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ORIGINAL PAPER

An accurate real-time PCR test for the detection and quantification of cauliflower mosaïc virus (CaMV): applicable in GMO screening

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Abstract Due to its very large use in the first generation of genetically modified organisms (GMO), the 35S promoter derived from the cauliflower mosaic virus (CaMV) is the most used PCR target for screening tests in GMO routine analysis, before any identification and quantification of GMOs. Accordingly, a specific detection of the virus donor organism is required to avoid false positives. A new qualitative and quantitative method based on real time polymerase chain reaction (PCR) techniques was developed for the detection and quantification of CaMV. The region targeted was an internal part of a qualitative test previously published using the ORFIII of the CaMV genome. It codes for a protein (P3) responsible for the pathogen infectivity and not in the ORFIV coding for a coat protein (P4), which can be used for GMO constructions. In this paper, we show the high reliability of the PCR test both in simplex and duplex (with P35S of the CaMV). This test shows high specificity and sensitivity (LODa $\leq$ 10 copies and LOQa $\leq$ 100 copies). Advantages and drawbacks of this test with a previously published test are discussed. Finally, the ORFIII PCR product was cloned in a pGEM-T vector for further use in the near future as an alternative calibrant for quantitative analyses through its availability from the international BCCM/LMBP Ghent plasmid bank.

Keywords Cauliflower mosaic virus (CaMV) · Figwort mosaic virus (FMV) · Carnation etched ring virus (CERV) · Real time PCR · Detection · Quantification · ORFIII · Specificity · Identification · Genetically modified organism (GMO)

Introduction

The controversy surrounding agribiotech crops since the 1990s has been particularly important in the European Union. It also led to the release of several European directive and regulations (2001/18/EEC, 1829/03/EC and 1830/03/EC) able to keep the consumers their freedom of choice. Therefore, processed food and feed containing or derived from genetically modified organisms (GMOs) have to be labelled above a threshold of fortuitous presence. For approved GMO, this threshold is 0.9% per ingredient in EU [1], 5% in Japan [2] and 3% in South Korea (http://www.doh.gov.tw/).

The development and the application of reliable and cost-effective methods based on molecular tools to detect GMOs are thus essential in order to precisely label and control food and feed for offering free choice to consumer and for ensuring the traceability from "farm to fork" and vice-versa [3] This traceability will allow the tracing of GMO materials and derived products along the supply

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chains [4]. All these aspects are studied in a recent European project called Co-Extra (Co-Existence and Traceability; 2005–2009), which should provide practical tools and methodologies for practically implementing co-existence.

The reference technique for gene quantification is real-time PCR. It is proved to be more suitable for the diagnostic laboratory than conventional PCR, due its quantitative performance, its sensitivity and the use of closed tube assays [5]. The real time PCR have been used in most of the methods developed in quantitative analysis of GMO [6]. Many authors looked for more sensitivity and reviewed the application of alternative techniques such as the ligation-dependent probe amplification (LPA) technique by Moreano et al. [7], a quartz crystal microbalance (QCM) DNA-sensor by Passamano et al. [8] or, recently, microarrays by Leimanis et al. [9].

To use the developed methods in routine analysis, its validation have to be performed through inter-laboratory studies which is the case for example of the P35S test validated in 15 French laboratories and based on the strategy promoted by the European Network of GMO Laboratories (ENGL, http://engl.jrc.it/) [10]. Also, in Europe, the Community Reference Laboratory for GM Food and Feed (CRL) has as a core task, the assessment and the validation of the GMO detection methods as part of the European Commission authorization procedure (http://gmo-crl.jrc.it/).

To maintain cost-effectiveness of detection methods, the routine detection labs use generally an initial step of "screening" which target elements present in the inserts of numerous GMOs [11]. In case of positive results, a second step of specific PCR attempts identifies and quantifies the GMO [12] relatively to the plant taxon [13]. The accuracy of the first step (screening) by, e.g., avoiding false positive or false negative results is a crucial requirement.

Screening tests are of major importance for routine analysis in food and feed but also in other kind of materials such as seeds and grains. According to the biotech crop databases BATS (Biosafety Assessment, Technology and Sustainability, http://www.bats.ch/gmo-watch/) and Agbios (http://www.agbios.com), promoters and terminators coming from the donor organisms *CaMV* and *Agrobacterium tumefaciens* are the most commonly used in the currently approved GMO. Some tests have to be developed to distinguish the presence of donor organism, i.e., the taxon from which the sequences of the GMO insert originated. While some of these controls are qualitative, quantitative tests are required to clarify the situation when both GMO and donor organism are present and for use, e.g., in quantitative differential PCR for assessing the presence of unknown GMOs (Ancel et al. unpublished data).

Cauliflower mosaic virus (CaMV) is a 8 kbp member of caulimovirus whose genome is composed of a double stranded circular DNA [14]. It is made of six open reading frames (ORF) and encodes for six proteins [15–17]. Our PCR targeted region is located in the ORFIII [10] and encodes a small basic protein organized into two functional domains located towards the N and C terminal regions. Both domains are required for CaMV infectivity [18] and are thus always present in CaMV strains, in particular the few strains of CaMV (W260, D4 and B29) known to infect solanaceous or both solanaceous and brassicacae (D/H, Bari and CabbS) [19]. As it is known, many genetically modified plants, such as potatoes or papaya, have been created to confer resistance to viruses (e.g., NewLeaf Y Russet Burbank potato) [20]. Since the ORFIV used by Cankar et al. codes for a 56 kDa viral capsid protein (P4), this target might be used later in the construction of GMOs. Thus, probable discordance in specificity results might be observed in the future causing false positives. To avoid such problems, we have chosen the ORFIII as target for designing our CaMV specific test. It codes for a 15 kDa (kilodalton) protein (P3) of 129 AA involved in the CaMV infectivity. Our work describes a quantitative assay that can distinguish the presence of CaMV in presence or not of GMO, and its advantages when compared to other previously developed tests. It demonstrates also the ability of this test to be used in a combination with our previously validated P35S method [10] used worldwide.

Materials and methods

Plant material

Samples from Solanaceae family such as pepper (*Capsicum annuum*, *Capsicum baccatum*, *Capsicum cardenasii*, *Capsicum chacoense* and *Capsicum chinense*) and tomato varieties (*Lycopersicon esculentum*, *Lycopersicon cerasiforme*, *Lycopersicon pimpinellifolium*) and eggplant (*Solanum melongena*), Mock tomato (*Solanum aethiopicum* subsp. *Kumba*, *Solanum aethiopicum* subsp. *aculeatum*, *Solanum aethiopicum* subsp. *Shum* and *Solanum aethiopicum* subsp. *Gilo*) were provided by the laboratoire de Génétique et d'Amélioration des Fruits et Légumes, (INRA, Domaine St Maurice, France). Brassicaceae species of the triangle of U [21] were also tested. Cauliflower (*Brassica oleraceae*), Chinese cabbage (*Brassica rapa*), mustard (*Brassica juncea*), black mustard (*Brassica nigra*) were provided by the Laboratoire d'Amélioration des Plantes et Biotechnologies Végétales (INRA; Rennes, France). The other species used for the specificity test were *Arabidopsis thaliana*, sugar beet (*Beta vulgaris* subsp. *vulgaris*), cotton (*Gossypium barbadense* and *Gossypium hirsutum*), Barley (*Hordeum vulgare*), Soybean (*Glycine max*), rice (*Oryza sativa*), Line (*Linum*

usitatissimum), alfalfa (*Medicago truncatula*), white bean (*Phaseolus aureus*), pea (*Pisum sativum*), cereal rye (*Secale cereale*), wheat (*Triticum durum* and *Triticum aestivum*), maize and teosinte (*Zea mays* and *Z. diploperennis*). Transgenic material used was maize T25, MON810, Bt176, Bt11, Bt10, GA21 and CBH351, soybean RR, sugarbeet GTSB77, rapeseed (GT73, RF1, RF2, RF3, MS1, MS8) and rice (LL62, provided for research only from JRC; Ispra, Italy). As previously reported, plants were grown in greenhouses and than collected and stored at −20°C until DNA extraction [22].

Donor organism material

The origin of CaMV, FMV and CERV strains used in this paper are provided in Table 1. Several types of DNA were then used for the tests: DNA extracted from infected leaves, pure CaMV virus and DNA, and plasmids either containing the targeted amplicon, or a wider overlapping fragment already published by our laboratory [22]. *Agrobacterium tumefaciens* (strain EHA105) was also used for specificity assessment in order to confirm the reliability even in the presence of a contamination with the promoter and terminator NOS donor organism.

Extraction of the CaMV virus and its DNA

Plants were infected using the classical method with mechanical transmission a of the virus inoculums on the whole surface of the leaves. The method used to extract the virus genome from the infected plants was carried out according to Hull et al. (1976) [23]. The leaves were grinded in a phosphate buffer (0.5 M, pH 7.5), 2.5% Triton 100× and urea 1 M and placed at 4 °C overnight. After a 20 min centrifugation at 4°C, 7,000*g*, the supernatant was filtered and centrifuged again for 90 min at 70,000*g* between 10 and 25 °C. Then, the supernatant was discarded and the pellet was homogenized in 0.5 mL of ultra

Table 1 List of the CaMV material

Type of material	Strains	Origin	Infecting host plant	Used as
Infected leaves	3 CaMV wild type strains (CK1, 2 and 3)	Camille Kerlan (INRA Rennes, France)	(Cabbage) *Brassica rapa*	Extracted DNA (CTAB)
	PV148	American Type Culture collection (**ATCC**, USA)	(Rapeseed) *Brassica napus*	Extracted DNA (Qiagen DNeasy kit)
	PV303	American Type Culture collection (**ATCC**, USA)	(Rapeseed) *Brassica napus*	Extracted DNA (Qiagen DNeasy kit)
	PV652 (FMV)	American Type Culture collection (**ATCC**, USA)	(Rapeseed) *Brassica napus*	Extracted DNA (Qiagen DNeasy kit)
	PV0227	Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH (**DSMZ**, Germany)	(Rapeseed) *Brassica napus*	Extracted DNA (Qiagen DNeasy kit)
	PV0229	Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH (**DSMZ**, Germany)	(Rapeseed) *Brassica napus*	Extracted DNA (Qiagen DNeasy kit)
	D4	**IBMP**, CNRS, Strasbourg, France	(Cauliflower) *Brassica oleracea*	Extracted DNA (Qiagen DNeasy kit)
	CERV	(NIB- Ljubljana, Slovenia)	–	Extracted DNA (CTAB
Virus purified DNA	CabbS	**IBMP**, CNRS, Strasbourg, France	Tomato (*Solanum lycopersicum*) and rapeseed (*Brassica napus*)	See protocol ("Materials and methods")
	Ji	**IBMP**, CNRS, Strasbourg, France	Tomato (*Solanum lycopersicum*) and rapeseed (*Brassica napus*)	See protocol ("Materials and methods")
Clones	261 bp	**INRA Rennes**	–	pGEM-T Easy vector (Promega)
	152 bp	PV148 from **ATCC**, USA	–	pGEM-T Easy vector (Promega)
	CabbS	**IBMP**, CNRS, Strasbourg, France	–	Complete CabbS CaMV genome cloned in pBR322 vector (in SalI restriction site)

Table 2 CaMV primers and probe sequences, universal plant DNA primers sequences

Oligonucleotide	Name	Sequences	References
Forward primer	CaMV-F1	5′-TGAAATCCTCAGTGACCAAAAATC-3′	This work
Reverse primer	CaMV-R1	5′-TACAAGGACAATCATTGATGAGC-3′	This work
Probe (FAM-TAMRA)	CaMV-pr1	5′-AAGCCGTTGCAGCGAAAATCGTTAATGA-3′	This work
Forward primer	SF	5′-CGTCTTCAAAGCAAGTGGATTG-3′	[22]
Reverse primer	SR	5′-TCTTGCGAAGGATAGTGGGATT-3′	[22]
Probe (VIC-BHQ)	35Spr	5′-TCTCCACTGACGTAAGGGATGACGCA-3′	[22]
Universal Forward primer	OGM-22	5′-CCTgATCTTCTGTGAAGGGTTCGAGT-3′	[25]
Universal Reverse primer	OGM-23	5′-CCTATACCCAAGTCAGACGAACGAT-3′	[25]

pure water. A chloroform extraction was then performed. After a 15 min centrifugation at 15 °C, 1,200*g*, the supernatant was loaded on a 25% saccharose gradient (kept at −20 °C overnight). A centrifugation was performed during 2 h at 10 °C, 90,000*g* and the virus particles were collected from one of the saccharose phases (white color). This extract was diluted twice in ultra pure water and centrifuged during 1 h 30 min at 200,000*g* at 10 °C. The pellet containing the virus particles was gently resuspended in 100–200 µL of ultra pure water. This pellet was treated with DNAseI (10 µg/mL) during 10 min at 37 °C to eliminate exogenous cellular DNA. EDTA (1 mM) was finally added to stop the reaction. As an average, 1 kg of infected leaves gives approximately 10 mg of virus DNA (unpublished data).

To extract the DNA from the virus particles, SDS buffer (0.5%) was added in a 1 mL final volume with proteinase K (50 µg/mL) and Tris 0.1 M. The mixture was incubated overnight at 37 °C. The day after, a phenol–chloroform extraction was performed with a 10 min centrifugation at 90,000*g*, 15 °C. The supernatant was collected and mixed with two volumes of cold absolute ethanol, 1 µL of glycogen (2 mg/mL), NaCl (1 M). A precipitation was carried on at −80 °C during 15 min minimum. Then, a centrifugation was performed at 4 °C during 30 min, 90,000*g*. After the supernatant was eliminated, the pellet was dried and resuspended in 50 µL of ultra pure water.

The CaMV particles and the resulting DNA were both quantified by a spectrophotometer at 260 nm wavelength: 7 OD units correspond to 1 mg of virus particles and 1 OD unit corresponds to 50 µg/mL of double-strand DNA. The quality of virus DNA was checked on a 0.8% agarose electrophoresis gel.

Extraction of plant material

The total DNA was extracted from plant leaves with a CTAB method [24]. In order to check and quantify the DNA extractions, the samples were loaded on 0.8% agarose gels stained with Ethidium Bromide and quantified by Bio1D software (Vilbert Lourmat, Marne la Vallée, France) in comparison with a calibration curve of commercial λDNA (MBI Fermentas, Vilnius, Lituania) with a concentration range (10, 20 and 30 ng). The DNA amplifiability was assessed by the universal test [25].

Sequencing of the ORFIII 260 bp-fragment

The sequencing of the 260 bp amplicons [22] was performed in a 20 µL reaction containing 3.2 pmol of primer (forward or reverse), 4 µL of BigDye Terminator Cycle Sequencing reaction mixture (Perkin–Elmer), 1.6 µL of 5× sequencing buffer, 3.2 µL of H_2O and 8 µL of the purified PCR product. The products of this reaction were purified using a G50 gel filtration [Sephadex® G-50 superfine (Amersham Biosciences, Hercules, CA, USA)] and loaded to an ABI 310 sequencer. Sequence alignments and detection of polymorphism were performed using the software Chromas version 2.32.

Primers and probe design and PCR conditions

The primers and probe (Table 2) were designed with the Primer Express software (version 2.1, Applied Biosystems, Foster, California) inside the 260 bp fragment previously described [10, 22], in to amplify a 152 bp amplicon. The DNA plant quality was checked with universal plant primers. All the simplex experiments were made with a TaqMan® probe labelled with FAM and black Hold quencher (BHQ).

The real-time amplifications were performed with the Real-time Master MIX provided by Applied Biosystems, 200 nM of the probe, 300 nM of the primers, and 2.5 mM of Mg^{2+}.

Cloning of the 152 bp target

The 152 bp amplicon was cloned from the PCR amplicon obtained from infected leaves of the PV148 CaMV strain in

a TA-cloning pGEM-T vector (Promega, Charbonnières, France). This plasmid is available after its deposit in the BCCM / LMBP Ghent (Belgium; http://bccm.belspo.be/) plasmid bank by Institut Scientifique de Santé publique, (ISP; Section de Biosecurité et Biotechnologie, SBB; Brussels, Belgium). It was used in this work for the sensitivity studies.

Results and discussion

Qualitative test with conventional PCR: specificity of the 152 bp test

The specificity is one of the PCR test performance criteria (EN ISO 24276) [26]. The Procedural Manual of the Codex Alimentarius and EN ISO 24276 defines this criterion as the ability of a method to respond exclusively to the characteristic or analyte. In other terms, it describes the absence of false positives. Theoretical and experimental specificity were assessed.

Theoretical specificity

Sequence specificity of the CaMV primers and probes was examinated by the Basic Local Alignment Search Tool (*Blast*) on the website of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov/BLAST/) using DDBJ (DNA Database of Japan; release 5.0 December 2002), EMBL (European Molecular Biology Laboratory) and GenBank (Accession number X79465). The results of *Blast* confirmed the theoretical specificity of the targeted region. It was almost conserved among the CaMV strains while no obvious homology was observed with other virus groups such as Figwort Mosaic virus (FMV) and Carnation Etched Ring virus (CERV) (data not shown).

Experimental specificity

Experimental specificity of the primers targeting the ORF III of the CaMV was assessed against several targets: *Agrobacterium tumefaciens*, CaMV, CERV and FMV strains, GMOs, and non-transgenic plants including especially the CaMV and the CERV hosts. Results are presented in the Table 3. Only samples containing the CaMV genome or the cloned 152 bp fragment were amplified (Fig. 1). Parallel tests with the primers SF/SR targeting P35S, were performed with all the templates containing this promoter. We concluded that the two tests are independent and no interferences can occur

Table 3 Assessment of the specificity of CaMV primers using different types of targets

Specificity targets	PCR result with CaMV test	Plant universal test
CaMV strains		
Strain C.K1	+	–
Strain C.K2	+	–
Strain C.K3	+	–
PV148	+	–
PV303	+	–
PV652	+	–
PV0227	+	–
PV0229	+	–
D4	+	–
Purified virus DNA		
CabSS	+	–
Ji	+	–
Clones	+	–
PV148	+	–
261 bp	+	–
152 bp	+	–
CabSS	+	–
Other donor organisms		
Figwort mosaic virus (FMV)	–	–
Agrobacterium tumifaciens	–	–
GMO events		
Maize T25	–	+
Maize MON810	–	+
Maize Bt11	–	+
Maize CBH351	–	+
Maize GA21	–	+
Maize Bt176	–	+
Maize Bt10	–	+
Rice LL62	–	+
Tomato Tg7TF	–	+
Rapeseed Topas19/2	–	+
Rapeseed RF1	–	+
Rapeseed RF2	–	+
Rapeseed RF3	–	+
Rapeseed MS1	–	+
Rapeseed MS8	–	+
Soybean RRS	–	+
Rapeseed GT73	–	+
Sugarbeet GTSB77	–	+
Plants of brassicaceae family		
Brassica napus var. westar	–	+
Brassica oleracea	–	+
Brassica nigra	–	+
Brassica juncea	–	+
Brassica rapa	–	+

Table 3 continued

Specificity targets	PCR result with CaMV test	Plant universal test
Plants of solanaceae family		
Capsicum annuum	–	+
Capsicum baccatum	–	+
Capsicum cardenasii	–	+
Capsicum chacoense	–	+
Capsicum chinense	–	+
Lycopersicon cerasiforme	–	+
Lycopersicon esculentum	–	+
Lycopersicon hirsutum	–	+
Lycopersicon pimpinellifolium	–	+
Solanum macrocarpon	–	+
Solanum melongena	–	+
Solanum aethiopicum Kumba	–	+
Solanum aethiopicum aculeatum	–	+
Solanum aethiopicum Shum	–	+
Solanum aethiopicum Gilo	–	+
Solanum tuberosum	–	+
Other species		
Arabidopsis thaliana	–	+
Beta vulgaris	–	+
Glycine max	–	+
Gossypium barbadense	–	+
Gossypium hirsutum	–	+
Hordeum vulgare	–	+
Oryza sativa	–	+
Linum usitatissimum	–	+
Medicago truncatula	–	+
Phaseolus aureus	–	+
Pisum sativum	–	+
Secale cereale	–	+
Triticum durum	–	+
Triticum aestivum	–	+
Zea diploperennis(teosinte)	–	+
Zea mays	–	+

The sign (+) means positive amplification and (−) means no amplification observed

between the two targets. According to these results, our qualitative PCR test is highly specific of the ORFIII of CaMV strains and does target neither CERV nor FMV viruses.

Confirmation of the specificity by sequencing

To further assess the specificity of our PCR test and to confirm the specificity of the internal probe, the PCR amplicons of seven CaMV strains (Fig. 2) namely, PV148, PV303, PV0227, PV0229, D4, Ji and CabbS were sequenced. The alignments show a conserved 152 bp sequence for the PV0227, PV0229, D4 strains, for the CabbS and Ji virus DNAs, while a 154 bp amplicon was observed for the PV148 and PV303 strains (data not shown). The main differences between viruses in the collection tested are the genome size and the infectivity spectra.

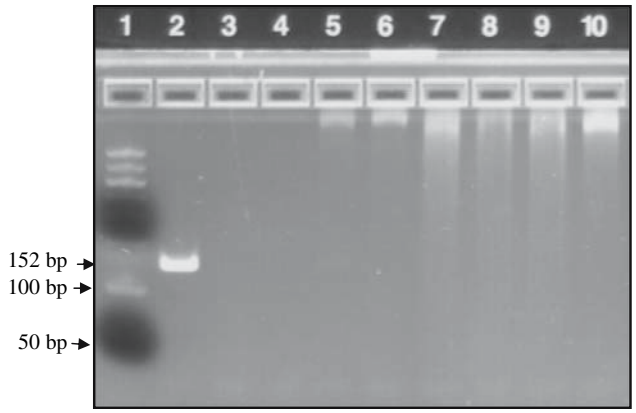

Fig. 1 Migration of the PCR products in 4% agarose gel. Experimental specificity with GMO samples *1* low marker, *2* Ji virus, *3* no template control, *4* cabbage, *5* event T25 maize, *6* Mon810 maize, *7* Bt11 maize, *8* GTS40-3-2 "RRS" soybean, *9* Starlink (CBH351) maize and *10* event 176 maize

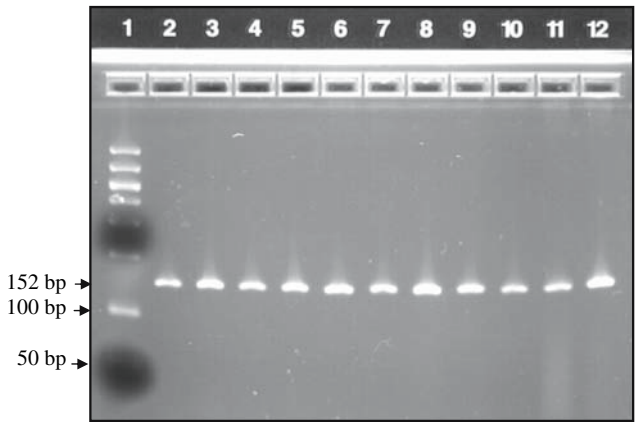

Fig. 2 Migration of the PCR products in 4% agarose gels. Single PCR amplification of the fragment ORFIII. Gel amplification products of PCR corresponding to: *1* biomarker low ladder, *2* virus Ji, *3* virus CabSS, *4* clone 148 bp, *5* clone CabbS, *6* PV148, *7* PV0229, *8* PV303, *9* PV0227, *10* C.K1, *11* C.K2

Cloning of the 152 bp amplicon

According to Mattarucchi et al. [27] the use of a linearised or not plasmid DNA as a standard in GMO detection and quantification could provide a cheaper and more flexible alternative to conventional reference materials (pure

CaMV DNA or DNA of infected leaves) for quantifying the GMOs and donor organisms. It can thus ensure the continuity and stability, necessary for reference material, provided commutability is demonstrated. The target amplicon of CaMV (152 bp) was thus cloned in a p-GEMT plasmid. This clone (pPV148) is available after its deposit to the international Ghent collection.

Quantitative real time PCR

Estimation of the DNA copy number in the samples

The copy number of a virus DNA solution is calculated as follow:

$$\frac{\text{copynumber}}{\mu\,\text{L}} = \frac{[\text{concentration in ng}/\mu\text{L}] \times N}{Ji\,\text{virus size} \times 649} \times 10^9$$

($N = Avogadro\ \ number = 6.023 \times 10^{23}$ molecules; *Ji*: CaMV virus strain). The virus genome size is assumed to be 8,030 bp [17]. As an example a DNA solution of 200 ng/µL contains:

$$\left(200 \times 6.023 \times 10^{23}\right)/\left(8030 \times 649 \times 10^9\right)$$
$$\cong 2.3 \times 10^{10}\text{copies}/\ \mu\ \text{L}.$$

Experiments were carried out to determine the relationship between DNA plasmid and leaves infected virus genome copies. Serial dilutions were performed from a 10^9 copies of either plasmid or infected leaves DNA for assessing the absolute limit of detection (LODa) of the test.

The ΔRn (fluorescence value obtained at the end of the PCR amplification curve) of the infected leaves, cloned 152 bp and CabbS and pure virus DNA ranged from 1.6 to 1.8, 1.5 to 2, 1.2 to 1.4 and 1 to 1.2, respectively. These values demonstrates the good amplification efficiency of the PCR reaction whose differences were not statistically significant.

Amplification efficiency of the TaqMan® assay

The PCR efficiency was evaluated with all the CaMV available materials: virus DNA, plasmids and DNA extracted from infected leaves. The mean efficiency was determined at 90 ± 5%. The squared coefficient of correlation (R^2) ranged from 0.993 to 0.998 (Fig. 3). This demonstrates that there is a high correlation between the original amount of genomic DNA in the template and the real-time PCR Ct values. As the experiment was performed at least three times with different batches of primers and probes, the test seems to be highly reproducible. Statistically speaking, no significant differences were observed in the efficiencies between plasmid and virus DNA (ANOVA, *t* test at 1%).

Determination of the dynamic range and the absolute limit of detection (LODa)

Generally, the sensitivity of a given test is determined using the limit of detection (LOD) performance criterium. There are two types of LOD, the absolute LOD calculated in our study using one target. Besides, relative LOD is

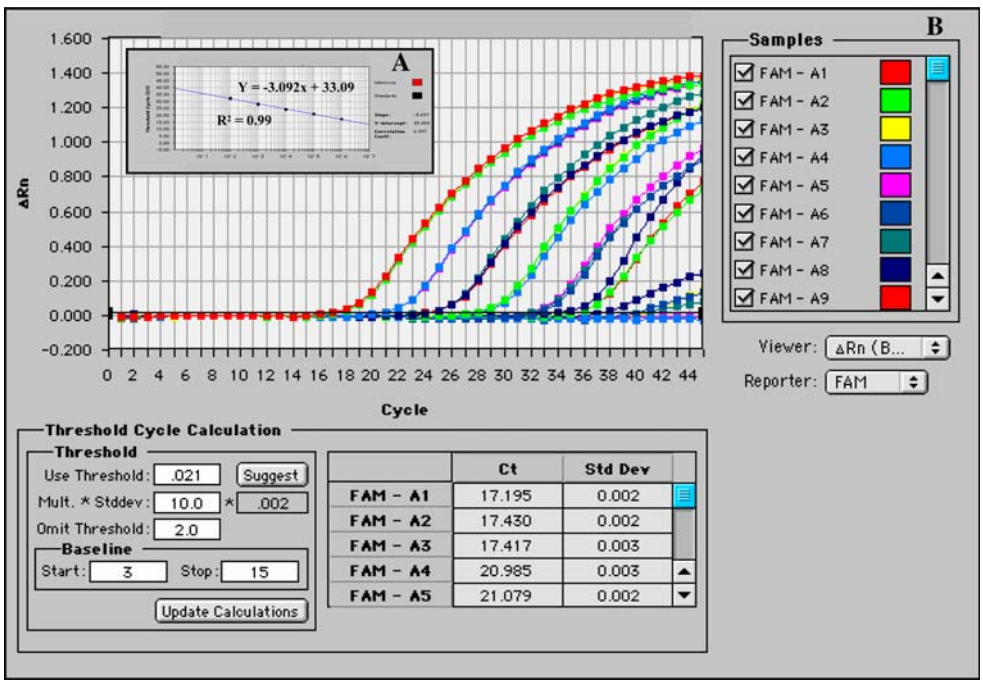

Fig. 3 Example of amplification plot and standard curve using as sample the DNA of the strain Ji. Tenfold dilution series ranged from 10^6 copies to 0.1 copies. Each point was analysed in triplicate and each real-time experiment was repeated three times. The absolute limit of detection was better than ten copies

calculated in presence of a template other than the targeted called ballast DNA [28] Here, the LODa was determined with qualitative and quantitative real time PCR on six dilutions in triplicates and done three times independently (9 data per dilution), using ranges of the targets varying from 10^6 copies to one copy per dilution. The LODa results are similar for the Ji and CabbS virus, as for the clone PV148: between one and ten copie(s) (Fig. 3). According to the amplification efficiency and LODa data, the correlation factor between plasmid and virus DNA copy numbers contents is therefore equal to one.

Determination of the absolute limit of quantification (LOQa)

The LOQ is defined as the lowest amount of the target to be reliably detected and quantified under ideal conditions with a probability ≥95% with a RSDr (relative standard deviation of the repeatability) ≤25% (EN ISO 21570) [28]. In order to validate the LOQ, we used two purified viruses Ji and CabbS DNA. The same templates for the determination of the limit of detection were used with tenfold dilution series from 10^6 to one copy of CaMV genome. Each dilution was tested in triplicate. The results are summarized in the Table 4. As expected, the SD values increased in the case of low amounts of CaMV genome copies. We were able to detect CaMV in all three parallels down to ten copies, whereas only one of the parallels was positive when we used an approximate average of one copy. From the results shown in the Table 4, we concluded that under our conditions, approximate 100 initial CaMV genome copies were required for reliable quantification.

Table 4 Determination of the absolute limit of detection (LODa) and limit of quantification (LOQa)

Template copies (CaMV genome)	Signal rate (positive signals)	Means Ct	SD (Ct values)	CV% (Ct values)	RSDr (%)
Virus Ji					
10^6	3/3	17.35	0.13	0.74	4.2
10^5	3/3	21	0.06	0.28	1.94
10^4	3/3	24.34	0.05	0.2	1.61
10^3	3/3	28.11	0.33	1.17	10.67
10^2 (LOQ)	3/3	32.29	0.3	0.92	9.7
10 (LODa)	3/3	35.09	0.79	2.25	25.56
1	1/3	43.67	2.29	5.24	74.11
Virus CabSS					
10^6	3/3	18.47	0.19	1.02	6.3
10^5	3/3	22.11	0.11	0.49	3.55
10^4	3/3	25.65	0.31	1.2	10.03
10^3	3/3	29.5	0.28	0.94	9.06
10^2 (LOQ)	3/3	32.92	0.23	0.69	7.44
10 (LODa)	3/3	37.05	0.59	1.59	19.09
1	1/3	40.63	2.52	6.2	81.55

Ct cycle threshold, *SD* standard deviation, *CV* coefficient of variation

Development of a qualitative and quantitative duplex CaMV/P35S

In addition to the simplex test described in this paper, we developed a new qualitative and quantitative test for the simultaneous detection of P35S (having either the virus or the GMO or both as origin) and CaMV. This test is of a great importance especially in quantitative trials, because it will allow the segregation between the quantity of P35S present in the construction of the GMOs and the P35S of the CaMV using a differential equation. The Fig. 4 shows the result of the qualitative duplex test with two infected leaves using PV148 strain. We have also developed a quantitative duplex test using the two specific sets of primers and probes, P35S and CaMV, for the detection of CaMV virus (strains CK1 and CK2, respectively) in rapeseed (*Brassica napus* var. *westar*) leaves and cauliflower (*Brassica oleracea*). Three dilutions of the total extracted

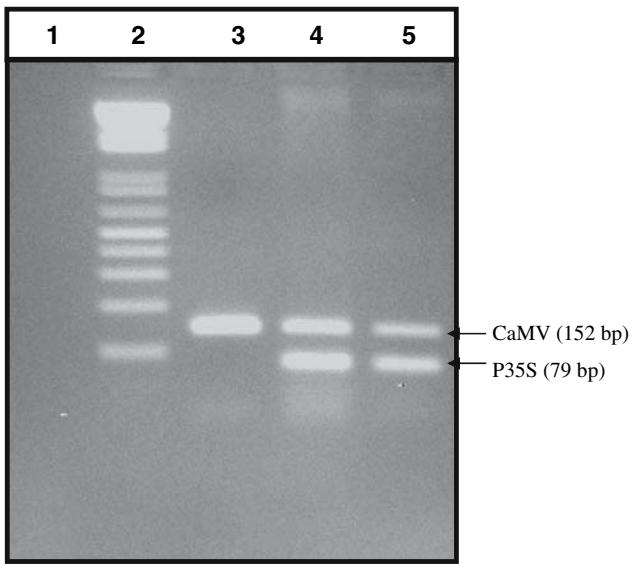

Fig. 4 Migration of the PCR products in 4% agarose gels. Example of end point amplification with the duplex assay combining the P35S (amplicon 79 bp) and the CaMV control [amplicon 152 bp. *1* water, *2* low marker, *3* CaMV positive control (Clone 152 bp PV148), *4* inoculated rapeseed (*Brassica napus*) with strain PV148, *5* inoculated cabbage (*Brassica rapa*) with the strain C.K1 (see Table of virus material)]

DNA (genomic plant DNA and virus DNA all together) were tested. The LODa and the PCR efficiencies of the duplexes were similar to the simplexes in presence of low content of both targets (data not shown). Thus, the combination of both targets, P35S and CaMV is feasible since the performance criteria do not look affected.

Conclusions

The P35S screening test is the most used one because of its sequence high frequency in the currently approved GMOs. Thus, the CaMV identification test is needed to discard false positive, a requirement in the French accreditation system currently reached by the change, laboratory by laboratory, of our previously published qualitative test. Although there is no published data about the frequency of false positive in P35S screening, numerous requests over the last years of enforcement laboratories to use the qualitative test let suspect that the presence of CaMV can occur more frequently than supposed even in plant samples without known GMO like sunflower seeds, probably contaminated by dusts.

After our previous development of a qualitative CaMV test already in use in numerous detection laboratories, we developed qualitative and real-time PCR systems suitable for detection, identification and, most of all, quantification of the CaMV virus. In comparison with CaMV tests already published which target the ORFIV [29] or ORFV and ORFVI [30] our primers and probes were assessed against a larger set of samples and showed a very good specificity altogether with a similar sensitivity. Moreover, as the capsid (ORFIV) protein sequence is often used in GMOs for plant protection, our ORFIII based test might be a better choice for long-term uses, since the ORFIV might be used in future virus resistant GM plants.

The absolute limit of detection (LODa) was estimated below 10 genome or plasmid copies and the absolute limit of quantification (LOQa) below 100 copies using TaqMan® chemistry which is less expensive than MGB probe while the two chemistries look to perform equally [31, 32]. The measured uncertainty, an important factor in quantitative analysis, is in good compliance with the current thresholds set in regulations [33]. This test looks quite robust as observed with the similar PCR efficiencies on DNA extracted by several methods. Moreover, plasmids prepared in this study can be used as alternative standards for the quantification of the CaMV virus (correlation factor of 1) as it is the case of many plasmids constructed for precise GMO quantification [34, 35].

Finally, a duplex qualitative and quantitative test (P35S and CaMV) is routinely working, thus providing a time- and cost-effective detection method. The average cost of 1 quantitative real-time PCR reaction is about 0.75 $ per tube and thus the cost is not the most important factor to be taken into account. However, the ability of directly detecting CaMV in a P35S positive sample could is time-effective and should provide rapid indications of cross-contamination even for plants, such as solanaceous, whose infection by the CaMV is generally unknown from the enforcement laboratories or in samples whose P35S positive character may result of dusts contamination as observed for sunflower seeds. The CaMV P35S tests quantitative duplex tests can thus be used for detecting unknown GMO by one the strategy under study in the European research program Co-Extra, namely the "quantitative differential PCR" (Ancel et al., unpublished data).

Finally, an inter-laboratory validation should be now started for the routine uses by the detection laboratories of our tests.

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