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DNA degradation in genetically modified rice with *Cry1Ab* by food processing methods: Implications for the quantification of genetically modified organisms

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ABSTRACT

Food processing methods contribute to DNA degradation, thereby affecting genetically modified organism detection and quantification. This study evaluated the effect of food processing methods on the relative transgenic content of genetically modified rice with *Cry1Ab*. In steamed rice and rice noodles, the levels of *Cry1Ab* were ⩾100% and <83%, respectively. Frying and baking in rice crackers contributed to a reduction in *Pubi* and *Cry1Ab*, while microwaving caused a decrease in *Pubi* and an increase in *Cry1Ab*. The processing methods of sweet rice wine had the most severe degradation effects on *Pubi* and *Cry1Ab*. In steamed rice and rice noodles, *Cry1Ab* was the most stable, followed by *SPS* and *Pubi*. However, in rice crackers and sweet rice wine, *SPS* was the most stable, followed by *Cry1Ab* and *Pubi*. Therefore, *Cry1Ab* is a better representative of transgenic components than is *Pubi* because the levels of *Cry1Ab* were less affected compared to *Pubi*.

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1. Introduction

In recent years, there has been a rapid growth in transgenic crops. According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), the area cultivated with genetically modified (GM) crops in 2013 was 175 million hectares, a 3% increase compared to 2012 and a 103-fold increase compared to 1996. Currently, there are more GM crops being utilized in food and feed production; therefore, transgenic components, such as genes and proteins from GM crops, have been directly or indirectly entering the human food chain. However, there have been public concerns about the safety of GM crops. Even though the potential transfer of exogenous genes to intestinal bacteria or animal cells has never been reported (Kosieradzka, Vasko, Szwacka, Przybysz, & Fiedorowicz, 2010; Lutz, Wiedemann, & Albrecht, 2006; Rizzi et al., 2012; Sieradzki et al., 2013; Świątkiewicz et al., 2010), the consequences of potential gene transfer have been the focus of public concerns (Gasson, 2000; Hertel, Probst, Cavadini, Meding, & Hammes, 1995; Metz & Nap, 1997).

The adequate management of GM crops and their products is of utmost importance in several countries. The labelling of GM foods has been mandated in several nations, including the European Union (EU), Korea, Japan, and Australia. According to the labelling regulations, the threshold level of unintended transgenic components in food products is 0.9–5% (Commission Regulation EC No. 1829/2003; Japan Notification No. 1775; Korea Notification No. 2000-31). As a result, several studies have focussed on the detection of transgenic components in food products. It has been reported that the detection limits of Roundup-Ready soybean in meat products and tofu are 1% (Meyer, Chardonnens, Hübner, & Lüthy, 1996) and 0.5% (Matsuoka et al., 1999), respectively. However, food processing methods, which involve physical treatments (e.g. mechanical treatment and high pressure), chemical changes (e.g. acid and alkali), and biological reactions (e.g. fermentation processes), have different effects on the degradation of endogenous and exogenous genes (Ballari & Martin, 2013; Chen, Ge, & Wang, 2007; Chen, Wang, Ge, & Xu, 2005; Chen, Wang, Xu, & Ge, 2005; Fernandes, Oliveira, & Mafra, 2013; Guan, Wang, Teng, Yang, & Wang, 2013; Zhang, Xing, Selvaraj, & Liu, 2014b). Therefore, the efficiency of GMO quantification will be affected by the relative degradation degrees of exogenous genes to endogenous gene.

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Conventional PCR is widely used to study the fate of genes during food processing (Murray, Butler, Hardacre, & Timmerman-Vaughan, 2007). Song et al. (2011) used conventional PCR to study the effects of different food processing methods on the degradation of endogenous and exogenous genes (*CAMV35S*, *NOS* and *Bar*) present in rice crackers and sweet rice wine made with *Bar*-transgenic rice. During the four-day fermentation of sweet rice wine, *Bar* gene was highly stable, followed by *Nos* terminator, *CAMV35S* promoter, and *SPS* gene. For rice crackers, frying was the most severe procedure, followed by baking, microwaving, drying and boiling. *CAMV35S* promoter was highly stable, followed by *SPS* gene, *NOS* terminator, and the *Bar* gene. In our previous study, we used conventional PCR to assess the effects of food processing methods on the degradation of endogenous and exogenous genes (*Pubi*, *Cry1Ab*, *NOS* and *Hpt*) in steamed rice, rice noodles, rice crackers and sweet rice wine and found that the endogenous and exogenous genes of GM rice with *Cry1Ab* were degraded (Zhang et al., 2014b). For steamed rice and rice noodles, the procedures were so mild that only the genes with $\geqslant$1500 bp were degraded. Frying is the most tough process in processing of rice crackers, followed by microwaving, baking and boiling. For sweet rice wine, fermentation had more impact on degradation of genes than had other processing procedures. Fernandes et al. (2013), who assessed the effects of fermentation and baking during bread making, reported that DNA from GM maize was degraded but $\leqslant$200 bp DNA fragments were still detected. Additionally, in all these studies, DNA fragments with <200 bp were stable and did not degrade, indicating that these fragments can be used to detect the presence of GMOs in foods. The above studies used conventional PCR to identify processing steps that result in the complete loss of target molecules but do not quantify DNA degradation. Furthermore, there is less evidence about the effects of food processing methods on the levels of exogenous genes. Real-time PCR is the preferred method for the quantification of GMOs and implementation of labelling regulations.

Real-time PCR was used to study the levels of GMOs in 2.5–5% GM soybeans and 25% GM cottonseed after different food processing conditions (Chen et al., 2007; Guan et al., 2013). Ballari and Martin (2013) evaluated the effect of various processing treatments, such as heating, baking, microwaving, autoclaving and ultraviolet (UV) irradiation, on the relative transgenic content of MON 810 maize, using pRSETMON-02, a dual target plasmid, as a model system. Autoclaving and UV irradiation resulted in the least recovery of the transgenic (*CAMV35S* promoter) and taxon-specific (*zein*) target DNA sequences. Although the treatments had a significant effect on DNA degradation, DNA can still be quantified by real-time PCR and food processing did not alter the relative quantification of the transgenic content. However, few studies have focussed on the degradation of exogenous genes in GM rice with *Cry1Ab* by food processing methods, using real-time PCR, even though two transgenic *Bt* rice lines were approved for field trials in 2009 and may become the first commercial transgenic rice variety cultivated at large in China. Steamed rice is a stable Chinese food that is consumed by >60% of the population and that contributes 40% of their total caloric intake (Cao et al., 2010). Rice noodles and rice crackers are popular snack foods, and sweet rice wine is a traditional fermented beverage in China. In this study, 100% GM rice with *Cry1Ab* was used to study the effects of food processing methods on the relative levels of the exogenous genes (*Pubi* promoter, *Cry1Ab*, *NOS* terminator, *Hpt*) to endogenous gene in steamed rice, rice noodles, rice crackers and sweet rice wine, using real-time PCR. *Cry1Ab* is the target gene in GM rice, the expression is controlled by the maize-specific ubiquitin promoter (*Pubi*) and *Agrobacterium tumefaciens* nopaline synthase (*NOS*) terminator that exists in GM rice. Hygromycin phosphotransferase (*Hpt*) gene was used as the selected marker gene.

2. Materials and methods

2.1. Materials

GM rice (*Oryza sativa* L.) with *Cry1Ab* and non-transgenic rice (cv. Minghui 86) were obtained from Fujian Academy of Agricultural Sciences (Fuzhou, China). Sucrose, potato starch, salt, and sweet rice wine starter (containing *Rhizopus* for fermentation) were purchased from a local market (Beijing, China). Real-time PCR was performed on all samples to ensure that they did not contain any transgenic components.

2.2. Reagents

SYBR® Green Real-time PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for real-time fluorescence PCR reactions. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and were diluted to 10 μM with distilled water.

2.3. Sample preparation

2.3.1. Preparation of certified reference material

GM rice with *Cry1Ab* and non-transgenic rice were milled, ground, and passed through a 100-mesh sieve. For the generation of standard curves, certified reference material samples, containing 100%, 50%, 25%, 12.5%, 5%, 1% and 0.5% GM rice (w/w), were prepared, using GM rice with *Cry1Ab* and non-transgenic rice, i.e., the certified reference material containing 50% GMO was prepared by mixing 50.0 g of GM rice with 50.0 g of non-transgenic rice. All foods were stored at −20 °C prior to further use.

2.3.2. Preparation of steamed rice

Steamed rice, rice noodles, rice crackers and sweet rice wine were prepared with GM rice in our laboratory according to traditional methods.

In this experiment, 200 g of rice were washed, mixed with 400 ml water, and steamed for 30 min. Samples were collected after 10, 15, 20, 25 and 30 min of steaming.

2.3.3. Preparation of rice noodles

Rice noodles were prepared according to the method reported by Zhang et al. (2014b). Briefly, 250 g of rice were washed, and mixed with 500 ml of water. After soaking for 6 h, the mixture was ground and squeezed. The rice was steamed for 5 min, moulded into a disc-like shape (2 mm thick), and cut into strips (8–10 mm wide). The rice noodles were steamed for 15 min, allowed to cool at room temperature (25 °C), and dried for 10 h at 45 °C. Samples were collected after each processing step, i.e., grinding, steaming for 5 min, steaming for 15 min and drying.

2.3.4. Preparation of rice crackers

Rice crackers were prepared according to the method described by Song et al. (2011) with minor modifications. In this experiment, 200 g of rice were washed and mixed with 3 g of sucrose, 9 g of potato starch and 1 g of salt. The mixture was boiled for 30 min, and moulded into a disc-like shape (2 mm thick). The rice disc was dried at 80 °C for 2.5 h, allowed to cool at room temperature for 24 h, and dried at 80 °C for 2 h. Rice crackers were baked (230 °C for 3 min), fried (180 °C for 30 s) or microwaved (800 W for 3 min). Samples were collected after each processing step (Fu, 2008).

2.3.5. Preparation of sweet rice wine

Sweet rice wine was prepared according to the method of Song et al. (2011). Briefly, 250 g of rice were boiled for 30 min, allowed to cool to approximately 35 °C and mixed with 1 g of fermentation

starter. The mixture was transferred to a covered bowl with a hole in the middle and incubated at 37 °C for 4 d. Samples were collected every 12 h during the fermentation process.

2.4. DNA extraction

DNA was extracted from GM rice and food products, using a Column Plant DNAout Kit (Tiandz Incorporation, Beijing, China), which provided the highest DNA quality at a lower cost and in a shorter period of time. Additionally, this kit provided the optimum DNA extraction method from GM rice and GM rice-derived products among six tested DNA extraction methods (Zhang, Xing, Selvaraj, & Liu, 2014a). Two independent DNA isolations of each sample were performed and three replicates were performed for each extract. The concentrations of the DNA extracts were determined by measuring absorbance at 260 nm (A_{260nm}) in a NanoDrop 2000 (Gene Company Limited, HongKong, China). The purity of the DNA extracts was assessed by A_{260nm}/A_{280nm}.

2.5. PCR primers

Primers of the rice endogenous *SPS*, exogenous *Pubi* promoter, *Cry1Ab* gene, *NOS* terminator and *Hpt* gene were designed with Primer 5.0 software (Premier Biosoft International, Palo Alto, CA) and Oligo 6.71 software (Molecular Biology Insights, Cascade, CO). The length of the amplifcons ranged from 142 bp to 165 bp. The nucleotide sequences were submitted to a BLASTn sequence similarity search to confirm the specificity of the primers. The primers were synthesized by Sangon Biotech (Shanghai, China). The primers of *SPS*, *Pubi*, *Cry1Ab*, *NOS* and *Hpt* are shown in Table 1.

2.6. Real-time PCR

The real-time PCR reactions (20 μl) contained 10 μl SYBR® Green Real-time PCR Master Mix, 0.3 μl of 10 mM concentrations of each primer and 20 ng of DNA extract. Non-template DNA controls were prepared with nuclease-free water (Promega Corporation, Madison, WI, USA).

Real-time PCR was performed in an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The PCR programme included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 25 s, and a final annealing step at 58 °C for 25 s. PCR products were analysed, using the sequence detection system software 1.9.1 (Applied Biosystems). The fluorescence signals obtained were continuously measured once per cycle after the annealing and extension step.

Table 1
The primers of *SPS gene*, *Pubi* promoter, *Cry1Ab gene*, *NOS* terminator and *Hpt* gene for real-time PCR.

Primer name		Primer sequence (5′–3′)	GC% of amplicons	Length (bp)
SPS	S-F	AAGCCACGGACTCCTCTAAT	50.6	160
	S-R	CGGCGAGGTTTGCTAATG		
Pubi	P-F	GGGCAAACCAAACCCTATG	48.6	142
	P-R	TGCGACCTGTACGTCAGACAC		
Cry1Ab	C-F	GTGGAGAACGCATTGAAACC	51.4	142
	C-R	TGAGATGGACCAAAGATACCC		
NOS	N-F	GAATCCTGTTGCCGGTCTTG	32.1	165
	N-R	CGCTATATTTTGTTTTCTATCGCGT		
Hpt	H-F	TCGGTTTCAGGCAGGTCT	56.4	140
	H-R	CGTTATGTTTATCGGCACTTT		

2.7. Construction of standard curves

For the generation of standard curves, endogenous gene (*SPS*) and exogenous gene (*Cry1Ab*, *NOS*, *Pubi* and *Hpt*) reactions of 100%, 50%, 25%, 12.5%, 5%, 1% and 0.5% GM rice certified reference material were performed in separate tubes; the Ct values of each gene obtained, ΔCt were equal to the difference between the Ct value of each exogenous gene and the Ct value of the endogenous *SPS* gene. Three replicates were performed for each sample and averaged. Standard curves were generated between log(GM rice%) and ΔCt of each exogenous gene.

2.8. Determination of the level of exogenous genes

The quantification of exogenous genes in steamed rice, rice noodles, rice crackers and sweet rice wine was performed by interpolation on the standard regression curve of ΔCt values generated from DNA samples of known concentration (%).

2.9. Statistical analyses

All the experimental results were compared by one-way analysis of variance (ANOVA). Tukey's test was used to assess differences among treatments, and statistical difference between treatment means was concluded when $P \leqslant 0.05$. All data were expressed as means ± SD (standard deviation).

3. Results and discussion

3.1. General

Rice is one of the most important crops in the world and is the staple food of more than half of the world's population (Chen, Lin, & Zhang, 2009). Rice is the staple food in China, feeding more than 60% of the population and contributing nearly 40% of total calorie intake (Cao et al., 2010). Rice is processed in diverse ways into various food fractions around the world. For instance, in China, rice is processed into traditional foods, such as steamed rice, rice noodles, rice crackers and sweet rice wine. A number of complex steps, such as heating, grinding, drying, boiling, frying, microwaving and fermentation are involved in the food processing. Those different processing procedures have more or less influences on the DNA degradation in products. Generally, the relative content of GMOs can be estimated from the ratio of copy numbers of exogenous gene and endogenous reference gene, using real-time PCR (Kakihara, Matsufuji, Chino, & Yamagata, 2007). However, different degradation levels of the exogenous gene and reference gene during food processing significantly affect the real-time PCR results, and such quantification results may not reflect the actual weight proportions of GM material contained in the food sample. Therefore, the levels of exogenous genes will vary among different food processing methods, thereby affecting the detection of GMOs in foods.

3.2. Construction of standard curves

Amplification by real-time PCR can be assessed by the cycle threshold (Ct) value. There is a linear relationship between Ct value and the logarithm of the initial template copy number. Therefore, a standard curve of log(GMO%) against ΔCt can be generated to study the levels of exogenous genes during food processing.

In this study, the Ct value of endogenous and exogenous genes was determined, using certified reference material containing 100%, 50%, 25%, 12.5%, 5%, 1% and 0.5% of GM rice (w/w). Fig. 1A and B show the standard curves of the *Pubi* promoter and *Cry1Ab*

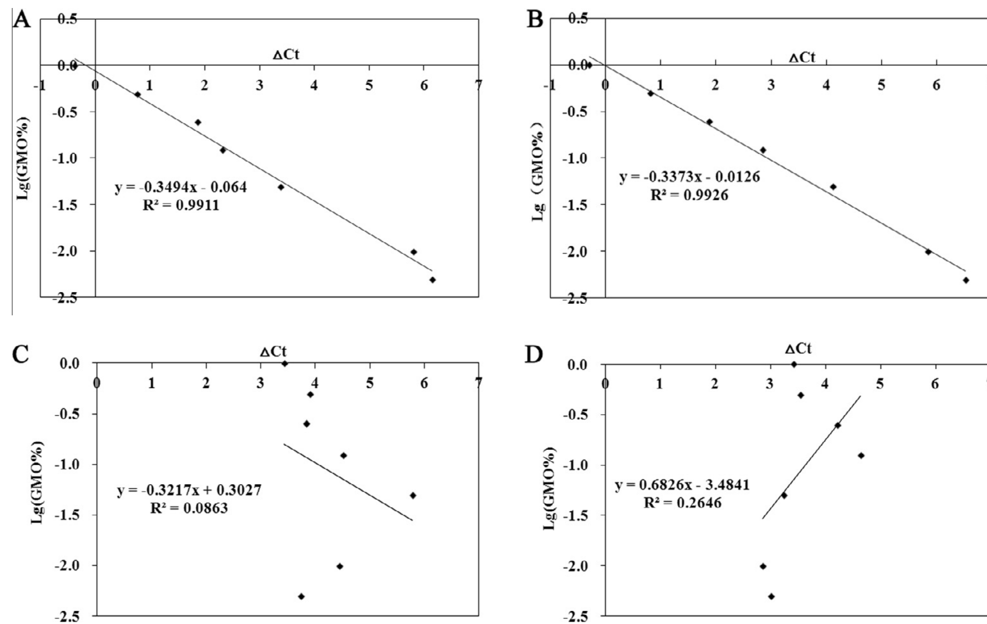

Fig. 1. Creterion curves of the content of exogenous genes for real-time PCR. (A) *Pubi* promoter; (B) *Cry1Ab* gene; (C) *NOS* terminator and (D) *Hpt* gene.

genes with correlations (R^2) of 0.9926 and 0.9911, respectively; therefore, there was an excellent linearity between the log(GMO%) and ΔCt (Ct of target genes-Ct of reference *SPS*).

However, the standard curves of *NOS* terminator and *Hpt* genes had R^2 values of 0.0863 and 0.2646, respectively. Therefore, in this study, we only used *Pubi* and *Cry1Ab* as the target genes, instead of *NOS* or *Hpt* to assess the levels of exogenous genes during food processing. Consequently, *NOS* and *Hpt* should not be selected as the target genes for the quantification of GMOs, using real-time PCR in foods derived from GM rice with *Cry1Ab*.

3.3. Levels of exogenous genes during the preparation of steamed rice

To study the levels of exogenous genes during food processing, 100% GM rice with *Cry1Ab* was used as raw material to produce steamed rice, rice noodles, rice crackers and sweet rice wine. The samples were collected after each processing step and the level of exogenous genes was calculated by interpolation on the generated standard curves.

The processing method of steamed rice involved heat treatment. As shown in Table 2, during the preparation of steamed rice, the level of *Pubi* decreased from 100% to 48.0% after steaming for 10 min and subsequently increased to 62.1% after steaming for 25 min. The level of *Pubi* was subsequently reduced to 51.8%, while the percentage in the final product was 59.7%.

However, the changes in the levels of *Cry1Ab* gene were different from those obtained with *Pubi*. The level of *Cry1Ab* in steamed rice was ⩾100% (104–131%), except after steaming for 15 min (98.2%), indicating that the degradation degree of *Cry1Ab* was lower than that of the endogenous *SPS* gene during the processing of steamed rice (Table 2).

In our previous study (Zhang et al., 2014b), qualitative PCR was used to investigate the degradation of endogenous and exogenous genes of GM rice after four food processing methods. The findings revealed that the processing methods of steamed rice had little impact on the degradation of endogenous and exogenous genes. Additionally, *Cry1Ab*, *Pubi* and *SPS* had similar stabilities. However, the results in this study were inconsistent with our previous study. In this study, we used a more sensitive detection method (real-time PCR) and found that the level of *Pubi* decreased from 100% to 59.7%

Table 2
The results of real-time PCR amplification of *Pubi* promoter and *Cry1Ab* gene during the processing of steamed rice.

Sample	Content of *Pubi* promoter (%)	Content of *Cry1Ab* gene (%)	
Steaming for 10 min	48.0 ± 3.80^{a}	131 ± 6.00^{a}	>100
Steaming for 15 min	58.3 ± 6.58^{b}	98.2 ± 4.02^{b}	≈100
Steaming for 20 min	62.1 ± 6.73^{c}	104 ± 3.16^{c}	>100
Steaming for 25 min	51.8 ± 1.58^{d}	111 ± 1.94^{d}	>100
Steaming for 30 min	59.7 ± 1.91^{b}	105 ± 2.75^{c}	>100

Different superscript lowercase letters in each column indicate a significant difference ($P < 0.05$).

during the processing of steamed rice. However, the percentage of *Cry1Ab* in steamed rice was ⩾100%. The results of this study revealed that *Pubi* degradation was more severe than that of *SPS* during the processing of steamed rice, while *Cry1Ab* degradation was less than that of *SPS*. Therefore, based on the levels of exogenous genes in steamed rice, *Cry1Ab* was the most stable, followed by *SPS* and *Pubi*. This result may be attributed to the GC content of *Cry1Ab* amplicon (51.4%), which is higher than that of *SPS* (50.6%) and *Pubi* (48.6%) amplicons. The stabilities of the three genes were positively correlated with their GC content during the processing of steamed rice. According to previous studies, the DNA fragments of similar size having high GC contents are generally considered to be stable when exposed to high temperatures. The DNA sequences between *CTP* and *CP4EPSPS* of GM soybeans would be more easily degraded than the sequences between *NOS* and part of *CP4EPSPS* during heat treatment, and this attributed to the GC contents of the PCR products of sequences between *CTP* and *CP4EPSPS* being lower than those sequences between *NOS* and part of *CP4EPSPS* (Kakihara, Matsufuji, Chino, & Takeda, 2006). Therefore, GC content in the target sequence may be an indicator of stability during food processings, especially heat treatment.

3.4. Levels of exogenous gene during the preparation of rice noodles

The preparation of rice noodles involved four processing steps: grinding, first steaming (5 min), second steaming (15 min) and

drying (45 °C, 10 h), of which grinding was the only mechanical treatment. Several studies have reported that mechanical treatment does not significantly affect the degradation of endogenous or exogenous genes (Chiter, Forbes, & Blair, 2000; Forbes, Blair, Chiter, & Perks, 1998). In our previous study, similar results were obtained by qualitative PCR (Zhang et al., 2014b). However, another study has reported that grinding resulted in obvious degradation of exogenous gene *epsps* and endogenous gene *lectin* during soymilk processing of Roundup Ready Soybean. After grinding, fragments of the endogenous gene *lectin* with the size of 1883 bp could not be detected, while those with the size of 800 bp (or below) could be detected. Fragments of the exogenous gene *epsps* with the size of 1512 and 807 bp could not be detected, and only those with the size of 400 bp (or below) could be detected (Chen et al., 2007). In this study, the levels of *Pubi* and *Cry1Ab* increased to 113% and 105% after grinding, respectively (Table 3), suggesting that the degradation of *Pubi* and *Cry1Ab* was less severe than that of *SPS*. This result might be attributed to the length of *SPS* (7,150 bp), which was significantly longer than that of *Pubi* (1,999 bp) and *Cry1Ab* (1854 bp); therefore, grinding resulted in a more severe degradation of *SPS* than of the smaller *Pubi* promoter or *Cry1Ab* genes. The degradation of these genes was so mild that their longest fragments could still be detected by qualitative PCR (Zhang et al., 2014b). The content of *Pubi* decreased by approximately 70% after the first and second steamings, suggesting that the degradation of *Pubi* was more significant than that of *SPS*. However, the drying step increased the level of *Pubi* to 82.6%. The levels of *Cry1Ab* in rice noodle samples were 105.35–153.03% (>100%), indicating that the processing methods of rice noodles resulted in a more severe degradation of *SPS* than of *Cry1Ab* (Table 3). In general, during the processing of rice noodles, *Cry1Ab* was the most stable, followed by *SPS* and *Pubi*.

3.5. Levels of exogenous genes during the preparation of rice crackers

The preparation of rice crackers involved six processing steps. The level of *Pubi* was reduced to 67.3% after boiling and to approximately 48% after the first and second drying steps. Frying, microwaving and baking had different effects on the levels of *Pubi*. Frying resulted in the most severe degradation of *Pubi* (from 47.9% to 33.4%), followed by baking and microwaving (Table 4).

The level of *Cry1Ab* was 109% after boiling, suggesting that the degradation of *SPS* by boiling was slightly more severe than that of *Cry1Ab*. Following the first drying steps, the level of *Cry1Ab* decreased to 55.2%, while the second drying process did not result in any significant changes. Frying contributed to the most severe degradation of *Cry1Ab* (from 58.0% to 30.9%), followed by baking and microwaving (Table 4).

In the preparation of rice crackers, frying, microwaving and baking treatments had different effects on the levels of exogenous genes. After frying, the two exogenous genes (*Pubi* and *Cry1Ab*) were significantly degraded; their levels quickly dropped to approximately 30%. Baking contributed to a slight reduction in *Pubi* and *Cry1Ab*. Microwaving caused a slight decrease in *Pubi* and a slight increase in *Cry1Ab*, probably due to different susceptibilities of the genes (Gryson, 2010). Our findings were in accordance with those obtained in our previous study (Zhang et al., 2014b). In general, frying was the most severe processing step, followed by baking and microwaving. With respect to gene stability, *SPS* was the most stable, followed by *Cry1Ab* and *Pubi*.

Table 3
The results of real-time PCR amplification of *Pubi* promoter and *Cry1Ab* gene during the processing of rice noodles.

Sample	Content of *Pubi* promoter (%)		Content of *Cry1Ab* gene (%)	
Grinding	113 ± 4.44^{a}	>100	105 ± 7.00^{a}	>100
First steaming	71.0 ± 8.94^{b}		143 ± 11.1^{b}	>100
Second steaming	70.4 ± 4.64^{b}		153 ± 12.6^{c}	>100
Drying	82.6 ± 6.20^{c}		107 ± 2.81^{a}	>100

Different superscript lowercase letters in each column indicate a significant difference ($P < 0.05$).

Table 4
The results of real-time PCR amplification of *Pubi* promoter and *Cry1Ab* gene during the processing of rice crackers.

Sample	Content of *Pubi* promoter (%)	Content of *Cry1Ab* gene (%)
Boiling	67.3 ± 1.50^{a}	109 ± 2.12^{a}
First drying	49.0 ± 2.56^{b}	55.2 ± 7.02^{b}
Second drying	47.9 ± 2.91^{b}	58.0 ± 8.23^{b}
Frying	33.4 ± 2.33^{c}	30.9 ± 4.50^{c}
Microwaving	47.0 ± 2.70^{b}	60.6 ± 2.01^{d}
Baking	43.3 ± 3.01^{d}	44.2 ± 4.49^{e}

Different superscript lowercase letters in each column indicate a significant difference ($P < 0.05$).

3.6. Levels of exogenous genes during the preparation of sweet rice wine

The levels of *Pubi* after boiling were similar to those obtained in steamed rice after steaming for 30 min (57.4% and 59.7%, respectively). Fermentation is a biotransformation process, resulting in different degradation degrees of endogenous and exogenous genes. After 12 h of fermentation, the level of *Pubi* was significantly reduced from 57.4% to 20.5%, and there were no significant changes from 12 to 24 h of fermentation. After 36 h of fermentation, the level of *Pubi* increased to 42.8%. However, after 48 h of fermentation, the level of *Pubi* decreased, reaching 17.6% after 60 h of fermentation. After 96 h of fermentation, the level of *Pubi* increased, reaching 28.6% in the final product (Table 5).

The changes in *Cry1Ab* levels were similar to those in *Pubi*. The level of *Cry1Ab* decreased slightly (96.0%) after boiling. After 12 h of fermentation, the level was significantly reduced to 13.4%. After 36 h, the levels increased to 47.5% and subsequently decreased to 21.8% after 60 h of fermentation. The levels subsequently increased, reaching 52.0% in the final product (Table 5). In general, the levels of *Pubi* and *Cry1Ab* in sweet rice wine samples were <100%, suggesting that the degradation of *Pubi* and *Cry1Ab* was more severe than that of *SPS* during the preparation of sweet rice wine. Based on the levels of exogenous genes, *SPS* was the most stable, followed by *Cry1Ab* and *Pubi*.

The processing methods of steamed rice and rice noodles were relatively simple. The levels of *Cry1Ab* during the processing of steamed rice and rice noodles were >100%, while those in rice crackers and sweet rice wine were <65% (except for the boiling step). Therefore, the processing methods of steamed rice and rice

Table 5
The results of real-time PCR amplification of *Pubi* promoter and *Cry1Ab* during the processing of sweet rice wine.

Sample	Content of *Pubi* promoter (%)	Content of *Cry1Ab* gene (%)
Boiling	57.4 ± 1.40^{a}	96.0 ± 2.48^{a}
Fermentation for 12 h	20.5 ± 1.66^{b}	13.4 ± 1.39^{b}
Fermentation for 24 h	21.2 ± 2.77^{b}	20.7 ± 4.15^{c}
Fermentation for 36 h	42.8 ± 3.87^{c}	47.5 ± 3.39^{d}
Fermentation for 48 h	36.3 ± 3.94^{d}	28.6 ± 3.83^{e}
Fermentation for 60 h	17.6 ± 1.34^{e}	21.8 ± 2.38^{c}
Fermentation for 72 h	18.0 ± 2.51^{e}	42.9 ± 3.09^{f}
Fermentation for 96 h	28.6 ± 3.11^{f}	51.9 ± 2.71^{g}

Different superscript lowercase letters in each column indicate a significant difference ($P < 0.05$).

noodles caused a smaller degradation in *Cry1Ab* than in *SPS*, while the processing methods of rice crackers and sweet rice wine resulted in a more severe degradation of the exogenous genes than that of *SPS*. Among all processing methods, fermentation had the most severe impact on the degradation of exogenous genes. This was consistent with the findings of our previous study (Zhang et al., 2014b), and may be attributed to DNA digestion by microbial DNases (Ogasawara, Arakawa, Akiyama, Goda, & Ozeki, 2003) and pH reduction during fermentation (Gryson, 2010).

According to the previous studies, the DNA fragments of similar size having high GC contents are generally considered to be stable when exposed to high temperatures (Song et al., 2011). The critical processing procedures were heat treatment during the processing of steamed rice and rice noodles. Therefore, the stabilities of *Cry1Ab*, *Pubi* and *SPS* genes were positively correlated with their GC content during the mild processing methods of steamed rice and rice noodles. The stability of the genes in steamed rice and rice noodles was found to be in the following decreasing order: *Cry1Ab* > *SPS* > *Pubi*. However, the differences between the GC contents of the amplicons did not completely correspond with the stability of the target gene. There may be other factors contributing to the different levels of DNA degradation, such as ingredients having different properties, and the different susceptibilities of DNA to various processing conditions (Gryson, 2010). The stability of the genes in rice crackers and sweet rice wine was inconsistent with steamed rice and rice noodles, and was found to be in the following decreasing order: *SPS* > *Cry1Ab* > *Pubi*. The changes in stabilities of genes may be attributed to the severe processing procedures, such as frying, microwaving, baking and fermentation during the processing of rice crackers and sweet rice wine. These results confirm that predicting the stability and degradation of exogenous genes, and finding detailed reasons for changes in their stability in food processing is quite difficult or almost impossible. Therefore it is indispensable to do analysis by using scientific and effective methods for GM food safety control.

Several quantitative detection methods of GMOs have been based on the copy numbers of exogenous and endogenous genes (Chen et al., 2007; Guan et al., 2013; Yoshimura et al., 2005). In this study, two exogenous genes, namely, *Pubi* promoter and *Cry1Ab* gene were used as target genes to assess the levels of transgenic components after four traditional food processing methods. When *Pubi* was selected as the representative of transgenic components, the contents of GMOs in steamed rice, rice noodles and sweet rice wine were 59.7%, 82.6% and 28.6%, respectively. In the frying, microwaving and baking steps involved in the preparation of rice crackers, *Pubi* levels were 33.4%, 47.0% and 43.3% respectively. When *Cry1Ab* gene was selected as the representative of transgenic components, the contents of GMOs in steamed rice, noodles and sweet rice wine were 105%, 107% and 51.9%, respectively. The levels of *Cry1Ab* after frying, microwaving and baking steps of rice crackers were 33.9%, 60.6% and 44.2%, respectively. The levels of these exogenous genes depended on their stability relative to that of the endogenous *SPS* gene. Therefore, the detection of GMOs in food products depends on the exogenous genes that are selected as representatives of transgenic components. In the case of foods from GM rice with *Cry1Ab*, the *Cry1Ab* gene is a better representative of transgenic components than is the *Pubi* promoter, because the levels of *Cry1Ab* were less affected than were those of *Pubi*.

4. Conclusions

Food processing methods have different effects on the levels of exogenous genes. During the processing of steamed rice and rice noodles, the levels of *Cry1Ab* were $\geqslant$100%, while the content of *Pubi* was <83% (ranging from 48.0% to 82.6%, except for the ground samples). In rice crackers, frying and baking significantly reduced the levels of *Pubi* and *Cry1Ab*; the effect of frying was significantly greater. Microwaving caused a mild decrease in *Pubi* (from 47.9% to 47.0%), and a mild increase in *Cry1Ab* (from 58.0% to 60.6%). Among all processing methods, the fermentation step of sweet rice wine had the most severe effect on the degradation of the exogenous genes. As to the relative stability, *Cry1Ab* was the most stable, followed by *SPS* and *Pubi* in steamed rice and rice noodles. However, during the processing of rice crackers and sweet rice wine, *SPS* was the most stable, followed by *Cry1Ab* and *Pubi*. Finally, the detection of GMOs in food products depends on the exogenous genes selected as representatives of transgenic components.

Conflict of interest

The authors declare that there are no conflicts of interest.

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