ddPCR strategy to detect a gene-edited plant carrying a single variation point: Technical feasibility and interpretation issues

Marie-Alice Fraiture, Emmanuel Guiderdoni, Anne-Cécile Meunier, Nina Papazova, Nancy Roosens

Food Control 137 (2022) 108904

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

ddPCR strategy to detect a gene-edited plant carrying a single variation point: Technical feasibility and interpretation issues

Marie-Alice Fraiture [a], Emmanuel Guiderdoni [b,c], Anne-Cécile Meunier [b,c], Nina Papazova [a], Nancy H.C. Roosens [a,*]

[a] Sciensano, Transversal Activities in Applied Genomics (TAG), rue Juliette Wytsman 14, 1050, Brussels, Belgium
[b] CIRAD, UMR AGAP institut, F-34398, Montpellier, France
[c] UMR AGAP institut, Université de Montpellier, CIRAD, INRAE, Institut Agro, F-34398, Montpellier, France

ARTICLE INFO

Keywords:
CRISPR/Cas9
Single nucleotide mutation
Transgenic rice
Detection
Digital droplet PCR
Food and feed chain

ABSTRACT

Gene-edited organisms and derived food and feed products commercialized on the European market falls within the scope of the Directive, 2001/18/EC. Therefore, the possibility to specifically detect and quantify them has become a priority. To this end, PCR-based approaches, such as real-time PCR and digital droplet PCR, targeting a single variation point carried by a gene-edited organism are expected to be suitable, even if potentially challenging at the technical level. However, additional issues related to the interpretation of the results can also be encountered. Indeed, given its possible spread, natural or through breeding programs, the presence of this single variation does not automatically prove the presence of the gene-edited organism. To overcome such critical issue, we proposed a general workflow to develop and validate a PCR-based method specific to a gene-edited organism in targeting its single variation point. First, based on *in silico* analyses, the possibility to technically design the PCR-based method as well as to discriminate the gene-edited organism using it single variation point are assessed. In case such parameters are confirmed, the performance of the developed PCR-based method are then tested in agreement with the minimum performance requirements for GMO testing. The use of the proposed general workflow was successfully illustrated through the development a 2-plex digital droplet PCR method targeting specifically a gene-edited rice carrying a single nucleotide insertion. The proposed workflow was thus considered as a key tool to support the competent authorities regarding the food and feed traceability.

1. Introduction

As ruled by the Court of Justice of the European Union in 2018, organisms produced by gene editing fall within the scope of the Directive, 2001/18/EC related to the deliberate release of genetically modified organisms (GMO) into the environment (Court of Justice of the European Union, 2018; Directive, 2001/18/EC). Consequently, similarly to GMO produced by classical genetic engineering, the commercialization of gene-edited organisms and derived food and feed products on the European market required a prior authorization based on their risk assessments. Moreover, an event-specific method should be provided by the applicant, allowing a control of the food and feed chain by GMO enforcement laboratories (Fraiture et al., 2015; Grohmann et al., 2019; Menz et al., 2020; Ribarits et al., 2021).

Using gene editing techniques (i.e., OMD and CRISPR/Cas9), a modification of only few nucleotides, or even one single variation point (i.e., substitution, insertion or deletion) is specifically introduced in the sequence genome of the organism of interest. Resulting gene-edited organism differs thus minimally from its parental organism. Therefore, the detection of such gene-edited organism as well as its discrimination with its parental organism is considered at the technical level as complex. In addition, even with a successful detection of such variations, their natural or artificial origins are highly challenging to determine (ENGL, 2020a; Grohmann et al., 2019; Menz et al., 2020; Ribarits et al., 2021).

No gene-edited organisms are nowadays authorized to be commercialized on the European market. However, their short-term presence is expected due to the increasingly use of gene editing techniques by biotech companies as well as the increasingly commercialization of gene-edited plants on non-European markets. For example, two gene-

* Corresponding author.
E-mail addresses: Marie-Alice.Fraiture@sciensano.be (M.-A. Fraiture), Emmanuel.Guiderdoni@cirad.fr (E. Guiderdoni), anne-cecile.meunier@cirad.fr (A.-C. Meunier), Nina.Papazova@sciensano.be (N. Papazova), nancy.roosens@sciensano.be (N.H.C. Roosens).

https://doi.org/10.1016/j.foodcont.2022.108904
Received 22 November 2021; Received in revised form 3 February 2022; Accepted 21 February 2022
Available online 9 March 2022

edited crops, a herbicide-tolerant canola and a soybean with a modified oil composition, are already commercialized in the United States of America (Chhalliyil et al., 2020) In addition, a gene-edited high-GABA tomato is commercialized in Japan (ISAAA). As gene-edited organisms are falling within the scope of the Directive, 2001/18/EC, the possibility to detect, identify and quantify them at the technical level has become a priority. To this aim, the different strategies have been theoretically investigated (Chhalliyil et al., 2020; ENGL, 2020b; Grohmann et al., 2019). Among available detection tools, PCR-based methods are mainly used by GMO enforcement laboratories and their minimum performance requirements for GMO testing are well established (ENGL, 2015; ENGL, 2021). Although real-time PCR is widely mastered by GMO enforcement laboratories, digital droplet PCR (ddPCR) is increasingly used for the detection and quantification of classic GMO. The major advantage of this technology is the absolute quantification without dependence on optimal reference materials and standard curves. This is especially interesting in case of no available reference material such as for example with unknown GMO. Moreover, this end-point PCR technology is less sensitive to inhibitors due to the partitioning of the sample into thousands of droplets by a water-oil emulsion. The measurement uncertainty is thus reduced, in particular at low target copy number (Cottenet et al., 2019; Dobnik et al., 2016; Dobnik, Spilsberg, Košir, Holst-Jensen, & Žel, 2015; Gerdes et al., 2016; Grelewska-Nowotko et al., 2018; Iwobi et al., 2016; Kosir et al., 2017; Kosir et al., 2019; Li, Li, et al., 2020; Li, Zhang, et al., 2020; Morisset et al., 2013; Wan et al., 2016).

Such PCR-based strategies are expected to be technically suitable to detect plants carrying a single variation point induced by gene-editing (Fraiture et al., 2015; Grohmann et al., 2019; Stevanato & Biscarini, 2016). Currently, at our knowledge, such crucial investigation was experimentally explored in only few studies. Indeed, real-time PCR and ddPCR methods were successfully developed to target a single nucleotide variation in an acetolactate synthase gene carried by the commercial gene-edited herbicide-tolerant canola (Chhalliyil et al., 2020), a single nucleotide variation in the *chlorophyllide a oxygenase 1 (CAO1)* gene harboured by a gene-edited rice (Zhang et al., 2021) and single nucleotide variations in the Os06g0623700 gene carried by gene-edited rice lines (Peng et al., 2018; Peng et al., 2020). Most of these PCR-based methods were developed in line with the current European guidelines for method validation ("Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories) (ENGL, 2015). Nonetheless, the successful identification of a specific gene-edited organism is not only depending on potential technical issues associated to the development of a PCR-based methods targeting a single variation point. Indeed, it is crucial to determine if the single variation point is naturally spread or exclusively found in a specific gene-edited organism. Nowadays, no investigations related to the natural distribution of these single variation points of interest were however included. Consequently, the presence of such gene-edited organisms cannot be unambiguously proved by the detection of such single variation points (ENGL, 2020a).

In this study, we proposed a general workflow to determine if a PCR-based method, such as real-time PCR and ddPCR, can be developed and validated in order to specifically detect and quantify a gene-edited organism carrying a single variation point. The applicability of this workflow, composed of two main successive steps, was illustrated using a 2-plex ddPCR targeting a gene-edited rice line, carrying a single nucleotide (adenosine) insertion in the *OsMADS26* gene. First, *in silico* nucleotide sequence analyses are performed using available prior knowledge associated to the specific single variation point and its flanking regions. On this basis, we assessed the possibility to design at the technical level oligonucleotides allowing to specifically target the single variation point. In addition, using publicly available data related to the natural distribution of the single variation point, we assessed and discussed the discrimination power of this single variation in order to specifically identify the gene-edited rice line from all other lines, cultivars, varieties and species. Second, in agreement with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" (ENGL, 2015; ENGL, 2021; ISO 20395; Pecoraro et al., 2019), the performance of the developed PCR-based method are evaluated in terms of specificity (inclusivity and exclusivity) and sensitivity. In addition, the applicability of the validated PCR-based method was investigated using different mixtures.

Based on the generated experimental results, the possible use of PCR-based methods, such as real-time PCR and ddPCR, to guarantee the traceability of the food and feed chain was discussed, allowing to support the enforcement laboratories and competent authorities.

2. Materials and methods

2.1. Materials

Rice (*Oryza sativa* L. *Nipponbare*) seeds from a gene-edited line and its parental line were used. The gene-edited rice line, carrying a single nucleotide (adenosine) insertion in *OsMADS26* (locus: Os08g02070), was generated using CRISPR/Cas9 (Fig. 1, Table 1) (Meunier A.C. et al., unpublished). This single nucleotide insertion was introduced in the coding region close to the codon start between genomic position 679646 and 679647 from the chromosome 8. This frameshift mutation is expected to inactivate the gene, thereby putatively increasing the biotic resistance and biotic stress tolerance of the gene-edited rice line as previously described (Khong et al., 2015). Third generation seeds of the gene-edited rice line with homozygous A insertion and devoid of T-DNA were used. Using Rice SNP-Seek Database (a core collection of more than 3000 rice accessions), neither single nucleotide polymorphism (SNP) nor indel polymorphism were found in the 679641–679663 region (CCGTCGCATCGAGAACCCGGTTC) targeted by the gRNA in the natural diversity of rice (Wang et al., 2018). DNA from homogenous powder of grinded rice seeds from the gene-edited and parental lines was extracted using a CTAB-based procedure (ISO 21571) in combination with the Genomic-tip20/G kit (QIAGEN, Hilden, Germany) as previously described (EULR, 2006; Fraiture et al., 2014; ISO 21571).

DNA from *Homo sapiens* (G3041 Promega), DNA from microbial species as well as DNA from several plant materials were obtained as previously described (Table 2) (Broeders et al., 2015; Fraiture et al., 2021). The plant materials included different transgenic and common wild-type crop species (*Beta vulgaris, Brassica napus, Glycine max, Gossypium hirsutum, Oryza sativa, Solanum tuberosum, Zea mays*). The transgenic crops comprise at least all 44 plant events currently authorized on the European market as well as some unauthorized ones (24 plant events) belonging to *Brassica napus*, *Glycine max*, *Gossypium hirsutum, Oryza sativa, Solanum tuberosum* and *Zea mays* species (Table 2).

DNA concentration was measured by spectrophotometry using the Nanodrop® 2000 (ThermoFisher, DE, USA) 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.

2.2. ddPCR assays

According to the manufacturer's instructions, a standard 20 μl reaction volume, containing 1X ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 900 nM of each primer (Eurogentec), 250 nM of the probe (Eurogentec) and 5 μl of DNA, as well as 70 μl of Droplet Generation Oil for Probes (Bio-Rad) were loaded into a QX200™ droplet generator (Bio-Rad). Using 40 μl of generated droplets per reaction, a PCR amplification was performed on a T100™ Thermal Cycler (Bio-Rad). The PCR program consisted of a single cycle at 25 °C for 3 min, a single cycle at 95 °C for 10 min (Taq polymerase activation), 40 cycles at 94 °C for 30 s (denaturation) and at 60 °C for 1 min (annealing-extension), and a single cycle at 98 °C for 10 min (Taq polymerase inactivation). For each ddPCR reaction, results were acquired via the HEX and FAM channel using a QX200 reader (Bio-Rad) and were analysed through the QuantaSoft software v1.7.4.0917 (Bio-Rad) in manually setting the threshold above

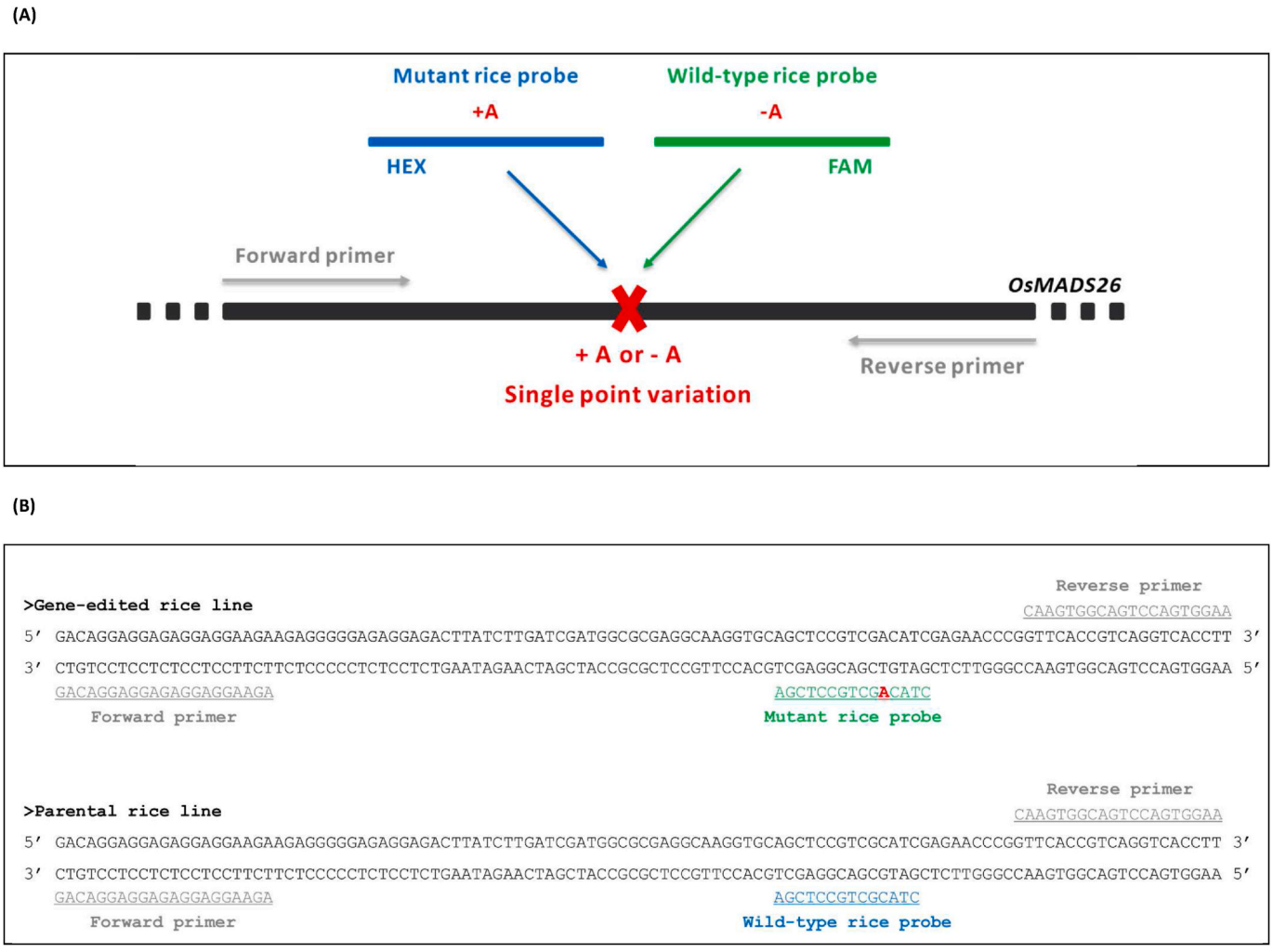

Fig. 1. Description of the developed 2-plex competing ddPCR strategy. (A) Schematic representation. (B) Location of the designed oligonucleotides on the targeted sequences. The single nucleotide variation (adenine insertion) is indicated in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Oligonucleotides of the developed 2-plex ddPCR method.

Oligonucleotide		Annealing temperature	Expected amplicon size
Name	Sequence		
OsMADS26 – Forward primer	GACAGGAGGAGAGGAGGAAGA	60 °C	113 bp for the gene-edited rice line 112 bp for the parental rice line
OsMADS26 – Reverse primer	AAGGTGACCTGACGGTGAAC		
Mutant rice – Probe	FAM-AGCTCCGTCGACATC-MGB-Eclipse®		
Wild-type rice - Probe	HEX-AGCTCCGTCGCATC-MGB-Eclipse®		

the cluster of negative partitions (threshold value of 2300; Supplementary data 3, 5, 7). The rejection criterion was set to exclude PCR reactions presenting less than 10,000 accepted droplets. For each ddPCR assay, a NTC (no template control) was included.

2.3. Development and validation of the 2-plex ddPCR method

Using the software Primer3, two primers (OsMADS26 – Forward primer and OsMADS26 – Reverse primer) were designed, covering 113 bp of the gene-edited rice line and 112 bp of the parental rice line (Fig. 1, Table 1). In addition, two hybridization probes labelled with a different dye (FAM or HEX) were designed to anneal on the single variation point, allowing to target either the gene-edited rice line (Mutant rice – Probe) or its parental rice line (Wild-type rice – Probe).

2.3.1. Specificity

2.3.1.1. In silico assessment. First, to investigate the natural distribution of the *OsMADS26* gene (locus: Os08g02070), its sequence was blasted against the NCBI nucleotide collection (nr/nt) with megablast/nblast default parameters (Supplementary data 1A). The NCBI nucleotide collection (nr/nt) consists of GenBank + EMBL + DDBJ + PDB + RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1 and 2 HTGS sequences and sequences longer than 100 Mb. The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry. The NCBI nucleotide collection (nr/nt) contained 76,336,016 sequences, including 385,132 items belonging to *Oryza sativa*. Second, the natural distribution of the single variation point of interest was investigated by blasting the amplicon sequences from the gene-edited rice line and its parental line generated by the developed 2-plex ddPCR method against the NCBI nucleotide collection (nr/nt) with megablast default parameters (Supplementary

Table 2
Specificity assessment of the developed 2-plex ddPCR method. The presence and absence of amplification are respectively symbolized by "+" and "-". For each result, the experiment was carried out in duplicates and the means of the measured copies are indicated in brackets. WT: wild-type.

Kingdom	Species	Collection number	Estimated haploid genome copy number	2-plex ddPCR targets	
				Mutant rice probe	Wild-type rice probe
Animalia	*Homo sapiens* (human)	/	6480	–	–
Bacteria	*Bacillus subtilis*	E07-505	200,000	–	–
Fungi	*Aspergillus niger*	IHEM 25485	260,000	–	–
Plantae	*Beta vulgaris* (sugar beet)	WT sugar beet[a]	12,500	–	–
		H7-100%[a,b]	12,500	–	–
	Brassica napus (rapeseed)	WT colza[a]	8000	–	–
		T45-100%[a,b]	8000	–	–
		Ms8-100%[a,b]	8000	–	–
		Rf3-100%[a,b]	8000	–	–
		GT73-100%[a,b]	8000	–	–
		Rf1-100%[a]	8000	–	–
		Rf2-100%[a]	8000	–	–
		Ms1-100%[a]	8000	–	–
		Topas 19/2–100%[a]	8000	–	–
		73496-10%[a]	4000	–	–
		MON88302-100%[a,b]	8000	–	–
	Glycine max (soybean)	WT soybean[a]	8620	–	–
		FG72-100%[a,b]	8620	–	–
		GTS40-3-2-10%[a,b]	4310	–	–
		A2704-12-100%[a,b]	8620	–	–
		MON89788-100%[a,b]	8620	–	–
		356043-10%[a,b]	4310	–	–
		305423-10%[a,b]	4310	–	–
		A5547-127 -100%[a,b]	8620	–	–
		MON87701-100%[a,b]	8620	–	–
		BPS-CV-127-9-100%[a,b]	8620	–	–
		MON87769-100%[a,b]	8620	–	–
		MON87705-100%[a,b]	8620	–	–
		DAS68416-4-10%[a,b]	4310	–	–
		MON87708-100%[a,b]	8620	–	–
		44406-1%[a,b]	2586	–	–
		SYHT0H2-100%[a,b]	8620	–	–
		DAS 81419-2-10%[a]	4310	–	–
		MON 87751-100%[a]	8620	–	–
	Gossypium hirsutum (cotton)	WT cotton[a]	4291	–	–
		MON1445-100%[a,b]	4291	–	–
		MON15985-100%[a,b]	4291	–	–
		MON531-100%[a,b]	4291	–	–
		LLCotton25-100%[a,b]	4291	–	–
		GHB614-100%[a,b]	4291	–	–
		281-24-236/3006-210-23-100%[a,b]	4291	–	–
		MON88913-100%[a,b]	4291	–	–
		GHB119-1%[a,b]	2575	–	–
		T304-40-1%[a,b]	2575	–	–
		MON88701-100%[a]	4291	–	–
		COT 102-100%[a]	4291	–	–
		DAS 81910-100%[a]	4291	–	–
	Oryza sativa (rice)	WT rice[a] (Japonica cv. Bengal)	20,000	–	+ (17,790 ± 70.7 copies)
		WT rice (Japonica cv. Ariete)	20,000	–	+ (921 ± 55.2 copies)
		Parental rice line (Japonica cv. Nipponbare)	20,000	–	+ (24,270 ± 325.3 copies)
		Bt rice-100% (Japonica cv. Ariete)	20,000	–	+ (3090 ± 14.1 copies)
		LLRICE62-100%[a] (Japonica cv. Bengal)	20,000	–	+ (18,120 ± 141.4 copies)
		Kefeng-6-100%[a] (Indica cv. Minghui 86)	20,000	–	+ (21,620 ± 311.1 copies)
		Gene-edited rice (Japonica cv. Nipponbare)	20,000	+ (19,610 ± 353.6 copies)	–
	Solanum tuberosum (potato)	WT potato[a]	5555	–	–
		EH92-527-1-100%[a]	5555	–	–
		AM04-1020-100%[a]	5555	–	–
		AV43-6-G7-1%[a]	2777	–	–
		PH048-100%[a]	5555	–	–
	Zea mays (maize)	WT maize[a]	3846	–	–
		MON87427-100%[a,b]	3846	–	–
		DAS59122–9.8%[a,b]	2261	–	–
		GA21–4.29%[a,b]	2475	–	–
		MON810–9.9%[a,b]	2885	–	–
		MON863–9.86%[a]	2275	–	–

(*continued on next page*)

Table 2 (*continued*)

Kingdom	Species	Collection number	Estimated haploid genome copy number	2-plex ddPCR targets	
				Mutant rice probe	Wild-type rice probe
		NK603–4.91%[a,b]	2833	–	–
		T25-100%[a,b]	3846	–	–
		TC1507–9.86%[a,b]	2275	–	–
		MON89034-100%[a,b]	3846	–	–
		MON88017-100%[a,b]	3846	–	–
		MIR604-100%[a,b]	3846	–	–
		Bt11-5%[a,b]	2885	–	–
		3272–9.8%[a]	2261	–	–
		98140-10%[a]	2308	–	–
		MIR162-100%[a,b]	3846	–	–
		Bt176 -5%[a]	2885	–	–
		MON87460-100%[a,b]	3846	–	–
		DAS40278-9-10%[a,b]	2308	–	–
		5307-100%[a]	3846	–	–
		VCO-Ø1981-5-100%[a]	3846	–	–
		4114-100%[a]	3846	–	–
		MON 87411-100%[a]	3846	–	–
		MON87403-100%[a]	3846	–	–
		MZHG0JG-100%[a]	3846	–	–

[a] CRM (Certified Reference Materials).
[b] Transgenic plant events authorized for use in food and feed on the European market (EC/1829/2003).

data 1B). A multiple sequence alignment analysis was then performed with these sequences using the Clustal Omega software with default parameters (Supplementary data 1C).

2.3.1.2. In vitro assessment. First, the presence of the sequences targeted by the developed 2-plex ddPCR method was experimentally investigated in transgenic and wild-type rice materials by conventional PCR followed by sequencing (Supplementary data 2). Using the software Primer3, a couple of primers (Forward: CCGGAGCTATCGATCATCA; Reverse: TGATGATGCCGATGTCGG) was designed to amplify a part of *OsMADS26* containing the amplicons targeted by the developed 2-plex ddPCR method. Each PCR assay was composed of a standard 25 µl reaction volume was applied containing 1X Green DreamTaq PCR Master Mix (ThermoFisher Scientific), 400 nM of each primer (Eurogentec) and 10 ng of DNA. The PCR run was performed on a Swift MaxPro Thermal Cycler (Esco) with a PCR program consisting of a single cycle of 1 min at 95 °C (initial denaturation) followed by 35 amplification cycles of 30 s at 95 °C (denaturation), 30 s at 60 °C (annealing) and 1 min at 72 °C (extension) and finishing by a single cycle of 5 min at 72 °C (final extension). The final PCR products were visualized by electrophoresis using the Tapestation 4200 device with the associated D1000 Screen Tape and reagents (Agilent). These PCR products were then purified using USB ExoSAP-IT PCR Product Cleanup (Affymetrix) and sequenced on a Genetic Sequencer 3500 (ThermoFisher) using the Big Dye Terminator Kit v3.1 (Applied Biosystems). The generated sequences were aligned using the Clustal Omega software with default parameters.

Second, the 2-plex ddPCR method was experimentally tested in duplicate on DNA from the gene-edited rice line and its parental line as well as on DNA from animal (*Homo sapiens*), bacteria (*Bacillus subtilis*) and fungi (*Aspergillus niger*) and plant (*Beta vulgaris, Brassica napus, Glycine max, Gossypium hirsutum, Oryza sativa, Solanum tuberosum, Zea mays*), including wild-type and transgenic crops. The latter comprises at least all events currently authorized on the European market as well as unauthorized ones (Table 2, Supplementary data 3). A minimum of around 2500 estimated haploid genome copies of non-targeted and targeted DNA were tested (Arumuganathan & Earle, 1991; Fraiture et al., 2021). The generated amplicons from the gene-edited rice line and its parental line were purified using USB ExoSAP-IT PCR Product Cleanup (Affymetrix) and sequenced on a Genetic Sequencer 3500 (ThermoFisher) using the Big Dye Terminator Kit v3.1 (Applied Biosystems). The generated sequences were aligned to the reference sequences using the Clustal Omega software (Supplementary data 4).

2.3.2. Sensitivity

Serial dilutions of DNA from gene-edited rice line and its parental line, ranging from 100 to 0 estimated target copies, were prepared and each dilution point was tested in 12 replicates (Table 3, Supplementary data 5). The calculation of the estimated target copy number was based on the size of the rice haploid genome (0.5 pg) (Arumuganathan & Earle, 1991). The limit of detection $LOD_{95\%}$, defined as the number of copies of the target required to ensure a 95% probability of detection (POD), was determined by using Quodata web application (Supplementary data 6) (Grohmann et al., 2016; ISO 16140-2; Uhlig et al., 2015; https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory).

2.3.3. Applicability

To prepare samples n°1–11, DNA from the gene-edited rice line and

Table 3
Sensitivity assessments of the developed 2-plex ddPCR method, using DNA from gene-edited rice line and its parental rice line. The presence and absence of amplification are respectively symbolized by "+" and "-". For each target copy number, 12 replicates were tested. The number of positive replicate (s) out of the 12 replicates tested is indicated and the means of the measured copies are indicated in brackets.

Estimated genome copy number	2-plex ddPCR targets	
	Mutant rice probe	Wild-type rice probe
100	+ (12/12) (99.2 ± 7.4 copies)	+ (12/12) (134.2 ± 13.9 copies)
50	+ (12/12) (50.8 ± 10.4 copies)	+ (12/12) (70.3 ± 9.3 copies)
20	+ (12/12) (17.5 ± 4.5 copies)	+ (12/12) (27.9 ± 5.0 copies)
10	+ (12/12) (8.9 ± 4.1 copies)	+ (12/12) (11.7 ± 3.2 copies)
5	+ (12/12) (4.4 ± 2.0 copies)	+ (11/12) (6.0 ± 3.9 copies)
1	+ (6/12) (1.3 ± 2.0 copies)	+ (8/12) (1.1 ± 0.9 copies)
0.1	- (0/12)	- (0/12)
0	- (0/12)	- (0/12)

its parental line were mixed to obtain samples containing 100%, 99.9%, 99.1%, 95%, 90%, 50%, 10%, 5%, 0.9%, 0.1% or 0% of the gene-edited rice line, ranging from 14,000 to 0 estimated target copies (Table 4). To prepare samples n°12–13, DNA from the gene-edited rice line and its parental line, both at 14 estimated target copies, were mixed in DNA from wild-type maize or wild-type soybean at 13,972 estimated haploid genome copies. The calculation of the estimated haploid genome copy number was based on the size of the rice (0.5 pg), maize (2.6 pg) and soybean (1.16 pg) haploid genomes (Arumuganathan & Earle, 1991). Each sample was tested in triplicate.

3. Results and discussion

3.1. ddPCR design

As a proof-of-concept, a ddPCR method was developed using a gene-edited rice line carrying a homozygous single nucleotide (adenosine) insertion in *OsMADS26* and its parental line (Fig. 1). This single nucleotide insertion was introduced in the coding region close to the codon start and expected to increase the stress resistance of the gene-edited rice line.

With the aim to detect a single variation point in gene-edit plants, the possible use of a 2-plex competing ddPCR strategy was investigated. In a single PCR assay, this multiplex strategy combines a couple of primers, amplifying a sequence of interest, and two hybridization probes, binding on the same region (Whale et al., 2016). To this end, a primer pair was designed to amplify a part of *OsMADS26* containing the single variation point of interest. Consequently, two different PCR amplicons can be generated, one with a length of 113bp using the gene-edited rice material and one with a length of 112 bp using the parental rice material (Fig. 1, Table 1). In addition, two probes, labelled with a different dye (FAM or HEX), were designed on the OsMADS26 region containing the single variation point of interest, allowing to discriminate the PCR amplicons generated from the gene-edited rice line and its parental line (Fig. 1, Table 1). The performance of the developed 2-plex competing ddPCR method was then assess for its specificity, sensitivity and applicability.

This ddPCR method was designed to detect and quantify the copy number of the gene-edited rice line in a given sample. This is currently sufficient because the gene-edited rice used in this study is unauthorized for commercialization on the European market. Therefore, its potential presence is associated to a "zero tolerance" policy. However, in case of authorization, the GM content per ingredient will need to be quantified. To this end, the use of a ddPCR method targeting an internal reference, such as previously developed for the rice-specific phospholipase D gene, will be needed (Corbisier et al., 2022; Mazzara et al., 2006).

Table 4
Applicability assessments of the developed 2-plex ddPCR method, using mixture samples containing different amount of gene-edited rice and its parental line as well as wild-type maize or wild-type soybean. The presence and absence of amplification are respectively symbolized by "+" and "-". Each different samples were tested in triplicated and the means of the measured copies are indicated in brackets.

Mixture samples	2-plex ddPCR targets	
	Mutant rice probe	Wild-type rice probe
1/Gene-edited rice line 100% - Parental rice line 0%	+ (13,733.3 ± 141.9 copies)	–
2/Gene-edited rice line 99.9% - Parental rice line 0.1%	+ (13,346.7 ± 213.8 copies)	+ (10 copies ± 6.1 copies)
3/Gene-edited rice line 99.1% - Parental rice line 0.9%	+ (13,273.3 ± 253.2 copies)	+ (87.3 ± 5 copies)
4/Gene-edited rice line 95% - Parental rice line 5%	+ (12,113.3 ± 375.4 copies)	+ (566.67 ± 16.3 copies)
5/Gene-edited rice line 90% - Parental rice line 10%	+ (10,753.3 ± 75.7 copies)	+ (1102.7 ± 27.1 copies)
6/Gene-edited rice line 50% - Parental rice line 50%	+ (5640 ± 87.2 copies)	+ (6240 ± 20 copies)
7/Gene-edited rice line 10% - Parental rice line 90%	- (1153.3 ± 33.2 copies)	- (12,160 ± 156.2 copies)
8/Gene-edited rice line 5% - Parental rice line 95%	- (528 ± 13.1 copies)	- (13,320 ± 72.1 copies)
9/Gene-edited rice line 0.9% - Parental rice line 99.1%	- (85.3 ± 8.3 copies)	- (13,706.7 ± 150.1 copies)
10/Gene-edited rice line 0.1% - Parental rice line 99.9%	- (10.8 ± 1.5 copies)	- (13,933.3 ± 197.3 copies)
11/Gene-edited rice line 0% - Parental rice line 100%	-	- (13,960 ± 69.3 copies)
12/Gene-edited rice line 0.1% - Parental rice line 0.1% - Wild-type maize 99.8%	+ (10.6 ± 1.6 copies)	– (9.7 ± 1.2 copies)
13/Gene-edited rice line 0.1% - Parental rice line 0.1% - Wild-type soybean 99.8%	+ (9.3 ± 2.8 copies)	– (7.3 ± 2.7 copies)

3.2. ddPCR specificity

3.2.1. In silico assessment

First, the natural distribution of the *OsMADS26* gene (locus: Os08g02070) was investigated (Supplementary data 1A). A total of 6 hits with a sequence coverage and identity ≥97% was observed from the megablast analysis. This gene was only found in rice species, including both *O. sativa* japonica and indica cultivars.

Second, the natural distribution of the single variation point in the *OsMADS26* gene was investigated using the PCR amplicon sequences from the gene-edited rice line (113 bp) and its parental line (112 bp) generated by the developed 2-plex ddPCR method (Supplementary data 1B-C). Among the entire NCBI nucleotide (nr/nt) collection, a total of 11 hits, all belonging to rice species, was observed and presented sequence coverage of 100% (Supplementary data 1B). All these hits showed a sequence identity of 100% with the parental rice line PCR amplicon and 99.12% with the gene-edited rice line PCR amplicon. Such difference was always associated to the single nucleotide insertion region (Supplementary data 1C). In line with these results, no SNP and indel polymorphism in the region of interest (CCGTCGCATCGAGAACCCGGTTC) were found in the natural diversity of rice, using the Rice SNP-Seek Database containing more than 3000 rice accessions, suggesting that the region of interest is conserved across rice varieties (Meunier A.C. et al., unpublished; Wang et al., 2018).

To our knowledge, the present study is the first one using sequence databases to investigate the frequency of a single variation point introduced by gene-editing. Such investigation is a crucial step during the development of a detection method targeting a specific gene-edited organism line. In this study, based on the current available information, as the single adenosine insertion was not found in any other rice genome sequences as well as in any other analysed species, these results indicated that this genetic modification carried by the gene-edited rice line seems not naturally spread, or at least infrequent in the natural rice population. These results indicate the possible discrimination between the gene-edited rice line and other rice lines using the developed 2-plex ddPCR method targeting the single variation point of interest. However, although rice is one of the most sequenced crop genome as well as accessions from more than 3000 rice varieties and species were in this study screened, such results cannot be confirmed regarding materials from commercial breeding programs. As the frequency of SNP in breeding germplasms is not from a random drift, their natural

occurrence is expected to be weak in the natural population without specific selection conditions. Nonetheless, there is no guarantee that the single point variation does not naturally exist or will appear in the future in reason of its advantage to confer a putatively biotic stress resistance.

3.2.2. *In vitro assessment*

First, the region amplified by the developed 2-plex ddPCR method in various transgenic and wild-type rice materials were compared (Supplementary data 2). The presence of the region of interest was confirmed in all these different rice materials and the single adenosine insertion was only observed in the gene-edited rice line, supporting the results from the *in silico* analysis (Supplementary data 1–2).

Second, the specificity of the developed 2-plex ddPCR method was experimentally assessed using animal (human) and microbial (*Bacillus subtilis* and *Aspergillus niger*) materials as well as several plant materials including different transgenic and common wild-type crop species (*Beta vulgaris, Brassica napus, Glycine max, Gossypium hirsutum, Oryza sativa, Solanum tuberosum, Zea mays*). The transgenic crops comprise at least all events currently authorized on the European market as well as some unauthorized ones belonging to (*Brassica napus, Glycine max, Gossypium hirsutum, Oryza sativa, Solanum tuberosum* and *Zea mays* species (Table 2, Supplementary data 3). As expected, among all tested materials, positive signals were only observed for the rice materials. More precisely, a positive signal for the mutant rice probe was only generated with the gene-edited rice line while a positive signal for the wild-type rice probe was only generated for all other tested rice materials, transgenic or not. In addition, the sequences of the generated PCR amplicons from the gene-edited rice line and its parental line materials presented a coverage and identity of 100% to their reference sequences (Supplementary data 4). Moreover, the observed percentages of the targeted rice materials were similar to the expected ones, except with the two rice materials belonging to the Japonica cv. Ariete (Table 2).

Based on these results, no false positive and false negative were observed. The specificity of the developed ddPCR method was thus confirmed and complied with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL, 2015). This 2-plex ddPCR method was also able to distinguish the presence and absence of a single nucleotide insertion in the *OsMADS26* gene, which is not trivial (Belousov et al., 2004; ENGL, 2020a).

3.3. *ddPCR sensitivity*

Using different estimated haploid genome copy number of either the gene-edited rice line or its parental line (100, 50, 20, 10, 5, 1, 0.1 and 0), the sensitivity of the developed 2-plex ddPCR method was investigated (Table 3, Supplementary data 5). For all 12 replicates, a positive signal as low as 5 estimated haploid genome copies was observed for the mutant rice probe while a positive signal as low as 10 estimated haploid genome copies was observed for the wilt-type rice probe. For both probes of the developed 2-plex ddPCR method, a positive signal for few replicates was also observed as low as 1 estimated haploid genome copy. As expected, no positive signal was also observed at 0.1 and 0 estimated haploid genome copy. In addition, the $LOD_{95\%}$ was determined at 4 estimated haploid genome copies for both probes of the developed 2-plex ddPCR method. As such $LOD_{95\%}$ were below 25 copies, the sensitivity of the developed ddPCR method was confirmed and complied with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL, 2015) (Supplementary data 6).

3.4. *ddPCR applicability*

The applicability of the developed 2-plex ddPCR method was assessed using several mixture samples. On the one hand, the mixture samples n°1–11 contained different percentages of the gene-edited rice line and its parental line. These mixture samples were prepared to mimic a large spectrum of possible contamination levels of a gene-edited plant in its wild-type, and vice-versa (Table 4, Supplementary data 7A). On the other hand, the mixture samples n°12–13 were prepared to mimic either a maize or a soybean matrix contaminated with a low amount of both the gene-edited rice and its parental line (Table 4, Supplementary data 7B).

For all these mixtures, the presence, both at high and low percentages, of the gene-edited rice line and its parental line was detected by the developed 2-plex ddPCR method. For example, this ddPCR method was able to detect a low amount of the gene-edited rice line (i.e., ~14 estimated haploid genome copies) spiked in a high amount of its parental line (i.e., ~14,000 estimated haploid genome copies), and vice-versa. Moreover, the presence of the gene-edited rice line and its parental line, both at low percentage (~14 estimated haploid genome copies), spiked in a high amount of untargeted materials (i.e., 14,000 estimated haploid genome copies of maize or soybean) was also successfully detected. Based on these results, the specificity and sensitivity of the developed 2-plex ddPCR method were confirmed. In addition, in all the tested mixtures, the expected and observed percentages of targeted materials were similar, suggesting that the developed 2-plex ddPCR method was not affected by competitive effects from PCR modules as well as by the presence of untargeted materials (Table 4, Supplementary data 8).

4. Conclusion

In the present study, the possibility to develop and validate a PCR-based method, such as real-time PCR and ddPCR, allowing to specifically detect and quantify a gene-edited organism carrying a known and characterized single variation point was investigated. Therefore, a general workflow, composed of two main successive steps, was proposed (Fig. 2) and its use was successfully illustrated through the development and validation of a 2-plex ddPCR method targeting unambiguously a gene-edited rice carrying a single nucleotide insertion.

The first step of the proposed workflow includes *in silico* analyses using available prior knowledge associated to the single variation point of interest and its flanking regions. Such analyses allow to determine the possibility to technically design oligonucleotides for the PCR-based method in the region of interest. In addition, based on the natural distribution of the single variation point, such analyses allow to determine the discriminative power of this single variation in order to unambiguously identify the gene-edited organism line from all other lines, cultivars, varieties and species. This is essential to prove the presence of a gene-edited organism in particular. Such key analysis step could be facilitated by a future access to a genome database representative of the diversity, natural or through breeding programs, of all the cultivars and varieties for each species of interest. However, although it is essential for any statement on the frequency of a particular single variation point, such database is at our knowledge not currently available for crops, even for rice being one of the most sequenced crops. This database should include at least all the whole-genome sequences from the authorized gene-edited organisms and their parental lines, both ideally provided by producers. For this reason, it can be difficult to prove the origin, natural or not, of a single variation point (ENGL, 2019; http://iric.irri.org/resources/rice-databases; Ribarits et al., 2021; Rice SNP-Seek Database).

In case such specificity criteria are met, the second step of the proposed workflow is applied. It consists to assess, in agreement with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Laboratories (ENGL, 2015; ENGL, 2021), the performance of the developed PCR-based method, including the specificity (inclusivity and exclusivity), sensitivity and applicability. If the gene-edited organism is commercialized on the EU market and therefore needs to be control by enforcement laboratories, a full validation of the PCR-based method is required. To this end, additional analyses need to be performed in order to assess additional

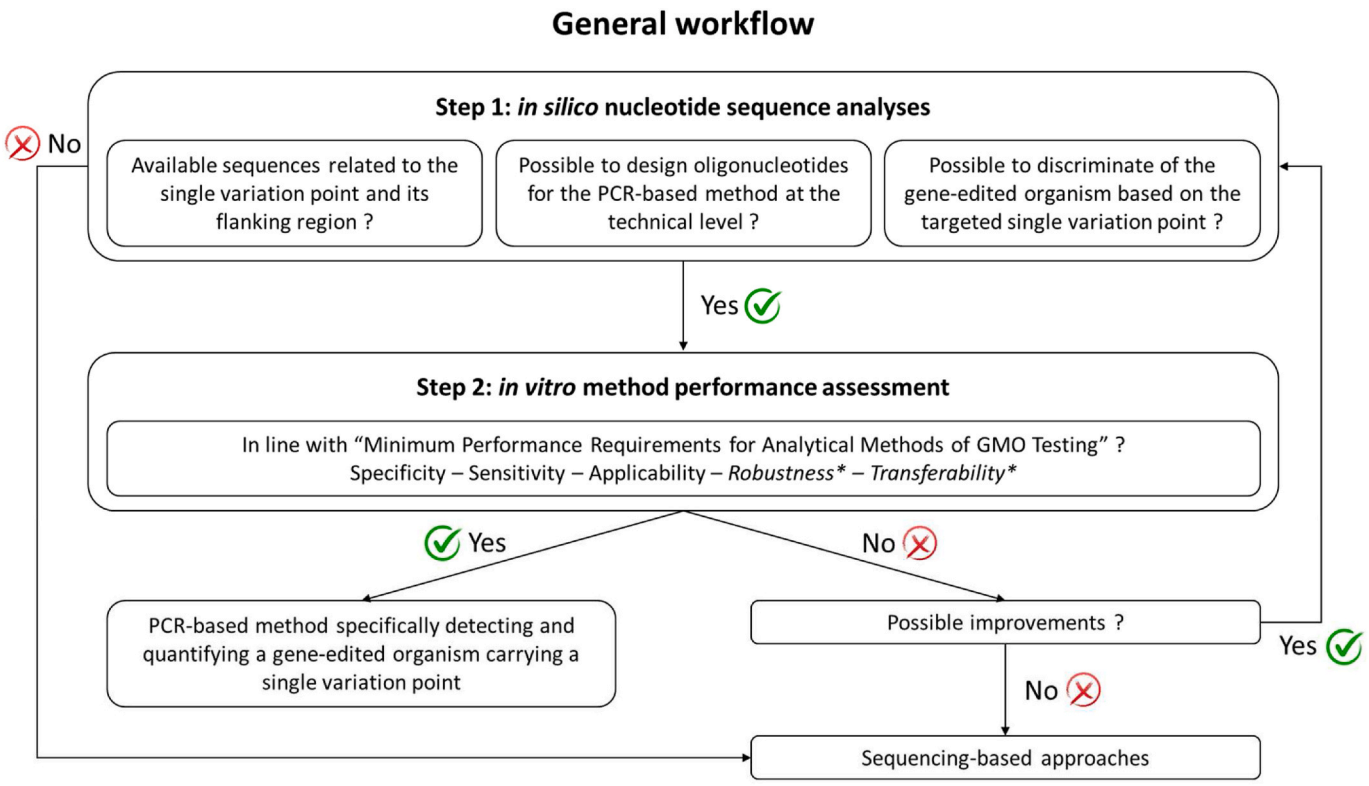

Fig. 2. Schematic representation of the general workflow proposed in this study.
* If the gene-edited organism is commercialized on the EU market, a full validation of the PCR-based method is needed, including the evaluation of additional parameters like robustness and transferability.

parameters, like robustness and transferability, of the PCR-based method allowing its dissemination within European enforcement laboratories. Given the gene-edited rice line used in this study is still at the research and development stage, the tested performance parameters (specificity, sensitivity and applicability) were sufficient.

However, using this workflow, the development of such PCR-based methods is a "case-by-case" process that can encountered difficulties. First, technical bottlenecks could be encountered with the design of the oligonucleotides due to the restricted region of interest to target. Such design, including primers to cover around 120 bp of the region of interest as well as probes annealing on the single variation point, is not always possible to achieve, especially considering the "Minimum Performance Requirements for Analytical Methods of GMO Testing methods" of the European Network of GMO Laboratories (ENGL, 2015). The design of probes able to unequivocally identify and discriminate a single nucleotide variation, which can be challenging, is also crucial in the proposed workflow. Second, the detection of a single variation point could be insufficient to unambiguously identify the presence of a specific gene-edited organism as well as to determine the origin of the variation (e.g., natural process or induced by gene-editing). Indeed, if the single variation point is naturally present, its detection by PCR-based method alone is not sufficient to prove the presence of the gene-edited organism. To overcome such issue, additional information on the surrounding sequences, genome background and off-targeting sites could for example be necessary in order to unambiguously prove the presence of the gene-edited organism. However, these information cannot be nowadays provided using such PCR-based strategy, amplifying only short fragments of few hundred base-pairs. Therefore, instead of PCR-based methods, the use of sequencing-based strategies supported by an appropriated database is needed. The comparison of a given sample to reference genome sequences will allow to verify the presence of SNP as well as genetic elements unique in the genetic background of a particular modified organism. Although sequencing-based approaches are promising, their implementation to control the food and feed chain are however currently highly challenging due to their expensiveness and technical bottlenecks related to the plant genome size and complexity as well as the need of specific reference genome sequences. In case of samples containing a single gene-edited plant, a whole-genome sequencing strategy is feasible, even if developments and optimisations are still required for an implementation at the level of enforcement laboratories. For mixtures composed of different gene-edited plants (expected to be frequently encountered), a targeted sequencing strategy or even a shotgun metagenomics strategy are more appropriated. However, the targeted sequencing strategy, including a prior enrichment of the regions of interest, through hybridization or PCR, represents, in a short time-frame, the most realistic option for mixtures. The enriched regions of interest should comprise both the introduced single variation point as well as the specific genetic background of the modified organism. Therefore, in case no information about the introduced single variation point is available, the presence of remaining off-targeting sites and genetic elements unique to a particular modified organism can for example be investigated (Narushima et al. (2021), Belousov et al., 2004; ENGL, 2020a; Fraiture et al., 2020; Grohmann et al., 2019; Narushima et al. (2021); Peng et al., 2020; Wang et al., 2021; Zhang et al., 2021).

Taking into account the above considerations, the proposed general workflow represents a key tool to support the competent authorities in their control of GMO produced by gene-editing in order to guarantee the traceability of the food and feed chain.

CRediT authorship contribution statement

Marie-Alice Fraiture: Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Emmanuel Guiderdoni:** Writing – review & editing. **Anne-Cécile Meunier:** Writing – review & editing. **Nina Papazova:**

Formal analysis, Writing – review & editing. **Nancy H.C. Roosens:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The research that yielded these results was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through GENEDIT (RF 20/6342). The Sanger sequencing was performed at the Transversal activities in Applied Genomics Service at Sciensano. The authors would like to thank Emmanuel Guiderdoni (CIRAD) for his kindness to provide seeds from the gene-edited rice line and its parental line. This material was produced in the frame of the ANR-11-BTBR-0001_GENIUS project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.108904.

References

Arumuganathan, K., & Earle, E. D. (1991). Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter, 9*, 208–218.

Belousov, Y. S., Welch, R. A., Sanders, S., Mills, A., Kulchenko, A., Dempcy, R., Afonina, I. A., Walburger, D. K., Glaser, C. L., Yadavalli, S., Vermeulen, N. M., & Mahoney, W. (2004). Single nucleotide polymorphism genotyping by two colour melting curve analysis using the MGB Eclipse Probe System in challenging sequence environment. *Human Genomics, 1*, 209–217.

Broeders, S., Fraiture, M. A., Vandermassen, E., Delvoye, M., Barbau-Piednoir, E., Lievens, A., & Roosens, N. (2015). New qualitative trait-specific SYBR®Green qPCR methods to expand the panel of GMO screening methods used in the CoSYPS. *European Food Research and Technology, 241*, 275–287.

Chhalliyil, P., Ilves, H., Kazakov, S. A., Howard, S. J., Johnston, B. H., & Fagan, J. A. (2020). Real-time quantitative PCR method specific for detection and quantification of the first commercialized genome-edited plant. *Foods, 9*, 1245.

Corbisier, C., Buttinger, G., Savini, C., Grazia Sacco, M., Gatto, F., & Emons, H. (2022). Expression of GM content in mass fraction from digital PCR data. *Food Control, 133*.

Cottenet, G., Blancpain, C., & Chuah, P. F. (2019). Performance assessment of digital PCR for the quantification of GM-maize and GM-soya events. *Analytical and Bioanalytical Chemistry, 411*, 2461–2469.

Court of Justice, E.. C-528/16-Judgement of 25 July 2018 on New Mutagenesis Techniques http://curia.europa.eu/juris/document/document.jsf?text=&docid=204387&pageIndex=0&doclang=EN&mode=lst&dir=&occ=first&part=1&cid=138460.

Directive/18/EC 2001 of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Official Journal of European Communities, L106*, (2001), 1–38.

Dobnik, D., Spilsberg, B., Košir, A. B., Holst-Jensen, A., & Žel, J. (2015). Multiplex quantification of 12 European Union Authorized genetically modified maize lines with droplet digital polymerase chain reaction. *Analytical Chemistry, 87*(16), 8218–8226.

Dobnik, D., Štebih, D., Blejec, A., Morisset, D., & Žel, J. (2016). Multiplex quantification of four DNA targets in one reaction with Bio-Rad droplet digital PCR system for GMO detection. *Scientific Reports, 6*, Article 35451.

ENGL. (2015). *Definition of minimum performance requirements for analytical methods of GMO testing*. https://gmo-crl.jrc.ec.europa.eu/guidance-documents.

ENGL. (2019). *Detection of food and feed plant products obtained by new mutagenesis techniques*. https://gmo-crl.jrc.ec.europa.eu/doc/JRC116289-GE-report-ENGL.pdf.

ENGL. (2020a). Evaluation of the Scientific Publication "A Real-Time Quantitative PCR Method Specific for Detection and Quantification of the First Commercialized Genome-Edited Plant" P. Chhalliyil et al. *Foods, 9*, 1245. https://gmo-crl.jrc.ec.europa.eu/ENGL/docs/ENGL%20Evaluation%20of%20the%20scientific%20publication%2002-10-2020.pdf.

ENGL. (2020b). *Detection of food and feed plant products obtained by new mutagenesis techniques*, 2020 https://gmo-crl.jrc.ec.europa.eu/doc/JRC116289-GE-report-ENGL.pdf.

ENGL. (2021). *Definition of minimum performance requirements for analytical methods of GMO testing – part* (Vol. 2). submitted for publication.

EULR. (2006). *Sampling and DNA extraction of cotton seeds. Report from the Validation of the "CTAB/Genomic-tip 20" method for DNA extraction from ground cotton seeds*. http://gmo-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf.

Fraiture, M. A., Gobbo, A., Marchesi, U., Verginelli, D., Papazova, N., & Roosens, N. (2021). Development of a real-time PCR marker targeting a new unauthorized genetically modified microorganism producing protease identified by DNA walking. *International Journal of Food Microbiology, 16*, Article 109330, 354.

Fraiture, M. A., Herman, P., Taverniers, I., De Loose, M., Deforce, D., & Roosens, N. H. C. (2014). An innovative and integrated approach based on DNA walking to identify unauthorised GMOs. *Food Chemistry, 147*, 60–69.

Fraiture, M. A., Herman, P., Taverniers, I., De Loose, M., Deforce, D., & Roosens, N. H. C. (2015). Current and new approaches in gmo detection: Challenges and solutions. *BioMed Research International, 2015*, Article 392872.

Fraiture, M. A., Papazova, N., Vanneste, K., De Keersmaecker, S. C. J., & Roosens, N. H. C. (2020). In Malcolm Burns, Lucy Foster, & Michael Walker (Eds.), *Chapter 8: GMO detection and identification using next-generation sequencing. DNA techniques to verify food authenticity* (pp. 96–106). RSC book.

Gerdes, L., Iwobi, A., Busch, U., & Pecoraro, S. (2016). Optimization of digital droplet polymerase chain reaction for quantification of genetically modified organisms. *Biomolecular Detection Quantification, 7*, 9–20.

Grelewska-Nowotko, K., Żurawska-Zajfert, M., Żmijewska, E., & Sowa, S. (2018). Optimization and verification of droplet digital PCR even-specific methods for the quantification of GM maize DAS1507 and NK603. *Applied Biochemistry and Biotechnology, 185*, 207–220.

Grohmann, L., Broll, H., Dagand, E., Hildebrandt, S., Hübert, P., Kiesecker, H., Lieske, K., Mäde, D., Mankertz, J., Reiting, R., Schulze, M., Speck, B., Uhlig, S., Wahler, D., Waiblinger, H. U., Woll, K., & Zur, K. (2016). Guidelines for the validation of qualitative real-time PCR methods by means of a collaborative study. *Techical Report, BVL, 1*, 1–17.

Grohmann, L., Keilwagen, J., Duensing, N., Dagand, E., Hartung, F., Wilhelm, R., Bendiek, J., & Sprink, T. (2019). Detection and identification of genome editing in plants: Challenges and opportunities. *Frontiers of Plant Science, 10*, 236.

International Standard ISO 20395. (2019). *Biotechnology - requirements for evaluating the performance of quantification methods for nucleic acid target sequences - qPCR and dPCR. Genève, Switzerland*.

International Standard ISO 21571. (2005). *Foodstuffs - methods of analysis for the detection of genetically modified organisms and derived products - nucleic acid extraction. Genève, Switzerland*.

International Standard ISO/DIS 16140-2. (2016). *Microbiology of food and animal feed - method validation - Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*. Genève, Switzerland: ISAAA. https://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=19024.

Iwobi, A., Gerdes, L., Busch, U., & Pecoraro, S. (2016). Droplet digital PCR for routine analysis of genetically modified foods (GMO) – a comparison with real-time quantitative PCR. *Food Control, 69*, 205–213.

Khong, N. G., Pati, P. K., Richaud, F., Parizot, B., Bidzinski, P., Mai, C. D., Bes, M., Bourrié, I., Meynard, D., Beeckman, T., Selvaraj, M. G., Manabu, I., Genga, A. M., Brugidou, C., Do, V. N., Guiderdoni, E., Morel, J. B., & Gantet, P. (2015). OsMADS26 negatively regulates resistance to pathogens and drought tolerance in rice. *Plant Physiology, 169*(4), 2935–2949.

Kosir, A. B., Demsar, T., Stebih, D., Zel, J., & Milavec, M. (2019). Digital PCR as an effective tool for GMO quantification in complex matrices. *Food Chemistry, 294*, 73–78.

Košir, A. B., Spilsberg, B., Holst-Jensen, A., Žel, J., & Dobnik, D. (2017). Development and inter-laboratory assessment of droplet digital PCR assays for multiplex quantification of 15 genetically modified soybean lines. *Scientific Reports, 7*(1), 8601.

Li, J., Li, L., Zhang, L., Zhang, X., Li, X., Zhai, S., & Wu, Y. (2020). Development of a certified genomic DNA reference material for detection and quantification of genetically modified rice KMD. *Analytical and Bioanalytical Chemistry, 412*, 7007–7016.

Li, J., Zhang, L., Li, L., Li, X., Zhang, X., Zhai, S., & Wu, Y. (2020). Development of genomic DNA certified reference materials for genetically modified rice Kefeng 6. *ACS Omega, 5*, 21602–21609.

Mazzara, M., Cordeil, S., & Van Den Eede, G. (2006). *Report on the verification of an event-specific detection method for identification of rice GM-event LLRICE601 using a real-time PCR assay*.

Menz, J., Modrzejewski, D., Hartung, F., Wilhelm, R., & Sprink, T. (2020). Genome edited crops touch the market: A view on the global development and regulatory environment. *Frontiers of Plant Science, 11*, 586027.

Morisset, D., Štebih, D., Milavec, M., Gruden, K., & Žel, J. (2013). Quantitative analysis of food and feed samples with droplet digital PCR. *PLoS One, 8*(5), Article e62583.

Narushima, J., Kimata, S., Shiwa, Y., Gondo, T., Akimoto, S., Soga, K., Yoshiba, S., Nakamura, K., Shibata, N., & Kondo, K. (2021). Strategy for detecting off-target sites in genome-edited rice. *bioRxiv, 2021*, 05.28.446070.

Pecoraro, S., Berben, G., Burns, M., Corbisier, P., De Giacomo, M., De Loose, M., Dagand, E., Dobnik, D., Eriksson, R., Holst-Jensen, A., Kagkli, D. M., Kreysa, J., Lievens, A., Mäde, D., Mazzara, M., Paternò, A., Peterseil, V., Savini, C., Sovová, T., Sowa, S., & Spilsberg, B. (2019). *Overview and recommendations for the application of digital PCR. EUR 29673 EN*, 2019, ISBN 978-92-76-00180-5. Luxembourg: Publications Office of the European Union. https://doi.org/10.2760/192883. JRC 115736.

Peng, C., Wang, H., Xu, X., et al. (2018). High throughput detection and screening of plants modified by gene editing using quantitative real-time polymerase chain reaction. *The Plant Journal, 95*, 557–567.

Peng, C., Zheng, M., Ding, L., Chen, X., Wang, X., Feng, X., Wang, J., & Xu, J. (2020). Accurate detection and evaluation of the gene-editing frequency in plants using droplet digital PCR. *Frontiers of Plant Science, 11*, 610790.

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. O. J. E. U., L268, 1-23.

Ribarits, A., Eckerstorfer, M., Simon, S., & Stepanek, W. (2021). Genome-edited plants: Opportunities and challenges for an anticipatory detection and identification framework. *Foods, 10*, 430.

Stevanato, P., & Biscarini, F. (2016). Digital PCR as new approach to SNP genotyping in sugar beet. *Sugar Technology, 18*(4), 429–432.

Uhlig, S., Frost, K., Colson, B., Simon, K., Made, D., Reiting, R., Gowig, P., & Grohmann, L. (2015). Validation of qualitative PCR methods on the basis of mathematical–statistical modelling of the probability of detection. *Accreditation and Quality Assurance, 20*, 75–83.

Wang, W., Mauleon, R., Hu, Z., et al. (2018). Genomic variation in 3,010 diverse accessions of Asian cultivated rice. *Nature, 557*, 43–49.

Wang, X., Tu, M., Wang, Y., et al. (2021). Whole-genome sequencing reveals rare off-target mutations in CRISPR/Cas9-edited grapevine. *Horticultural Research, 8*, 114.

Wan, J., Song, L., Wu, Y., et al. (2016). Application of digital PCR in the analysis of transgenic soybean plants. *Advances in Bioscience and Biotechnology, 7*(10), 403–417.

Whale, A. S., Huggett, J. F., & Tzonev, S. (2016). Fundamentals of multiplexing with digital PCR. *Biomolecular Detection Quantification, 10*, 15–23.

Zhang, H., Li, J., Zhao, S., Yan, X., Si, N., Gao, H., Li, Y., Zhai, S., Xiao, F., Wu, G., & Wu, Y. (2021). In , *10. An editing-site-specific PCR method for detection and quantification of CAO1* (p. 1209). Rice. Foods.