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RESEARCH ARTICLE

Development of a rapid detection method for genetically modified rice using the ultra-fast PCR system

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Abstract Genetically modified (GM) rice varieties containing traits such as tolerance to abiotic stress and resistance against pests and diseases continue to be developed. However, contamination incidents from unauthorized GM rice varieties have been encountered. To date, no GM rice crop has been authorized for consumption and/or commercialization in Korea. Therefore, to enhance safety management of unauthorized genetically modified organisms (GMOs), accurate and reliable detection methods are needed to identify GMOs in crops or products. In this study, we developed rapid detection methods for GM rice events (Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62) using ultra-fast PCR system. Ultra-fast PCR is a state-of-the-art technology and decreases PCR run-times dramatically. However, the ultra-fast PCR is not widely used in GMO analysis. Thus, we designed a detection method for five events of GM rice and confirmed them by performing specificity, sensitivity, and applicability assays. All results demonstrate that the ultra-fast PCR system is a specific, sensitive, and reliable method to identify and monitor GM rice events. Additionally, it can be utilized as a rapid and simple method for GMO analysis in crops or processed products. This study can be used as a reference for future research on new analysis methods of unauthorized GMOs.

Keywords *Oryza sativa* · Plasmid reference · Event specific qualification · Unauthorized GM rice

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Introduction

Rice is one of the major crops cultivated in the world and is a principal food consumed by the global population. China is the largest rice producer and consumer in the world and devotes approximately 20% of its cultivated area to rice production (Chen et al., 2011). In Korea, rice production reached approximately 3.74 million tons in 2019, according to Statistics Korea.

Genetically modified (GM) rice was first developed in 1988 through electroporation or polyethylene glycol-mediated protoplast transformation methods (Fraiture et al., 2016). Since 1988, various traits such as tolerance to abiotic stresses, resistance against pests and diseases, and increased nutritional values have been introduced into rice (Bajaj and Mohanty, 2005). In 2020, according to the GM Approval Database (www.isaaa.org/gmapprovaldatabase/default.asp) of the International Service for the Acquisition of Agri-biotech Applications (ISAAA), seven GM rice events have been developed, namely GM shanyou63, GR2E, Huahui-1/TT51-1, LLRice06, LLRice601, LLRice62, and Tarom molaii + cry1Ab.

The transgenic rice lines Bt63 (TT51-1), Kemingdao1 (KMD1), Kefeng6, and Kefeng8 are widely known events developed in China. The Bt63 includes a hybrid *Cry1Ab/Ac* gene (an insect-resistant trait) that was granted the safety certificate by China in 2009. The KMD1 rice line is

resistant to lepidopteran pest species (a synthetic *cry1Ab* gene) and has the potential to be approved in China. The Kefeng6 and Kefeng8 contain two insect-resistant genes, *cry1Ac* and cowpea trypsin inhibitor (*CpTI*) gene (Lu et al., 2016; Wang et al., 2012). The LLRice62 contains glufosinate herbicide-tolerant *bar* gene and phosphinothricin-*N*-acetyltransferase (PAT) proteins (Oberdoerfer et al., 2005).

Unauthorized GM rice varieties have been found in many countries. The number of GM rice contamination incidents were the highest among all other crops (Cotter and Price, 2014). Illegal cultivation of unapproved GM rice in China was reported in 2005 (Zi, 2005). In 2006, Bayer's LLRice601 and LLRice62 have been discovered across the world (Greenpeace International, 2006). Bt63 was also detected in Europe and identified in imported food products according to the Rapid Alert System for Food and Feed (RASFF) (Lu et al., 2016; Price and Cotter, 2014). For these reasons, accurate and reliable detection methods are needed for the identification and analysis of unauthorized GMOs crops or products.

Numerous studies have been conducted to identify detection methods for GM rice. Detection methods for GM Bt63 have been developed using real-time PCR (Grohmann and Mäde, 2009; Wu et al., 2010) and droplet digital PCR (Wang et al., 2019). Furthermore, the qualitative and quantitative assays for KMD1, Kefeng6, LLRice62 and LLRice601 analysis have been previously established (Babekova et al., 2009; Guertler et al., 2012; Wang et al., 2011; Watanabe et al., 2007). The aim of most studies investigating screening assays for GM rice explored simple and cost-effective methods to identify GMOs (Safaei et al., 2019; Zang et al., 2015). Recently, the detection methods for other various GM rice such as M12 and G6H1 have been developed (Deng et al., 2020; Xu et al., 2019). Conventional PCR and real-time PCR are the classical and most widely accepted GMO detection methods.

Time and cost are important factors in the analysis. The use of the ultra-fast PCR system can decrease the PCR run time dramatically. Also, the amount of reagent used for analysis can be reduced. For this reason, the ultra-fast PCR system has recently been studied and utilized in various fields. The recent examples of the ultra-fast PCR system are COVID-19 causative virus detection, tuberculosis diagnosis, and the authenticity determination of food raw materials.

While the ultra-fast PCR system based on Rapi:chip^{TM} is a state-of-the-art technology, not enough research has been conducted using this method in order to establish assay for GMO detection. Therefore, this study aimed to develop a simple, rapid, and effective detection method for five GM rice events (Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62).

Materials and methods

Standard plasmid and reference materials

The standard plasmid (pUC-RICE5) used in this study was developed through research conducted by the Ministry of Food and Drug Safety (MFDS) in Korea. Owing to a limited access to unauthorized GM rice Certified Reference Materials (CRMs), plasmid DNA was used for positive reference of PCR assays. The plasmid that was cloned into a pUC19 vector with ampicillin resistance contained the endogenous sucrose phosphate synthase (*SPS)* gene and five GM rice events (Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62) sequences (Fig. 1).

Reference materials of GM soybean (RRS, MON89788, A2704-12, DP-356043-5, DP-305423-1, A5547-127, MON87701, CV127, MON87769, MON87705, MON87708, FG72, SYHT0H2, DAS-44406-6, DAS-68416–4, DAS-81419-2, and MON87751) and maize (MON810, GA21, TC1507, NK603, MON863, Bt11, T25, Bt176, DLL25, DAS-59122-7, MON88017, MIR604, Bt10, MON89034, MIR162, DP-098140–6, 3272, MON87460, 5307, MON87427, DAS-40278-9, DP-004114-3, MON87411, VCO-01981-5, MON87403, and MON87419) were kindly provided by the developers (Table 1). Non-GM (NGM) seeds of soybean, maize, canola, rice, wheat, potato, and barley were also used in this study.

DNA extraction

NGM crops, GM soybean, and maize seeds and processed products were separately milled with a grinder. GM soybean mix (17 events) and maize mix (26 events) samples were produced by mixing 0.1 g of ground powder from each event. Genomic DNA was extracted from the powder samples using a PowerPrep^{TM} DNA Extraction from Food and Feed kit (Kogenebiotech, Korea) according to the manufacturer's instructions.

DNA concentrations were determined by QUBIT^{TM} 3.0 Quantitation Kit (Thermo Fisher Scientific, Wilmington, DE, USA). DNA samples were diluted in nuclease-free water to a final concentration of 10 ng/μL and stored at −20 °C.

Primers

The event-specific primers of the five GM rice events were used in the ultra-fast PCR assays. The *SPS* gene was used as the endogenous reference gene. OsSPS-F/R primer sets were previously shown to detect the *SPS* gene (Kim et al., 2010). The Bt63-KF/KR, KMD1-KFr/KRr, Kef6-KF/KR,

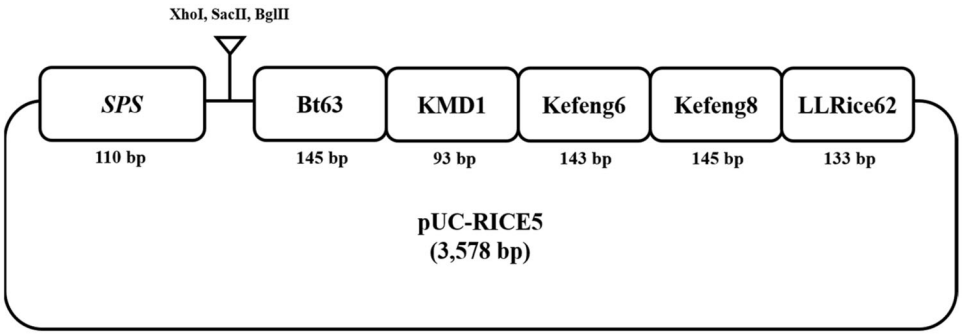

Fig. 1 Schematic diagram of pUC-RICE5 plasmid which contained the endogenous sucrose phosphate synthase (*SPS)* gene and five GM rice events (Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62) sequences with restriction site of XhoI, SacII and BglII were cloned into a pUC57 vector with the ampicillin resistance

Table 1 Reference materials used in this study

Crop	Event Name	Developer	Crop	Event Name	Developer
Soybean	RRS	Monsanto	Maize	Bt11	Syngenta
Soybean	MON89788	Monsanto	Maize	T25	Bayer CropScience
Soybean	A2704-12	Bayer CropScience	Maize	Bt176	Syngenta
Soybean	DP-356043-5	Pioneer	Maize	DLL25	Monsanto
Soybean	DP-305423-1	Pioneer	Maize	DAS-59122-7	Dow AgroScience
Soybean	A5547-127	Bayer CropScience	Maize	MON88017	Monsanto
Soybean	MON87701	Monsanto	Maize	MIR604	Syngenta
Soybean	CV127	BASF plant Science	Maize	Bt10	Syngenta
Soybean	MON87769	Monsanto	Maize	MON89034	Monsanto
Soybean	MON87705	Monsanto	Maize	MIR162	Syngenta
Soybean	MON87708	Monsanto	Maize	DP-098140-6	Pioneer
Soybean	FG72	Bayer CropScience	Maize	3272	Syngenta
Soybean	SYHT0H2	Syngenta	Maize	MON87460	Monsanto
Soybean	DAS-44406-6	Dow AgroScience	Maize	5307	Syngenta
Soybean	DAS-68416-4	Dow AgroScience	Maize	MON87427	Monsanto
Soybean	DAS-81419-2	Dow AgroScience	Maize	DAS-40278-9	Dow AgroScience
Soybean	MON87751	Monsanto	Maize	DP-004114-3	Pioneer
Maize	MON810	Monsanto	Maize	MON87411	Monsanto
Maize	GA21	Syngenta	Maize	VCO-01981-5	Genective
Maize	TC1507	Pioneer	Maize	MON87403	Monsanto
Maize	NK603	Monsanto	Maize	MON87419	Monsanto
Maize	MON863	Monsanto			

Kef8-KF/KR, and LLR62-KF/KR primer pairs were based on the 3′ integration flanking sequence between the T-DNA and the host rice genome of GM rice EU880444 (Bt63), EU980363 (KMD1), HQ161057 (Kefeng6), HQ161059 (Kefeng8), and JQ406881 (LLRice62), respectively (Kim et al., 2017). The primers were synthesized by Bioneer (Daejeon, Korea) and the oligonucleotide sequences have been listed in Table 2.

Ultra-fast PCR system

The ultra-fast PCR analyzes on the same principle as SYBR green based real-time PCR, except that Evagreen dye is used instead of SYBR green as an intercalating dye.

The PCR analysis was performed on the GENE-CHECKER UF-150 Ultra-fast Real-Time qPCR system (Genesystem, Daejeon, Korea) with Rapi:chip™. For the final reaction volume 10 µL, 5 µL of SSO Fast™ Eva-Green Supermixes (Bio-Rad Laboratories, Berkeley, CA, USA), 1 µL of each forward and reverse primer (0.8 µM), and 2 µL of the DNA template were used. All ultra-fast PCR assays were performed under the following conditions: 1 cycle of initial denaturation at 95 °C for 1 min; 40 cycles of denaturation at 95 °C for 5 s; annealing at 58 °C for 5 s; extension at 72 °C for 5 s; 1 cycle of elongation at 72 °C for 5 s. No template control (NTC) was used as the negative control in the ultra-fast PCR reaction.

Table 2 Oligonucleotide primers used in this study

Target	Name	Sequence (5′ → 3′)	Length (bp)	GeneBank accession no.
SPS	OsSPS-F	GAT CGG TTC CGC CAT TAG CA	110	U33175
	OsSPS-R	AAC CGA GCG CGA TCA CTT GC		
Bt63	Bt63-KF	CGC GCC ACA TAG CAG AAC TT	145	EU880444
	Bt63-KR	CAC TCG TCC GGG ATC CTC TA		
KMD1	KMD1-KFr	CAT TAA AAA CGT CCG CAA TGT G	93	EU980363
	KMD1-KRr	TAC GCC GAT ATG CCT GCC CA		
Kefeng6	Kef6-KF	GAG GCC ATG ATC TGG TGT CC	143	HQ161057
	Kef6-KR	TTG CGG TGG AAC ACC GAA GC		
Kefeng8	Kef8-KF	GCC GGT CTT GCG ATG ATT AT	145	HQ161059
	Kef8-KR	GAC CAT GAT GCT GTT CTG CC		
LLRice62	LLR62-KF	AAC ACG CAC ACT CAC CTA CT	133	JQ406881
	LLR62-KR	CAG CTG GCG TAA TAG CGA AG		

Specificity, sensitivity, and application test

The specificity of the designed detection method was evaluated using various crops and GM events. A sensitivity test was conducted to identify detectable concentration for each target event. We compared the sensitivity results of genomic DNA and plasmid using endogenous gene (*SPS*) primers. Additionally, the designed method was used in commercial rice containing foods to investigate its applicability in processed products.

To confirm the specificity and sensitivity of the ultra-fast PCR detection method for five GM rice events, plasmid DNA samples were prepared. The copy number of the pUC-RICE5 plasmid was calculated using Formula (1) mentioned below:

$$m = [n]\left[1.096 \times 10^{-21} g/copy\right] \quad (1)$$

where m is mass and n is the recombinant plasmid or genome size (bp).

For the sensitivity analysis, plasmid DNA was serially diluted 10 times in TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) to four concentrations ranging from 2×10^4 to 2×10^1 copies per reaction. The genomic DNA concentrations of NGM rice was ranged from 2 to 0.002 ng per reaction and the dilution was performed in nuclease-free water.

Results and discussion

Specificity

To evaluate the specificity of the ultra-fast PCR detection method, the assays involving all primers were performed using genomic DNA templates of NGM crops (seven crops; soybean, maize, canola, rice, wheat, potato, and barley), GM soybean (mix of seventeen events), and GM maize (mix of twenty-six events). The results showed a single peak detected from each target GM rice event, and there were no specific amplifications for non-target crops, events, and NTC (Fig. 2). Additionally, the melting temperature (Tm) of the amplification products obtained from each target was 85.60 °C, 78.47 °C, 83.01 °C, 76.85 °C, and 80.09 °C for Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62, respectively (Table 2). Five GM rice events can be selectively distinguished from each other by Tm (°C) values since the amplicons have specific Tm values.

Consequently, these results showed that the detection methods established in this study had high specificity for the target primer sets. This method can be utilized to identify and monitor GM rice events, and rapid analysis results can be obtained in approximately 20 min.

Sensitivity

Unauthorized GMOs encounter zero-tolerance or low-percentage tolerance policies in many countries (Fraiture et al., 2017). Therefore, the development of a sensitive detection method is necessary for the identification of the presence of unapproved GMOs. To assess the sensitivity of the ultra-fast PCR method, pUC-RICE5 plasmid was serially diluted 10 times to concentrations ranging from 20,000 to 20 copies per reaction. The Ct and Tm values of the amplification results for each event were analyzed.

For all GM rice events, primer sets for 20 copies of target DNA were sufficient for their identification. Based on the copy number of rice (One copy number for haploid genome of rice (*Oryza sativa*) is considered 0.47 pg

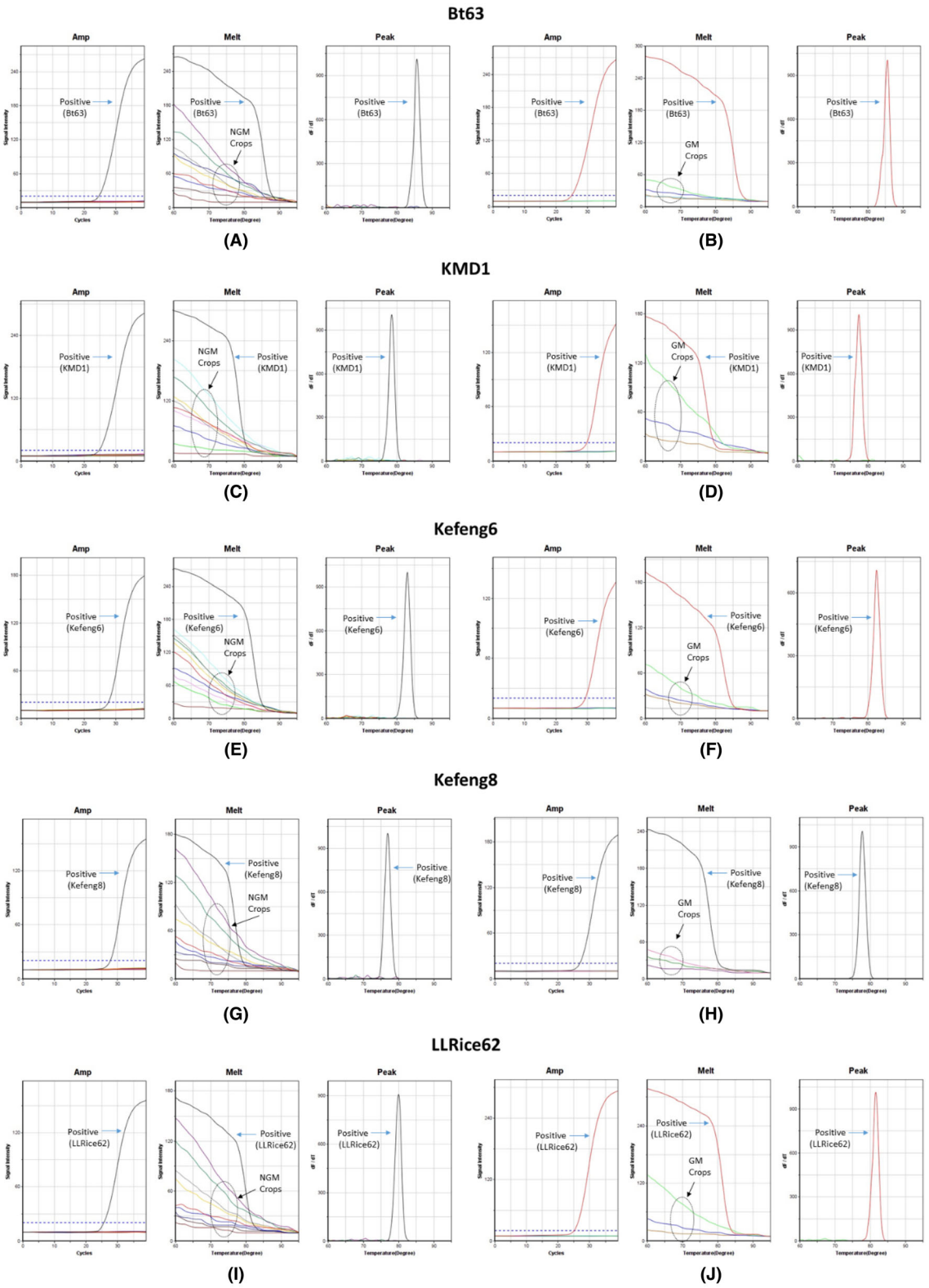

Fig. 2 Primer specificity for five GM rice events using Ultra-fast PCR. (**A, C, E, G, I**) Specificity results for Non-GM crops (seven crops; soybean, maize, canola, rice, wheat, potato, and barley) DNA; (**B, D, F, H, J**) Specificity results for GM soybean (mix of seventeen events) and GM maize (mix of twenty-six events) DNA

(Arumuganathan and Earle, 1991)), 20 copies can be calculated to approximately 0.009 ng.

To test the sensitivity for genomic DNA, extracted from NGM rice was used due to the limited access to unapproved GM rice varieties. The PCR assays were performed using *SPS* primer pairs and the results were compared to the results obtained from plasmid DNA. The results indicated that genomic and plasmid DNA were detected in 0.002 ng and 20 copies, respectively (Table 3).

These results demonstrated that rapid detection method used in this study was highly sensitive and accurate. Furthermore, we confirmed that these assays were applicable for genomic DNA templates and compared its sensitivity to the sensitivity for plasmid DNA. While the Tm value of Bt63 (85.68 °C) was similar to the Tm value obtained for the *SPS* primer set, the specificity results showed no NGM rice detection for these primer sets.

Table 3 Sensitivity results of the Ultra-fast PCR method

Events	Conc.	Ct value		Tm (°C) value	
		Mean	SD	Mean	SD
SPS	20,000 copies	25.12	0.44	85.08	0.24
	2000 copies	28.43	0.01		
	200 copies	32.38	1.29		
	20 copies	34.82	0.70		
Bt63	20,000 copies	23.98	0.62	85.68	0.41
	2,000 copies	27.84	0.62		
	200 copies	31.29	0.03		
	20 copies	35.23	0.04		
KMD1	20,000 copies	25.10	0.02	78.03	0.34
	2000 copies	29.58	0.68		
	200 copies	32.59	0.69		
	20 copies	36.69	0.84		
Kefeng6	20,000 copies	26.75	0.54	83.37	0.32
	2000 copies	32.15	1.41		
	200 copies	33.70	0.64		
	20 copies	36.25	1.44		
Kefeng8	20,000 copies	25.73	0.64	77.70	0.38
	2000 copies	29.66	0.63		
	200 copies	33.15	0.01		
	20 copies	35.69	2.09		
LLRice62	20,000 copies	26.41	0.01	80.05	0.36
	2000 copies	29.40	0.01		
	200 copies	33.68	0.64		
	20 copies	35.73	0.76		
SPS	2 ng	24.97	0.04	85.16	0.24
(gDNA)	0.2 ng	26.53	0.71		
	0.02 ng	31.96	0.01		
	0.002 ng	33.92	0.01		

SD standard deviation

Application of ultra-fast PCR in commercial processed products

To evaluate the application of ultra-fast PCR assays, a total of thirteen commercially available rice processed products were selected. The selected products included noodles, cookies, ready-made meals, and other items. Genomic DNA extracted from processed products was used as DNA template for the ultra-fast PCR analysis. The applicability of this method for commercially available products is shown in Table 4. For all products, the *SPS* primers led to amplification and a Tm value of approximately 84.5 °C was observed. The primer sets for GM Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62 events did not lead to amplicon production in any of the products. We confirmed that the rice from the processed products used in this study did not contain any of the above-mentioned five GM events. One of the main limitations of this study is represented by the lack of available products containing unauthorized GM rice. However, the ultra-fast PCR system used in this study has potential applications in the analysis of processed products based on the results of amplification of endogenous *SPS* gene in rice.

A rapid detection method for five GM rice events using the ultra-fast PCR system with Rapi:chip^{TM} was designed to selectively detect target primer sets that amplified at a specific melting temperature. Furthermore, this method was sensitive enough to detect a low concentration of GM rice DNA. Moreover, the wide applicability of the ultra-fast PCR method was demonstrated by its capacity to detect and monitor rice DNA in processed products. Thus, it can be utilized as a rapid and simple method for GMO analysis as it only used the event-specific primer sets without the need for a specific probe. The ultra-fast PCR assay established in this study is a specific, sensitive, and reliable method for GM rice event identification, which delivers the results within approximately 20 min.

Compared to the existing detection methods, the ultra-fast PCR method is an economical and eco-friendly method that uses a smaller amount of reagents. Also, the inspection takes a very short time, comparatively, it is 18% of the conventional PCR inspection time and 23% of the real-time PCR. There is an advantage in that the test results can be confirmed in a short time due to short duration between cycles and high thermal conductivity of the device. In addition, it is possible to apply the test not only in a laboratory but also in the field, as a portable type small analyzer is able to be supported. In conclusion, we developed a rapid and sensitive PCR method for five non-approved GM rice events, Bt63, KMD1, Kefeng6, Kefeng8, and LLRice62, which are under strict safety management. The ultra-fast PCR system has an economic advantage by using half the reaction reagent compared to the current PCR

Table 4 Ultra-fast PCR results for rice DNA in processed products

Products	Tm (°C) value					
	SPS	Bt63	KMD1	Kefeng6	Kefeng8	LLRice62
PHO BO XUA & NAY BEEF FLAVOUR	+ (84.95)	ND	ND	ND	ND	ND
JIA YUAN SNACK(CUMIN)	+ (84.31)	ND	ND	ND	ND	ND
NON GLUTINOUS BROWN RICE	+ (84.31)	ND	ND	ND	ND	ND
PHO GA XUA & NAY CHICKEN FLAVOUR	+ (84.95)	ND	ND	ND	ND	ND
HU TIEU NAM VANG	+ (84.95)	ND	ND	ND	ND	ND
RICE STICK	+ (83.63)	ND	ND	ND	ND	ND
BANH DA CUA	+ (83.98)	ND	ND	ND	ND	ND
UNRIPED PEPPER PREPARATION	+ (83.98)	ND	ND	ND	ND	ND
HOT KID BABY MUM-MUM RICE RUSK VEGETABLE	+ (84.63)	ND	ND	ND	ND	ND
GLUTINOUS RICE BALLS WITH BLACK SESAME FILLING	+ (84.95)	ND	ND	ND	ND	ND
INJEOLMI COOKIE	+ (84.31)	ND	ND	ND	ND	ND
PAD THAI STIR-FRY NOODLE MEAL	+ (84.63)	ND	ND	ND	ND	ND
KRA YA SART CRISPY RICE COOKIE	+ (84.95)	ND	ND	ND	ND	ND

ND not determined

method, and the reaction time is also very short, by about one fourth to one fifth of the existing methods, and the analysis equipment is portable so that analysis can be done in the field. Therefore, these advantages are expanding the scope of application.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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