Development of an in-house Plasmid Control for *Cauliflower Mosaic Virus* (CaMV) for the Detection of Genetically Modified (GM) Chinese Rice Lines

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Summary

On 12th January 2012 Commission Implementing Decision 2011/884/EU was adopted which describes emergency measures regarding unauthorised genetically modified rice in rice products originating from China. All rice consignments imported into the EU from China are subject to testing for the presence of molecular markers and elements often associated with genetic modification. At present there are no genetically modified (GM) rice varieties approved for use in the European Union, and upon detection consignments containing these genetic elements must be re-dispatched to the country of origin or destroyed. The P-35S promoter sequence, derived from Cauliflower Mosaic Virus (CaMV), is one of the genetic elements routinely screened for to infer the presence of GM rice. Guidance in support of the Commission Implementing Decision 2011/884/EU provided by the European Union Reference Laboratory (EURL) for GMO's in food and feed advocates that appropriate follow-on tests be conducted to ensure that the detection of P-35S is not a false positive due to the natural occurrence of CaMV present with the test sample. However, the EURL Guidance does not provide further instruction on what control material can be used to facilitate such a test, creating an analytical void in the correct application of such a test for false positives.

The present study aimed at developing a suitable plasmid control DNA for CaMV. This was analysed alongside appropriate samples that contained P-35S only, using a validated and EU approved real-time PCR assay that could be used as a follow on test for the detection of Chinese GM rice varieties. The assay was successful in amplifying the DNA target in the CaMV plasmid control with a limit of detection of approximately four copies of the plasmid target. All other sample templates that contained just P-35S produced no detectable amplification. This illustrates the use of the CaMV plasmid DNA as an appropriate control material in conjunction with EU-approved tests for the detection of false positives arising from the application of the P-35S test for the detection of Chinese GM rice varieties in support of the relevant legislation.

Keywords

GMOs, Chinese GM rice, Plasmid Control, CaMV

Introduction

The Commission Implementing Decision 2011/884/EU[1], which came into force early in 2012, describes emergency measures regarding unauthorised genetically modified rice in food products originating from China. There are reportedly over 25 genetically modified rice varieties that may be a source of contamination of rice products imported from China. There are currently no GM rice varieties approved for use in the European Union. In support of the repeal of Commission Decision 2008/289/EC of the 3rd April 2008, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) was requested to carry out an in-house verification study to assess the performance of a broad screening approach for the detection of genetically modified rice in food products[2]. The screening tests were performed by real-time PCR according to the method published by the EURL-GMFF for the following genetic elements: DNA sequences characteristic of the 35S promoter derived from Cauliflower Mosaic Virus (P-35S), the nopaline synthase terminator (T-NOS) derived from *Agrobacterium tumefaciens* and the genetically engineered CryIAb/CryIAc.

The P-35S genetic element is derived from the Cauliflower Mosaic Virus (CaMV), which is often used in GMO transfection. Any sample that exhibits detection of P-35S must therefore also be tested for the presence of naturally occurring CaMV in order to rule out any false positive detection of a GM variety due to the presence of the naturally occurring virus. In the event that P-35S is detected in a test sample, a CaMV Taqman® assay can be used as advocated in the EURL Guidance Document and Commission Implementing Decision 2011/884[2], such as that fully described in Cankar *et al*, 2005[3].

No CaMV positive control DNA is currently commercially available for this distinct purpose, and EU Commission Implementing Decision 2011/884[1] or the EURL Guidance Document[2] do not advocate the use of a specific control material or recommend what sample should be used. Live virus strains are available from commercial sources and service providers (e.g. ATCC-LGC Standards) however it may be inappropriate or beyond a laboratory's capability to implement these as positive controls on a routine basis without the relevant specialised equipment and appropriate procedures in place for microbiological work. The current study was therefore aimed at developing an appropriate positive control for use in the CaMV TaqMan® assay, so as to help afford greater confidence in tests conducted in accordance with EU Commission Implementing Decision 2011/884[1]. The control DNA template was synthesised *de novo* and sub-cloned into a standard pUC19 vector. Following linearization, the CaMV plasmid control was analysed using an EU-approved approach for the detection of CaMV[3] alongside other test samples known to be positive for P-35S, in order to assess the applicability of the assay as a follow-on test from the P-35S SYBR Green assay advocated in the EURL Guidance Document[2].

Safeguards for businesses subject to official controls are an integral part of European food law[4] and are provided in the UK by the National Measurement Office Government Chemist Programme at LGC. This statutorily-based route of technical appeal, the "referee function", has been invoked in a number of recent cases on disputed results on genetically modified rice in rice products originating from China. These cases prompted the development of a control material, which tests positive for the presence of CaMV, and it is suggested that this will be of practical significance in support of the relevant EU legislation for the testing and detection of GM rice originating from China.

This Technical Article describes the development and testing of such a control material in the form of plasmid DNA.

Materials and Methods

Synthesis of DNA Control

Part of the Cauliflower Mosaic Virus (CaMV) genome (Accession number V00141 / J02048; 3041bp to 3940bp) which incorporates the 89bp CaMV target region referenced in Cankar *et al*, 2005[3], was synthesised *de novo* and sub-cloned into a standard pUC19 vector. The sequences consisted of a 900bp region of the CaMV genome that did not incorporate the P-35S promoter. Multiple cloning sites are present just before and after the insert in the pUC19 vector. BioEdit (Ibis Biosciences) and NEBcutter V2.0 (New England BioLabs) software programmes were used to search for restriction enzyme sites in the multiple cloning sites that were not present in the insert, and the SmaI restriction endonuclease was found to fit this criterion.

DNA Samples

CaMV plasmid control DNA was synthesized *de novo* as described above. The CaMV plasmid control contained part of the CaMV genome which was designed to be detected by the CaMV assay described in Cankar *et al*, 2005[3], but not by the P-35S assay. Non-modified rice leaf [AOCS 0306-C] and LLRice62 [AOCS 0306-I4 99.99% GM (m/m)] were obtained as pre-extracted genomic DNA samples from AOCS [IL, USA]. Bt11 Maize DNA was extracted from 5% GM maize powder obtained from IRMM [IRMM ERM-BF412c 5% GM (m/m)] using a CTAB approach. LLRice62 and Bt11 Maize are positive for the P-35S genetic element but negative for the naturally occurring CaMV DNA sequence. Non-modified rice DNA does not contain either the CaMV or P-35S sequence (Table 1). Sterile nuclease free water (Ambion) was used as a no-template control.

Table 1 - Target DNA Sequences for “CaMV” and “P-35S” Present in the Templates

Template	CaMV	P-35S
CaMV plasmid control	+	-
Non-modified rice	-	-
LLRice62	-	+
Bt11	-	+

Restriction Digest

The CaMV plasmid control was digested with the restriction enzyme *SmaI*. The digestion reaction consisted of nuclease-free water (Ambion), *SmaI* enzyme (5 units) and 1XNEBuffer4 (New England BioLabs). Lyophilised plasmids were re-suspended in 10mM TE, 10µl of which was added to 40µl of the reaction mixture and left to incubate at 25°C for 2 hours. The restriction enzyme was inactivated at 65°C and the product run on a pre-stained agarose “Flash Gel” (Lonza) to assess the relative success of each reaction and the integrity

of the products. Each linearised control template was diluted to a final concentration of approximately 1 million copies per µl.

PCR Analysis

Two real-time PCR assays were used to test the efficacy of the CaMV plasmid control. The first assay was the SYBR®Green assay to test for the presence of P-35S as described in the EURL Guidance document[2] for detection of Chinese GM rice varieties. This consisted of a standard 20µl PCR reaction volume containing 25ng of DNA, 1 x Power SYBR®Green PCR Master Mix and 250nM of each primer (Table 2). PCR was carried out on an ABI Prism 7900HT Real Time PCR system under the following cycling conditions: 95°C for 10mins, 40 cycles of 95°C for 15sec and 60°C for 60sec. A final dissociation stage was also included for the purpose of melt curve analysis of the obtained product.

The second real-time PCR assay was for the detection of CaMV and was performed as described by Cankar *et al*, 2005[3]. Each 25µl reaction consisted of 1X the TaqMan® Universal PCR Master Mix (Life Technologies), 0.9µM of each primer and 0.2µM MGB probe as listed in Table 3. The linearized CaMV plasmid control was diluted to 250 copies per reaction. PCR was carried out on an ABI Prism 7900HT Real Time PCR system under the following cycling conditions: 50°C for 2mins, 95°C for 10mins, 50 cycles of 95°C for 15sec and 60°C for 60sec.

Table 2- Primers and Probes used for the Detection of P-35S

Name	Sequence 5' to 3'	Source
35S_N3Fwd	AAAGCAAGTGGATTGATGTGATA	EURL Guidance document[2]
35S_N3Rev	GGGTCTTGCGAAGGATAGTG	

Table 3 - Primers and Probes used for the Detection of CaMV

Name	Sequence 5' to 3'	Source
CaMVF (sense)	GGCCATTACGCCAACGAAT	Cankar *et al*, 2005[3]
CaMVR (antisense)	ATGGGCTGGAGACCCAATTTT	
CaMV-MGB Probe	FAM-TTCTCCGAGCTTTGTC-NFQ	

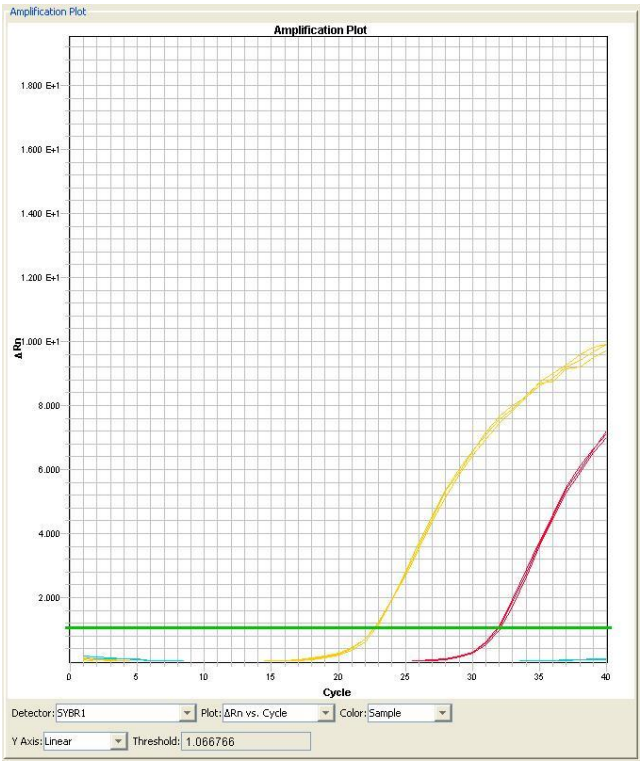
Determination of the Limit of Detection (LOD) and Amplification Efficiency

The linearised CaMV plasmid control DNA was used to construct a standard curve to determine the LOD and amplification efficiency of the assay, in line with Cankar *et al*, 2005. DNA was serially diluted (4-fold) from 1000 to 1 copy per reaction for inclusion in a six-point standard curve at a PCR replication level of 4.

Results

Figure 1 shows the linear amplification plots following TaqMan® qPCR analysis with the SDS software (Applied Biosystems) using the P-35S assay on the four templates of the CaMV plasmid control, non-modified rice, LLRice62, Bt11, and the PCR no-template control. PCR replicates were run in triplicate and the mean Cq values calculated where appropriate, as shown in Table 4. The results show that P-35S was detected in the LLRice62 and Bt11 templates, but was not detected in the CaMV plasmid control, wild-type rice and the no-template controls, all in line with expectations based on the targets present in each of the templates.

Figure 1 - Linear Amplification Plot of the P-35S assay



P-35S was detected in the Bt11 template (red) and the LLRice62 templates (yellow)

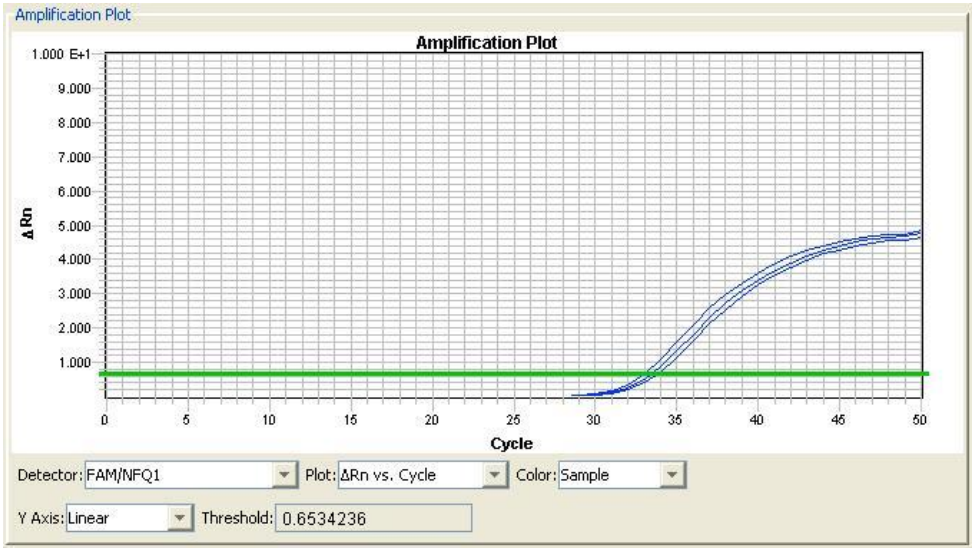
Table 4 - Results Obtained Following qPCR Analysis using the P-35S Assay

Sample Name	Colour in Fig1	Mean Cq	[DNA]	Outcome
CaMV plasmid control	Blue	None	250 copies	Not detected
Bt11 Maize (5% GM, IRMM)	Red	31.96	25ng	Detected
Non-modified Rice (AOCS)	Aqua	None	25ng	Not detected
LLRice62 (AOCS)	Yellow	22.76	25ng	Detected
No template control	Black	None	n/a	Not detected

The Bt11 and the LLRice62 templates were assigned Cq values

Figure 2 shows the linear amplification plots following TaqMan® qPCR analysis with the SDS software (Applied Biosystems) applied to the same samples for the CaMV assay. PCR replicates were run in triplicate and the mean Cq values calculated where appropriate, as described in Table 5. The results show that only DNA from the CaMV plasmid control was detectable by PCR, as expected. No other DNA templates were assigned a Cq value, and all no-template controls (NTCs) were negative.

Figure 2 - Linear Amplification Plot for Cankar *et al*, 2005 CaMV PCR Assay



CaMV DNA was detected in the CaMV plasmid control (blue) only

Table 5 - Results Obtained Following qPCR Analysis using the CaMV Assay

Sample Name	Colour in Fig 2	Mean Cq	[DNA]	Outcome
CaMV plasmid control	Blue	33.39	250 copies	Detected
Bt11 Maize (5% GM, IRMM)	Red	None	25ng	Not detected
Non-modified Rice (AOCS)	Aqua	None	25ng	Not detected
LLRice62 (AOCS)	Yellow	None	25ng	Not detected
No template control	Black	None	n/a	Not detected

The CaMV plasmid control was the only template to be assigned a Cq value

The Limit of Detection (LOD) and amplification efficiency of the assay were assessed using a dilution series of the CaMV plasmid control. The working range of the standard curve was approximately 1000 to one copy per reaction, and each point on the curve was assayed at a PCR replication level of four. The approximate LOD was determined as the lowest concentration of the CaMV plasmid control where the CaMV target was still detected in all PCR replicates. The LOD was determined as approximately four copies of the target sequence, which is comparable to the estimates described in Cankar *et al*, 2005[3].

Discussion

The synthesis of a *Cauliflower Mosaic Virus* (CaMV) DNA control in the form of a plasmid construct has proven to be a cost and labour-effective way of producing a suitable positive control for this scope of work. The plasmids were stored at -70°C before being linearised in a simple enzymatic digest and diluted for use in qPCR. This illustrates the simplicity of obtaining, storing and handling plasmid DNA in comparison to, for example, live virus strains for use as positive controls.

The CaMV plasmid control was challenged by the assay described in Cankar *et al*, 2005[3] for the detection of naturally occurring CaMV DNA, alongside other sample templates of Bt11 maize, LLRice62 and non-modified rice. Approximately 250 copies of CaMV DNA were added to each reaction replicate, and upon analysis it was found that DNA from the CaMV plasmid control was amplified around 33 cycles. This is comparable with the results obtained by Cankar *et al*, 2005[3] where approximately 100 copies (molecules) of DNA gave a Cq value of around the same cycle number. No other templates included in the analysis were assigned Cq values, including Bt11 maize and LLRice62. Both of these GM lines are positive for the P-35S CaMV promoter sequence and are commonly used as positive controls in the detection of this element in suspected samples. The absence of the CaMV target in these controls suggests that the assay is specific for the appropriate DNA target, but also that this set of controls is suitable for use in future tests for the naturally occurring virus in suspected GM-positive samples. The LOD was determined as approximately four copies of the target sequence per reaction, which compares well to the LOD described in Cankar *et al*, 2005[3].

Conclusions

The synthesised CaMV oligonucleotide sequence, derived from a 900bp region of the CaMV genome, appears to be fit for purpose for use as a positive control in the CaMV test described by Cankar *et al*, 2005[3] after cloning into a suitable vector. The present study has therefore demonstrated how a plasmid-based positive control for CaMV could potentially be used in follow-on tests for detection of naturally occurring CaMV in support of the current legislation for the testing and detection of GM rice strains originating from China[1,2].

Acknowledgements

The work described in this Technical Article was funded by the UK National Measurement Office as part of the Government Chemist Programme 2011-2014.

References

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