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ORIGINAL ARTICLE

Detection of *Cauliflower Mosaic Virus 35S Promoter* in Soybean Collected from Sarawak Using Polymerase Chain Reaction

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ABSTRACT - A genetically modified (GM) soybean is a legume that has been modified using genetic engineering techniques to be resistant to herbicides and insects, improve nutritional content, and palatability. Despite the first GM soybean has been consumed as food more than two decades ago, public perception of GM soybean still differs. Even now, there is an ongoing debate over genetically engineered foods, with the main concerns focusing on their long-term impact on human health. According to the Malaysia Food Regulations 1985, food obtained through biotechnology must be labelled. Nevertheless, labelling information was unavailable in certain raw soybeans sold in supermarkets, grocery stores, and traditional markets in Sarawak. Hence, this preliminary study was conducted to detect the presence of GM soybean in Sarawak, Malaysia using polymerase chain reaction (PCR) by targeting a specific region of the *Cauliflower Mosaic Virus 35S promoter (CaMV P35S)* gene sequence. To attain the objective, 20 raw soybean samples were collected from traditional markets, grocery stores, and supermarkets located in four main cities of Sarawak. The soybean DNA was extracted using a commercial DNA extraction kit and subsequently utilized as a template in the PCR. The PCR analysis on 3 % agarose gel identified 17 of 20 raw soybean samples were positive for 108 bp of *CaMV P35S* sequence. The PCR results were validated by nucleotide sequencing, and the BLAST (NCBI) analysis revealed 95 % similarity to cloning and expression vectors driven by the *CaMV P35S*. The preliminary study highlighted about 85% of the soybean in Sarawak market were dominated by the genetically modified type.

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INTRODUCTION

Soybean (*Glycine max. L*) is the world's fourth most important grain crop after wheat, maize, and rice [1]. Examples of foods produced from the soybean are oil, milk, tofu, and flour. The raw soybean also can be processed into soy sauce and *tempeh* through fermentation process. An immature soybean known as *Edamame* is served as an appetizer in the Japanese restaurant. The economic significance of soybean as a source of food for humans and animals, as well as for industrial applications [2] made this crop a target for genetic improvement since 1988 [3].

Genetically Modified (GM), Genetically Engineered (GE), or Transgenic, is the term used to describe an organism that has been genetically altered through genetic engineering techniques [4]. As of 2019, one of the most cultivated GM crops worldwide is soybean [5]. The Roundup Ready® (RR) soybean or transgenic soybean line GTS-40-3-2 is a variety of GM soybean that expresses the CP4 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS) protein which confers resistance to the glyphosate herbicide [6]. It was created by Monsanto company based in the USA and has been approved as a food product in 1996 [7]. Since then, numerous studies have been conducted to develop a variety of GM soybeans with different enhanced characteristics, such as drought tolerance [8] increased carotene content [9], elimination of immunodominant allergen [10] and so forth. This is made possible by inserting a gene of interest (GOI) into the soybean genome. In order to drive the constitutive expression of the GOI,

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the transcriptional promoter derived from virus, such as the *Cauliflower Mosaic Virus* (*CaMV*) has been widely used [11]. Currently, more than 80 % of engineered genetic constructs in the GM plants grown globally were created with the *P35S* from *CaMV* and/or the *NOS terminator* (*TNOS*) from the soil-borne bacteria, *Agrobacterium tumefaciens* [12]. Figure 1 illustrates the general genetic construct in a GM plant. The *CaMV P35S* and *TNOS* are incorporated at the 5' and 3' of the GOI, respectively.

GM foods could provide various advantages, including the absence of allergic traits, better palatability, higher nutritional content, and the ability to survive adverse environmental conditions such as drought and grow rapidly. Moreover, lower cost of GM crop production [4] provides food security to support the world's expanding population [13]. It is expected that the world population increase to 9 million by 2050, leaving the world short of food supply by over 70 % [14]. Despite all of the advantages mentioned above, consumers' perceptions regarding GM foods vary, with consumers in the European Union (EU) having more difficulties adopting GM foods than citizens in the United States (US) [15]. The main concern posed by GM foods is their long-term effects on human health [4]. According to Shen *et al.* [16], the effect associated with GM consumption includes mortality, tumor or cancer, a significant reduction in fertility, poor learning, and reaction abilities as well as several organ abnormalities. In contrast, an assessment conducted by Lin *et al.* [17] revealed that commercial GM soybeans are similar to non-GM soybean in rats in terms of safety. Consumers, on the other hand, have the right to choose what they would purchase according to their knowledge, concerns, diet, and preferences. Customers normally rely on the information provided in food labelling to assist their purchasing decision. However, some of the raw food such as soybean sold in the market and supermarkets in Sarawak were packed or sold without labelling information.

Therefore, a preliminary study to identify the existence of GM soybeans in Sarawak has been conducted. To attain the objective, the raw soybean samples were collected from traditional markets, grocery stores, and supermarkets in Kuching, Sibu, Bintulu, and Miri (Sarawak). Subsequently, DNA was extracted using a commercial kit and specific primer sequences were employed in PCR to amplify the 108 bp specific region of *CaMV P35S* gene sequence. The results were validated through nucleotide sequencing and analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST) that is available at National Center for Biotechnology Information (NCBI).

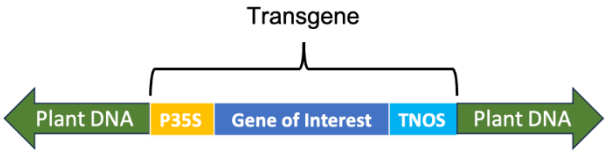


Figure 1. General genetic constructs in a GM plant. The GOI (blue) in a GM plant is driven by *CaMV P35S* (yellow) and *TNOS* (blue) (Bak and Emerson, 2020)

MATERIALS AND METHODOLOGY

Samples Collection

A total of 20 raw soybean samples labelled as S1-S20 were purchased from traditional markets, grocery stores, and supermarkets in four main cities in Sarawak, Malaysia; namely Kuching, Sibu, Bintulu, and Miri.

Extraction of DNA from Soybean Samples

The soybean DNA extraction was carried out according to NucleoSpin® Food (Macherey Nagel, Germany) guideline with minor modifications. Approximately 1 g of raw soybeans was soaked overnight with sterilized distilled water in a 50 mL tube. On the following day, the water was discarded, and the raw soybeans were homogenized using a mortar and pestle. Twenty milligrams of the resulting wet powder were weighed and transferred into a 1.5 mL microcentrifuge tube. Approximately, 1100 µL of preheated Buffer CF was added and mixed well for 15 s. Next, 20 µL of Proteinase K was added and mixed by inverting the tube for 3 – 5 times.

After that, the tube was incubated at 65°C for 4 h and centrifuged at 10,000 x g for 15 min to pellet the cell debris. The resulting clear supernatant was carefully transferred into a new microcentrifuge tube and

added with one volume of Buffer C4 and one volume of molecular-grade ethanol. The tube containing the mixture was then vortexed for 30 s. Next, 700 µL of the mixture was transferred into a NucleoSpin® Food Column, centrifuged at 11,000 x g for 1 min and the flow-through was discarded. This step was repeated for the remaining mixture.

Next, 400 µL of Buffer CQW was pipetted into the column, centrifuged at 11, 000 x g for 1 min and the flow-through was discarded. The washing step was continued by 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 the traces of wash buffers. Finally, the column was transferred into a new 1.5 mL microcentrifuge tube and 50 µL of preheated Elution Buffer CE was pipetted on the center of the microcentrifuge tube. The column was incubated for 10 - 15 min at room temperature and centrifuged for 1 min at 11,000 x g to elute the DNA.

DNA Integrity Analysis by Agarose Gel Electrophoresis

The integrity of the extracted gDNA was analyzed on 1 % agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, United States). The electrophoresis was run in a 1X TBE buffer solution (Promega, United States) at 110 V for 40 min and visualized under an E-Box UV transilluminator (Vilber, France). The GeneRuler 1 Kb DNA Ladder (Thermo Scientific, United States) was used as a reference standard.

Detection of 108 bp *CaMV P35S* sequence region using PCR

A total of 25 µL PCR mixture was prepared. The components of the PCR mixture were 12.5 µL of 2X GoTaq Green Master Mix (Promega, United States), 1 µL of DNA, 0.5 µL of 10 µM P35S – F primer: 5'-ATT GAT GTG ATA TCT CCA CTG ACG T-3', 0.5 µL of 10 µM P35S – R primer: 5'- CCT CTC CAA ATG AAA TGA ACT TCC T-3' [18] and 10.5 µL of nuclease-free water (Promega, United States). The PCR amplification profile used was 1 cycle of 95°C for 3 min, 50 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 1 cycle of 72°C for 5 min. Negative control was prepared by using 1 µL of nucleus-free water as a template. The PCR was performed in Mastercycler 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 electrophoresis was run in a 1X TBE buffer solution (Promega, United States) at 100 V for 45 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 standard.

Verification of PCR products via Nucleotide Sequencing

Approximately 60 µL of the PCR product (S12) was sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for verification via nucleotide sequencing. The 35S promoter-F/R primers were used for the verification. The Nucleotide Basic Local Alignment Search Tool (BLASTN) available at the National Center for Biotechnology Information (NCBI) database was used to analyze the sequencing results.

RESULTS AND DISCUSSION

The extraction of DNA from raw soybeans using NucleoSpin® Food (Macherey Nagel, Germany) was successful as evidenced by the presence of a band with size of more than 10 Kb on 1 % agarose gel. Figure 2 shows a representative of the result.

The GM detection methods can be categorized into two types: DNA-based detection method and protein-based detection method [19]. The protein-based detection method offers several advantages, such as rapidity and simplicity; however, its reliability depends on the degree of expression of the targeted protein [20]. In addition, identifying the presence of GM protein in highly processed food is challenging due to the protein degradation that occurs during processing [20]. Hence, DNA-based detection methods such as PCR have been widely used to screen for the presence of foreign genetic elements [21; 22]. In this study, the PCR was conducted by targeting a specific region of the *CaMV P35S* gene sequence using a combination of specific primers; P35S – F/R primers. The analysis of PCR products on 3 % agarose gel shows the appearance of an intact and bright band with a size of approximately 108 bp from 17 out of 20 samples tested (S1-S8; S10-S18). Figure 3a and Figure 3b are representative of the results. This finding shows a higher yield of *CaMV P35S*-positive samples than the studies reported by Abdullah *et al.* [23] and

Tung Nguyen *et al.* [24]. The discrepancy is attributable to sample locations, PCR profiles, and DNA quality due to different reagents used for DNA extraction. The analysis of sequencing results using BLAST (NCBI) revealed a similarity of 95 % to cloning and binary vectors such as pRATIO3212-SMXL7, pRATIO3212-SMAX1, and pRATIO1212-SMAX1D2. All of these binary vectors are driven by the *CaMV P35S* [25].

The PCR results for samples S9, S19, and S20 were negative for *CaMV P35S* (the result of agarose gel not shown for samples S19 and S20). Both S19 and S20 soybeans were sold in Sibu's supermarket under organic and GM-free. However, further investigation by Sani *et al.* [26] using the same DNA extracted from S19 and S20 in this study has detected approximately 118 bp *cp4 epsps* and 115 bp *TNOS* gene sequences in both samples, thus revealing that S19 and S20 are glyphosate-resistant GM soybean varieties. This finding is in line with the information provided in GenBit and ISAAA database as not all of the GM soybeans approved in Malaysia carried both the *CaMV P35S* and *TNOS,* thus suggesting the mislabelled of S19 and S20 as organic soybean products [26]. A similar mislabelling case was reported in Japan in 2002, where 25 tofu and natto sold under organic labels were found to contain GM soybean [29]. Mislabelling is categorized as a technical food fraud risk that involves intentional mislabelling of product content [30]. Mislabelling led to unfair trade and violating the consumer's rights.

Although Malaysians consume soybeans as food and drink, the country does not cultivate this legume [31]. The largest supplier of soybeans to Malaysia is the United States [32] and the most widely cultivated type of GM soybean in the US is herbicide-tolerance (HT) [33]. Other cultivated GM varieties in the US are insect resistant (Bt) and stacked varieties, which combine both HT and Bt traits [33]. The U.S Soybean Export Council estimated that the soybean traded to Malaysia for human consumption is around 70, 000 tons [32]. As of July 2023, 11 events of GM soybeans have been approved in Malaysia for food and feed; either for direct use or as an additive [28].

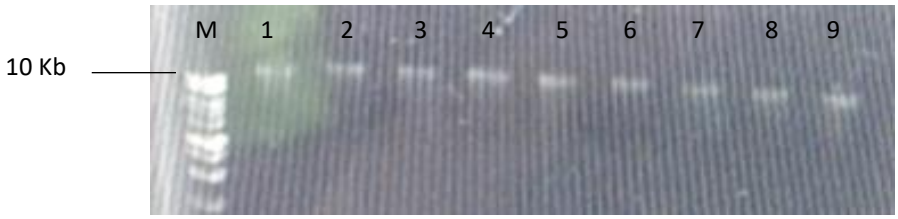
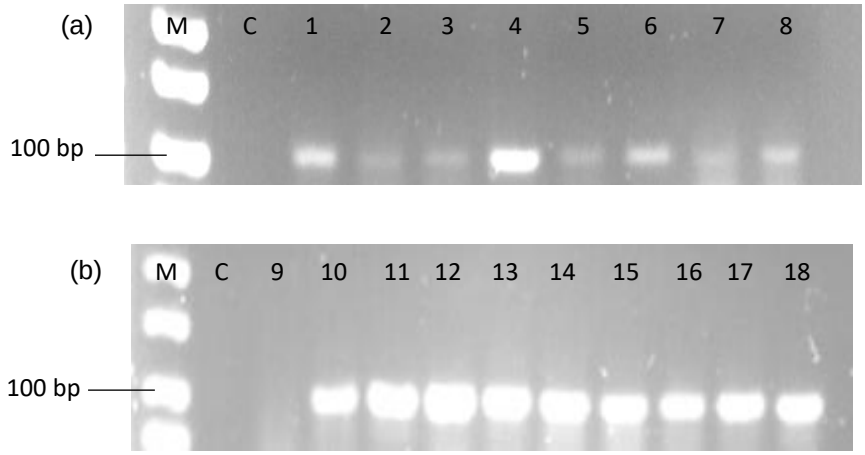


Figure 2. Analysis of DNA extraction on 1% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, United States). The DNA extraction was carried out using NucleoSpin® Food (Macherey Nagel, Germany) with minor modifications. Lane M: GeneRuler 1 Kb DNA Ladder (Thermo Scientific, United States). Lanes S1-S9: Soybean sample 1 – Soybean sample 9.

Since GM foods are not produced naturally, the public perception of GM food differs. Based on statistics as of September 2018, 60 % of Malaysians stated they would never consume genetically modified foods, while 33 % stated they would [34]. It is believed that the most serious risk posed by GM foods is that they are harmful to human health, such as the development of antibiotic-resistant diseases [4]. Considering the prevalence of GM foods in our food chain, concerns regarding their safety are valid [35]. In response to consumer concerns, several states have been working on legislation to require labelling of GMO-containing products [35]. The GM food labelling is important to provide information so that consumers could have informed choices [36]. The adoption of the threshold level for mandatory GM labelling varies from country to country with levels ranging from 0.9 – 5 % [37]. In Malaysia, for food that is composed of genetically modified organisms the words "genetically modified (name of the ingredient)" shall appear on the label. However, the labelling requirements shall not apply to foods that contain, consist of, or are produced from GMOs in a proportion of not more than 3 % of the food ingredients, considered individually, or foods consisting of a single ingredient, provided that this presence is accidental or technically unavoidable [38]. This guideline is provided following the requirements of Malaysia Food Regulation 1985, Regulations 11(3A), 11(6), 11(7), and 17(3A) [39]. This requirement also serves as a guideline for food industries, consumers, and authorized or regulatory officers who work closely with GM food products [39]. Nonetheless, our investigation in this study observed that 90% of the samples collected from four major cities in Sarawak lacked labelling information.



Figures 3(a) and 3(b). Analysis of PCR products on 3% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, United States). DNA amplification was conducted using a combination of primers P35S-F and P35S-R to detect the presence of the 108 bp *CaMV P35S* gene sequence in DNA extracted from 20 raw soybeans (S1- S20). Lane M: GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific, USA). Lane -ve: Negative control. Lanes 1-18: Soybean Sample 1 – Soybean Sample 18. Result for S19 – S20 are not shown.

CONCLUSION

In this study, out of 20 raw soybean samples tested, (17) 85 % of samples were positive for 108 bp of *CaMV P35S* sequence, hence preliminarily indicating the prevalence existence of GM soybean in Sarawak's traditional markets, grocery stores, and supermarkets. The finding suggests that GM labelling should be strictly enforced in Sarawak to protect consumers' rights. Future works include using more advanced, specific, and sensitive DNA-based methods such as real-time PCR or drop digital PCR for detection.

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