

Improved Diagnostic Tools for the Certification of Strawberry Propagation Material – The Use of PCR and NASBA for Detection of *Strawberry Vein Banding Virus* (SVBV)

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Abstract

Control of SVBV relies completely on the use of virus-free planting material, that can be tested either by biological indexing or by molecular methods. A NASBA-based amplification was developed for the detection of SVBV. NASBA is a method based on the primer-dependent, specific amplification of RNA by concurrent activity of a special enzyme mix (AMV-reverse transcriptase, RNaseH, T7 RNA polymerase) at a single temperature (41°C). Specific and sensitive detection of the amplified sequence can be performed in the same tube using molecular beacons. Sensitivity of SVBV-NASBA was 10² molecules of *in vitro* RNA detected per reaction. Results of the NASBA-based detection of SVBV in indicator strawberry plants were well comparable to the results of PCR.

Keywords: *Strawberry vein banding virus*; SVBV; Caulimovirus; *Fragaria* spp.; nucleic acid sequence based amplification; NASBA; molecular beacon

INTRODUCTION

SVBV is a double stranded DNA virus belonging to the genus Caulimovirus. It is transmitted by grafting or by aphids in a semipersistent manner (FRAZIER & CONVERSE 1980) and is probably distributed worldwide on cultivated strawberries (PETRZIK *et al.* 1998). Infection of SVBV in the field strawberries is mostly symptomless and thus it escapes the visual selection. However when occurring in mixed infections with other viruses, the symptoms are more expressed and more deleterious (BOLTON 1974). There are no commercially available antibodies for serological detection of SVBV, thus the immuno assays are excluded from its detection. Moreover the concentration of Caulimovirus particles in plant tissue is generally low (LAWSON *et*

al. 1977; SHEPHERD 1981), which intensifies the need of a sensitive and reliable detection method.

A PCR-based detection system for SVBV was developed in 1998. However its sensitivity was not sufficient, so it had to be supplemented by a special detection step, where the amplified PCR products were blotted and hybridised to an SVBV-specific probe (MRÁZ *et al.* 1998).

Recently a sensitive, nucleic acid sequence based amplification (NASBA) method was developed for the detection of many plant and animal pathogens (for example HEIM & SCHUMANN 2002; JEAN *et al.* 2002; KLERKS *et al.* 2001). NASBA is a homogenous, isothermal RNA amplification process involving the action of three enzymes: reverse transcriptase, T7 RNA polymerase and RNase H, as well as two tar-

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get sequence-specific oligonucleotide primers (one of which bears a T7 promoter sequence at its 5' end) acting in concert to amplify target sequences more than 10^8 -fold (COMPTON 1991). The single stranded NASBA product can be detected by means of the molecular beacon (MB) technology. MB are hairpin-shaped oligonucleotide probes that report the presence of specific nucleic acid in homogenous solutions. They become fluorescent when they bind to perfectly complementary nucleic acids, whereas in the absence of their target they remain non-fluorescent (TYAGI & KRAMER 1996). The real-time, one-tube detection of NASBA amplicons by MB offers a sensitive, simple and rapid detection technique. Such a technique was developed for the detection of SVBV and a comparison to a PCR-based method was performed.

MATERIALS AND METHODS

Virus isolates. The SVBV isolates (9010 and 9093) used in this study were obtained from the National Clonal Germplasm Repository, Corvallis, Oregon, USA. They represent the American western-type isolates. Viral RNA was extracted from the leaves of strawberry indicator clones (Alpine, UC4, UC6) by the RNeasy Plant Mini Kit (Qiagen). It was eluted in RNase free water and stored at -60°C .

Synthesis of *in vitro* RNA. Positive-sense SVBV RNA was used as a positive control. Primers RP (TTT CTC CAT GTA GGC TTTGA) and T7FP (aat tct aat acg act cac tat agg gag AGT AAG ACT GTT GGT AAT GCC A) were used to amplify the 424 bp long region of the capsid protein (CP) gene in a standard PCR reaction. The template was a full-length clone of the American SVBV in pUC plasmid designated pSVBV-E3 (STENGER *et al.* 1988) obtained from the American Type Culture Collection (ATCC; product No. 45058, Rockville, MD). After initial denaturation at 94°C for 10 min, the following cycle was repeated 40 times: 15 s at 94°C , 30 s at 60°C and 60 s at 72°C . The PCR product was checked by electrophoresis, purified from low melting agarose and then transcribed (detailed protocol can be found in SZEMES *et al.* 2002). The synthesised RNA was purified by the RNeasy Mini Kit (Qiagen). Its purity and concentration were determined by measuring the UV absorption at 260 and 280 nm on a Beckman spectrophotometer.

Design of amplification primers and probes. Target sequences for the sense P₁B (AAT TCT AAT ACG ACT CAC TAT AGG GAG AGC ATA TCC AAG TGA TCC TTT A) and antisense P₂B (GAG AAA GCT GTT CAA GAA GCT AGA) primers and the

molecular beacon MB1 (5' Fluorescein – GCT GCA GCA ATC TCT GTT CAC TAG AAT GCA GC – Dabcyl 3') were selected within the CP gene. The MB1 was synthesised by Isogene Bioscience BV (The Netherlands), other oligonucleotides by the Amersham Pharmacia Biotech.

NASBA. NASBA was performed as described previously (SZEMES *et al.* 2002) using premixed enzymes, buffers and nucleotide provided in the NASBA amplification kit (Organon Teknika, Turnhout, Belgium). The real-time monitoring of NASBA amplification was assessed by adding MB1 to the reaction mix (9 ng of MB1 per reaction) and incubating the reaction for 90 min at 41°C in an aluminium block in the SpectraFluor Plus spectrophotometer (Tecan). The reactions were excited at 492 nm and fluorescence was measured at 535 nm.

PCR. 0.1 μM primers RP and T7FP were used in a standard PCR reaction. After initial denaturation at 94°C for 10 min, the following cycle was repeated 35 times: 30 s at 94°C , 30 s at 52°C and 90 s at 72°C . The final extension step lasted for 10 min at 72°C . PCR products were analysed on a 1% agarose gel.

RESULTS

Real-time NASBA

The real-time NASBA developed in this study was designed to specifically detect SVBV by amplification of its nucleic acid. Detection of the amplified products was done by means of the molecular beacon technology. For assessment of the detection limit of this one-tube assay a ten-fold dilution of *in vitro* RNA was prepared as a template and NASBA was accomplished in presence of molecular beacon MB1. The increase of fluorescence was followed in real-time conditions and it was plotted as a function of time (Figure 1). The lowest detection limit repeatedly achieved was 10^2 molecules of *in vitro* RNA per reaction.

Performance of the real-time NASBA on strawberry material

To test whether the sensitivity achieved in real-time NASBA is sufficient for strawberry testing, two strawberry plants infected with different isolates of SVBV were used. RNA isolated from these plants was diluted 5, 10 and 100 times to eliminate possible effects of strawberry inhibitory compounds. All the three dilutions of the both samples gave positive results in the real-time NASBA, while the negative

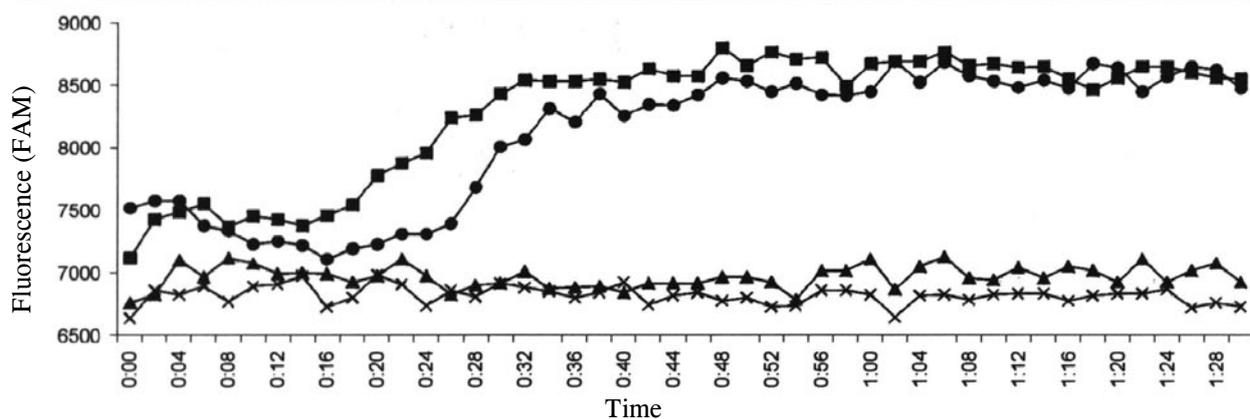


Figure 1. Real-time detection of dilution series of *in vitro* RNA. Three concentrations were tested and water was used as the no-template control. (■)10³, (●)10², (▲)10¹ molecules per reaction, (×) water

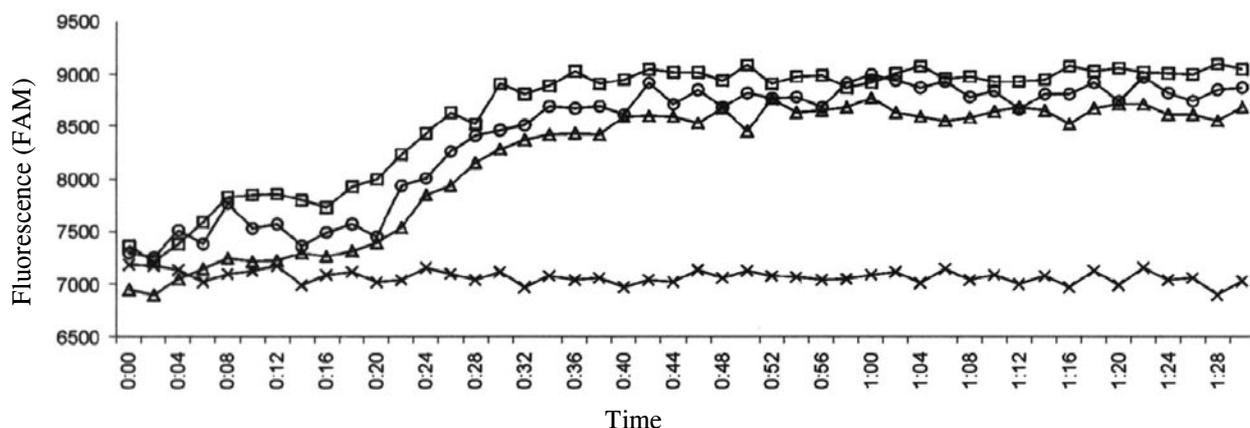


Figure 2. Real-time NASBA on diluted RNA isolated from infected strawberry plant (isolate 9010) – (□) diluted 5 times, (○) diluted 10 times, (▲) diluted 100 times. RNA isolated from a healthy plant (diluted 10 times) was used as the negative control (×)

strawberry control gave a negative result (Figure 2 for the 9010 isolate).

Table 1. Comparison of PCR and NASBA

Isolate	NASBA	PCR
Dossenheim – February 2001	+	+
Dossenheim – August 2001	-	-
9016	-	-
9043	-	-
9044	-	-
9010	+	+
9093	+	+

Comparison of NASBA and PCR

Results of NASBA and the simplified PCR assay (hybridisation step in the detection omitted) were well comparable. The agreement of the results on a small pool of samples tested was in fact absolute (Table 1).

DISCUSSION

The real-time NASBA showed to be a good alternative to current PCR tests used for detection of SVBV in strawberries. Its performance on indicator *Fragaria* clones was satisfactory, but the fields samples usually contain much lower amount of SVBV than the indica-

tor clones do. Thus the assay may need some more optimisation to increase the sensitivity achieved. But even though, the real-time NASBA has shown great promise in the detection of SVBV in strawberry plants. It has several advantages over the standard, PCR-based assay: its one-tube, gel-free nature decreases labour and false positives dramatically (risk for contamination of other reactions while manipulating with amplicons) and it enables a full automation of the process, thus enabling a high throughput screening. The possibility of development of a multiplex NASBA assay for the most important strawberry viruses makes this method even more attractive.

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