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Validation of a sensitive DNA walking strategy to characterise unauthorised GMOs using model food matrices mimicking common rice products

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ABSTRACT

To identify unauthorised GMOs in food and feed matrices, an integrated approach has recently been developed targeting pCAMBIA family vectors, highly present in transgenic plants. Their presence is first assessed by qPCR screening and is subsequently confirmed by characterising the transgene flanking regions, using DNA walking. Here, the DNA walking performance has been thoroughly tested for the first time, regarding the targeted DNA quality and quantity. Several assays, on model food matrices mimicking common rice products, have allowed to determine the limit of detection as well as the potential effects of food mixture and processing. This detection system allows the identification of transgenic insertions as low as 10 HGEs and was not affected by the presence of untargeted DNA. Moreover, despite the clear impact of food processing on DNA quality, this method was able to cope with degraded DNA. Given its specificity, sensitivity, reliability, applicability and practicability, the proposed approach is a key detection tool, easily implementable in enforcement laboratories.

1. Introduction

In the European (EU) market, to preserve freedom of choice for the consumer and to protect the food and feed chain, authorisations for genetically modified (GM) food or feed products are subjected to the EU legislation (Dir. 2001/18/EC; Reg. EC No. 1829/2003; Reg. EC No. 1830/2003). The commercialisation as well as the detection of genetically modified organisms (GMOs) make up an integrated part of these EU regulations. In this context, several methods to detect GMOs in food and feed matrices have been developed. These methods, based essentially on real-time PCR technologies, target the most common elements present in GMOs (Broeders, De Keersmaecker, & Roosens, 2012b). Screening methods, usually used as the first step in GMO analysis, allow the detection of GMOs in a given food/feed sample. In case of positive responses for some GM targets, it allows to narrow down the number of EU-authorised GM events to be identified using event-specific methods in a second step (Broeders, Papazova, Van den Bulcke, & Roosens, 2012a; GMOMETHODS: EU Database of Reference Methods for GMO Analysis). The screening step can also indirectly indicate the potential presence of EU-unauthorised GM events. Indeed, if the transgenic elements identified during the screening step cannot be explained by EU-authorised events, the presence of unauthorised GMOs can be suspected (Broeders et al., 2012b; Reg. EC No. 619/2011). However, screening methods are only able to provide an indirect proof of GMO presence. In addition, targeted screening elements often originate from natural organisms, such as the Cauliflower Mosaic Virus (CaMV). As a consequence, an "unexplained" transgenic element identified in the screening (e.g. p35S) might also find its origin in the presence of the corresponding donor organism (e.g. CaMV) in a food/feed sample (Broeders et al., 2012a, 2012b). Therefore, the presence of GMO in food and feed matrices can only be confirmed by the identification of the junction between the transgenic integrated cassette and the plant genome, which represents the unique signature of a GMO.

In order to prove indubitably the presence of an unauthorised GMO, different DNA walking methods have been carried out on

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transgenic plants (Fraiture et al., 2014 and references therein; Spalinskas, Van den Bulcke, & Milcamps, 2013; Zhang et al., 2012). However, most of these DNA walking strategies are not easily implementable in GMO routine analysis by the enforcement laboratories for several reasons. First, some techniques are laborious, complex or insufficiently specific. Second, although some of them are based on PCR, which is simple, mastered and frequently used by the enforcement laboratories, DNA walking approaches are not integrated into the screening strategy. In addition, their ability to detect low amounts of target is expected to be weak (Spalinskas, Van den Bulcke, Van den Eede, & Milcamps, 2012). To design a strategy corresponding to the need of the enforcement laboratories, we have recently developed an integrated approach to rapidly detect and identify unauthorised GMOs in food and feed matrices in two main steps (Fraiture et al., 2014). First, a qPCR SYBR®Green screening allows to determine the potential presence of unauthorised GMOs in a given sample. Second, their presence is confirmed by DNA walking. This last method is based on PCR, which is applied using a first reverse primer followed by two semi-nested PCR rounds using primers that are each time nested to the previous reverse primer. This approach is integrated since the same primer sequences are used for both the unauthorised GMO detection (screening method) and its characterisation (DNA walking method). Moreover, the use of two semi-nested PCR rounds allows us to increase the yield and the specificity of the method, especially in the case of a low level presence of GMOs.

The detection of a target using DNA-based methods implies limitations related to the nature of the tested food/feed sample. Two different categories of limitations can affect this detection system (Ballari & Martin, 2013; Fernandes, Oliveira, & Mafra, 2013). On the one hand, the detection of a weak concentration of the target in food and feed matrices, which is usually the case for unauthorised GMOs, requires a method sufficiently sensitive (Broeders et al., 2012b; ENGL *ad hoc* working group on "unauthorised GMOs", 2011). On the other hand, the performance of the detection method could also be affected by the state of the sample. Indeed, food processing, defined as any food manipulation step (physical, chemical or mechanical) from the raw material to the final product, is known to induce DNA damage. This process could thus reduce the size of the obtained amplicons due to the fragmentation of high molecular weight DNA strands (Arun, Yılmaz, & Muratoglu, 2013; Ballari & Martin, 2013; Fernandes et al., 2013; Gryson, 2010; Lipp et al., 2001; Peano, Samson, Palmieri, Gulli, & Marmiroli, 2004; Ruttink et al., 2010). To our knowledge, few of these potential impacts (quantity and quality of the target) on DNA walking methods have been examined to date (Nielsen, Berdal, & Holst-Jensen, 2008).

Previously, we have developed an integrated approach, based on DNA walking, to identify unauthorised GMOs (Fraiture et al., 2014). In the extension of this study, the sensitivity of the method has been assessed using different amounts of Bt rice. In addition, the method has been tested on model food matrices mimicking rice food mixtures (rice and maize) and processed rice food (rice flour and rice noodles). In this way, the present study will provide the enforcement laboratories with crucial information concerning the applicability, the practicability and the dynamic range of the proposed method (International Standard ISO 24276, 2006). To our knowledge, the performance criteria measured in the present study, applied to unauthorised GMOs, have never been described for DNA walking methods published so far.

2. Materials and methods

2.1. Reagents and devices

Genomic-tip20/G was purchased from QIAGEN (Hilden, Germany). SYBR®Green PCR Mastermix was provided by Diagenode (Liège, Belgium). APAgene™ GOLD Genome Walking Kit was obtained from BIO S&T (Montréal, Canada). Agarose was bought from INVITROGEN (CA, USA). Wizard® SV Gel and PCR Clean-Up System and pGEM®-T Easy Vector Systems were purchased from PROMEGA (WI, USA). Big Dye Terminator Kit v3.1 was obtained from Applied Biosystems (CA, USA). Nanodrop® 2000 (Thermo-Fisher, DE, USA), iQ™5 real-time PCR detection system (BioRad, Hemel Hempstead, UK) and Genetic Sequencer 3130XL were used.

2.2. Plant materials and sample preparation

As previously described, transgenic Bt rice grains (*Oryza sativa L. Japonica cv Ariete*), transformed with the binary vector pCAMBIA1300 harbouring the synthetic Cry1B gene to confer an insect resistance, and its wild-type (WT) were used in this study to develop and optimise the methodology (Breitler et al., 2004; Fraiture et al., 2014).

From these rice grains, in-house rice flour and noodles were prepared by mixing WT rice grains and Bt rice grains to obtain rice products containing 0%, 0.1% or 1% of Bt rice (w/w) (Fig. 2). First, to prepare the rice flour, one volume of rice grain samples, containing 0%, 0.1% or 1% of Bt rice, mixed with two volumes of Milli-Q water were incubated at room temperature (RT) overnight. The mixtures were then ground, filtered and dried at RT. Second, to prepare rice noodles, the previously described rice flours were mixed with warm water to obtain a homogenous dough allowing us to form noodles (Tram's kochen). The fresh noodles were then dried at 30 °C with ventilation for 30 min in order to mimic the traditional sun-drying method (Hsieh & Luh, 1991). As control, unprocessed WT grain samples, containing 0%, 0.1% or 1% of Bt rice grains compared to WT rice grains (w/w), were also prepared.

The Certified Reference Materials (CRM) of the WT maize (non GM MON810 maize counterpart (ERM-BF413ak)), in the form of seed powders, were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). This material was characterised as previously described (Broeders et al., 2012c).

2.3. DNA extraction, concentration and purity

DNA, from homogeneous powder of ground rice grains, rice flour and rice noodles, was extracted using a CTAB-based procedure (ISO 21571), in combination with the Genomic-tip20/G, which was adapted from the EU-RL GMFF (European Union Reference Laboratory for GM Food and Feed) validated method (European Union Reference Laboratory., 2006; International Standard ISO 21571, 2005). DNA concentration was measured by spectrophotometry using the Nanodrop® 2000 device and DNA purity was evaluated as falling within the acceptance criteria according to the A260/A280 (~1.8) and A260/A230 (~2.0–2.2) ratios. DNA extraction, concentration and purity of WT maize were carried out as previously described (Broeders et al., 2012c).

2.4. qPCR SYBR®Green assay

All qPCR assays were carried out in a standard 25 µl reaction volume containing 1X SYBR®Green PCR Mastermix, 250 nM of each primer and 5 µl of DNA. The qPCR cycling programme consisted of a single cycle of DNA polymerase activation for 10 min at 95 °C, followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing-extension step). Melting curve analysis was performed by gradually increasing the temperature from 60 to 95 °C over 20 min (±0.6°/20 s) (Barbau-Piednoir et al., 2010; Broeders et al., 2012c). All runs were performed on an iQ™5 real-time PCR detection system. For each assay, a "No Template Control" (NTC) was included.

As a decision support system to identify pCAMBIA unauthorised GM rice, the *p35S* (Promoter of the 35S cauliflower mosaic virus; Forward: AAAGCAAGTGGATTGATGTGATA; Reverse: GGGTCTTGCG AAGGATAGTG), *tNOS* (Terminator of the nopaline synthase gene; Forward: GATTAGAGTCCCGCAATTATACATTTAA; Reverse: TTATCC TAGKTTGCGCGCTATATT), *pld* (Phospholipase D gene from rice; Forward: GCTTAGGGAACAGGGAAGTAAAGTT; Reverse: CTTAGCAT AGTCTGTGCCATCCA) and *t35S* pCAMBIA (Forward: CGGGGGATCT GGATTTTAGTA; Reverse: AGGGTTCCTATAGGGTTTCGCTC) markers were used on 100 ng of DNA (Barbau-Piednoir et al., 2010; Fraiture et al., 2014; Mbongolo Mbella et al., 2011).

In addition, the potential presence of inhibitors in the analysed rice (grains, flours and noodles) and maize materials was evaluated via an inhibition test, based on the difference of C_t values (ΔC_t) as a function of the DNA concentration (Broeders et al., 2012a). To this end, all rice and maize materials were analysed at two different DNA concentrations (100 and 10 ng) by qPCR, using the *pld* and *adh* (Alcohol dehydrogenase I gene from maize; Forward: TCTCTTCCTCCTTTAGAGCTACCACTA; Reverse: AATCGATCCAAAGCG AGATGA) markers, respectively (Broeders et al., 2012b; Mbongolo Mbella et al., 2011). The inhibition was excluded if the calculated $\Delta\Delta C_t$ value, based on the difference between the observed ΔC_t value and the theoretical ΔC_t value (corresponding to 3.3 for a dilution 10), was equal to or less than 0.5 (Broeders et al., 2012a).

Moreover, qPCR assays, using the *pld* and *t35S* pCAMBIA markers, were carried out on the samples analysed by DNA walking. Only the DNA from WT maize was tested at 100 ng instead of 520 ng, due to the presence of inhibitors (data not shown).

2.5. DNA walking approach

2.5.1. General

As previously described, the present DNA walking strategy was performed using a first reverse target-specific primer (*t35S* pCAMBIA a-R: AGGGTTCCTATAGGGTTTCGCTC) and a degenerated random tagging primer (DRT). Two semi-nested PCR rounds were then applied using target-specific primers (*t35S* pCAMBIA b-R: GTGTTGAGCATATAAGAAACCC; *t35S* pCAMBIA c-R: TACTAAAATCC AGATCCCCCG), that are each time nested to the previous reverse target-specific primer, combined with universal tagging primers (UAP-N1 and UAP-N2) (Fraiture et al., 2014). PCR mixes and conditions were carried out according to the manufacturers' instructions of APAgene™ GOLD Genome Walking Kit. The final PCR product was separated by electrophoresis on a 1% agarose gel (100 V, 400 mA, 60 min).

2.5.2. Assessment of the method sensitivity, the impact of food mixture and food processing

First, to determine the limit of detection (LOD), the DNA walking approach was carried out on 100 ng of rice DNA templates composed of 100% of Bt rice (200 000 haploid genome equivalents (HGEs) of Bt rice) or WT rice containing 1% (2000 HGEs of Bt rice), 0.1% (200 HGEs of Bt rice), 0.05% (100 HGEs of Bt rice), 0.025% (50 HGEs of Bt rice), 0.001% (20 HGEs of Bt rice), 0.005% (10 HGEs of Bt rice) or 0% of Bt rice. In addition, as the sensitivity of the DNA walking method may be limited by the visualisation of obtained amplicons on agarose gel, a cloning strategy of direct ligation was carried out on final PCR products from the four different DRT primer mixes at a Bt rice concentration of 0.025% (50 HGEs). Second, the potential effect of background DNA from food mixtures was tested by mixing WT maize DNA with 200 000 HGEs, 2000 HGEs, 200 HGEs or 0 HGE of Bt rice DNA. Finally, to evaluate the potential impact of food processing, the DNA walking method was applied to 100 ng of DNA from "home-made" WT rice flour and noodles containing 1%, 0.1% or 0% of Bt rice. The WT rice grain samples containing 0%, 0.1% or 1% of Bt rice were used as a control. The HGE contents of the DNA extracts were calculated according to the size of the rice genome (0.5 pg) and the maize genome (2.6 pg) (Arumuganathan & Earle, 1991). A NTC was included in the assay.

2.5.3. Cloning and sequencing

Two different sequencing methods were applied. On the one hand, the amplicons were excised from the gel and were purified using the Wizard® SV Gel and PCR Clean-Up System. The purified amplicons were directly sequenced using the *t35S* pCAMBIA c-R primer or cloned into the pGEM®-T Easy Vector Systems, according to the manufacturers' instructions, in order to be then sequenced. On the other hand, the final PCR products from the DNA template 0.025% (50 HGEs) and 0% (WT rice) of Bt rice were cloned into the pGEM®-T Easy Vector Systems. A PCR was carried out on colonies using pGEM®-T Easy Vector (T7: TAATACGACTCACTATAGGG; SP6: ATTTAGGTGACACTATAGAAT) combined with rice primers (Rice chromosome II: CGAAAAGAAGATGGCAGGAT; Rice chromosome III: TTTCTTTCGCTTCTGCAGGT) and was analysed by electrophoresis on a 1% agarose gel (100 V, 400 mA, 60 min). The colonies presenting a fragment of the correct size were then sequenced. All sequencing reactions were performed on a Genetic Sequencer 3130XL using the Big Dye Terminator Kit v3.1. The sequences were aligned via the software "ClustalW2" and analysed using the software "Nucleotide BLAST NCBI" (ClustalW2; Nucleotide BLAST NCBI).

3. Results and discussion

3.1. General

To assess the potential impact of the quality and quantity of targeted DNA on the DNA-based method, the developed DNA walking strategy identifying pCAMBIA-transformed unauthorised GMOs was thoroughly assessed. To this end, three different assays have been established in order to evaluate the limit of detection (LOD) of this method as well as the effect of food mixtures and food processing. All tested samples were first analysed by qPCR using a decision support system to confirm the identity of the materials (Fraiture et al., 2014). This system, based on three screening markers (p35S, tNOS and t35S pCAMBIA), allows to effectively confirm the presence of the pCAMBIA target in the sample. In addition, an inhibition test was applied on the rice and maize materials using the endogenous *PLD* and *ADH* markers respectively. Based on the obtained C_t values, the calculated $\Delta\Delta C_t$ values of rice grains (0.23), rice flour (0.19), rice noodles (0.48) and maize (0.24) were less than 0.5, indicating that none of these samples at 100 ng of DNA was subject to inhibition.

3.2. Sensitivity assessment

In order to determine the LOD of the DNA walking approach, DNA samples containing 100% to 0% of Bt rice were prepared and analysed (Table 1).

As the first step of GMO analysis in enforcement laboratories, all these DNA samples were submitted to a qPCR SYBR®Green analysis using the *PLD* and *t35S* pCAMBIA screening markers in order to identify potential unauthorised GMOs. As expected, all samples containing Bt rice (100% to 0.005%) presented a positive signal, inversely proportional to the DNA target amount, for the two markers. Moreover, the WT rice (0%) showed only a positive signal of the same amplitude for the *PLD* marker and no signal was observed for the NTC with these screening markers (Table 1; data not shown).

As a second step to confirm the presence of unauthorised GMOs, the DNA walking approach was applied to these samples using the

Table 1
Sensitivity analysis of the DNA walking method using the four different mixes of DRT primers (A–D). For each tested sample, the Bt rice amount in WT rice is indicated in percentage (100% to 0%) with its corresponding Bt rice HGEs. The obtaining of these samples was based on the HGEs. Following the qPCR analysis using the *PLD* and *t35S* pCAMBIA screening markers, the observed C_t values with the standard deviations are indicated for each sample. The detection of transgene flanking regions on the chromosomes II (X2) or III (X3) is symbolised by + (3/3), (+) (1–2/3) or – (0/3). For each result, the experiment was carried out in triplicate. The approximate size of amplicons, only obtained three times, is indicated between brackets in base-pair under the corresponding signal.

		100%	1%	0.1%	0.05%	0.025%	0.01%	0.005%	0%
		200 000 HGEs	2000 HGEs	200 HGEs	100 HGEs	50 HGEs	20 HGEs	10 HGEs	0 HGE
PLD		17.2 ± 0.4	17.3 ± 0.4	17.4 ± 0.4	17.9 ± 0.7	17.4 ± 0.3	17.3 ± 0.4	17.3 ± 0.3	17.3 ± 0.3
t35S pCAMBIA		21.8 ± 0.3	27.6 ± 0.5	31.9 ± 0.1	33.9 ± 0.2	34.9 ± 0.3	36.0 ± 0.6	37.1 ± 0.1	0.0 ± 0.0
Mix A	X2	+ *(950)*	+ *(950)*	+ *(950)*	+ *(950)*	–	–	–	–
	X3	+ *(700; 500)*	+ *(500)*	+ *(700)*	–	–	–	–	–
Mix B	X2	+ *(450)*	(+)	–	–	–	–	–	–
	X3	+ *(800)*	+ *(800)*	+ *(800)*	–	–	–	–	–
Mix C	X2	+ *(650)*	+ *(650)*	(+)	–	–	–	–	–
	X3	+ *(450)*	–	–	–	–	–	–	–
Mix D	X2	–	–	–	–	–	–	–	–
	X3	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850)*	+ *(850)*	+ *(850)*	+ *(850)*	–

four different DRT primers (A–D) (Fig. 1; Table 1). The first analysis of the 100% Bt rice (200 000 HGEs) had shown the presence of two different insertions of the pCAMBIA vector in the rice genome. Indeed, as previously mentioned, the transgenic cassette was integrated in the *Oryza sativa* japonica genome in chromosome III (OSJNBb0111B07) and chromosome II (OSJNBa0016G10) (Fraiture et al., 2014). In the present study, the sensitivity of this method was assessed using samples containing 1% (2000 HGEs) to 0.005% (10 HGEs) of Bt rice in order to determine its LOD. Up to 100 HGEs of Bt rice (0.05% Bt rice), the transgene flanking regions were detected on both chromosomes II and III via DRT A and D primers, respectively. At this Bt rice concentration, the size of the remaining amplicons was approximately 950 and 850 bp corresponding to the junctions on chromosomes II and III, respectively. With a lower amount of Bt rice, only the transgene flanking region on the chromosome III was identified as low as 10 HGEs of Bt rice (0.005% Bt rice) using DRT D primer mix. Among the tested materials, DRT B and C primers presented a weaker sensitivity compared to DRT A and D primers. Indeed, the transgene flanking regions on chromosomes II and III were detected as low as 200 HGEs of Bt rice (0.1% Bt rice) using DRT C and B primers, respectively. The decrease of target concentration thus seems to affect the detection power of the DNA walking approach. This performance drop was related to the affinity of the DRT primers mixes used and not to the size of the amplicons.

As the sensitivity of the method may be limited by the visualisation of PCR amplicons on agarose gel, a cloning strategy of PCR products by direct ligation was applied on a Bt rice amount of 0.025% (50 HGEs) where no amplicon was obtained with the different DRT primers, except for the D mix. However, a maximum of 3.3% of tested colonies from the A, B and C mixes (1 over 30) allowed to detect the transgene flanking regions on the chromosome II (DRT A primer) and chromosome III (DRT B primer) while 80% of tested colonies from the DRT D mix presented the transgenic insertion on the chromosome III. This strategy did not thus allow to improve the sensitivity of the present method since the majority of the cloned amplicons corresponded to an aspecific amplification when no PCR products were visible on agarose gel.

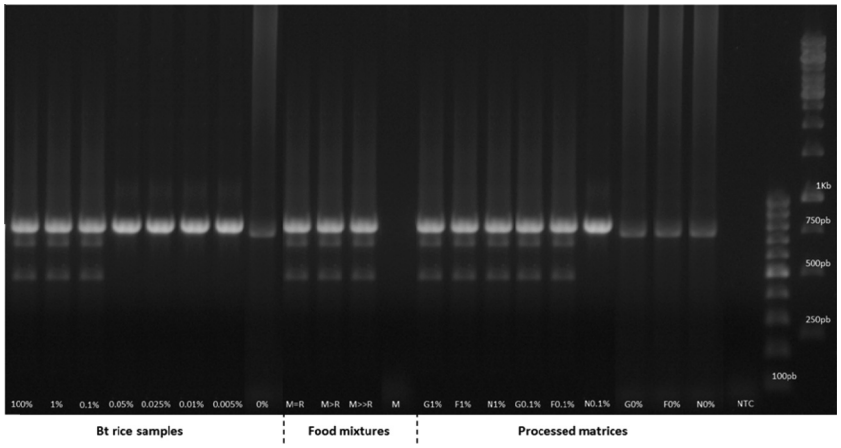

Fig. 1. Visualised amplicons on 1% agarose gel from all the tested matrices using the DRT D primer mix: (i) Bt rice (200 000 HGEs; 100%) and WT rice containing 2000 (1%), 200 (0.1%), 100 (0.05%), 50 (0.025%), 20 (0.01%), 10 (0.005%) or 0 HGEs (0%) of Bt rice; (ii) WT maize containing 200 000 (M = R), 2000 (M > R), 200 (M ≫ R) or 0 HGEs (M) of Bt rice; (iii) WT rice grains (G), flour (F) and noodles (N) containing 1%, 0.1% or 0% of Bt rice. The "No Template Control" is symbolised by NTC.

Table 2
Performance analysis of the DNA walking method tested on food mixtures (rice/maize) using four different mixes of DRT primers (A–D). For each sample, the amount of Bt rice and WT maize is based and indicated in HGEs (200 000, 2000, 200 or 0 HGEs). The indicated percentage corresponds to the Bt rice HGEs tested as in point 3.2. Following the qPCR analysis using the *PLD* and *t35S* pCAMBIA screening markers, the observed C_t values, with the standard deviations are indicated for each sample. The detection of transgene flanking regions on the chromosomes II (X2) or III (X3) is symbolised by + (3/3), (+) (1–2/3) or – (0/3). For each result, the experiment was carried out in triplicate. The approximate size of amplicons, only obtained three times, is indicated in base-pair under the corresponding signal.

WT Maize		200 000 HGEs	200 000 HGEs	200 000 HGEs	200 000 HGEs
Bt Rice		200 000 HGEs (100%)	2000 HGEs (1%)	200 HGEs (0.1%)	0 HGE
PLD		17.5 ± 0.1	25.1 ± 0.1	29.8 ± 0.7	0.0 ± 0.0
t35S pCAMBIA		21.2 ± 0.2	28.3 ± 0.5	31.9 ± 0.2	0.0 ± 0.0
Mix A	X2	+ *(950)*	+ *(950)*	+ *(950)*	–
	X3	+ *(700; 500)*	+ *(700)*	+ *(500)*	–
Mix B	X2	+ *(450)*	–	–	–
	X3	+ *(800)*	+ *(800)*	+ *(800)*	–
Mix C	X2	+ *(650)*	+ *(650)*	(+)	–
	X3	+ *(450)*	–	–	–
Mix D	X2	–	–	–	–
	X3	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850; 800; 500)*	–

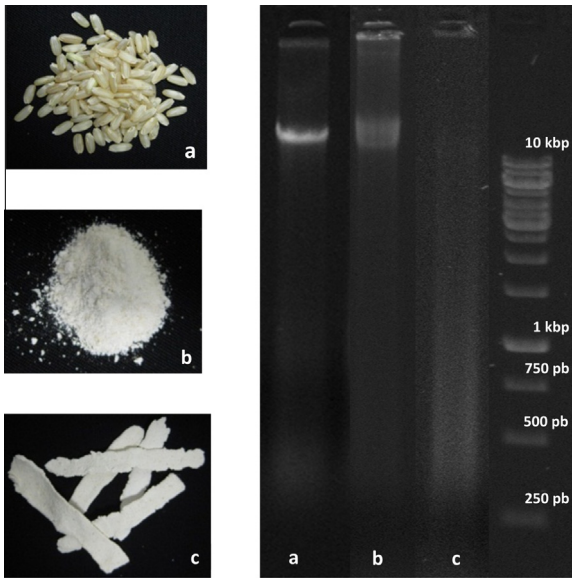

Fig. 2. Genomic DNA (100 ng) extracted from rice grains (a), flour (b) and noodles (c).

These results demonstrate a high sensitivity of the present DNA walking method (as low as 100 HGEs and 10 HGEs for the junction on chromosomes II and III, respectively) that is crucial for detecting traces of unauthorised GMOs in food matrices. In addition, the dynamic range of the proposed method depends essentially on the DRT primer mixes used and not on the size of the obtained amplicons. For instance, only mix A was still able to identify the pCAMBIA insertion on the chromosome II at 100 HGEs of Bt rice while the transgenic junction on the chromosome III was detected only by the mix D from 100 to 10 HGEs of Bt rice (Table 1). Moreover, this last DRT mix cannot provide the junction on the chromosome II at any concentration of the target, as previously observed by Fraiture et al. (2014). These data highlight the importance of using the four different DRT primers, as the sensitivity of the method depends mainly on the affinity of the primers, in order to increase the probability of detecting the two different transgene flanking regions, especially in the presence of low amounts of target. Concerning the specificity of the method, no aspecific amplification was observed, except for the WT rice (0% Bt rice) combined with the DRT D primer mix (Fig. 1). The obtained amplicon corresponds to an *Oryza sativa* Japonica genomic sequence of chromosome X (OSJNBa0050N08.1) which codes for a putative retro-element protein. It was generated by the adventitious presence of the UAP-N2 integrated tag near a rice sequence which is its reverse complement in order to allow this amplification using the UAP-N2 primer (data not shown). In addition, as expected, the NTC presented no amplification, suggesting that no reaction between the used primers was generated, independently of the DRT primers used.

3.3. Study of potential food mixture impact on DNA walking method

In order to evaluate the potential impact of a food mixture on the DNA walking approach, DNA samples composed of 200 000 HGEs of WT maize mixed with 200 000 HGEs, 2000 HGEs, 200 HGEs or 0 HGE of Bt rice were prepared. The WT maize was chosen as DNA background given its frequent presence in rice-based products, such as vermicelli. In addition, the qPCR SYBR®Green analysis using the *PLD* and *t35S* pCAMBIA screening markers, was applied (Table 2). The obtained signals were inversely proportional to the DNA target amount present in the tested matrices. As expected, the results were comparable in the presence and absence of maize (Table 1).

A DNA walking assay was carried out on these samples using the four different DRT primers (A–D) (Fig. 1; Table 2). For all the samples containing Bt rice, the transgene flanking regions on the chromosome II and III were detected similarly to samples composed exclusively of rice (Table 1). However, only the pCAMBIA insertion on the chromosome II was not identified at 2000 HGEs (1%) of the target using the DRT B mix. Furthermore, no aspecific amplification was generated.

Moreover, the sizes of the obtained amplicons were similar to the corresponding samples without maize (Fig. 1; Tables 1 and 2).

Table 3
Performance analysis of the DNA walking method tested on unprocessed (grains) and processed rice food (flour and noodles), using four different DRT primers (A–D). For each tested sample, the amount of Bt rice in WT rice, indicated in percentage (1%, 0.1% or 0%), is based on the rice grain weight. Following the qPCR analysis using the *PLD* and *t35S* pCAMBIA screening markers, the observed C_t values with the standard deviations are indicated for each sample. The detection of transgene flanking regions on the chromosomes II (X2) or III (X3) is symbolised by + (3/3), (+) (1–2/3) or − (0/3). For each result, the experiment was carried out in triplicate. The approximate size of amplicons, only obtained three times, is indicated in base-pair under the corresponding signal.

		Grains 1%	Flour 1%	Noodles 1%	Grains 0.1%	Flour 0.1%	Noodles 0.1%	Grains 0%	Flour 0%	Noodles 0%
PLD		17.6 ± 0.1	18.4 ± 0.5	19.9 ± 0.8	17.2 ± 0.2	18.6 ± 0.4	18.7 ± 0.6	17.3 ± 0.3	18.7 ± 0.6	19.6 ± 0.7
t35S pCAMBIA		27.2 ± 0.2	29.3 ± 0.3	30.1 ± 0.5	30.3 ± 0.2	31.6 ± 0.2	31.9 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Mix A	X2	+ *(950)*	+ *(950)*	+ *(950)*	+ *(950)*	+ *(950)*	(+)	–	–	–
	X3	+ *(500)*	+ *(500)*	–	+ *(500)*	+ *(500)*	–	–	–	–
Mix B	X2	(+)	–	–	–	–	–	–	–	–
	X3	+ *(800)*	–	–	+ *(800)*	–	–	–	–	–
Mix C	X2	+ *(650)*	+ *(650)*	(+)	(+)	(+)	(+)	–	–	–
	X3	–	–	–	–	–	–	–	–	–
Mix D	X2	–	–	–	–	–	–	–	–	–
	X3	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850; 800; 500)*	+ *(850)*	–	–	–

These results thus indicate the high specificity and reliability of the developed DNA walking approach applied to a food matrix, independently of the addition of another ingredient.

3.4. Study of potential food processing impact on DNA walking strategy

As food processing is well-known to cause DNA damage, the DNA walking approach was carried out on rice matrices processed in-house (Ballari & Martin, 2013). In order to gradually test the potential effect of food processing, rice flour and rice noodles were generated. These products are composed of WT rice and 1%, 0.1% or 0% of Bt rice. As a control, unprocessed WT rice samples (rice grain), containing 1%, 0.1% or 0% of Bt rice, were also prepared.

To evaluate the impact of food processing on DNA quality, extracted DNAs were observed on 1% agarose gel (Fig. 2). Although the DNA yields were similar, DNA degradation followed the level of food processing. Indeed, rice flour and rice noodles presented a DNA slightly and strongly degraded compared to the unprocessed materials, respectively.

First, these processed food products were analysed by qPCR SYBR®Green using the *PLD* and *t35S* pCAMBIA markers (Table 3). As expected, the Bt rice samples presented a positive signal for the two screening markers (*PLD* and *t35s* pCAMBIA), inversely proportional to the amount of target. A difference of C_t was observed between the unprocessed and processed samples, suggesting an impact of food processing.

Second, the DNA walking approach was evaluated on processed rice products (Fig. 1; Table 3). Compared to the unprocessed materials, the detection power of this system had decreased according to the level of DNA damage caused by food processing. However, the intensity of this effect differed in function of the DRT mix used. Indeed, no amplicon was generated on processed food with the DRT B primer while the mix D was able to detect the junction localised on the chromosome III similarly to unprocessed materials. Concerning the obtained amplicons, their sizes were in the same range as those of unprocessed materials (Fig. 1; Table 1). Nonetheless, the DNA degradation had implied a disappearance of some of them, such as in mix D tested on the 0.1% Bt rice noodles sample. As a whole, based on the combination of results from the four different DRT primer mixes (A–D), the DNA walking approach was able to confirm the presence of the target at its lowest tested concentration (0.1%), in both rice flour and noodles, identifying the transgene flanking regions on chromosomes II and III. Regarding the specificity, no additional aspecific amplification was observed.

The high sensitivity and specificity of the proposed DNA walking approach was confirmed by all these results. This method presents the important advantage to be able to cope with processed food that is essential for all analysis applied to food matrices. It should be noted, however, that, although the DNA from rice noodles was strongly degraded by the food processing, the impact of higher temperatures was not investigated in this study (Fig. 2).

In addition, the sensitivity of the method is clearly linked to the affinity of DRT primers used. Indeed, a primer with a poor affinity for the targeted sequence, such as mix B, presents an obvious difficulty to detect the transgene flanking regions in a given sample submitted to food processing. Therefore, the importance of using the four different DRT primers is highlighted in order to maximise the detection power of the DNA walking method, independently of the processing state of the tested matrix.

4. Conclusion

An integrated PCR-based DNA walking approach has recently been developed to identify unauthorised GMOs including a pCAMBIA family cassette that is frequently present in transgenic plants (Fraiture et al., 2014). For the first time, the analytical performance of this method has been here thoroughly assessed, in terms of sensitivity as well as applicability to a range of model food samples mimicking common rice-based mixtures and processed products.

The results obtained in this study suggest the good specificity, sensitivity, reliability, practicability and applicability of the developed DNA walking strategy on food mixtures, processed food matrices and low amounts of target, especially interesting in the case of unauthorised GMOs present at trace level.

At the moment, other new high-throughput technologies to identify unauthorised GMOs are emerging, such as Next-Generation Sequencing (NGS). Recently, this last technology has successfully characterised the transgene flanking region of GM soybean and rice (LLRice62, TT51-1 and T1c-19) (Kovalic et al., 2012; Wahler, Schauser, Bendiek, & Grohmann, 2013; Yang, Wang, Holst-Jensen, Morisset, Lin, & Zhang, 2013). However, these approaches were tested only on unprocessed materials composed of 100% targeted GMOs and the detection of low amounts of GMO mixture and

processed food, although crucial, remains challenging and extremely expensive. At the present time, NGS technology is not easily implementable routinely in the enforcement laboratories and still requires a long time-frame to get results, high-cost and qualified bioinformaticians for dealing with NGS data. Therefore, the proposed DNA walking strategy is currently a key molecular tool to easily prove, without significant additional cost and equipment, the presence of unauthorised GMOs in any given food/feed matrix.

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