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ORIGINAL PAPER

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Characterisation of the 5′ integration site and development of an event-specific real-time PCR assay for NK603 maize from a low starting copy number

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Abstract Genetically modified (GM) maize event NK603 is in the pipeline for authorisation in the EU and is likely to become a dominating GM maize on the world market. The 5′ integration junction was characterised from a small quantity of commercially available genomic DNA. Specific primers and a probe were designed targeting the 5′ integration junction in the plant, amplifying a 102 bp DNA fragment. A method for specific detection and quantification of NK603 using event-specific real-time PCR based on the identified and cloned sequence is described. The assay was specificity tested against five different GM maizes, one non-GM maize and one GM soya bean. Plasmids were constructed for use as external standards in calibration curves. Due to the lack of certified or other suitable commercial reference material for quantification of NK603, the quantifications were performed on non-target DNA spiked with plasmid DNA containing the cloned NK603 5′ integration junction. The plasmid was detectable down to one copy per PCR. The limits of detection (LOD) and quantitation (LOQ) are estimated to be comparable to those of state-of-the-art methods applied for other GM events.

Keywords Event-specific PCR · Genetically modified organism · Integration site · NK603 · Quantification

Introduction

In 2002 the global area of genetically modified (GM) crops was 58.7 million hectares [1]. The present share of GM crops constitutes a considerable part of the total crop production and this share will probably increase in the coming years.

NK603 is a RoundupReady maize produced by Monsanto and intended for food and feed use, and is presently pending for authorisation in the European Union (EU). NK603 is likely to become one of the dominating GM maize lines on the market in forthcoming years, as the successor of another RoundupReady maize (GA21) from Monsanto.

The currently applied transformation technology for plants causes random integration of the recombinant DNA into the plant genome; the insert-genome junctions are therefore unique to each particular transformation event and each junction will be present in only one copy per haploid genome [2]. NK603 maize is transformed with a construct consisting of two expression cassettes [3]. The first expression cassette contains a modified gene for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*cp4 epsps*) isolated from the soil bacterium *Agrobacterium tumefaciens* strain CP4 under the control of the rice actin (*Act*) promoter and rice actin intron. The second cassette, which is fused to the first, consists of the *cp4 epsps* gene regulated by the enhanced cauliflower mosaic virus (CaMV) 35S promoter (e35S) and intron from the maize heat shock protein 70. Both expression cassettes incorporate the 3′ untranslated region of the nopaline synthase gene (*nos* 3′) also from *A. tumefaciens*.

The principles of legislation in the EU concerning genetically modified organism (GMO) foods [4] are as follows. A GMO or its derived product can become authorised for food use if it is deemed safe by the European Food Safety Authority (EFSA). If authorised, then labelling is required if the presence is not adventitious or technically unavoidable, as well as if the amount exceeds a legally defined threshold (presently 0.9%). A transitional threshold for unauthorised GMOs exists if the GMO has benefited from a favourable opinion from the Community Scientific Committee(s) or the EFSA before 18 April 2004 if certain other criteria are met. Any other presence of unauthorised GMO is by default illegal.

According to Regulation (EC) No. 1829/2003, article 36 [4]: "To facilitate controls on genetically modified food and feed, applicants for authorisation should propose appropriate methods for sampling, identification and quantification, and deposit samples of the genetically modified food and feed with the Authority; methods of

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sampling and detection should be validated, where appropriate, by the Community reference laboratory." Even though the regulation requires that methods for detection of the GMOs are provided by the applicants, as of March 2004 no such method had yet been published. In order to have available methods for detection of GMOs in food and feed, and especially methods for detection of unauthorised GMOs, independent research in the field will continue to be important.

The recently finalised EU-funded research project QPCRGMOFOOD (QLK1-1999-01301) has provided valuable new knowledge and a new generation of methods in the area of GMO detection [5]. Methods developed in the project include event-specific detection and quantification methods for genetically modified GTS 40-3-2 soya bean [6, 7, 8], and Mon810 [9, 10], Bt11 [11], GA21 [12], T25 [13] and CBH-351 (StarLink) [14] maize, as well as unpublished methods for Event 176 (Bt 176) and DBT418 (BtXtra) maize, and Ms8, Rf3 and GT73 rapeseed. All of the above-mentioned methods are based on amplification of event-specific sequences with a probe designed to cover the junction between the inserted fragment and the plant DNA or DNA rearranged during the transformation, thus making each method specific for the desired transformation event. For a review of recent developments in detection technology, see e.g. [15]. In this paper we will present such a method for detection and quantification of NK603 maize, based on a cloned sequence from the 5′ integration site of NK603 maize. Development of an NK603-specific method was made possible by the availability of commercial DNA samples containing small quantities of NK603 genomic DNA.

The aim of the study was to characterise the insertion site in the NK603 and develop an event-specific detection method for this GMO. In order also to assess the method transferability, the performance of this method was assessed on the two major types of real-time thermal cyclers represented by the LightCycler (Roche, airflow thermal cycler) and the ABI 7900 HT (Applied Biosystems, block thermal cycler). For relative quantification purposes, a maize alcohol dehydrogenase I gene (*Adh*)-specific real-time PCR system [16] was adopted.

Materials and methods

Reference materials

Powdered genomic maize DNA containing approximately 1% of transgenic NK603, GA21 and CBH-351 maize (Fluka, prod. ref. 69407) was purchased from Sigma and resuspended in ddH_2O according to the manufacturer's recommendation. This was expected to yield a concentration of 1,000 haploid copies/μl of maize genome equivalents in a total volume of 100 μl and approximately five haploid copies/μl of NK603 genome equivalents. These estimates rely on the assumption that DNA had been extracted from 1% of heterozygous NK603 maize material. Genomic DNA was isolated from Event 176 (IRMM-411), Bt11 (IRMM-412) and Mon810 (IRMM-413) certified reference materials (CRMs) produced by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and from GTS 40-3-2 soya bean leaf material provided by Matforsk (Ås, Norway), using a DNeasy Plant Mini Kit (Qiagen). Genomic DNA was also isolated from a soya bean sample (22A) provided by the Genetically Modified Materials Analyses proficiency testing scheme (GeMMA; CSL, UK) using a DNeasy Plant Maxi Kit (Qiagen). DNA was quantified with an SYBR Green I method [6].

Characterisation of the NK603 5′ integration junction

The 5′ integration junction was characterised using a modified version of the AFLP technique [17]. First, a tube containing DNA (expected quantity of 250±50 ng) with approximately 1% NK603 DNA was resuspended in 22.2 μl ddH_2O. The DNA was successively digested with 3 U of Hpy CH4 IV (New England Biolabs) in a total volume of 25 μl, containing restriction/ligation buffer (10 mM Tris–acetic acid, 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM DTT, pH 7.5) for 4 h at 37 °C. The digested DNA was ligated to a double-stranded adapter (50 pmol) in a total volume of 30 μl with 0.8 U of T4 DNA ligase (Fermentas) and 0.33 mM ATP (Sigma) for 4 h at 37 °C in the restriction/ligation buffer described above. The adapters were designed to ligate to the digestion site of the Hpy CH4 IV enzyme, and to prohibit re-establishment of the restriction site after ligation (Table 1).

The ligated product was first PCR amplified on a PTC-200 (MJ Research) thermal cycler with the primer pair APGAR1 [12] and Ap1 (Table 1), targeting the rice *Act* promoter and the adapter sequence, respectively. One volume of ligation mix was diluted in

Table 1 Oligonucleotides used

PCR system	Name	Type	Sequence	Source
Junction mapping	APGAR1	Reverse	5′-gAC TAT CCC gAC TCT CTT CTC AAg C-3′	[12]
	APGARn	Reverse	5′-TCT TCT CAA gCA TAT gAA TgA CC-3′	This study
	Ap1	Forward	5′-TgC TCT CgT AgA CTg CgT-3′	This study
	Ap2	Forward	5′-CTC gTA gAC TgC gTA CCC gT-3′	This study
	Adapter	Double stranded	5′-TgC TCT CgT AgA CTg CgT ACC-3′ II III III III III 3′-CA TCT gAC gCA Tgg gC-5′	Modified from [17]
	SP6	Reverse	5′-ATT TAg gTg ACA CTA TAg AA-3′	Promega
	T7	Forward	5′-gTA ATA CgA CTC ACT ATA ggg-3′	Promega
TaqMan NK603	NK603-5JFor	Forward	5′-CTg CTC ggC CAg CAA gCC t-3′	This study
	NK603-5JRev	Reverse	5′-AAg CAT ATg AAT gAC CTC gAg TA-3′	This study
	NK603-5JFT	TaqMan probe[a]	5′-TAg Cgg CCC ACg CgT ggT AC-3′	This study
TaqMan *Adh*	Adh-1	Forward	Sequence in press	[16]
	Adh-2	Reverse	Sequence in press	[16]
	Adh probe	TaqMan probe[a]	Sequence in press	[16]

[a] TaqMan probes were labelled with 5′-Fam and 3′-Tamra

two volumes of ddH_2O and 2.5 μl of the dilution was used as template in 30 μl final volume containing 25 pmol of each primer, 0.2 mM dNTP (Applied Biosystems), 1 U AmpliTaq Gold polymerase (Applied Biosystems), 10 mM Tris–HCl, 50 mM KCl and 3.0 mM $MgCl_2$. The resulting amplification products (2 μl) were used as a template in a nested PCR applying the same reagents and conditions as in the first PCR, except that the nested primers for the rice *Act* promoter (APGARn) and for the adapter (Ap2) were used (Table 1). The touchdown PCR program for both PCR amplifications was the following: initial heating for 10 min at 94 °C to activate the polymerase and to denature the template DNA, followed by one cycle of (25 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C), 11 touchdown cycles (25 s at 94 °C, 30 s at an annealing temperature 0.7 °C lower than for each previous cycle, starting at 64.3 °C, 55 s at 72 °C), followed by 27 cycles of (25 s at 94 °C, 30 s at 56 °C and 55 s at 72 °C), and finally 3 min at 72 °C before cooling to 4 °C. All primers and probes were purchased from DNA Technology (Århus, Denmark).

Amplification products were subjected to electrophoresis on a 2% agarose gel at constant voltage (70 V) in 0.5% TBE buffer. To evaluate the size of the amplification products a pUC Mix Marker 8 (Fermentas) was used. The PCR fragments were visualised using a UV transilluminator and ethidium bromide-stained gels. The PCR fragment was cycle sequenced using a Big Dye terminator v.3.1 kit (Applied Biosystems) with the nested PCR primers as sequencing primers on an ABI Prism 3100 automated sequencer (Applied Biosystems).

Cloning of DNA sequences

The PCR fragments produced with the primer pairs APGARn/AP2 and Adh-1/Adh-2 (Table 1) were cloned into the pGEM-T Easy Vector (Promega) to be used as calibrant reference materials (pFNM-001 and pFNM-002). Plasmid DNA was purified using a Wizard plasmid purification kit (Promega) and analysed with restriction enzymes and sequenced as described above using SP6 and T7 primers (Table 1) corresponding to regions flanking the cloning site of the pGEM-T Easy Vector for verification. The plasmid concentration of the solutions containing the pFNM-001 (NK603) and pFNM-002 (*Adh*) were quantified after linearisation with NcoI (Fermentas), using an SYBR Green I method [6].

Quantitative PCR

PCR primers and a corresponding TaqMan probe for a real-time PCR detection and quantification assay targeting event NK603 (Table 1) were designed using the Primer3 software [18].

The PCR reactions were performed in a total reaction volume of 25 μl containing 5 μl template DNA. Reaction conditions for the NK603 assay were: 0.75 μM of each primer, 0.25 μM probe, 1.5 U of DNA polymerase (AmpliTaq Gold), 0.3 U uracil *N*-glycosylase (AmpErase UNG, Applied Biosystems), 0.1 mg/ml BSA (Fermentas), 0.2 mM dATP, dCTP and dGTP (Applied Biosystems) and 0.4 mM dUTP (Applied Biosystems), 4 mM $MgCl_2$ and 1× PCR Buffer II (100 mM Tris–HCl, pH 8.3, 500 mM KCl). Amplification reactions were performed on LightCycler (Roche) and ABI Prism 7900HT (Applied Biosystems) real-time thermal cyclers. Fluorescence measurements were analysed with LightCycler software version 3.5 and ABI SDS software version 2.1, respectively. For relative quantification, a maize-specific reference gene system targeting the *Adh* of maize was used [16]. PCR conditions for the *Adh* assay were: 0.3 μM of each primer, 0.2 μM probe, 1.5 U DNA polymerase (AmpliTaqGold), 0.3 U uracil *N*-glycosylase, 0.1 mg/ml BSA, 0.2 mM dATP, dCTP and dGTP, and 0.4 mM dUTP, 3.5 mM $MgCl_2$ and 1× PCR Buffer II. The *Adh* assay was only run on the ABI Prism 7900HT real-time thermal cycler.

The amplification program included an initial decontamination step for 2 min at 50 °C to allow optimal UNG enzymatic activity, followed by 10 min at 95 °C in order to activate the DNA polymerase, to deactivate the UNG and to denature the double-stranded template, and successively 50 cycles of 15 s denaturation at 95 °C and elongation for 30 s (LightCycler) or 60 s (ABI Prism 7900HT) at 64 °C.

For quantitative estimates and to mimic the situation in real food samples, a background of 10,000 soya bean genome equivalents (GeMMA 22A) was spiked with tenfold dilutions (10^5–10^2 copies) of plasmid pFNM-001 (NK603) and 10^5 copies of pFNM-002 (*Adh*) per 5 μl template.

Results

Characterisation and cloning of DNA

Anchored PCR performed with the primers APGAR1 and Ap1 yielded a smear, with most fragments in the size range between 100 and 400 bp, when separated on an agarose gel. After a second (nested) PCR with the primers APGARn and Ap2, a discrete product of approximately 140 bp was obtained. This fragment was sequenced. Thirty-three bases of the sequence were identified as a part of the rice *Act* promoter using an “nr” blastn sequence similarity search which should retrieve all GenBank and RefSeq nucleotide sequences, and EMBL, DDBJ and PDB sequences [19]. The rest of the sequence (from the adapter to rice *Act*) showed no homology to known sequences. The remaining bases were assumed to correspond to maize genomic DNA and transformation-derived vector sequence.

The NK603 5′ integration junction fragment and the fragment from maize *Adh* were cloned and sequenced to be used further in this study. The cloned NK603 sequence (Fig. 1) showed a perfect match with the NK603-derived sequence from nested PCR. Finally, a new search using WU-Blast2 (searching the EMBL database) was performed [20]. The cloned sequence gave a 100% match with a sequence (EMBL/Genbank accession number AX342368, sequence 7 from patent EP1167531) published by Monsanto. This sequence had not been retrieved by the previously performed blastn search. Probing with the sequence of the cloned maize *Adh* fragment showed a perfect match with the *Adh*-1-1F from maize (EMBL/GenBank accession number X04050).

Development of a real-time PCR method for NK603

The obtained sequence information was used to design a real-time PCR assay with the forward primer located in the plant genomic DNA and the reverse primer annealing to the 5′ region of the insert, yielding a 102 bp amplicon, with a binding site for a TaqMan probe spanning the junction between the maize genomic DNA and the insert (Fig. 1). This probe location provides extra specificity to the detection assay. Specificity testing was done only on the ABI Prism 7900HT real-time thermal cycler using the NK603 DNA from the Fluka reference material, with the cloned plasmid DNA as positive controls, and DNA from genetically modified Bt11, Event 176, Mon810, GA21, CBH-351, non-GM maize and genetically modified GTS

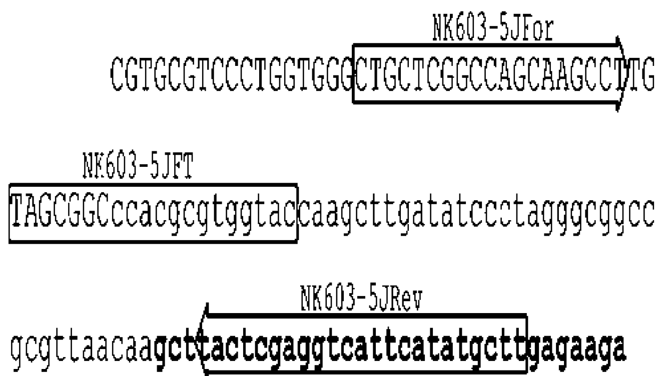

Fig. 1 The 5'-integration junction sequence obtained from the NK603 material. The identified sequence was identical with a part of a sequence published online by Monsanto (accession number AX342368). The identified 5′ sequence of the rice *Act* promoter is indicated with ***bold letters***. The vector-derived DNA used is shown in *small letters* while the plant genomic DNA is represented by *capitals* (inferred from the sequence published online by Monsanto). The primers and the probe for the quantitative PCR assay are framed by *arrows* and a *box*, respectively

40-3-2 soya bean as negative controls. Positive signals were only obtained with DNA from NK603 and the plasmid pFNM-001 (data not shown).

Assessment of method transferability

The NK603 assay was tested on two different types of thermal cycler systems. The LightCycler system applies glass capillaries, and very short ramp times are achieved through regulation of temperature by heating and cooling air around the capillaries. The ABI Prism system applies conventional PCR tubes or microtitre plate formats, and regulation of temperature by heating and cooling of metal blocks surrounding the tubes/plates. Reactions were set up in quadruplicate using tenfold dilutions of plasmid pFNM-001 (10^6–10^1 molecules) as template. The performance on the ABI Prism 7900HT was slightly better than on the LightCycler (Table 2), with a lower mean threshold cycle (Ct) value and standard deviation (SD). A tenfold dilution of the template would theoretically yield an increase in Ct (ΔCt) of 3.32. For both types of thermal cycler the differences in ΔCt between the different tenfold dilutions in the range 10^6 to 10^2 assigned copies per PCR (expected dynamic range) were close to this expected value (3.27–3.58; Table 2). The slope of the linear regression curves indicated overall high amplification efficiency, and a good correlation coefficient was observed (0.999) for both. Based on the amplification curves and regression analyses, the amplification efficiency of the NK603 assay was estimated to be 94.8 and 94.6% on the LightCycler and on the ABI Prism 7900HT, respectively.

Quantitative estimates and lowest detectable copy number

Repeated estimations of the DNA concentration in two separate vials of the NK603 reference material from Fluka using the SYBR Green I method [6] determined the DNA concentration to be approximately one fourth of the amount stated by the manufacturer. When the manufacturer's protocol for resuspension of the DNA was followed, our estimated yield was approximately 125 copies of the NK603 target sequence per vial and an average of 1.25 copies/μl in the resuspended solution. Resuspension in a smaller volume of ddH_2O to increase the concentration resulted in inhibition of PCR reactions (data not shown). For quantitative real-time PCR reactions, the absolute LOQ is normally around 100 copies [11]. Consequently, we concluded that the concentration and purity of the commercial reference material was too low to allow it to be used for validation of the real-time PCR assay. In the absence of suitable genomic DNA we chose to use the cloned DNA as reference material for calibration and to spike heterologous DNA isolated from a soya bean-derived sample (GeMMA 22A) with the linearised plasmid to mimic real food samples. Under mimic conditions the estimated copy numbers showed a high degree of correlation with the assigned copy numbers (Table 3). For relative quantitation of NK603 under mimic conditions, the copy number of pFNM-002 (*Adh*) was kept constant while the pFNM-001 (NK603) copy number was diluted in a tenfold series (10^5–10^2 copies per PCR). The relative quantitative estimates for the spiked (mimicked) samples (Table 4) corresponded well with the assigned concentrations and overall performance was very good, with a low standard deviation (SD) and error for all samples.

Table 2 Amplification data used to determine the absolute limits of detection and quantification (LOD and LOQ) for the dilution series of plasmid pFNM-001 (NK603), based on assigned (estimated) initial template copy numbers

Estimated no. of template molcules	10^6	10^5	10^4	10^3	10^2	10	1	0.1
Number of positives[a]	4/4	4/4	4/4	4/4	4/4	4/4	4/4	0/4
	4/4	4/4	4/4	4/4	4/4	4/4	3/4	0/4
Mean Ct value (of positives)	20.24	23.62	27.15	30.54	34.01	37.85	40.02	–
	19.45	22.97	26.55	30.04	33.67	36.90	41.05	–
SD of observed Ct values	0.15	0.11	0.16	0.17	0.30	0.73	0.89	–
	0.08	0.05	0.10	0.15	0.14	0.57	2.05	–
ΔCt[b]	na[c]	3.38	3.53	3.40	3.47	3.83	2.18	–
	na	3.52	3.58	3.49	3.27	3.23	4.15	–

[a] Upper/lower values: LightCycler, and ABI Prism 7900HT, respectively
[b] Difference between two successive dilutions. Expected value from tenfold dilutions ca. 3.32
[c] *na*=Not applicable

Table 3 Assessment of the effect of background DNA on the absolute quantitation. Four different dilutions of pFNM-001 (NK603) were mixed with DNA isolated from a sample from a GMO proficiency testing scheme (GeMMA 22A) to mimic real food samples. The same amount of background DNA was added to each sample, and each sample was analysed four times (A—D)

Sample	Assigned copy number	Measured NK603 target sequence copy number					$Error_r$ (%)	SD	RSD_r (%)[a]
		A	B	C	D	Mean			
1	10^5	104,252	106,949	108,375	113,459	108,259	7.62	3,865	3.57
2	10^4	9,694	7,936	8,868	11,025	9,381	6.60	1,310	13.96
3	10^3	1,003	945	946	1,030	981	1.93	42	4.28
4	10^2	114	114	102	122	113	11	8	7.07

[a] RSD_r=Repeatability relative SD (within lab)

Table 4 Assessment of the effect of background DNA on the relative quantitation. Each of four samples (1–4) was mixed with 10^5 copies of the pFNM-002 (*Adh*) target sequence and a constant amount of DNA isolated from a GMO proficiency testing scheme (GeMMA 22A) to mimic real food samples. A decreasing amount of pFNM-001 (NK603) was added to the samples for quantification, and each sample was analysed four times (A—D)

Sample	Assigned concentration (%)	Measured relative NK603 concentration (%)					$Error_r$ (%)	SD	RSD_r[a]
		A	B	C	D	Mean			
1	100	105.99	110.80	130.61	106.08	113.37	11.79	11.71	10.33
2	10	10.59	8.39	10.18	11.06	10.06	0.59	1.17	11.63
3	1	1.17	1.14	1.15	1.01	1.12	10.71	0.07	6.25
4	0.1	0.11	0.09	0.08	0.07	0.09	11.11	0.02	22.22

[a] RSD_r=Repeatability relative SD (within lab)

To find the lowest detectable copy number of the target sequence, an invariant quantity of DNA from the GeMMA soya bean sample was mixed with pFNM-001 (tenfold dilution series 10^6–10^0 copies per PCR). Plasmid pFNM-001 was detectable in all samples at all concentrations down to one copy for which four (LightCycler) and three (ABI Prism 7900HT) out of four samples were positive, respectively. We conclude that the LOD is near five copies per PCR and that the lowest detectable copy number is close to one.

Discussion

Characterisation of the NK603 transgene 5′ integration junction

On the basis of the rice *Act* promoter sequence [21] and a primer used for characterisation of GA21 (another RoundupReady maize) [12], we designed additional oligonucleotides to amplify the 5′ integration junction. The method used for isolation and characterisation of the NK603 5′ integration junction worked well on the commercially available reference material. From estimation of the DNA concentration in the vials of NK603, the target sequence copy number used in the reaction performed to capture and characterise the NK603 target sequence was calculated to be approximately three to four copies. This indicates that the applied method for capturing and characterising the integration junction was very sensitive, and that it may prove valuable also for other GMOs. For detection and identification of junction sequence(s) between insert and plant genomic DNA for a particular GMO, the availability of reference material is a problem. Even if a reference material is available, the amount of DNA derived from the target GMO and present in the material may limit the possibility of isolating and characterising the integration site. For comparison, the target sequence copy number in the starting material for other GMOs recently characterised has been in the range of 5,000 to 25,000 [10, 11, 22]. Thus we demonstrate that with an efficient method it is possible to characterise the integration sites of GMOs and clone reference materials even with minute quantities of starting materials.

After sequencing of the cloned fragment obtained from NK603 genomic DNA, and changing to another similarity search program (WU-Blast2), we retrieved a sequence published online by Monsanto (EMBL/GenBank accession number AX342368) that matched the characterised sequence. Given that no sequence was retrieved with the blastn search, this result came as a surprise to us and should be taken as a warning to others relying on the effectiveness of similarity searches. Comparison of the two sequences indicated that the cloned sequence was derived from NK603. The part of the sequence initially identified as a 5′ end of the rice *Act* promoter was verified by comparison with the published sequence. As expected, the rest of the cloned sequence consisted of a short stretch from the vector used for transformation of NK603 as well as a short stretch of genomic maize DNA. This study is therefore an independent verification of the online published sequence. Another sequence (EMBL/GenBank accession number AX342369) matching parts of the cloned sequence was also retrieved in the same search. This sequence contains a part of the rice *Act* promoter region and vector DNA inversely linked to the 3′ terminus of the NK603 insert. The NK603 sequences published online are to our knowledge the first sequences of inte-

gration sites for a commercial GMO published online by the company producing it.

Specificity of the developed assay

The NK603 real-time PCR assay yielded a positive signal only with genomic DNA from NK603 (Fluka reference material) and the cloned fragment pFNM-001. However, visual inspection of amplification products on agarose gel, using DNA from other GM plants as template, occasionally led to the detection of unspecific amplification products of variable signal intensity. This is common to event-specific methods targeting integration junctions, and probably is due to the fact that one of the primers matches a sequence of the host genome, i.e. a sequence present in all lines of the species of interest. Similarity searches probing with the assay-specific primers and probe did not retrieve any non-target sequences in the database. False positive signals on agarose gel may be caused by occasional binding of the primers to genomic maize DNA and may be avoided if the reaction is terminated after 40 to 45 cycles instead of 50. The part of the rice *Act* promoter and vector DNA present in the 3′ terminus covers the hybridisation site for the NK603-5JRev primer and a part of the hybridisation site for the probe (NK603-5JFT). Analyses of NK603 genomic DNA could therefore result in hybridisation of the NK603-5JRev primer to both the 5′ and 3′ end of the insert. Nevertheless, because the binding site for the NK603-5JFor primer is in the plant DNA, we believe that this sequence duplication has only an insignificant effect on the specificity of the developed method.

Assessment of method transferability

In this study the NK603 assay was tested on the two main types of thermal cyclers that are on the market today, namely airflow cyclers (here LightCycler) and block cyclers (here ABI Prism 7900HT). Our results show that the assay is robust and that adaptation of the system, e.g. to different thermal cyclers, may be easy, facilitating implementation of the method in different GMO testing laboratories.

The NK603 assay applied an elongation temperature of 64 °C, but performance is also very good at 60 °C (data not shown), i.e. the standard temperature for TaqMan assays including most GM testing assays. When absolute concentrations of NK603 and *Adh* are determined by application of calibration curves and calibrant reference materials, both elongation temperatures may be acceptable because the PCR reactions are performed independently in separate reaction vessels, and the relative GMO concentration is calculated on the basis of the ratio of these two separate targets.

Quantitative estimates and lowest detectable copy number

The results from our estimations of the DNA concentration in the commercial reference material led us to mimic samples for quantitation by mixing plasmid DNA (pFNM-001 and pFNM-002) with genomic DNA from soya beans (GeMMA 22A). Amplification curves for the mimicked samples were overlapping with the standard curves (based on plasmid DNA), which indicates that the added genomic DNA did not contribute to any significant inhibition of the assay when the number of plasmid copies was 100 or above. Quantitative estimates of the mimicked samples yielded a low relative error of independent measurements, demonstrating the accuracy of the assay. The experiments show that the variation in Ct values among parallels of the same template concentrations increase with decreasing copy number, corresponding to recently published results [11]. Comparison of the performance of the NK603 method with published methods for quantification of other GMOs [6, 7, 8, 9, 10, 11, 12, 13, 14, 23] indicates that the performance of the NK603 method is similar to that of other state-of-the-art methods.

The decision to use plasmids to establish calibration curves was a consequence of the lack of other appropriate quantitative reference material. For routine GMO testing there is currently an ongoing discussion on the choice between plasmid, genomic DNA and matrix-matched calibration reference materials [2]. Calibration curves established on the basis of cloned target plasmid DNA may be more suitable to quantify GMOs in a wide range of food-derived processed samples [7, 24], but correspondence between plasmid-based reference materials and typical food-derived DNA has only been confirmed in some cases [e.g. 10]. Current Japanese GMO testing standards rely on the use of cloned plasmid DNA as calibration reference material, and these methods have recently been included in the draft European (CEN) and international (ISO) standards for GMO testing prEN ISO 21570 [25]. Even though preparation of plasmid DNA calibrants may be simple and inexpensive and such calibrants may yield a wider dynamic range than genomic DNA-based calibrants, the latter may be more representative of the analytical situation, especially in terms of amplification efficiency.

In conclusion, we report the sequence of the 5′ integration site of GMO maize event NK603 identified from a low amount of starting material. Based on this sequence, a real-time PCR method for reliable and accurate detection of NK603 maize was developed. The specificity of the method is due to amplification of a region that is unique to the event, more specifically the junction between genomic plant DNA and the rice *Act* promoter sequence. In the absence of a suitable CRM, plasmids containing the NK603 and a maize endogenous reference target were constructed and applied for construction of calibration curves for quantitative PCR. The presented method is a new contribution to the family of event-specific methods for detection and quantitation of GMOs in food and feed

products, and may rapidly find its implementation in GMO testing laboratories.

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