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Real-time NASBA for detection of Strawberry vein banding virus

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Abstract

An assay for the detection of Strawberry vein banding virus (SVBV) in *Fragaria* spp. based on nucleic acid sequence based amplification (NASBA) and real-time detection using molecular beacons (real-time NASBA) is described. This assay was compared both with biological indexing, the current method for certification of SVBV, and a newly optimised PCR-based detection method. Performance of the assay was tested on three SVBV isolates in *Fragaria* indicator plants and 317 field strawberries from five European countries. The assay was shown to be SVBV-specific, testing negative for other common aphid-borne strawberry viruses. The virus was detected in purified total RNA preparations diluted a millionfold, which is an amount equivalent to ∼1 ng of fresh material. The real-time NASBA method developed here offers the potential of a fast, sensitive and reliable approach for the routine diagnosis of strawberry stock material.

Introduction

Strawberry vein banding virus (SVBV) is a viral pathogen infecting *Fragaria* species. It belongs to the genus *Caulimovirus* (retroid viruses), having a double stranded circular DNA genome. SVBV is transmitted by grafting or by aphids in a semi-persistent manner (Frazier and Converse, 1980) and its presence in strawberry plants has been reported from many countries worldwide (Frazier and Morris, 1987; Converse, 1992; Honetšlegrová et al., 1995; Petrzik et al., 1998a). Symptoms of SVBV in field strawberries are usually not pronounced thereby escaping visual selection. However, when occurring in mixed infections, the symptoms are more severe and more deleterious (Bolton, 1974). Since 1978, SVBV has been listed as a quarantine pest by the European and Mediterranean Plant Protection Organisation (OEPP/EPPO, 1978).

Until recently, routine SVBV detection was solely based on biological indexing on sensitive indicator clones: the only method accepted by inspection services during certification procedures. However, the use of greenhouse indexing is not ideal due to the time, space, money and staff-experience requirements and the resulting limited number of plants tested. Since no commercial antibodies for serological detection of SVBV are available, no immunological assays can be used for its detection. Moreover, the concentration of *Caulimovirus* particles in plant tissue is generally low (Lawson et al., 1977; Shepherd, 1981), intensifying the need for a more sensitive detection method.

In 1997, a PCR-based detection system for SVBV was developed that greatly reduced the testing-time needed (Mráz et al., 1997). Due to repeated smears in amplification products a combination of PCR and subsequent hybridisation to a homologous probe was highly recommended for routine detection of SVBV. Hybridisation to a non-amplified DNA isolated from field strawberries was not sufficient for detection of SVBV and in 1999 a combination of

PCR and hybridisation to an SVBV-specific probe was re-recommended as the only reliable detection method for SVBV (Mráz et al., 1999). The PCR technique enabled a determination of sequence variability of SVBV (Mráz et al., 1998).

Recently, sensitive nucleic acid sequence based amplification (NASBA) methods were developed for the detection of several plant and animal pathogens (e.g. Klerks et al., 2001a; Heim and Schumann, 2002; Jean et al., 2002). NASBA is a sensitive transcription-based amplification system for the specific replication of nucleic acids *in vitro* (Compton, 1991; Kievits et al., 1991). The amplification in the NASBA reaction involves two specific oligonucleotide primers and the concurrent activity of three enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase. NASBA is usually applied to amplify a single stranded RNA target. The amplification of DNA is also possible, however only after special modifications of the procedure (Voisset et al., 2000; Yates et al., 2001). SVBV is a DNA virus, but its RNA intermediates serve as template in protein synthesis as well as in replication of its genome. Existence of the pre-genomic RNA is a characteristic feature of pararetroviruses (Pooggin et al., 1999), a group SVBV belongs to.

Several NASBA formats were described using gel-based detection of amplification products: enzyme linked gel assays (van der Vliet et al., 1993; Darke et al., 1998), denaturing gel electrophoresis (Jean et al., 2001) and Northern blotting (Heim and Schumann, 2002). These time-consuming post-amplification detection methods limit the automation and a high-throughput use of NASBA. They also introduce the risk of a carry-over contamination of the samples. These disadvantages can be avoided by the use of a real-time detection of NASBA amplicons by means of the molecular beacons (called AmpliDet RNA, Leone et al., 1998).

Molecular beacons are a class of oligonucleotides that can report the presence of specific nucleic acids in homogenous solutions (Tyagi and Kramer, 1996). They become fluorescent when they bind to perfectly complementary nucleic acids, whereas in the absence of their target they remain non-fluorescent. The coupling of NASBA and molecular beacons enables a simultaneous amplification and detection of the specific amplicons in a sealed tube under real-time conditions.

In this paper, the development of the real-time SVBV NASBA assay and its application for detection of SVBV in strawberries is reported and compared to PCR.

Materials and methods

Virus isolates

The SVBV isolates (9010, 9044 and 9093) used in this study were obtained from the National Clonal Germplasm Repository, Corvallis, Oregon, USA. They represent the American western-type isolates. The isolates were maintained on *Fragaria vesca* var. Alpine and *Fragaria ananassa* var. UC4 and UC6. Evaluation of the method was done on 317 strawberry field plants (*F. ananassa*) originating from the Czech Republic, Slovak Republic, Poland, Latvia and Italy. Strawberry Crinkle Virus (SCV) and Strawberry Mottle Virus (SMoV) isolates originated from the Czech Republic. They were maintained on *F. ananassa* var. UC4 and var. Cacanska rana, respectively. Alpine plants raised from seeds were used as healthy control. All plants were maintained in an insect proof air-conditioned greenhouse.

Isolation of nucleic acids

Homogenisation of strawberry leaves was done by means of the Bioreba (Reinach, Switzerland) Extraction bags and the Bioreba HOMEX 6 homogeniser in the sample extraction buffer (0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_20$ (pH 7.4), 0.05% v/v Tween-20, 2% w/v polyvinylpyrrolidone 40, 0.2% w/v ovalbumin, 0.5% w/v bovine serum albumin, w/v 0.05% sodium azide) added in a ratio 1 : 10 (w : v). Hundred microlitre of this solution was used for extraction of nucleic acids by the RNeasy Plant Mini Kit or Dneasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). RNA was eluted in 100 µl of RNase free water and stored at −60 °C. DNA was eluted in 200 µl of low salt DNeasy dilution buffer and stored at −20 °C.

Synthesis of in vitro *RNA*

Primers RP (5′ TTT CTC CAT GTA GGC TTT GA 3′) and T7FP (5′ aat tct aat acg act cac tat agg gag AGT AAG ACT GTT GGT AAT GCC A 3′ – lower case letters indicate the T7 RNA polymerase promoter sequence overhang) were used to amplify a 435 bp long region within the capsid protein (CP) gene. The template was a full-length clone of an American SVBV isolate in pUC plasmid designated pSVBV-E3 (Stenger et al., 1988), obtained from the American type culture

collection (product No. 45058, Rockville, MD). DNA of the full-length clone was added to a 100 µl reaction mixture containing 1× Taq-buffer, 4 mM $MgCl_2$, 100 nM dNTP each, 100 nM primers and 1 U AmpliTaq Gold polymerase. The amplification mix was pre-heated for 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 60 s at 72 °C. The PCR product generated was purified from low melting agarose and transcribed for 3 h at 37 °C. The reaction mixture consisted of 1× T7 RNA polymerase transcription buffer, 1 mM rNTP each, 100 mM DTT, 20 U RNA guard and 100 U T7 RNA polymerase. The synthesised RNA was purified by the RNeasy Mini Kit (Qiagen) and its amount was determined by using a Beckman spectrophotometre. The serial dilutions of *in vitro* RNA in NASBA water (BioMerieux BV, Boxtel, the Netherlands) were prepared freshly from an aliquoted stock solution stored at −60 °C prior to each NASBA experiment.

Amplification primers and probes

Target sequences for the antisense P_1B (5′ aat tct aat acg act cac tat agg gag AGC ATA TCC AAG TGA TCC TTT A 3′ – lower case letters indicating the T7 RNA polymerase promoter-binding region) and sense P_2B (5′ GAG AAA GCT GTT CAA GAA GCT AGA 3′) primers were selected within the CP-coding region (nucleotides 2520–2751) of SVBV (GenBank Acc. No. NC_001725, Petrzik et al., 1998b). The molecular beacon (5′ Texas Red-GCT GCA GCA ATC TCT GTT CAC TAG AAT GCA GC – Dabcyl 3′; underlined letters indicate nucleotides hybridising to the NASBA product, which are identical to hybridising nucleotides of Bio1) and the biotin-labelled probe Bio1 (5′ Biotin-TGC AAT CTC TGT TCA CTA GAA 3′) were designed to hybridise to the NASBA amplification product. The molecular beacon was supplied by Isogene Bioscience BV (The Netherlands), other oligonucleotides were supplied by Amersham Pharmacia Biotech (Freiburg, Germany).

NASBA

The NASBA reactions were performed as described originally (Kievits et al., 1991) with some minor modifications. Three microlitre of sample (water; negative control) were added to 12 µl NASBA pre-mixture (final concentration in 20 µl reaction mixture: 40 mM Tris–HCl, pH 8.5, 0.5 mM DTT, 12 mM $MgCl_2$, 70 mM KCl, 15% v/v DMSO, 1 mM dNTPs, 2 mM each of ATP, UTP and CTP, 1.5 mM GTP and 0.5 mM ITP, 0.2 µM of each primer). For each real-time NASBA, 9 ng of molecular beacons per reaction mixture were added. After an initial denaturation of this mixture at 65 °C for 5 min, the temperature was adjusted to 41 °C (5 min), then pre-mixed enzymes (375 mM sorbitol, 2.1 µg bovine serum albumin, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-RT) (BioMerieux BV, Boxtel, The Netherlands) were added, and the reaction was incubated for 90 min at 41 °C. The real-time measurement were performed using the iCycler iQ™ (Real-Time PCR detection system) from BIO-RAD (Hercules, USA). The fluorophores were excited at 575 nm and fluorescence emission was measured at 620 nm every 2 min.

Post-NASBA analyses

Analyses performed as described previously (Leone et al., 1998). In short, 3 µl of NASBA amplification products were separated using a non-denaturing gel electrophoresis, blotted on a Z-probe nylon membrane, hybridised to SVBV-specific biotinylated probe (Bio1) and visualised using enhanced chemi-luminescent (ECL) detection (Amersham Pharmacia Biotech). Finally the membrane was exposed to X-ray film.

PCR

In total 0.1 µM primers RP and FP (5′ AGT AAG ACT GTT GGT AAT GCC A 3′) were used in a standard PCR reaction (1× RedTaq PCR Reaction Buffer, 200 nM dNTP each and 1 U RedTaq DNA polymerase). 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 2% agarose gel.

Results

Assay design

The NASBA assay developed in this study was designed to amplify part of the RNA coding the CP of SVBV. Variability within this region was previously determined among six different

American and European isolates (Mráz et al., 1998), and found to be only 0.7% in the 431 bp long sequence. The primers and molecular beacons were designed on basis of conserved segments within this region.

Initial optimisation of the SVBV NASBA assay involved *in vitro* produced template RNA (435 bp long region of the SVBV CP gene). Three forward primers with three reverse primers were tested in every combination. The efficiency of each of the nine primer sets was assessed by the determination of the detection threshold, using 10-fold dilution series of *in vitro* RNA; serial dilutions were made ranging from 10^4 to 10^0 *in vitro* RNA copies per reaction. The NASBA amplification products were detected by Northern blotting, hybridisation to a biotin-labelled probe and subsequent ECL detection. The highest sensitivity was obtained with probe Bio1 and the primer set P_1B–P_2B, enabling the detection of 10^1 molecules of *in vitro* template per reaction (Figure 1). All following experiments were performed using this primer set.

Real-time NASBA

To allow for a gel-free detection, a molecular beacon-based detection system was developed. Molecular beacons were designed to anneal to the same target sequence as the biotin-labelled probes. In order to determine the sensitivity of the real-time NASBA, the same dilution series of *in vitro* RNA were used as described above. The increase of fluorescence was followed in real-time during amplification and was plotted as a function of time (Figure 2). Results indicate a detection limit of 10^1 molecules of *in vitro* RNA per reaction, with no apparent reduction in sensitivity caused by incorporation of molecular beacons into the NASBA system.

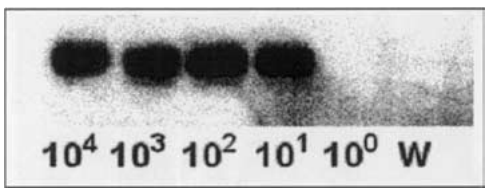

Figure 1. Northern blot analysis using SVBV-specific biotinylated probe for NASBA products of a dilution series of *in vitro* generated SVBV partial RNA. 10^4–10^0 indicates the number of *in vitro* RNA molecules per reaction mixture, W: water as a negative control.

Evaluation of the assay on a collection of SVBV isolates

The performance of the SVBV real-time assay was tested using total RNA from indicator strawberry plants infected with SVBV isolates 9010, 9044 and 9093. Total RNA extracts were diluted several times to investigate the effect of potential inhibitory compounds. As no inhibition was observed (Figure 3), results indicate that no inhibitory compounds were present after RNA extraction and that no additional post-extraction dilution step was needed. Results were similar for all the three SVBV isolates, thus only data for one of them (isolate 9093) are presented here (Figure 3).

To determine if the real-time assay was reproducible and accurate, infected and healthy indicator strawberry plants were tested throughout the year. We have focused mainly on autumn and winter months, when the detection of plant viruses is generally difficult (August–February). The data showed consistence in time indicating an independence on sampling period (results not shown) for these indicator plants. The levels of relative fluorescence achieved for plants infected with SVBV were not significantly different for any season of the year.

Specificity of the assay evaluated on a collection of SVBV isolates and field samples

The real-time NASBA for SVBV was used for a large-scale field screening experiment. Three hundred and seventeen strawberry plants from the Czech Republic, the Slovak Republic, Poland, Latvia and Italy were tested for the presence of SVBV. No strawberry field plant was found to be infected with SVBV. These results were confirmed by a concurrent SVBV-specific RT-PCR assay, which was in a multiplex design described by Thompson et al. (2003).

To determine the specificity of the real-time NASBA, RNA was extracted from healthy strawberry plants or plants infected either with SVBV, SCV or SMoV and tested using the real-time assay. No increase in fluorescence was observed in the case of healthy, SCV- or SMoV-infected plants tested by the SVBV-specific NASBA, whereas tests on SVBV-infected plant material resulted in a high fluorescence increase (Figure 4). Presence of SCV and SMoV in control plants was proven by SCV-specific NASBA and SMoV-specific NASBA, respectively (unpubl. results). Since no cross-reactions were observed, the developed assay appears to be specific for SVBV.

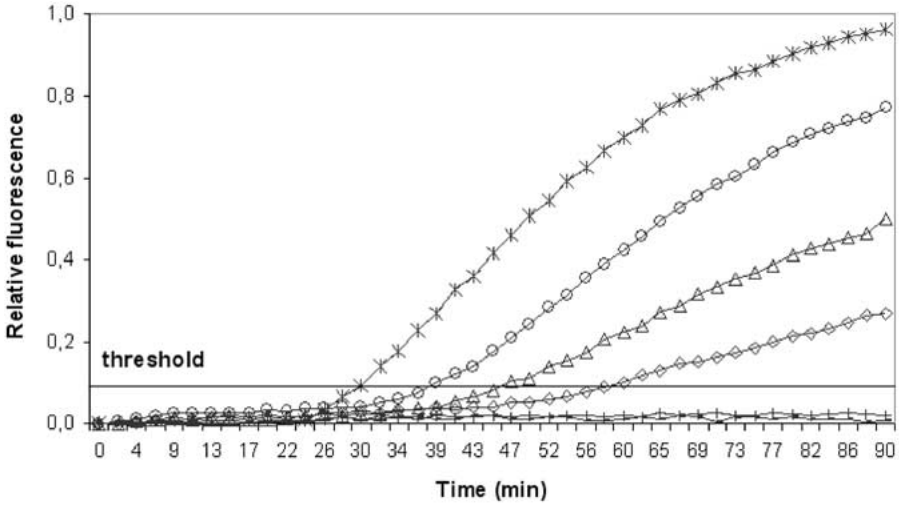

Figure 2. Relative fluorescence with time of a dilution series of *in vitro* generated SVBV partial RNA tested by real-time SVBV NASBA. Number of molecules per reaction mixture: (∗) 10^4, (◯) 10^3, (Δ) 10^2, (◊) 10^1, (−) 10^0; (+) water as a no-template control. The threshold line indicates the point at which the reaction reached a fluorescent intensity above background.

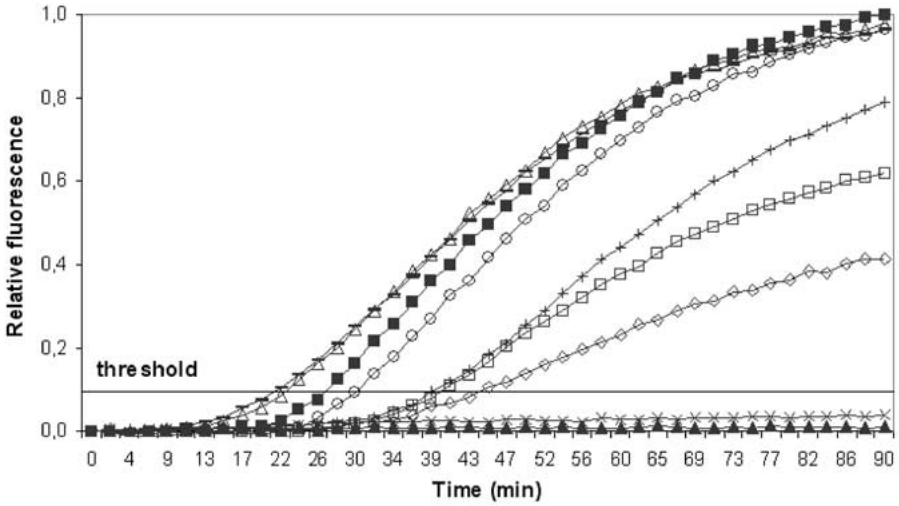

Figure 3. Monitoring of potential inhibition of purified RNA preparation: real-time SVBV NASBA on the 10-fold water dilution series of RNA isolated from infected strawberry plant (isolate 9093). Dilution coefficients: (−) 10^0, (Δ) 10^{-1}, (■) 10^{-2}, (◯) 10^{-3}, (+) 10^{-4}, (□) 10^{-5}, (◊) 10^{-6}, (×) 10^{-7}; (▲) water as a no-template control.

Comparison to PCR

The sensitivity of PCR was determined after a due optimisation of the assay (testing different extraction methods, primers, annealing temperatures and elongation times). The performance of both NASBA and PCR was evaluated by 10-fold dilution series of RNA and DNA isolated from an SVBV-infected plant. The RNA and DNA were isolated concurrently using the Qiagen RNeasy and DNeasy method, respectively. The dilution was done in total RNA and DNA preparations isolated from a healthy strawberry plant. Using both the NASBA and PCR assay, a dilution of 10^5 times of total nucleic acid extracted from infected strawberry plant showed to be positive (Figure 5).

In order to compare the performance of NASBA and PCR in a diagnostic setting, five suspicious SVBV-infected strawberry plants were tested in parallel using

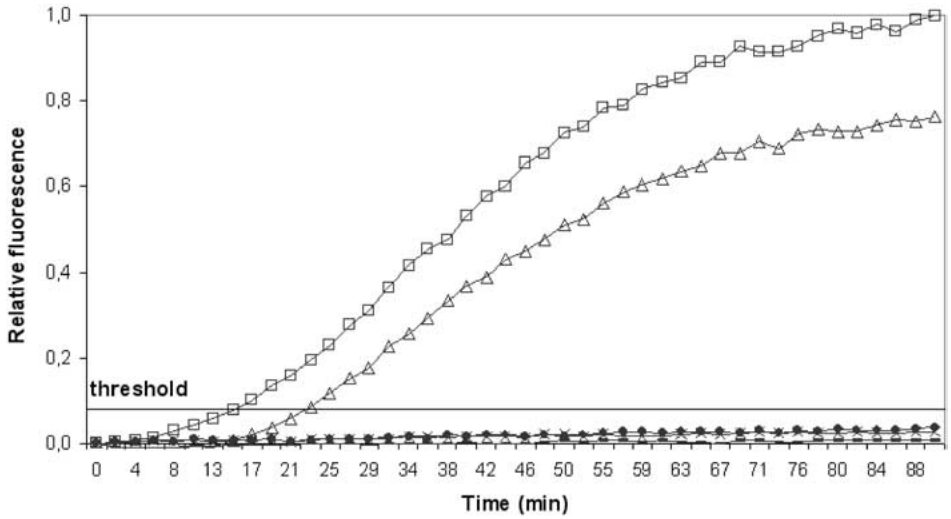

Figure 4. Specificity of the real-time SVBV NASBA: relative fluorescence in time for (Δ) SVBV-infected strawberry (isolate 9010), (●) SCV-infected strawberry, (×) SMoV-infected strawberry, (−) negative control – healthy strawberry plant, (□) positive control – 10^8 molecules of *in vitro* RNA per reaction mixture.

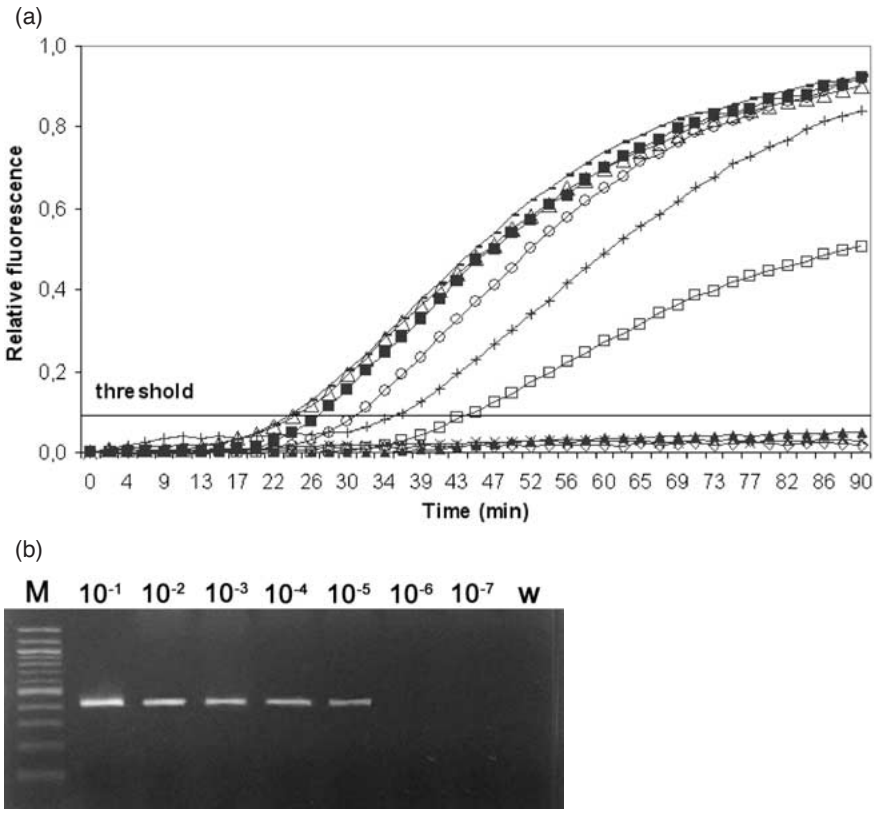

Figure 5. Ten-fold dilution series of nucleic acids isolated from infected strawberry plant (isolate 9044). Dilution was done in healthy plant sap and tested by: (a) real-time SVBV NASBA. Dilution coeficients: (−) 10^0, (Δ) 10^{-1}, (■) 10^{-2}, (◯) 10^{-3}, (+) 10^{-4}, (□) 10^{-5}, (◇) 10^{-6}, (×) 10^{-7}; (▲) water as a no-template control. (b) PCR. Dilution coeficients: 10^{-1}–10^{-7}, W – water as a no-template control.

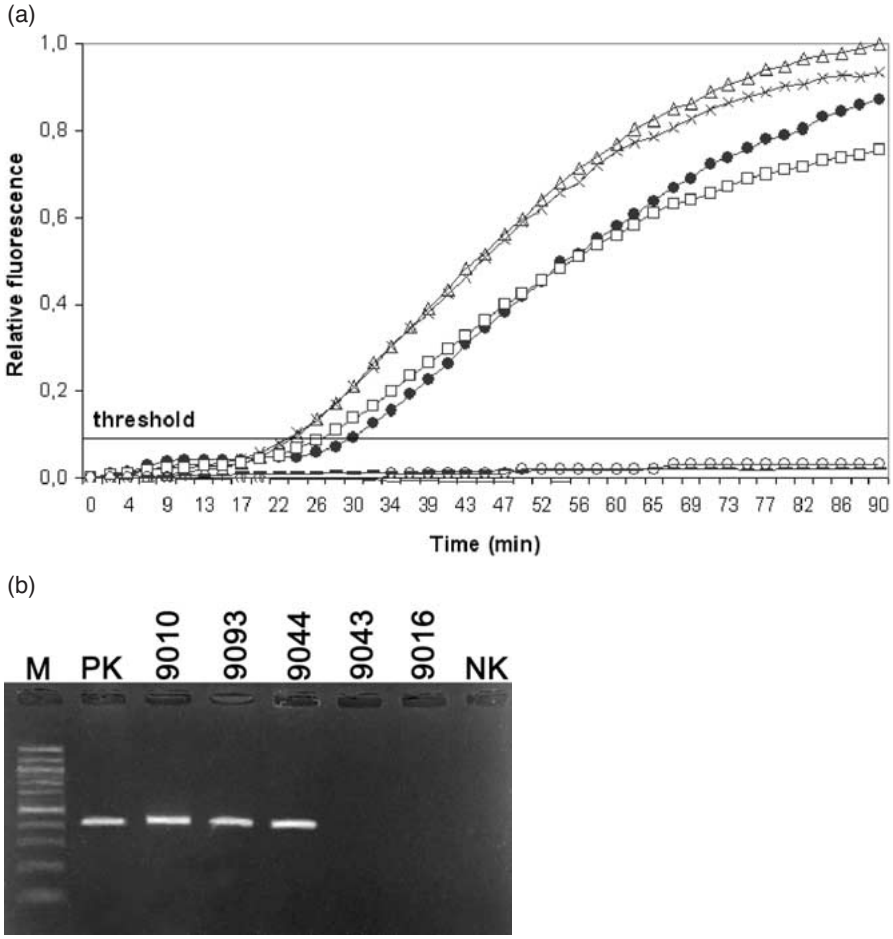

Figure 6. Detection of SVBV in strawberry plants by: (a) the real-time NASBA. Relative fluorescence with time for isolates (△) 9010, (×) 9044, (□) 9093, (◯) 9043, (+) 9016, (−) negative control (healthy strawberry plant), (●) positive control (10^3 molecules of *in vitro* RNA per reaction mixture). (b) PCR detection. M – 100 bp molecular marker, PK – positive control (DNA of a full-length SVBV clone), NK – negative control (healthy strawberry plant).

the real-time NASBA, PCR and biological indexing. Both NASBA and PCR clearly detected SVBV in three samples (Figure 6), confirmed by biological indexing. The remaining two samples were identically negative for all three methods.

Discussion

An SVBV detection assay is described, which is an alternative to current SVBV detection systems. It couples the NASBA amplification technology to the real-time detection with molecular beacon probes. Although SVBV is a DNA virus, the results showed that its detection by NASBA is possible. Results of NASBA were in a good agreement with the results of the biological indexing, and its sensitivity was similar to that of the PCR-based assay.

Reproducibility of the results throughout the year showed that the applicability of the assay may not be limited only to the period of active plant growth and pronounced virus replication. *Caulimoviruses* are closely related to the hepatitis B virus for which a production of detectable RNA intermediates even during the latent infection was demonstrated (Mason et al., 1998; Marusawa et al., 2000).

Almost 320 strawberry plants were tested by the newly developed real-time NASBA, but the presence of SVBV could only be confirmed in strawberry plants infected with three different SVBV isolates

kept in the National Clonal Germplasm Repository, Corvallis, Oregon, USA. The field survey on strawberry plantations in the Czech Republic, Slovak Republic, Poland, Latvia and Italy resulted in no positives, probably because of the use of certified strawberry seeds in the countries of the central and eastern Europe, which is nowadays more common than in 1980s and 1990s. Moreover, as SVBV has been listed as a quarantine virus, its elimination in Europe has been probably enhanced by the employment of the EPPO recommended certification scheme.

Performance of the evaluation tests of the SVBV real-time NASBA on only three SVBV could potentially hamper the widespread use of the method, if the virus was too variable. However, the variability of SVBV seems to be quite low. The results of Mráz et al. (1998) showed that variability among six European and American isolates was only 0.7% in the region, which is amplified by the PCR and NASBA primers. In contrary the variability of closely related Cauliflower mosaic virus was six times higher (2.7%) in the corresponding region. Mráz et al. (1997) also reported that no substantial differences were registered in the hybridisation signal in all combinations of SVBV field samples and probes prepared from different SVBV isolates. The pool of isolates detectable by the FP-RP PCR primer pair was in 2003 enriched by three isolates taken from wild strawberry (*Fragaria chiloensis* (L.) Duch.) from Chile (Thompson et al., 2003). These data indicate that a reliable performance of the newly designed primers is not limited to known Europe and North American isolates. The optimisation of the PCR assay in comparison with older PCR SVBV assays (e.g. Mráz et al., 1997) reclines upon a more efficient isolation method and a selection of a better primer pair.

Recently, the development of multiplex real-time NASBA (AmpliDet RNA) has been described (Klerks et al., 2001b). Application of such a multiplex assay for the simultaneous detection of major strawberry aphid-borne viruses like SCV, SMoV, Strawberry mild yellow edge virus and SVBV, might increase the efficiency of strawberry propagation material testing and virus elimination during routine certification.

One of the major goals of this study was a comparison of the newly developed assay to other SVBV detection methods, such as biological indexing and PCR. The sensitivities of both real-time NASBA and the newly optimised PCR were well comparable, making them progressive alternatives to biological indexing. The real-time NASBA has, however, several advantages over PCR. Results of amplification are available immediately (in contrast to PCR, where a gel electrophoresis step is required for detection) and consequently the hands-on time is reduced considerably. A potential carry-over contamination in the post-amplification processing steps is thus eliminated, too. The reduction of handling and the homogenous nature of the real-time NASBA assay also enable full automation and a high-throughput use of NASBA. The fluorescence of molecular beacons may also be measured in less sophisticated and less expensive machines than real-time cyclers, e.g., in spectrofluorometres with temperature control. NASBA could then be performed in standard ELISA format commonly used in certification laboratories. Future use of NASBA or PCR in certification laboratories depends on the availability of suitable equipment: (ELISA) fluorometres with temperature control or real-time readers for NASBA or thermal cyclers for PCR.

In conclusion, the results indicate that NASBA, the RNA amplification method, can be successfully used for the detection of SVBV, a plant pararetrovirus with a DNA genome. The results of our experiments accomplished on the complete available collection of SVBV isolates suggest that the real-time NASBA assay offers a good potential in terms of an accurate, fast and informative detection of SVBV. The number of positive isolates was very limited however, and the ability of this method to detect other SVBV isolates is not guaranteed therefore. At least, it seems to be a promising addition to existing methods (PCR, hybridisation to an SVBV-specific probe and biological indexing) for the detection of this quarantine virus. A field survey on more than 310 strawberry field plants did not confirm the presence of SVBV in the five inspected European countries.

Acknowledgements

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