

DETECTION OF NEMATODE-TRANSMITTED NEPOVIRUSES BY THE NOVEL, ONE-TUBE AMPLIDET RNA ASSAY

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Abstract

A novel highly sensitive isothermal AmpliDet RNA system is described for specific detection of *Arabid mosaic virus* (ArMV), *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV), *Tomato black ring virus* (TBRV) and *Tomato ringspot virus* (ToRSV) in *in vitro* plant material. Viruses identified using a molecular beacon directed to a selected virus specific sequence within the amplicon formed during NASBA.

1. Introduction

Large scale micropropagation of fruit plant species is routine now, but evaluation of plant health awaits resolution before full commercial exploitation of the technology will be achieved. In the certification of fruit plants there is an urgent need for the development of rapid, reliable, sensitive and user-friendly methods for detection and identification of harmful organisms. The release of fruit plant cultivars to fruit plant growers takes several years until all known as well as diseases of unknown etiology related with a plant species are checked by indexing methods currently in use. The combination of disease elimination and disease-indexing on *in vitro* plants, using reliable laboratory diagnostics, would considerably reduce the efforts and contribute to savings of time, money and labour.

AmpliDet RNA is a recently developed detection method and is based on the combination of two technologies: NASBA amplification of RNA and simultaneous fluorescent detection of the amplification product by means of a molecular beacon probe (Leone *et al.*, 1998). NASBA (Kievits *et al.*, 1991) is an isothermal amplification method which allows, by the concurrent activity of AMV reverse transcriptase, RNase H and T7 RNA polymerase in combination with target specific primers, an exponential amplification of RNA in 90 minutes at one temperature (41°C). Molecular beacons (Tyagi and Kramer, 1996) are a new class of oligonucleotides capable of forming a hairpin loop with a fluorophore and a quencher attached to the two ends of the stem. Molecular beacons emit an intense fluorescent signal only when hybridised to their target RNA molecules (Fig. 1). This provides excellent selectivity for the analysis of RNA molecules and the fluorescence produced can be measured in real-time as well as at the end of the reaction in unopened vessels, making the AmpliDet RNA suitable for routine applications.

In this paper we describe the development of isothermal AmpliDet RNA systems for the detection of nematode-transmitted nepoviruses of fruit crops in *in vitro* plant material. Detection systems for *Arabid mosaic virus* (ArMV), *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV), *Tomato black ring virus* (TBRV) and *Tomato ringspot virus* (ToRSV) are designed. We show that a separate gel-based detection of amplification products is no longer needed, eliminating the high risk of cross-contamination and thus giving rise to a reliable diagnosis of viruses.

2. Materials and methods

2.1. Virus isolates

Fifteen virus isolates of ArMV, RpRSV, SLRSV, TBRV and ToRSV present in the plant virus collection of Plant Research International were used for the development of NASBA. Purified virus (not available for ArMV and SLRSV) or infected leave material was used for extracting (total) RNA using the RNeasy isolation kit following the manufacturer's instructions (Qiagen).

2.2. Primers and probes

For the amplification of viral RNA by NASBA, virus-specific primers (P1 and P2) were designed within the coat protein (CP) -encoding region of each virus, which is located on RNA-2. The P1 down stream primers consists a 3' target complementary sequence and a 5' T7 polymerase recognition sequence site. The P2 forward primers, are enclosing a virus specific domain with their P1 counterpart of about 150-300 nt. Biotinylated probes were developed to detect the different virus-specific NASBA amplicons.

2.3. Molecular beacons

For the real-time detection of ArMV, RpRSV, SLRSV, TBRV and ToRSV amplicons, virus-specific molecular beacons were designed. Each molecular beacon has a 5' and a 3' arm sequence of 6 nucleotides and a sequence of 18-22 nucleotides complementary to the specific amplicon. The arm sequences of the molecular beacons form a double-stranded structure at 41°C to ensure nonhybridised molecular beacons to be 'dark' and were designed to hybridise with each other and not with the amplicon. To detect the specific NASBA amplicons of TBRV, SLRSV and ToRSV the molecular beacons were coupled with fluorescein (FAM) and the non-fluorescent quencher DABCYL. For detection of RpRSV and ArMV amplicon the molecular beacons were coupled with tetrachloro-6-carboxyfluorescein (TET) and the non-fluorescent quencher DABCYL. The emission maximum of the fluorescent labels FAM (530 nm) and TET (538 nm) are discriminated by using the ABI Prism 7700 thermal cycler. The used molecular beacons were HPLC-purified (Isogen, The Netherlands).

2.4. NASBA

For each reaction the NASBA was performed as previously described (Kieviets *et al.*, 1991; Compton, 1991). The NASBA reaction mix consisted of 4 µl 5x NASBA-buffer (200 mM Tris-HCl, pH 8.5, 60 mM MgCl₂, 350 mM KCl, 2.5 mM DTT, 5 mM of each dNTP, 10 mM each of ATP, UTP and CTP, 7.5 mM GTP and 2.5 mM ITP), 4 µl 5x primer mix (75% DMSO and 1 µM of each primer) and 2 µl of RNase-free water per reaction. In case of a multiplex NASBA reaction the primer mix consisted of two different target-specific primers pairs. A volume of 5 µl of sample solution (purified virus or total RNA extract) or water (negative control) was added to the NASBA reaction mix. The reactions were then pre-incubated at 65°C followed by 41°C for 5 min. The NASBA reaction was started by adding 5 µl of enzyme-mix (375 mM sorbitol, 2.1 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase) per reaction, incubated for 5 min at 41°C, shortly centrifuged and incubated again for 90 min at 41°C.

Real-time amplification and detection using AmpliDet RNA was carried out as described above, except the 2 µl RNase-free water was replaced by 1 µl 8 pmol ROX [5-(and -6)-carboxy-X-rhodamine]/µl and 1 µl of molecular beacons solution (9-18 ng/µl). The 90 min incubation at 41°C was performed in the ABI Prism 7700 thermal cycler and

the emission spectrum of either one or both labels FAM and TET was measured real-time in each sample every 2 min. If needed the reactions were stored at -20°C afterwards.

2.5. Detection of NASBA amplicons by enhanced chemiluminescence (ECL).

NASBA products were analysed by electrophoresis using a 1 % pronarose gel containing $0.5\ \mu\text{g} / \text{ml}$ EtBr. Gels were run at 100 V for 15 min in buffer containing 40 mM Tris-acetate and 1 mM EDTA, pH 8.0 (1x TAE). The gel was blotted onto a Z-probe nylon membrane in 0.3 M NaCl and 30 mM Na-citrate (2x SSC) solution for 20 min. Nucleic acids were cross-linked onto the Z-probe by UV exposure (280 nm) for 2 min. Hybridisation of the amplicon specific biotinylated probe solution ($3\ \mu\text{M}$) to the NASBA products occurred at 50°C for 30-60 min in hybridisation-mix (5x SSC, 7 % SDS, 20 mM Na-phosphate, pH 6.7, 10x Denhardt's solution). The blots were washed twice with 3x SSC, 1 % SDS at 50°C for 5 min and once with buffer containing 0.1 % SDS with 20 mM Na_2HPO_4 , 0.36 M NaCl and 2 mM EDTA (2x SSPE) at room temperature for 10 min. The blot was then incubated for 30 min with $2\ \mu\text{l}$ streptavidin / peroxidase conjugate in 5x SSPE with 0.5 % SDS, followed by washing three times with 2x SSPE with 0.1 % SDS for 1 min (twice) and 10 min, respectively. Then the blots were washed twice with 2x SSPE for 2 min, followed by incubation of the blots in substrate solution for 60 sec and then exposed to X-ray films.

3. Results

3.1. Development of virus specific NASBA amplification systems

For NASBA primer design we compared the available sequence data of ArMV, RpRSV, SLRSV, TBRV and ToRSV. Based on the absence sequence homology of RNA-2, the only component of which sequence information is available of all viruses involved, we developed the virus-specific primers. To optimise virus-specific NASBA amplification systems *in vitro* RNA was produced from viral RNA of ArMV, RpRSV, SLRSV, TBRV and ToRSV. These RNA transcripts were used to test the sensitivity of the NASBA primers. For each virus two forward and two reverse primers were developed and their combinations tested in the developed NASBA procedure. The most optimal primer set was determined based on the ultimate detection level of each combination of primers using dilution series of transcript RNA. For each virus the NASBA amplicons were revealed by northern blotting (gel based system) using a virus-specific biotinylated probe (ECL probe) (Fig. 2). The most optimal combinations of primers allowed the detection of 10^4 RNA transcripts of TBRV, 100 of RpRSV, ArMV, ToRSV and 10 of SLRSV. Fifteen virus isolates present in the plant virus collection of Plant Research International were tested using the virus specific NASBA systems. Data in table 1 show that all isolates were readily detected in the gel-based system.

3.2. Development of virus-specific molecular beacons for detection of the NASBA-amplicons

To allow virus specific detection in a gel-free system (one-tube assay), molecular beacons were designed to be specific for all virus isolates. The optimal concentrations of the molecular beacons and other components in the reaction mix were determined to develop a robust amplification and detection system (Fig. 2). All isolates of TBRV, ArMV, SLRSV, ToRSV and RpRSV were detected in the one-tube assay using molecular beacons (Table 1). The sensitivity reached with these MB systems on average is about 1-10 times less sensitive as compared to ECL detection although further optimisation for ToRSV and ArMV is in progress.

3.3. Multiplex detection

For multiplex detection of RpRSV/TBRV or ArMV/SLRSV the originally designed primers and molecular beacons from the individual AmpliDet RNA were combined. To determine if these viruses could be detected and distinguished from each other by these multiplex assays, three separate reactions were carried out. Each reaction contained 10 pg *in vitro* RNA of ArMV; SLRSV or ArMV together with SLRSV or water. The same was performed for RpRSV/TBRV (not shown). The fluorescence of the molecular beacons complementary to the expected virus specific amplicons clearly detected the virus originally present in each reaction tube (Fig. 3). The four primers and two molecular beacons to detect both viruses did not interfere with each other in one closed tube. These results demonstrate that each molecular beacon binds specifically to its complementary target amplicon and the emission spectra of both molecular beacons could be easily distinguished from each other in this multiplex AmpliDet RNA system.

4. Discussion

We have developed and evaluated the effectiveness of the NASBA system for the detection of the nematode-transmitted nepoviruses ArMV, RpRSV, SLRSV, TBRV and ToRSV. Based on sequence homology of RNA-2, five different virus-specific primer pairs were designed. A large number of virus isolates from the plant virus collection of Plant Research International were readily detected using NASBA and molecular beacons. Due to the lack of global sequence homology between the different nepoviruses, the development of group specific primer sets was not feasible. However introduction of more probes with different fluorescent labels in the reaction enabled simultaneous detection of ArMV/SLRSV or RpRSV/TBRV. These results show that AmpliDet RNA is a powerful tool for virus detection. By simultaneous amplification and detection of RNA in a single reaction tube, carry-over contamination introduced by gel analysis and northern blotting procedures are prevented. No separate detection is necessary anymore reducing 'hands on' time without losing sensitivity. This indicates that NASBA combined with the molecular beacon technology might be very suitable for routine diagnostics. Currently, material costs involved with AmpliDet RNA are still higher than those of antibody- or PCR-based assays. Therefore at this moment, the application fields of AmpliDet RNA include primarily those situations where detection by antibody-based techniques are difficult or impossible, clean stock programs of very high-quality propagative material, and detection of viable pathogens.

Acknowledgements

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References

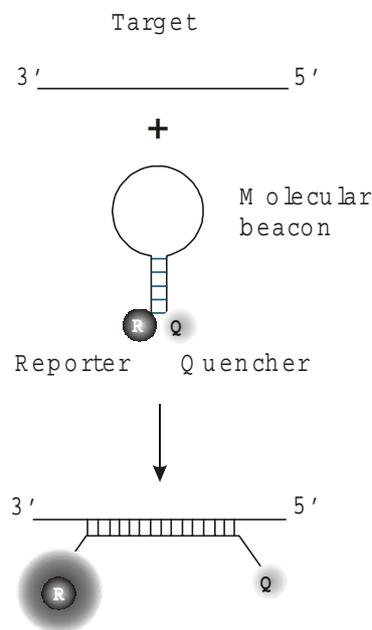
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Tables

- NASBA of nepovirus RNA. NASBA amplicon is detected with both virus-specific ECL probes and Molecular beacons. The tested virus isolates derived from the plant virus collection of Plant Research International. The sensitivity for ECL and MB detection for the indicated viruses was measured by the number of detectable copies in vitro RNA and, if available also with viral RNA. (*): Not fully optimised yet.

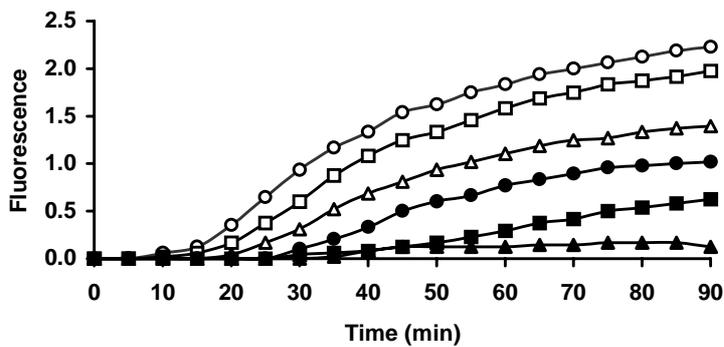
Virus	Isolates	ECL (RNA COPIES)	MB (RNA COPIES)
TBRV	TBRV AC170II TBRV B21 TBRV SLA TBRV Chen2	10 ⁴	10 ⁵
ToRSV	ToRSV Pelargonium ToRSV Fulton	10 ²	10 ³ (*)
SLRSV	SLRSV R44 SLRSV B143III	10	10
RpRSV	RpRSV Lepelblad RpRSV Hollings RpRSV v/d Meer	10 ²	10 ²
ArMV	ArMV Baskoop ArMV RA-5 ArMV Tu-4 ArMV Ac180	10 ²	10 ⁴ (*)

Figures

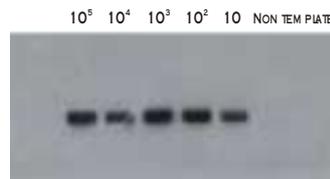


- Structure of a molecular beacon before (top) and after target binding (bottom). Molecular beacons in the closed state show a typical stem-and-loop structure. When the molecular beacon is not hybridised to its target, the light emitted by the fluorescent dye (R) is quenched by the quencher DABCYL (Q). Upon hybridisation to the complementary target, the stem structure is forced to open and the fluorescence is no longer quenched.

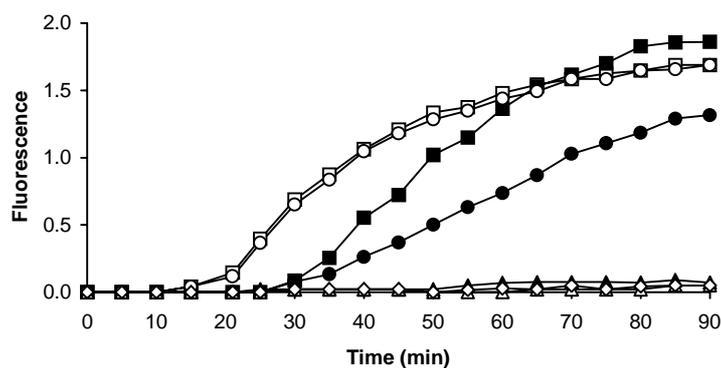
Real-time detection of SLRSV (A)



ECL detection of SLRV (B)



2. (A). Real-time detection of SLRV in the presence of a FAM labelled MB and (B). ECL detection with a biotinylated detection probe for SLRV after NASBA amplification. Dilution series of SLRV *in vitro* RNA were tested, each containing 10^5 (○), 10^4 (□), 10^3 (△), 10^2 (●) or 10 (■) or none (▲) RNA copies.



3. Real-time multiplex AmpliDet RNA on purified plant RNA spiked with 10 pg *in vitro* RNA of ArMV (■,▲); SLRSV (□,▲) or ArMV together with SLRSV (○,●) measured for TET (filled symbols) and FAM signal (open symbols). (◇) Represents the control incubation without added virus measured for both TET and FAM signals. During the different AmpliDet reactions all specific primers and molecular beacons were present for both viruses.