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Rapid and homogenous detection of *Apple stem pitting* virus by RT-PCR and a fluorogenic 3′ minor groove binder-DNA probe

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Abstract

Real-time PCR with fluorogenic hydrolysis probes (5′ nuclease assay) is increasingly used for the detection of pathogens for diagnostic purposes. Nevertheless, the size of the probes, usually 25–40 nucleotides, might limit their use to detect pathogens with high genome variability between isolates, where an identical sequence cannot be found without multiple mismatches. In this report, we describe a 5′ nuclease assay, to detect *Apple stem pitting* virus, based on the use of a shorter probe which is chemically modified with a minor groove binder in order to increase duplex stability and raise the melting temperature to a value suitable for real-time analysis. The short size of the probe, which is critical to target a conserved cluster sequence of 14 nucleotides in the RNA polymerase gene, circumvents the genome variability of the virus. The assay correlates at 96 percent with gel analysis and is more reliable than biological indexing to detect *Apple stem pitting* virus field isolates. It is fast and fully compatible with automation, and therefore particularly suitable for plant certification.

Introduction

Apple stem pitting virus (ASPV; genus *Foveavirus*) is a latent virus of apple trees, frequently infecting commercial cultivars (Stouffer, 1989). *Pear vein yellow* virus (PVYV) which induces vein yellowing and, in some cases, stony pits in pear, corresponds to strains of ASPV. The green crinkle and star crack diseases of apple are also associated with ASPV (Desvignes, 1999). The full sequence of the 9.3 kb ssRNA virus genome has been reported (Jelkmann and Keim-Konrad, 1997). Sequences at the 3′ end of the genome, including the coat protein gene, were highly variable amongst isolates (Nemchinov et al., 1998; Schwarz and Jelkmann, 1998; Yoshikawa et al., 2001). ASPV is found worldwide and frequently occurs in combination with other latent viruses such as *Apple chlorotic leafspot* virus (ACLSV) and *Apple stem grooving* virus (ASGV). Commercial apple tree varieties remain symptomless after infection, but growth and crop yield can be reduced. Symptoms only occur on a few indicators and on some ornamental *Malus* species. Biological indexing by grafting on woody indicators constitutes the baseline tests to certify fruit tree planting material, but appears cumbersome, time-consuming and expensive to perform and does not respond to the actual constraints of production of certified material. In these conditions, reliable and fast detection protocols are important for the implementation of sanitary controls for fruit propagation material.

The number of applications based on specific real-time detection using dual-labeled hybridization or hydrolysis probes is increasing rapidly (Bustin, 2000; Fang et al., 2000; Holland et al., 1991; Lie and Petropoulos, 1998; Livak et al., 1995; Thelwell et al., 2000; Tyagi and Kramer, 1996; Whitcombe et al., 1999). These techniques are particularly attractive since they combine the specificity of PCR amplification with DNA hybridization. The 5′ nuclease assay

is based on the hydrolysis of a probe that hybridizes specifically to the target PCR product during amplification. The probe carries both a fluorescent reporter dye at the 5′ end and a quencher at the 3′ end. During strand elongation, the probe is cleaved by the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase, resulting in the separation of the fluorescent dye and the quencher, and a subsequent increase in fluorescence. Repeated cycles result in the exponential amplification of the target sequence with a parallel increase in fluorescence, which is measured at each cycle of the PCR. The critical parameter is the threshold cycle (C_T) which is defined as the cycle number at which fluorescence crosses a fixed threshold value above the baseline emission. Interpretation of the data can be achieved within minutes at the end of the run. As reaction tubes remain closed throughout the assay, the risk of PCR carry-over contaminations is reduced dramatically.

For some diagnostic applications, one could be faced with several problems related to requirements in the design of the probes. Indeed, hydrolysis probes must necessarily hybridize prior to the primers and the duplex must be stable at the working temperature of *Taq* polymerase. Therefore, probes with melting temperature of 65–72 °C are optimal (Livak et al., 1996). Usually, such a probe would require 25–40 nucleotides. While probes of this size are generally suitable for most applications, they could be too long when targeting pathogens with high genomic variability between strains, where an identical sequence of 25–40 nucleotides cannot be found. Indeed, mismatches between the probe and the target dramatically decrease the signal, leading to false negative results. A strategy to circumvent this problem would be the design of shorter probes within highly conserved clusters in order to minimize the probability to have mismatches between the probe and the target. Such an approach has been made possible by the work of Kutyavin et al., who reported the synthesis of oligonucleotide primers (Kutyavin et al., 1997) and fluorogenic hydrolysis probes (Kutyavin et al., 2000) conjugated to a minor groove binder (MGB). The covalent attachment of molecules like dihydropyrroloindole tripeptides (DPI3) that bind the minor groove of double strand DNA stabilize the duplexes and the hybridization strength through van der Waals contacts, hydrophobic and electrostatic interactions. As a consequence, the melting temperature of the oligonucleotide increases, so that a value of 65–70 °C can be reached with very short probes of 12–15 nucleotides. Since the addition of an MGB moiety at the 3′ end of a probe does not inhibit PCR and has no effect on the exonuclease activity of *Taq* polymerase (Kutyavin et al., 2000), 3′ MGB DNA probes could be of particular interest for the development of fluorogenic 5′ nuclease assays to detect viruses with strong sequence variability between strains.

Here we describe the development of a fast homogenous fluorogenic 5′ nuclease RT-PCR assay to detect ASPV from infected trees and emphasize the usefulness of 3′ MGB DNA probes to circumvent the variability of virus isolates. Although MGB probes have been used recently to detect mutations associated with hereditary hemochromatosis (Walburger et al., 2001), this is the first report that describes the use of a fluorogenic 3′ MGB DNA probe for detection of a pathogen.

Materials and methods

Plant material

The plant material used for the development and the design of the fluorogenic probe consisted of ASPV-infected reference material from the laboratory: A1, A4, C4, 10291, T3058 and LP680 correspond to apple trees grown in the field, the Golden material originating from IAM Vienna (Austria) is propagated *in vitro* and isolate 3536 received from IPO/DLO Wageningen (The Netherlands) was in *Nicotiana occidentalis* 37B. For the validation of the real-time assay, 3 types of pome fruit materials were used: 13 reference trees from the orchard of the laboratory (A1 to 91325 in Table 1); 50 samples received from J.C. Desvignes (CTIFL, Lanxade, France) consisting of twigs of dormant wood taken from trees containing apple latent viruses: ASPV, ASGV, ACLSV in single or mixed infections (samples numbered from 1 to 55 in Table 1) and 8 apple cultivars taken in a nursery in La Hulpe, Belgium (Elstar, Jonathan, Jonagold, Melrose, Quastresse, Reinette France, Reinette Etoilee and Reinette Descadre). All the material was from apple trees except F1, H1 and I1 (from pear).

RNA extraction

Total RNA from bark tissues was extracted according to the technique of Bugos et al. (1995) or using the Qiagen RNEasy Plant RNA kit (Qiagen, Hilden, Germany) and a slightly modified protocol for woody plants, according to MacKenzie et al. (1997).

Table 1. Detection of *Apple stem pitting* virus from field isolates

Sample ID	Indexing	Gel	C_T (1)	C_T (2)	C_T (3)	Av.	Std. dev.	Sample ID	Indexing	Gel	C_T (1)	C_T (2)	C_T (3)	Av.	Std. dev.
A1	+	+	22	22	23	22.3	0.58	28	+	+	24	25	25	24.7	0.58
A4	+	+	21	21	22	21.3	0.58	29	+	+	23	26	26	25.0	1.73
B3	−	+	22	22	21	21.7	0.58	30	+	+	25	27	28	26.7	1.53
B4	−	−	37	37	37	37.0	0.00	31	−	+	26	28	28	27.3	1.15
C4	−	+	25	26	26	25.7	0.58	32	+	+	26	27	27	26.7	0.58
C7	+	+	24	26	26	25.3	1.15	33	+	+	29	30	30	29.7	0.58
D4	−	−	40	36	40	38.7	2.31	34	+	+	25	27	26	26.0	1.00
F1	−	+	22	21	23	22.0	1.00	35	−	+/−	40	40	38	39.3	1.15
H1	+	+	19	18	19	18.7	0.58	37	+	+	25	27	26	26.0	1.00
I1	+	+	20	20	21	20.3	0.58	38	+	+	24	26	26	25.3	1.15
LP680	+	+	35	40	40	38.3	2.89	39	−	+	26	28	28	27.3	1.15
T3058	+	+	35	40	40	38.3	2.89	40	+	+	26	27	28	27.0	1.00
91325	+	+	32	33	34	33.0	1.00	41	+	+	24	NT	NT		
10291	+	+	21	22	23	22.0	1.00	42	−	+/−	40	36	36	37.3	2.31
1	−	−	40	38	39	39.0	1.00	43	+	+	28	28	28	28.0	0.00
2	−	−	40	39	40	39.7	0.58	44	+	+	25	25	26	25.3	0.58
3	−	−	40	40	40	40.0	0.00	45	+	+	29	31	31	30.3	1.15
4	−	−	40	39	40	39.7	0.58	46	−	+	34	37	36	35.7	1.53
7	−	+/−	37	37	37	37.0	0.00	47	+	+	24	27	27	26.0	1.73
8	−	+/−	40	38	39	39.0	1.00	48	+	+	24	26	26	25.3	1.15
9	+	+	28	34	33	31.7	3.21	49	+	+	22	23	23	22.7	0.58
10	−	+	23	23	23	23.0	0.00	50	+	+	25	27	26	26.0	1.00
11	−	+/−	39	37	37	37.7	1.15	51	−	+	26	25	25	25.3	0.58
12	+	+	22	24	24	23.3	1.15	52	+	+	28	30	30	29.3	1.15
13	−	+	25	27	28	26.7	1.53	53	+	+	NT	31	NT		
14	+	+	25	28	28	27.0	1.73	54	−	+	34	34	34	34.0	0.00
15	+	+	29	32	33	31.3	2.08	55	+	+	26	28	28	27.3	1.15
16	−	+	25	26	27	26.0	1.00	Elstar	NT	−	NT	40	40	40.0	0.00
17	+	+/−	40	40	39	39.7	0.58	Jonathan	NT	−	NT	40	40	40.0	0.00
18	+	+	24	26	26	25.3	1.15	Jonagold	NT	−	NT	40	40	40.0	0.00
19	+	+	22	24	24	23.3	1.15	Melrose	NT	−	NT	40	40	40.0	0.00
20	+	+	24	28	27	26.3	2.08	Quastresse	NT	+	NT	23	23	23.0	0.00
22	+	+	40	40	40	40.0	0.00	R. France	NT	−	NT	40	40	40.0	0.00
23	+	+	25	27	27	26.3	1.15	R. étoilée	NT	+/−	NT	38	37	37.5	0.71
24	+	+	24	27	27	26.0	1.73	R. Descadre	NT	+	NT	24	24	24.0	0.00
25	+	+	25	28	28	27.0	1.73	NTC		−	40	40	40	40.0	0.00
27	+	+	24	26	27	25.7	1.53								

The table summarizes data from biological indexing, gel analysis and real-time detection. +/− indicates weak amplifications with ambiguous detection on gel. For real-time analysis, the threshold cycles are given from three separate experiments, i.e. C_T (1), C_T (2) and C_T (3), as well as the average values (Av.) and standard deviations (Std. dev.). By convention, a C_T of 40 means there is no amplification of the target sequence (no fluorescence above the threshold). NTC: no template control; NT: not tested.

RNA concentrations were calculated after determining the absorbance at 260 nm.

Cloning and sequencing of amplification products

RT-PCR products from infected apple trees were cloned into the pCR2.1 vector following the instructions of the supplier (TA cloning, Invitrogen, Groningen, The Netherlands). All inserts were sequenced by MWG Biotech (Ebersberg, Germany).

Probe synthesis

The 3′ MGB DNA probe (MGB 148) was selected by following the general rules outlined by Livak et al. (1996), on the basis of sequences aligned using PILEUP (Wisconsin Package Version 10.1, Genetic Computer Group, USA). The Primer Express 5.1 software (Applied Biosystems, Forster City, USA) was used for MGB probe validation and accurate Tm calculations. The probe (5′ TTTGCCATAATGCG)

was supplied by Applied Biosystems, with 6-carboxyfluorescein (FAM) as the reporter dye covalently attached at the 5′ end, a non-fluorescent quencher and the MGB moiety at the 3′ end. The MGB prevented the 3′ extension of the probe.

5′ nuclease RT-PCR assay

RT-PCR amplifications were performed by the OneStep RT-PCR Kit (Qiagen, Hilden, Germany) using primers 4F and 4R (Kummert et al., 2000). The system uses a hotstart polymerase and allows RT and PCR to be performed in a single tube. A 25-µl reaction mixture containing total RNA (200–400 ng), 0.4 µM of each dNTP, 0.6 µM of both primers, 0.2 µM of the MGB 148 probe and the reagents from the kit (5× buffer, enzyme mix) was submitted to cDNA synthesis (30 min at 50 °C), *Taq* polymerase activation (15 min at 95 °C) and amplification with 40 cycles (30 s at 95 °C, 1 min at 55 °C and 1 min at 60 °C). The GeneAmp 5700 Sequence Detection System (Applied Biosystems, Forster City, USA) was used for thermal cycling and to record fluorescence at the extension step of each amplification cycle. The threshold cycles were calculated using the 5700 SDS software. In most experiments, 10 µl of amplified products were analyzed on agarose gel after electrophoresis and ethidium bromide staining.

Results

Probe design

Conserved regions amongst available ASPV strains were identified within the 3′ end of the genes encoding RNA polymerase and coat protein, and in the 3′ non-coding region. Therefore, primers 4F and 4R were selected to amplify a target sequence of 251 bp within the 3′ end of the RNA polymerase gene (Kummert et al., 2000). Good amplifications were observed by gel analysis from all trees diagnosed as ASPV-infected by biological indexing.

Although primers allow efficient amplification even if they do not perfectly match the target, hydrolysis probes are much more specific and fluorescence intensity dramatically decreases if mismatches occur between the probe and the target. Therefore, a probe which is dedicated to detect all or most ASPV isolates should be designed on the basis of multiple target sequence alignments. Thus RT-PCR products from 8 infected trees (A1, A4, C4, Golden, 10291, T3058, 3536, LP680) available at the Gembloux University were cloned into pCR2.1 and sequenced. The alignment of these sequences together with the one published by Jelkmann (1994; accession ID: D21828) demonstrated strong sequence heterogeneity between isolates (Figure 1). As a result, we were unable to define a TaqMan probe of 25–35 nucleotides without multiple mismatches between the probe and the target sequence of most isolates from the 8 reference trees. Therefore, we designed a shorter probe of 14 nucleotides that perfectly matched 7 target sequences. The covalent attachment of an MGB moiety at the 3′ end of the probe raised the Tm to a range suitable for real-time PCR (66.3 °C).

Sensitivity of the 5′ nuclease assay compared to agarose gel electrophoresis

Serial dilutions in DEPC-treated water of total RNA extracted from tree 10291 were prepared and used as starting material to determine the relative sensitivity of the 5′ nuclease assay and agarose gel electrophoresis. As demonstrated in Figure 2 (panels A and B), the expected correlation between the amount of starting material and the corresponding C_T was observed in the 5′ nuclease assay. The dynamic range of the assay proved to be between 200 ng and 20 pg of starting RNA material. Gel analysis was at least equally sensitive (Figure 2C).

Detection of Apple stem pitting *virus from field isolates*

The effectiveness of the 5′ nuclease assay using the fluorogenic MGB 148 probe was assessed by applying the protocol for the detection of ASPV from a large collection of infected trees from the CTIFL, the Gembloux greenhouse and a nursery. Results were correlated with gel analysis. Twelve tests that were negative on gel were used for the calculation of a positive–negative threshold (B4, D4, 1, 2, 3, 4, Elstar, Jonathan, Jonagold, Melrose, Reinette France and the no template control) which was fixed at 36.9 as the mean $C_T - 3 \times$ St. Dev (Chebychev's equivalent). Seventy-two samples were tested in triplicate both in real-time and on gel in separate experiments (except sample 41 and 53 which were tested in a single run). The results are summarized in Table 1. Out of 72 samples tested, 51 were strongly positive in real-time. Three additional samples

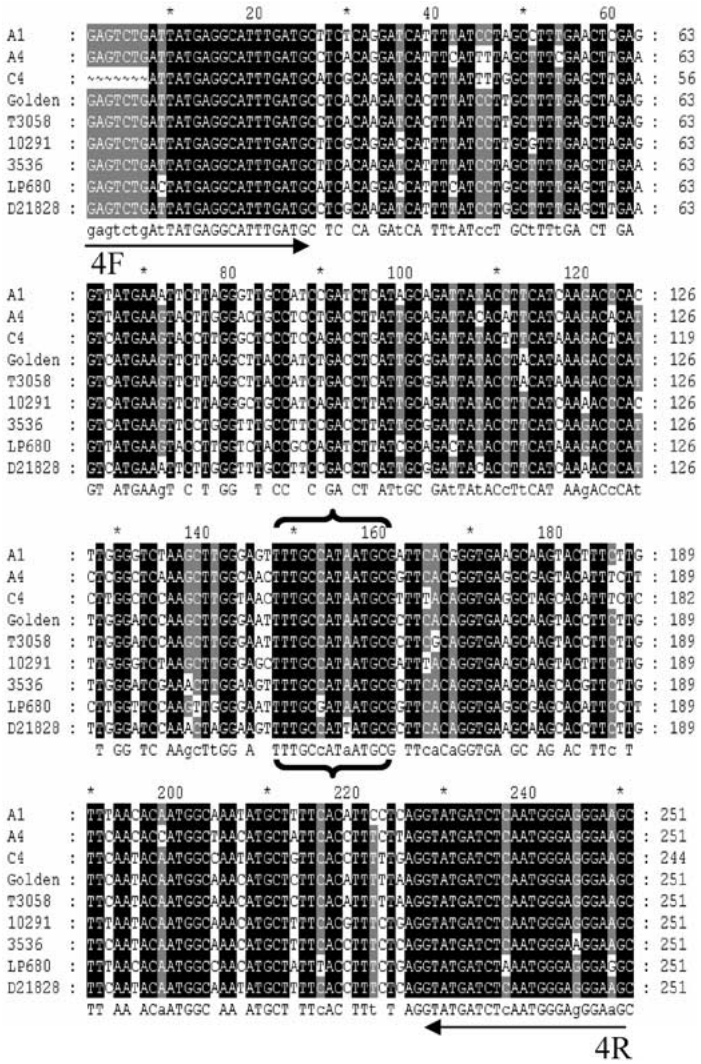

Figure 1. Apple stem pitting virus sequence alignments. The nucleotide sequences from height ASPV-infected reference trees are compared with the corresponding region of D21828 (Jelkmann, 1994). Sequence of MGB 148 is indicated by asterisks and primers 4F and 4R are underlined.

were detected on gel (LP680, T3058 and 22). Eleven trees were negative using both techniques. For 7 samples however, amplification was so weak that interpretation of the data was difficult on gel as well as in real-time (7, 8, 11, 17, 35, 42 and Reinette Etoilee). Except for sample 17 which was indexed positive and Reinette Etoilee which was not indexed, these samples were negative by indexing, which might reflect a weak viral load. Compared to biological indexing (42 positive trees out of 64 tested), real-time analysis proved to be more sensitive, as ASPV was detected unequivocally in 11 trees that were formerly identified as ASPV-free by indexing (B3, C4, F1, 10, 13, 16, 31, 39, 46, 51, 54).

Discussion

In the certification of fruit plants, there is a demand and an urgent need for the development of reliable, sensitive and user-friendly methods for detection and identification of viruses. Biological indexing, which currently represents the reference test for the certification of fruit tree planting material, remains lengthy

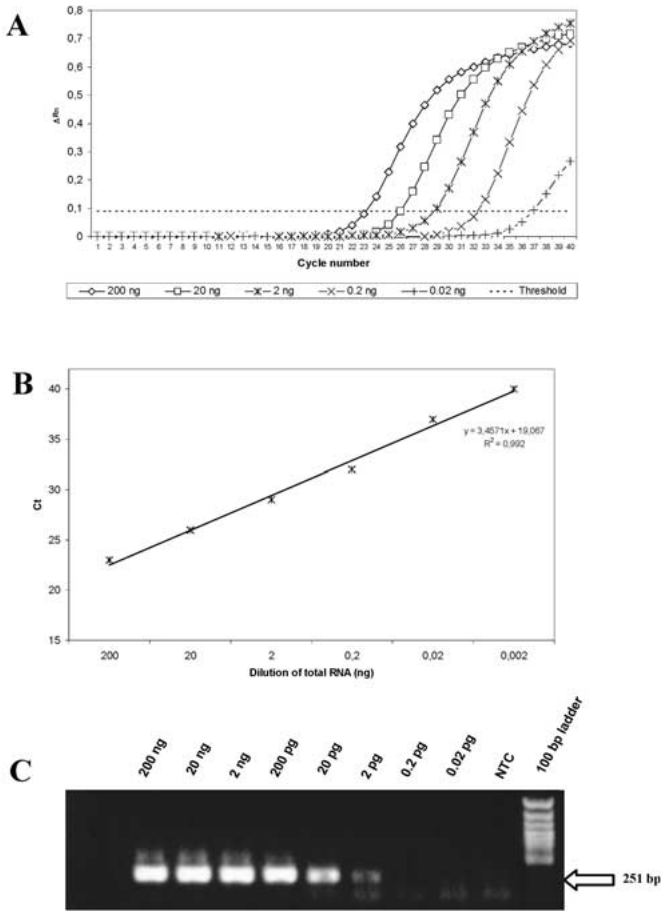

Figure 2. Relative sensitivity of the 5′ nuclease RT-PCR assay and gel analysis to detect ASPV from serial dilutions of total RNA from 10291 reference tree. A: Amplification plot showing cycle number versus normalized fluorescence (ΔRn) in the range of 200 ng–20 pg of total RNA. B: Plot of the amount of starting RNA versus threshold cycle (C_T). C: Gel analysis.

and does not respond to the actual constraints of the producers. Serological detection by enzyme-linked immunosorbent assay (ELISA) using commercially available antisera is the first alternative to biological indexing. However, for ASPV, good quality commercial antisera to be used in ELISA tests are not available so far. Gene amplification techniques represent a second alternative for indexing (Jelkmann and Keim-Konrad, 1997; Nemchinov et al., 1998; Schwarz and Jelkmann, 1998). Although these techniques are widely accepted in research laboratories, cumbersome and time-consuming post-PCR detection processes like gel analysis or microplate hybridization remain a major drawback in scaling-up for routine certification purposes. A new assay that combines nucleic acid sequence-based amplification (NASBA) with molecular beacons has been described recently (Klerks et al., 2001). Five biologically characterized isolates from a field trial and 12 out of 14 isolates from a plant virus collection were detected using this test (89 percent).

In this paper, we describe the development of a new 5′ nuclease RT-PCR assay for the real-time detection of ASPV, and the use of a fluorogenic probe of 14 nucleotides conjugated to a MGB. The covalent attachment of the MGB moiety stabilized the probe/target duplex and raised the Tm to a range suitable for the assay. This approach allowed us to circumvent genome variability of ASPV by targeting a short cluster sequence within the RNA polymerase gene which we found to be conserved for most ASPV isolates. The test uses bark shavings as a starting material for RNA isolation, and therefore, enables ASPV detection throughout the year.

To determine the threshold of sensitivity, we used serial ten-fold dilutions of total RNA from an ASPV-infected reference tree. The 5′ nuclease assay proved to be roughly as sensitive than gel analysis, with a threshold between 20 and 2 pg of plant RNA. As expected, the C_T values correlated with the amount of target input material: when plotting C_T versus sample dilutions, we observed that the C_T increased linearly while the amount of target was decreasing. Therefore, although not quantitative, the assay provides a crude estimation of the concentration of the virus in the sample tested. The same experiment was performed with a TaqMan probe of 27 nucleotides that perfectly matched the target sequence (data not shown). Compared to the MGB probe, TaqMan sensitivity was identical, with equivalent C_T values, but relative fluorescence was lower. We assume the quenching process to be more efficient with MGB probes. Indeed, the use of a non-fluorescent quencher in the MGB probe instead of a fluorescent quencher in the TaqMan oligonucleotide, might decrease the baseline fluorescence, with a higher signal to noise ratio for the MGB probe.

For assay validation, 72 plants of various geographical origins from collections or a nursery were tested in triplicate. The assay proved to be more sensitive and reliable than biological indexing as 11 samples from trees identified as ASPV-free by biological indexing were scored as positive. Only 3 isolates were not detected in the fluorogenic assay compared to gel analysis. For two of them, i.e. LP680 and 22, sequence data revealed mismatches between the probe and the corresponding target sequence (data not shown), which probably prevented the fluorogenic reaction to occur. Although there is no experimental evidence to support this hypothesis, one can imagine that a mismatch not only disrupts duplex stability and decreases the Tm, but also interferes with the enzymatic digestion of the probe by *Taq* polymerase. On another hand, it has been demonstrated that a mismatch within the 3′ terminal MGB binding domain reduces dramatically the Tm leveling effect provided by the MGB and therefore prevents hybridization and further processing of the probe at annealing and extension temperatures (Kutyavin et al., 2000). Thus, although the likelihood of having a mismatch is reduced by targeting a conserved cluster sequence, only one nucleotide difference could prevent the fluorogenic reaction to proceed and generate false-negative results. The position of the mismatch is believed to be critical. Therefore, as a general guideline, MGB probes should be designed carefully using multiple sequence alignments and validation should include enough field isolates from various geographical origins to be representative of the virus variability.

In conclusion, this paper described the first 5′ nuclease assay based on MGB DNA probes for the detection of plant viruses. The assay, which targets ASPV, correlated with gel analysis, except for 3 isolates that were not detected. The test offers multiple additional benefits being of primary importance in plant diagnosis: it can be performed within 3 h, occurs in a single closed tube, does not require any post-PCR detection step, and therefore dramatically reduces the risk of carry-over contamination and misdiagnosis in routine use. Moreover, unlike gel analysis, the assay is fully compatible with high-throughput analysis and automation. We also proved that 3′ MGB DNA probes targeting very short but strongly conserved sequences can be used to improve the detection of plant, animal or human viruses with high genome variability between strains.

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