (Schoen et al., submitted). Products obtained with RT-PCR that showed consistent results on a 1% pronarose gel with all SCV isolates tested, were cloned and sequenced. Nucleotide sequencing was performed at the Wageningen University and Research Center (Wageningen, the Netherlands).

NASBA and AmpliDet RNA

The NASBA was performed as previously described (Compton, 1991; Kievits et al. 1991). For each reaction the NASBA reaction mix consisted of 5 µl NASBA-reagents (160 mM Tris–HCl, pH 8.5, 48 mM $MgCl_2$, 2 mM DTT, 4 mM dNTP, 8 mM of each ATP, UTP and CTP, 6 mM GTP and 2 mM ITP), 0.7 µl 350 mM KCL, 4 µl 5× primer mix (75% DMSO and 1 µM of each primer) and 2.3 µl of RNase-free water. Three microlitre of sample solution was added to 12 µl of NASBA reaction mix. The reactions were pre-incubated for 5 min at 65 °C and 5 min at 41 °C. Each NASBA reaction was started by the addition of 5 µl of enzyme mix (375 mM sorbitol, 2.1 µg BSA, 0.08 units of RNase H, 32 units of T7 RNA polymerase and 6.4 units of AMV-reverse transcriptase), and incubated for 95 min at 41 °C. Detection of specific amplification products was performed by Northern blotting followed by ECL using the biotin-labelled probe Bio-1 (methodology performed as described by Leone et al., 1998). Real-time amplification and detection using AmpliDet RNA was carried out as described above, except that the 2.3 µl RNase-free water was replaced by 1 µl of molecular beacon solution (10 ng μl^{-1}), and either 1 µl of 10 µM ROX [5-(and -6)-carboxy-X-rhodamine] and 0.3 µl of RNase-free water, or 1.3 µl of RNase-free water. The ROX solution and molecular beacon solution were prepared in RNase-free water. The 95 min incubation at 41 °C was performed in an ABI Prism 7700 (Applera Corporation, Norwalk, CT) thermal cycler (presence of ROX is required) or the Fluoroskan FL thermostatic fluorometer (ROX is omitted). Using both types of equipment the emission spectrum of FAM

was measured in real-time for each sample every 2 min. All reactions were performed at least twice.

Results

Sequence distance of SCV isolates within the RNA-dependent RNA polymerase-coding region

A sequence of 1638 nucleotides was selected within the RNA-dependent RNA polymerase (L)-coding region to investigate the sequence heterogeneity among different SCV isolates. This region covered the GDN-motif, which is present in all negative sense viruses and constitutes the evolutionary and functional equivalent of the (G)DD motif in the polymerases of positive-strand viruses (Kamer and Argos, 1984; Argos, 1988; Poch et al., 1989; Koonin, 1991; Sleat and Banerjee, 1993). Several isolates produced different symptoms on strawberry UC-5 indicator plants (Table 1). These isolates (1554, KG, 37-1, 37-2, Hb-A1, 1553) were sequenced by using several PCR primer sets which covered the selected region. Subsequently, phylogenetic analysis were performed from this region within the L protein, including the extant sequence, called Post (Accession no. AY005146; Posthuma et al., 2002) (Figure 1). Results indicate that for both the nucleotide sequence and the amino acid sequence the isolates KG and 1554 closely resemble the extant isolate Post (∼98%), whereas isolates 37-1, 37-2, Hb-A1 and 1553, showed high heterogeneity (∼11%). Interestingly, the latter isolates show a sequence homology of at least 98% if compared with each other. This suggests the possibility of two distinct groups of SCV isolates.

Table 1. Isolate characteristics by means of origin, symptomatology on strawberry UC-5 indicator plants and molecular detection

Isolate	Origin	Symptom development[a]	PCR[b]	AmpliDet RNA[c]	Group I	Group II	Random PCR[d]	
							Pattern I	Pattern II
37-1	Netherlands	Weak	+	+		+		+
37-2	Netherlands	Weak	+	+		+		+
1554	Netherlands	Moderate	+	+	+		+	
KG	Germany	Moderate	+	+	+		+	
1553	Netherlands	Moderate	+	+		+		+
Hb-A1	Germany	Severe	+	+		+		+
R2-7	Netherlands	Moderate	+	+	n.t.	n.t.	+	
Post	United Kingdom	Moderate	n.t.[e]	n.t.	+	n.t	n.t.	n.t.

[a]Symptom development is indexed by the level of stunting, crinkling, the amount of local lesions and infection rate.
[b]Gel-based detection of SCV in strawberry plant material using PCR.
[c]Gel-free real-time detection of SCV in strawberry plant material using NASBA combined with molecular beacons (AmpliDet RNA).
[d]When using different primer sets directed to different regions within the SCV genome, either one of both specific patterns were always obtained when testing the present isolates with PCR.
[e]n.t.: not tested.

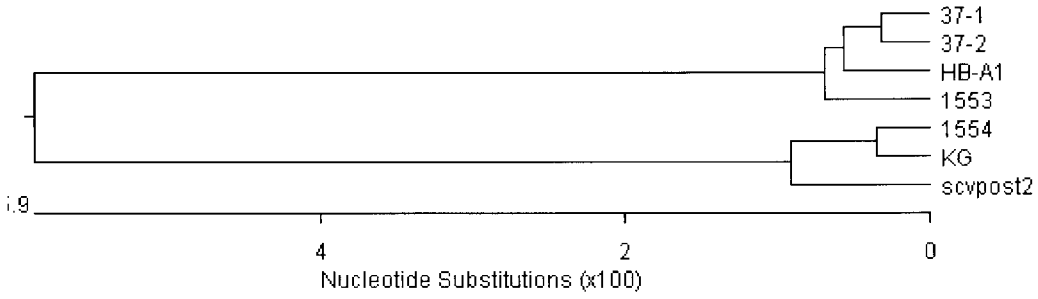

Figure 1. Phylogenetic tree of a partial RNA-dependent RNA polymerase-coding region of SCV isolates 1554 (Accession no. AY331385), KG (Accession no. AY331386), Hb-A1 (Accession no. AY331389), 1553 (Accession no. AY331390), 37-1 (Accession no. AY331387), 37-2 (Accession no. AY331388), and the extant sequence Post (Accession no. AY005146; Posthuma et al., 2002). The phylogenetic tree is obtained using the clustalW method of the MegAlign programme (DNAStar software, Lasergene, DNASTAR Inc, Madison, WI, USA).

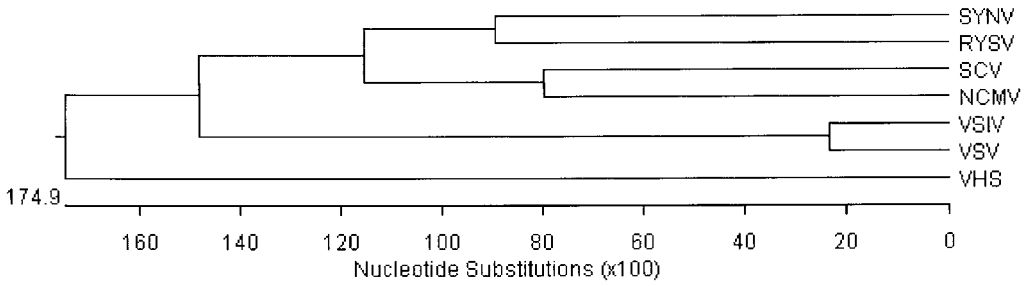

Figure 2. Phylogenetic tree of RNA-dependent RNA polymerase-coding region of SCV and the complete L protein of the Rhabdoviruses *Vesicular stomatitis Indiana virus* (VSIV) (Accession no. J02428), *Vesicular stomatitis virus* (VSV) (Accession no. J02428), *Sonchus yellow net virus* (SYNV) (Accession no. L32603), *Viral hemorrhagic septicemia virus* (VHSV) (Accession no. Y18263), *Northern cereal mosaic virus* (NCMV) (Accession no. AB030277) and *Rice yellow stunt virus* (RYSV) (Accession no. AB011257). The phylogenetic tree is obtained using the clustalW method of the MegAlign programme (DNAStar software, Lasergene, DNASTAR Inc, Madison, WI, USA).

Sequence distance of SCV within the RNA-dependent RNA polymerase-coding region of other rhabdoviruses

A comparison of the partial L protein of SCV with other rhabdovirus L protein sequences from the database was performed. A relatively close relationship with the cytorhabdovirus *Northern cereal mosaic virus* (24.7%) was found, and a more distant relationship was found with the nucleo-rhabdoviruses *Rice yellow stunt virus* (19.6%) and *Sonchus yellow net virus* (18%) (Figure 2). These results are in concurrency with previously suggested classification of the virus into the group of *Cytorhabdoviridae*, mainly based on biochemical and morphological data (Hunter et al., 1990; Richardson et al., 1972; Schoen and Leone, 1995).

Tentative grouping of SCV isolates

To determine the divergence between the isolates tested, a phylogenetic tree was obtained using the partially sequenced L protein-coding region. A clear grouping of the isolates 37-1, 37-2, 1553 and Hb-A1 was observed for the partial L protein. Also, the isolates 1554, KG and Post appeared to be closely related, but presented a distinct group from the other isolates. Both tentative groups were evaluated further to obtain more evidence leading towards separation of the isolates into two groups. Results from several RT-PCR assays that amplified different regions within the SCV genome, always showed two distinct patterns (Table 1), each displaying the same grouping as presented in the phylogenetic tree. Though, a comparison of isolate classification with symptomatology on strawberry UC-5 indicator plants did not reveal strong correspondence (Table 1). Nevertheless, these results assume a grouping of SCV isolates into group I (isolate 1554, KG and Post), and group II (isolate 1553, Hb-A1, 37-1 and 37-2), and should therefore be investigated further.

Development of a gel-based SCV-specific RT-PCR

To develop SCV-specific RT-PCR, the primers MKC-R and MKC-F were selected in a conserved region within the RNA-dependent RNA polymerase gene. The annealing temperature of the primers was tested and set at 58 °C. After RT-PCR, the amplification products were analysed on a 1% pronarose gel. For each SCV isolate tested, a specific band of 345 bp was clearly visible. To compare the results of the RT-PCR assay with AmpliDet RNA, the same RNA extracts of *P. pubescens* and strawberry UC-5 indicator plants were used with both methods for detection of SCV. Results showed that the RT-PCR assay enabled the detection of each SCV isolate originally present in both strawberry UC-5 indicator plant (Figure 3) and herbaceous host *P. pubescens*.

Development of a gel-free SCV-specific AmpliDet RNA

To enable a robust amplification of all SCV isolates, primers RPC and FPC were designed in a conserved region close to the GDN-motif in the RNA-dependent RNA polymerase gene. To determine the specificity of the NASBA, total RNA of *P. pubescens* (each plant infected with one SCV isolate) was tested using NASBA. Specific amplification products were detected

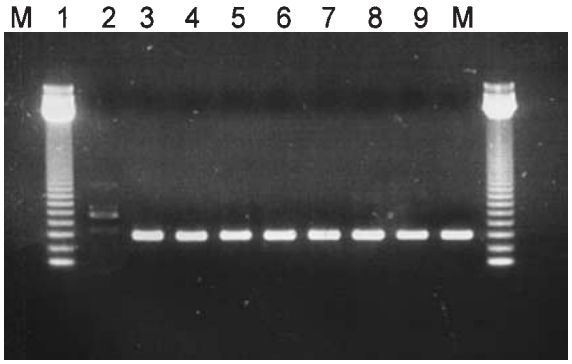

Figure 3. RT-PCR detection of all SCV isolates using a pronarose gel (1%). PCR products were generated by using SCV-specific primers MKC-F and MKC-R. The expected product size is 345 bp, and the DNA marker (M) is a 123 bp DNA marker. Samples 1–9 resemble the healthy control (1), isolate 1554 (2), isolate 1553 (3), isolate KG (4), isolate Hb-B2 (5), isolate Hb-A1 (6), isolate R2-7 (7), isolate 37-2 (8) and isolate 37-1 (9).

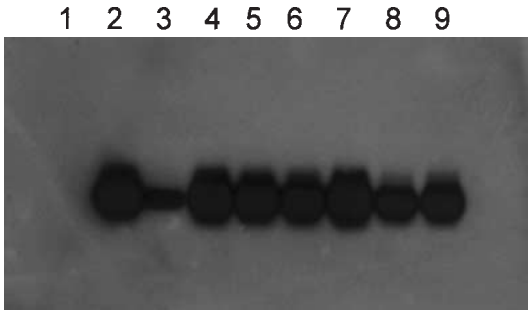

Figure 4. Detection of SCV amplification products using Northern blotting and enhanced chemiluminescent detection. The samples resemble the healthy control (1), isolate 1554 (2), isolate 1553 (3), isolate KG (4), isolate Hb-B2 (5), isolate Hb-A1 (6), isolate R2-7 (7), isolate 37-2 (8) and isolate 37-1 (9).

using ECL and the biotin-labelled probe Bio-1. Results showed that all SCV isolates were detected using NASBA followed by Northern blotting and ECL detection (data not shown).

To test whether NASBA could detect SCV in strawberry plant material, total RNA was extracted from strawberry UC-5 indicator plants and tested with NASBA. Results revealed that, after ECL detection using Bio-1, all SCV isolates were clearly detected (Figure 4).

To provide for a real-time detection during NASBA, a molecular beacon was incorporated. The molecular beacon was designed to hybridise to the same region within the amplification product as the probe Bio-1. This AmpliDet RNA (NASBA with molecular beacons incorporated) was tested using both total RNA from each SCV isolate maintained in *P. pubescens* and total RNA from each SCV isolate maintained in strawberry UC-5 indicator plants. The RNA extracts were also used for RT-PCR (as described in the previous section) to provide a clear comparison of both methods. From the real-time measurements, an increase of fluorescence was observed in all samples, except for the healthy controls. These results suggest that AmpliDet RNA is able to detect all SCV isolates maintained in both the strawberry host (Figure 5) and the herbaceous host *P. pubescens*.

Discussion

In this paper, a partial sequence of the putative RNA-dependent RNA polymerase of SCV has been described. Alignment of the partially sequenced regions within the L protein, all obtained from isolates that caused different symptom development in strawberry plants, indicated a homology of 89%. In addition, the partially elucidated putative L protein appears to be distantly related to other rhabdovirus L proteins, like *Northern cereal mosaic virus, Rice yellow stunt virus* and *Sonchus yellow net virus*. This is in agreement with previously published data (Posthuma et al., 2002).

Moreover, comparison of the partially sequenced region of all selected isolates within the L protein showed a clear separation into two groups of closely related isolates, resulting in 98% homology or higher within each group. This gives rise to the possibility of classification of the SCV-isolates into two tentative groups, namely group I (isolates 1554, KG and Post), and group II (isolates 1553, Hb-A1, 37-1 and 37-2). Morphological reinforcement of the tentative grouping might be symptom development on susceptible hosts, like strawberry UC-5 indicator plants. Unfortunately, the comparison of both tentative groups with their symptomatology on strawberry UC-5 indicator plants did not lead to a clear differentiation.

Both tentative groups might be the result of a previous geographical separation of two closely related SCV isolates, each evolving further in time. Since sequence heterogeneity within the isolates of a group is very low (2%), it is assumed that the geographical separation might have occurred only a few decades ago. According to this hypothesis, the isolates originating from other

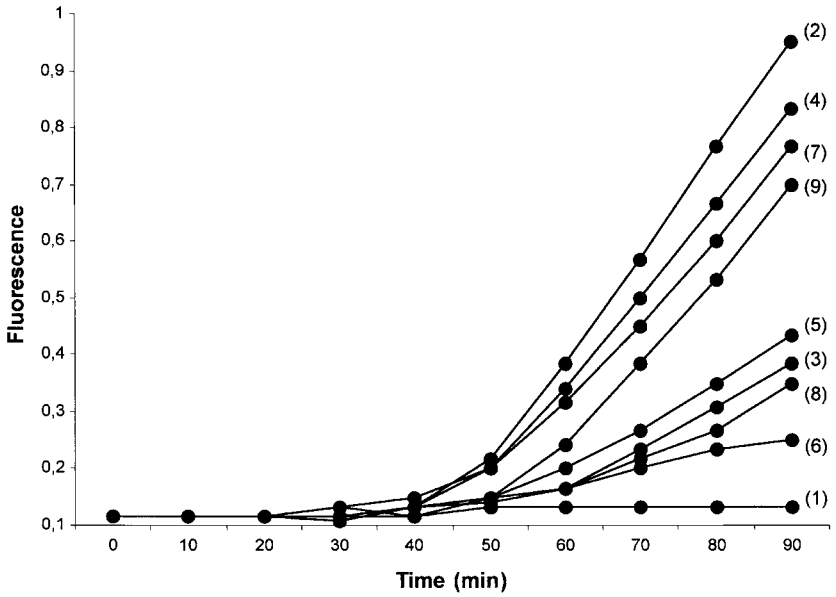

Figure 5. Real-time amplification and detection of all SCV isolates using the AmpliDet RNA system. The samples resemble the healthy control (1), isolate 1554 (2), isolate 1553 (3), isolate KG (4), isolate Hb-B2 (5), isolate Hb-A1 (6), isolate R2-7 (7), isolate 37-2 (8) and isolate 37-1 (9).

parts of Europe might not enable classification into group I or group II. Nevertheless, preliminary data indicate that no large differences are to be expected when testing isolates originating from other European countries (unpublished results). Therefore, the presence of two groups of SCV isolates is still convincing and should be investigated further by means of sequence, geographical distribution and host susceptibility.

At present, detection of the virus is still based on grafting onto strawberry UC-5 indicator plants, requiring at least one week until symptom development (Frazier et al., 1987). Recently, a PCR method was developed to allow for the detection of SCV directly from strawberry plants (Posthuma et al., 2002). Unfortunately, the method was sensitive to inhibitory agents originating from the RNA extraction. This resulted in inconsistent results when infected strawberry plants without symptoms were tested. In this paper, the RNA extraction was improved to allow for RNA extracts virtually free of inhibiting compounds. A new gel-based RT-PCR detection method and a gel-free AmpliDet RNA assay were developed, based on conserved regions within the L protein. Both methods clearly detect all SCV isolates present in herbaceous host *P. pubescens* and strawberry UC-5 indicator plants. In addition, preliminary results indicate that SCV in different strawberry field samples can be detected when the described RT-PCR or AmpliDet RNA is used. This indicates that introduction of these methods might greatly improve the detection of SCV during certification procedures and testing of *in vitro* propagation material.

In conclusion, detection of all present SCV isolates directly from strawberry plant material was obtained by a newly developed gel-based RT-PCR and a gel-free AmpliDet RNA assay. Both methods have the potential to provide a rapid, sensitive and specific detection of SCV in *in vitro* propagation material. Moreover, this study suggests the possibility of two distinct groups of SCV isolates, classified by means of partial nucleotide sequences of the putative L protein of SCV.

Acknowledgements

This paper was part of the EU project QLK5-1999-1553, improved diagnostic tools for the certification of strawberry propagation material.

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