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Research article

Detection of Genetically Modified Glyphosate-Resistant Soybean Sold in Sarawak

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Abstract

A genetically modified (GM) crop is an organism whose genetic makeup has been altered to express the desired physiological traits. Soybean (*Glycine max*) is a common GM crop. Its genome has been genetically engineered to confer resistance to herbicides, pests and extreme environmental conditions. Mislabelling of food products as GM-free has triggered insecurity among consumers. In addition, the health effects due to consumption of GM foods remains controversial. Therefore, this study aimed to identify the presence of GM soybean in animal feeds and several food products such as raw soybean, tempeh, and tofu collected from Sarawak traditional markets, grocery stores, and supermarkets. The presence of the regulatory elements CaMV 35S Promoter (P35S) and *NOS* Terminator (T*NOS*) were initially screened using conventional Polymerase Chain Reaction (PCR). Then, all samples were subjected to the PCR-based construct-specific method by targeting the *cp4 epsps* gene, which confers glyphosate-resistance. Positive samples were validated through DNA sequencing. The result demonstrated that 56 out of 65 samples including 17 soybean, 12 animal feeds, 7 tofu and 20 tempeh samples were positive for *cp4 epsps*. Furthermore, 2 out of 20 raw soybean samples were labelled as GM-free. However, validation using DNA sequencing indicates 100% identity to *cp4 epsps* gene in comparison with the Genbank database. This study demonstrated the significance of GM detection in soybean and the importance of accurate food labelling.

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1. Introduction

Soybean (*Glycine max*) is rich in protein and can be found in various food products such as soya milk, tempeh, miso, edamame, soy flour, soya oil, soya sauce, and textured soy protein (TSP). Malaysian widely utilize soybean as food and feed; however, Malaysia relies on supply from other countries [1] due to the lack of local cultivation of this legume [2]. In 2019, Malaysia imported 96% of soybean meal and 80% of soybean seed from Argentina and the United States (US), respectively [1]. The statistical data showed that 94% of the soybean crops in the US were genetically modified to be herbicide-tolerant [3]. A genetically modified (GM) organism is defined as an organism whose genome has been genetically engineered to favor the expression of certain physiological traits. In agriculture, GM crops are genetically modified to express certain traits including resistance to diseases, pests, herbicides, or the ability to adapt to challenging environments such as extreme temperature, drought, salinity, or acidity of the soil [4]. The soybean event GTS 40-3-2, or commercially known as Roundup Ready (RR^{TM}), was the first GM soybean approved in 1996 for food production [5]. It was developed by the Monsanto Company (USA) to harbor the *5-enol-pyruvyl shikimate-3-phosphate* (*epsps*) gene from *Agrobacterium tumefaciens* strain CP4, which is responsible for the synthesis of glyphosate-insensitive protein (CP4EPSPS). This protein is an enzyme that confers crop resistance to the glyphosate herbicide, which is the active ingredient of the RR herbicide [6-9].

Genetically modified foods are a new invention and little is known about their long-term effects on humans, animals, and the environment. Consequently, the introduction of these GM food products into the food chain has increased public concern and many people prefer to avoid consuming these food products [10]. Nowadays, consumers constantly rely on food labeling to find information about food ingredients, nutrition, country of origin, and statements about health benefits in order to assist in their purchasing decisions [11]. In accordance with the requirement of Regulations 11 3(A), 11(6), and 11(7) of Food Regulation 1985 and Food Act 1983 [12], the Ministry of Health (MOH) published a guideline on Genetically Engineered (GE) labelling in 2013, which stipulated that labelling was mandatory for foods that contained, consisted, and were produced from genetically modified organisms (GMOs) in a proportion of more than 3% of the food ingredients [13].

Analytical methods for the detection of the GMOs in various food products are necessary due to compliance with labelling requirements. The molecular identification of GMOs can be performed at different stages, i.e. deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein stages [14, 15]. Nevertheless, DNA is more stable [13], and hence better allows the detection of transgene(s) in highly processed food than does protein, which can easily be degraded or denatured through food processing [16]. The polymerase chain reaction (PCR) is one of the widely used DNA-based methods in GMO identification because it is fast, accurate, and highly sensitive for screening of transgene(s) in raw and processed food products [15-17]. Generally, more than 80% of engineered genetic constructs in GM crops are built with cauliflower mosaic virus (CaMV) 35S promoter (P35S) and *Agrobacterium tumefaciens nopaline synthase NOS* terminator (T*NOS*) [18]. Thus, the first step of GMO detection using PCR usually involves screening for the presence of these regulatory elements [15, 19]. Following this initial step, samples containing GMOs are analysed for a specific transgene in order to identify the particular strain of GMO [17].

The distribution of GM soybean in peninsular Malaysia has been reported in a few studies. For example, Abdullah *et al.* [14] showed that 18 out of 85 samples consisting of raw soybean, tofu, and tempeh were positive for the presence of P35S, T*NOS* and EPSPS genes. In contrast, Tung Nguyen *et al.* [20] found none of the GM materials present in all 10 raw soybean samples purchased from Malaysia. The study demonstrated that either one or both of the P35S promoter and T*NOS* were detected in 16 samples of animal feed [20]. Meanwhile, a preliminary study conducted by Sani *et al.* [21] showed the distribution of GM soybean in the Sarawak markets where 17 out of 20

samples were positive for the presence of the P35S promoter. However, reports on the presence of GM soybean in animal feed and food products sold in Sarawak are scarce. Therefore, the aim of this research was to identify the presence of GM soybean in animal feed and several food products such as raw soybean, tempeh, and tofu collected from Sarawak traditional markets, grocery stores, and supermarkets by initially screening for the presence of the regulatory elements P35S and T*NOS* using conventional PCR. Subsequently, all samples were subjected to the PCR-based constructs-specific method by targeting the *cp4 epsps* gene.

2. Materials and Methods

2.1 Sampling

A total of 45 samples consisting of animal feeds (AF1-AF12), tempeh (TH1-TH25), and tofu (TF1-TF8) were bought from the traditional markets, grocery stores, and supermarkets in four main cities in Sarawak; Kuching, Sibu, Bintulu, and Miri. Meanwhile, the genomic DNA (gDNA) soybean (S1-S20) was obtained directly from Sani *et al.* [21]. These soybean samples were also collected from the cities mentioned above.

2.2 Genomic DNA (gDNA) extraction

Each sample was homogenized using a mortar and pestle. Twenty milligrams of the resulting wet powder were transferred into a 1.5 ml microcentrifuge tube, and 550 µl of preheated Buffer CF was added. The mixture was mixed carefully for 15 s, and then added with 10 µl of Proteinase K. The mixture was mixed again carefully and subsequently incubated at 65°C for 3 h. After that, the mixture was centrifuged at 10,000 x g for 10-15 min to pellet contaminants and cell debris. The clear supernatant was transferred into a new microcentrifuge tube, and added with one volume of Buffer C4 and one volume of ethanol. Then, the tube was vortexed for 30 s. Next, 700 µl of the mixture was pipetted into a NucleoSpin® Food Column and centrifuged at 11,000 x g for 1 min. The flow-through was discarded and this step was repeated for the remaining mixture. Next, in the washing step, 400 µl of Buffer CQW was pipetted into the column and centrifuged at 11, 000 x g for 1 min. The flow-through was discarded. This step was repeated using 700 µl and 200 µl of Buffer C5, respectively. At the end of the washing step, the column was centrifuged at 10, 000 x g for 2 min to remove traces of Buffer C5. Finally, the column was placed in a new 1.5 ml microcentrifuge tube and added with 50 µl of preheated Elution Buffer CE. The column was incubated for 10 min at room temperature and then centrifuged for 1 min at 11,000 x g to elute genomic DNA (gDNA).

The gDNA extraction procedure was performed according to NucleoSpin® Food (Macherey Nagel, Germany) with minor modification. The integrity of the extracted gDNA was analysed on 1% agarose gel (1st Base, Malaysia).

2.3 Detection of P35S, T*NOS* and *cp4 epsps* using conventional polymerase chain reaction

The first and second PCR amplifications were conducted by targeting soy-specific *lectin* gene and P35S in extracted gDNA of 12 animal feeds (AF1-AF12), 25 tempeh (TH1-TH25), and 8 tofu (TF1-TF8) samples. After that, PCR amplification was performed to detect the presence of the T*NOS* and *cp4 epsps* in all samples including the soybeans (S1-S20) using combination of primers HA-F/R and EPSPS-F/R, respectively. The PCR amplification was carried out in 25 µl of total volume. The components of the PCR mixture were 12.5 µl of 2X GoTaq Green Master Mix (Promega, United

States), 1 µl of genomic DNA, 0.5 µl of 10 µM of forward and reverse primers, and 10.5 µl of nuclease-free water (Promega, United States). Tables 1, 2 and 3 show the amplification profile for each primer, and the list of primer sequences used in this study, respectively. PCR was performed in Mastercycle NEXUS GX2 (Eppendorf, Germany). The PCR products were analyzed on 3% agarose gel (1st Base, Malaysia) stained with SYBR Safe DNA gel stain (Invitrogen, United States). The agarose gel electrophoresis was run in a 1X TBE buffer solution (Promega, United States) at 100V for 35-40 min and visualized under an E-Box UV transilluminator (Vilber, France). The Generuler Ultra Low Range DNA Ladder (Thermo Scientific, United States) was used as a reference.

Table 1. The PCR profile used to detect the presence of soy-specific *lectin*, P35S and *cp4 epsps*

Elements	Temperature	Duration	Cycle
Initial denaturation	95°C	3 min	1x
Denaturation	95°C	30 s	50x
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1x

Table 2. The PCR profile used to detect the presence of the T*NOS*

Elements	Temperature	Duration	Cycle
Initial denaturation	95°C	10 min	1x
Denaturation	95°C	25 s	50x
Annealing	62°C	20 s	
Extension	72°C	45 s	
Final extension	72°C	7 min	1x

Table 3. List of primer sequences used in PCR to detect the presence of *lectin*, P35S, T*NOS*, and *cp4 epsps*

	Primers	Gene specificity	Size of amplicon, bp	References
Lec-F	5 - GTG CTA CTG ACC AGC AAG GCA AAC TCA GCG-3	*Lectin*	164	[22]
Lec-R	5- GAG GGT TTT GGG GTG CCG TTT TCG TCA AC-3			
P35S-F	5 - ATT GAT GTG ATA TCT CCA CTG ACG T - 3	CaMV 35S Promoter (*P35S*)	101	[23]
P35S-R	5 - CCT CTC CAA ATG AAA TGA ACT TCC T - 3			
HA-F	5 - GAC ACC GCG CGC GAT AAT TTA TCC - 3	*NOS* terminator (T*NOS*)	118	[24]
HA-R	5-GCA TGA CGT TAT TTA TGA GAT GGG - 3			
EPSPS-F	5 - TGG CGC CCA AAG CTT GCA TGG C - 3	*cp4 epsps*	118	[23]
EPSPS-R	5 -CCC CAA GTT CCT AAA TCT TCA AGT-3			

2.4 Verification of PCR products via nucleotide sequencing

The PCR products were sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for verification via nucleotide sequencing. Three sets of primers used for the verification were 35S promoter-F/R, HA-F/R and EPSPS-F/R. Nucleotide Basic Local Alignment Search Tool (BLASTN) at National Center for Biotechnology Information (NCBI) was used to analyze the sequencing results.

3. Results and Discussion

The presence of soybean in all animal feed, tofu, and tempeh samples was evidenced by PCR using soy-specific *lectin* primers-F/R. Figure 1 shows a representative of the result in which approximately 164 bp of a PCR product was clearly visible on 3% agarose gel, thus indicating the presence of soybean gDNA in all animal feed samples; AF1-AF12. This step was also useful in order to discriminate between negative and positive results due to inhibitors in the PCR amplification [25].

The second step involves amplification of the P35S and T*NOS* sequence from all samples. Most of commercialized GM crops worldwide contain either one or both of the P35S and T*NOS* [18, 26]. Only a few GM products have neither 35S nor T*NOS* sequences [18]. Therefore, a common practice in GMO detection begins with the screening of these regulatory elements [18].

A preliminary study conducted by Sani *et al.* [21] demonstrated that 17 out of 20 raw soybeans collected from Sarawak's traditional markets, grocery stores, and supermarkets tested positive for the 101 bp of P35S sequence [21]. When the same gDNA was used as the PCR template in this study, 13 out of 20 samples tested positive for the 118 bp T*NOS* sequence. Similarly, the PCR using gDNA extracted from animal feed, tempeh, and tofu samples produced a positive result for either one or both of the P35S and T*NOS* (refer to Table 4). The BLAST (NCBI) analysis of the sequencing result for both PCR products that employed P35S and T*NOS* specific primers revealed a high similarity of 96-100% identities to plant binary vectors and cloning vectors, such as pRATIO3212-SMXL7, 35S-GFP, and pNC-Cam1304-SubN. These cloning vectors contained genes under the control of the regulatory elements P35S and T*NOS* [27-29]. Furthermore, a similarity of 100% identity was obtained in comparison with the *NOS* terminator sequence [accession number: MK078637.1] and CaMV genome sequence [accession no: V00141] in the NCBI database, respectively.

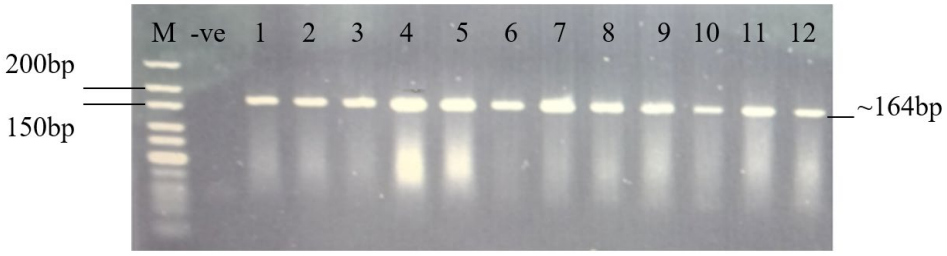

Figure 1. Analysis of PCR products on 3% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, United State). DNA amplification was conducted using a combination of primers Lec-F/R to detect the presence of the 164 bp *lectin* gene in gDNA extracted from 12 animal feed samples (AF1-AF12). Lane M: GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific, USA). Lane -ve: Negative control. Lanes 1-12: AF1-AF12

Table 4. Detection of 35S CaMV promoter (P35S), *NOS* terminator (T*NOS*), and *cp4 epsps* in gDNA extracted from soybean, animal feeds, tempeh and tofu

Sample	No of sample	No of positive sample		
		35S CaMV promoter	***NOS* terminator**	***cp4 epsps***
Soybean	20	17	13	17
Animal feeds	12	12	11	12
Tofu	8	8	7	7
Tempeh	25	24	20	20

Next, PCR was carried out to target the construct-specific 118 bp *cp4 epsps* sequence. The analysis of the PCR product on 3% agarose gel revealed that 56 out of 65 samples (S1-S20, AF1-AF12, TH1-TH25, TF1-TF8) tested positive for the presence of *cp4 epsps* [refer to Figure 2(a-f)]. From these results, two samples were raw soybeans (S19 and S20) that were labelled as GM-free and organic products [refer to Figure 2(b)]. The BLAST analysis of the sequencing results for the PCR products such as raw soybeans S19 and S20 showed a similarity of 100% identity to the *cp4 epsps* gene that was available in the Genbank database (NCBI). This result was in agreement with the current situation in which the main glyphosate-resistant soybean commercialized in the local market contains the glyphosate-resistant gene *cp4 epsps* [30]. In addition, Malaysia has authorized the importation of several events of GM soybeans that contain the *cp4 epsps* gene in their genome, including the GTS 40-3-2 as of 2022 [9].

It is undeniable that the GM crop provides numerous advantages. Nevertheless Bøhn *et al.* [31] demonstrated that organic soybeans possessed the healthiest nutritional profile compared to conventional and GM soybeans. In addition, concerns about the negative effects of glyphosate and its primary breakdown; aminomethylphosphonic acid (AMPA) on plants, animals, and human health have emerged as a result of glyphosate's extensive use and its build-up in the environment and food products globally [32]. A high level of glyphosate and AMPA has been discovered in glyphosate-resistant soybean [31]. Traces of glyphosate, which have been linked to endocrine, neurological, and intestinal disorders, have also been identified in human urine samples [33, 34]. The increasing prevalence of numerous neurological disorders such as Alzheimer's, senile dementia, Parkinson's, attention deficit hyperactivity disorder (ADHD), and autism in the United States was also found to be significantly associated with increased glyphosate use over time [35]. In 2015, the International Agency for Research on Cancer (IARC) reclassified glyphosate as possibly carcinogenic to humans. Additionally, the IARC concluded that there is significant genotoxic proof for both pure glyphosate and glyphosate formulations [36].

Generally, consumers are concerned about the food they consume, particularly its origin. Consumers may also be more cautious and apprehensive about buying GM food products [37]. Thus, food labeling is a crucial tool for informing consumers and protecting their health in terms of food safety and nutrition [38]. Furthermore, section 61 of the Biosafety Act, 2007 (Act No. 678) [39] stipulated that it is mandatory to label products that contain, consist, and are produce from GMOs. This is done to ensure the consumers can confidently choose whether or not to consume GM food in accordance with their cultural and dietary preferences [10]. Based on our study, it was discovered that all of the samples were not labeled with the claim, possibly because the manufacturer believed it would harm their business [41]. Moreover, product mislabeling was also identified in two soybean samples (S19 and S20).

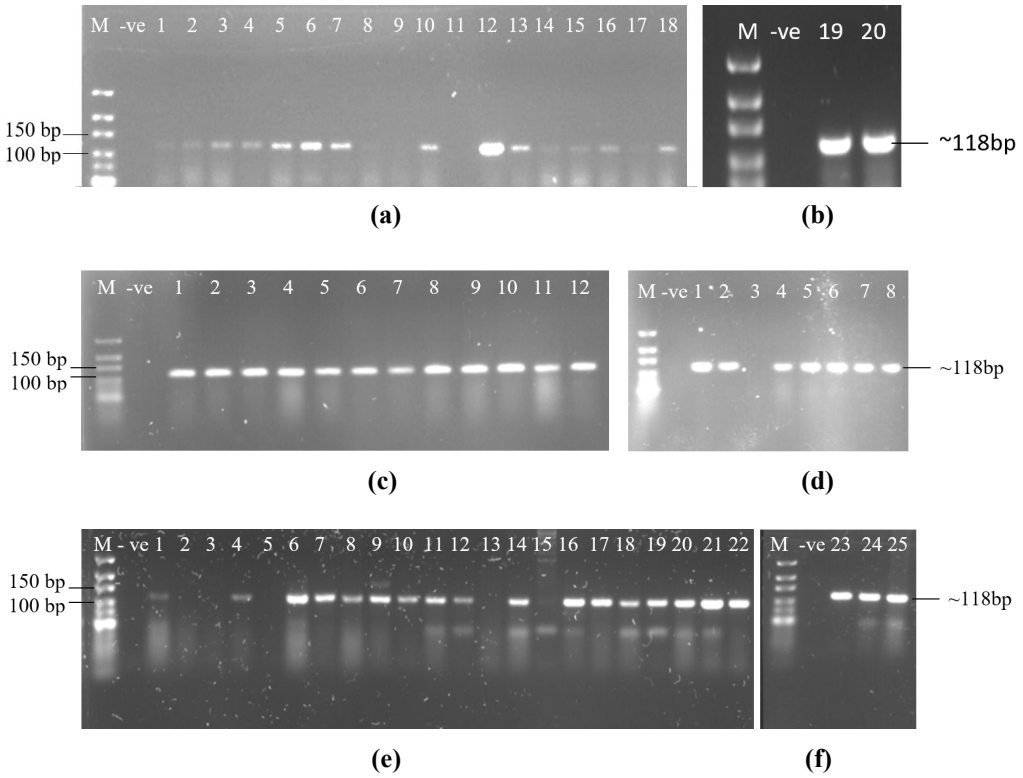

Figure 2. Analysis of PCR products on 3% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, United State). DNA amplification was conducted using a combination of primers EPSPS-F/R to detect the presence of the *cp4 epsps* in gDNA extracted from 20 raw soybeans (S1-S20), 12 animal feeds (AF1-AF12), 25 tempeh samples (TH1-TH25) and 8 tofu samples (TF1-TF8). Lane M: GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific, USA). Lane -ve: Negative control (PCR without the template). Figure 2(a): Lanes 1-18 are samples S1-S18. Figure 2(b): Lanes 19-20 are samples S19 and S20, Figure 2(c): Lanes 1-12 are samples AF1-AF12, Figure 2(d): Lanes 1-8 are samples TF1-TF8, Figure 2(e): Lanes 1-22 are sample TH1-TH22 and Figure 2(f): Lanes 23-25 are samples TH23-TH25

4. Conclusions

This study demonstrates a procedure of GM soybean detection in food and animal feed products in Sarawak using a PCR-based screening method and a constructs-specific method. The presence of GM soybean with glyphosate-resistant traits driven by P35S and T*NOS* in Sarawak, Malaysia was high with a frequency of 56 out of 65 samples, or about 86%. In addition, 2 out of 20 raw soybean samples that had been labelled GM-free were found to be positive for *cp4 epsps*. Although the health effects of consuming GM soybeans remain controversial, it is important to protect consumer rights through the accurate labelling of all foods that consists, contains, or are produced from GMOs. Strict laws and legislation particularly on the mislabelling of GM foods should be imposed and penalties should be given to the food manufacturers who violate the stipulated act. Further research should include the use of real-time PCR techniques for specific and sensitive detection of these samples.

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