International Food Research Journal 15(2): 155-166 (2008)

Detection of Genetically Modified Organisms (GMOs) Using Molecular Techniques in Food and Feed Samples from Malaysia and Vietnam

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Abstract: Food labeling in accordance with Novel Food Regulation has been enforced in the European Community since 1997 with a series of updated legislations namely, EC/258/97, EC/1139/98, EC/49/2000, EC/50/2000 and EC/1829/2003. Guidelines and labeling regulations for the use of GMOs materials in food and feed products has also been introduced in Malaysia and Vietnam. Therefore, the demand for the establishment and development of a robust and rapid operation procedure for GMO detection has increased recently in both countries. The procedure of GMO detection emphasizes not only on detection tests but also on confirmation assays. This study employed PCR technology for detection and direct DNA sequencing for confirmation procedures respectively. The results demonstrated for the first time the presence of GM plants with glyphosate-resistant trait led by the control of P35S promoter and NOS terminator in either Malaysian or Vietnamese feed with high frequency (20 positive samples out of 24 analyzed samples). The P35S promoter, EPSPS gene and NOS terminator sequences obtained showed some mutations on single-stranded and double-stranded targeted sequences caused by single nucleotide insertion or single nucleotide changes. These results reinforce the need for development of detection procedures to comply with food/feed labeling system.

Keywords: GMO detection, PCR, DNA sequencing, animal feed, food

INTRODUCTION

The necessity for labeling of food derived from genetically modified organisms (GMOs) to comply with the Novel Food Regulation (EC/258/97, EC/1139/98, EC/49/2000, EC/50/2000 and EC/1829/2003) has been updated continuously in European countries. In the past 9 years, there has been a dramatic increase in the numbers of GMOs being commercialized. Similarly, the area of land used for the cultivation of GM plants has also increased steadily with the estimated global area of 81 million hectares in 2004 compared to 67.7 million hectares in 2003 (James, 2003; 2004). According to Ahmed (2002), most of the currently available GMOs worldwide contain any of three genetic elements: the cauliflower mosaic virus (CaMV) P35S promoter, the nonpalin synthase (NOS) terminator or the kanamycin-resistance marker gene (nptII). In addition, herbicide resistance led by the introduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene into plant genome is the dominant trait in GM crops (Hemmer, 1997; James *et al.*, 2003). The two most cultivated GM plants globally are the biotech soybean (60% of the global biotech area) and biotech maize (23% of the global area) (James, 2004), which represent the staple constituents of many food types (Gachet *et al.*, 1999; Cardarelli *et al.*, 2005). As a result, the use of GMOs as food or in food products is becoming more and more popular and widespread. Again, this issue has gained the most public attention in 2004 when the GM materials were detected in ten out of 25 GM-

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free/organic food samples in the United Kingdom. Both cases, GM-free and organic foods, implied the absence of GM ingredients (Partridge and Murphy, 2004). This led many governments especially those of the European Union to implement the legislation of mandatory labeling for GMOs derived food/feed and food ingredients to control the possible impact of GMOs on public health and the environment (Cardarelli *et al.*, 2005; Hernandez *et al.*, 2005). Malaysia drafted a Biosafety Law in 1997 while Vietnam has applied the labeling system for 72 GMO derived products in September 2005 (http://www.vnexpress.net/Vietnam/Khoa-hoc/2005/09/3B9E1C95/). Despite these developments, there are few investigations, conducted on the distribution of GMO or contamination of GMO in food and feedstuffs in Malaysia and Vietnam. Nevertheless, the implementation of any labeling system will require the development of reliable and accurate detection methods for GMOs. Polymerase chain reaction (PCR) is the method of choice used by most analytical laboratories for the detection of GMOs and GMO-derived materials because of its high sensitivity, specificity and its capability to detect wide range of constructs (Giovannini and Concillio, 2002; Holst-Jensen *et al.*, 2003; James *et al.*, 2003; Forte *et al.*, 2005; Hernandez *et al.*, 2005). In addition, a confirmatory assay of the identity of the amplicon is required to ensure that the amplified DNA product actually corresponds to the chosen target sequence and is not non-specific binding of primers during PCR amplification (Wolf *et al.*, 2000; Anklam *et al.*, 2002). DNA sequencing is the most reliable method to confirm the authenticity of PCR products (Gachet *et al.*, 1999; Anklam *et al.*, 2002).

This study was conducted to survey the distribution and contamination of GMO in Malaysia and Vietnam. The complete procedure of GMO detection in food and animal feed (AF) stuffs from DNA extraction, screening for promoter and terminator elements and detection of glyphosate-resistant trait by polymerase chain reaction (PCR) and confirmatory assays by DNA sequencing is described in this paper.

MATERIALS AND METHODS

Sample Collection

A total of 60 food and feed samples containing soybean and maize were gathered randomly from the local markets and supermarkets around Serdang and Seri Kembangan, Malaysia. Similarly, all the samples from Vietnam were collected at local markets and animal feed agencies around Cantho city. There were 12 soybean samples labeled S1 to S12, 24 maize samples labeled M1 to M24 and 24 animal feed samples labeled AF1 to AF24. Among them, S11, S12, M21 to M24, AF17 to AF24 originated from Vietnam while the others came from Malaysia. Roundup Ready soybean powder (RR soybean) was used as standard for glyphosate-tolerant soybean at 5% w/w of GM soybean. The certified reference material developed by the Institute for Reference Materials and Measurement (IRMM, Geel, Belgium) was purchased from Fluka Chemical Co. (Switzerland) and stored at -18°C until use.

DNA Extraction and Quantification

CTAB (cetyltrimethylammonium bromide) extraction, the basis for the official German method, proposed by the CEN (European Committee for Standardization) in 2002 for the detection of genetically modified foods was used to extract genomic DNA from all samples. Homogenized samples of up to 350 mg were mixed with 500 µl CTAB buffer [20 g/L CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8), 20 mM EDTA] and incubated at 65°C for 30 min. The samples were then centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a new 1.5 ml tube, extracted with 200 µl chloroform and centrifuged again for 10 min at 13,000 rpm. The upper phase was transferred into a new 1.5 ml tube, precipitated with 1 volume of isopropanol and centrifuged for 10 min at 13,000 rpm. The supernatant was discarded and the pellet was washed once with 500 µl of 70% ethanol before air-drying for approximately 45 min. The pellet was dissolved in 100 µl distilled water and stored at -18°C until use. The quality and quantity of DNA extracted from samples were determined by using a Eppendorf Biophotometer model 6131 spectrophotometer at 260 nm (A_{260}) and 280 nm (A_{280}) absorbance. The DNA purity was determined by using A_{260}/ A_{280} ratio.

PCR Amplification

PCR amplification was carried out in a PCR mix of 25 µl on a PTC-200 thermal cycler (MJ Research, Watertown, MA). The final concentrations of each PCR were as follows: 2.5 µl of 10 x PCR buffer (Finnzymes, Finland); 100 ng of genomic DNA; 0.5 µM of each primers; 200 µM of dNTPs mix; 0.625 unit/reaction of DyNAzyme II DNA polymerase. Oligonucleotide primers were synthesized by Research Biolabs Sdn Bhd. (Malaysia). All the oligonucleotide primers were diluted to working concentration of 10 pmol/µl with deionized water

Table 1: Sequences of oligonucleotide primers used in this study

Primer	Sequence	Gene specificity	Amplicon (bp)	Reference
LEC1	5'-GTG CTA CTG ACC AGC AAG GCA AAC TCA GCG-3'	Soybean *lectin*	164	Vollenhofer *et al.*, 1999
LEC2	5'-GAG GGT TTT GGG GTG CCG TTT TCG TCA AC-3'			
ZE03	5'-AGT GCG ACC CAT ATT CCA G-3'	Maize *zein*	*277*	Pauli *et al.*, 2000
ZE04	5'-GAC ATT GTG GCA TCA TCA TTT-3'	CaMV P35S		
P35S 1-5'	5'-ATT GAT GTG ATA TCT CCA CTG ACG T-3'	promoter	101	Matsuoka *et al.*, 2002
P35S 2-3'	5'-CCT CTC CAA ATG AAA TGA ACT TCC T-3'	NOS terminator		
HA-nos118-F	5'-GCA TGA CGT TAT TTA TGA GAT GGG-3'	*CP4-EPSPS*	118	Lipp *et al.*, 2001
HA-nos118-R	5'-GAC ACC GCG CGC GAT AAT TTA TCC-3'		118	Matsuoka *et al.*, 2002
EPSPS 1-5'	5'-GCC TCG TGT CGG AAA ACC CT-3'			
EPSPS 3-3'	5'-TTC GTA TCG GAG AGT TCG ATC TTC-3'			

and stored at -18°C until use. The sequences and amplification conditions are presented in Tables 1 and 2.

DNA Sequencing

The amplicons of P35S promoter, EPSPS gene and NOS terminator were excised from the agarose gels and purified by QIAquick gel extraction kit (Qiagen, Germany) according the manufacturer's instructions. Purified products were sequenced using the ABI PRISM 7700 sequencer. The BLAST (Basic Local Alignment Search Tool) software (http://www.ncbi.nlm.nih.gov/) was used to analyze the sequence. The alignment of the sequences with several closely related genes was carried out using the Clustalw program from the BioEdit software version 6.0.

Agarose Gel Electrophoresis

Amplicons were analyzed in 1.8% agarose gel electrophoreses in a 1 x TBE [10 mM Tris-base (pH 8); 2.75 g Boric acid/L; 1mM EDTA (pH 8)] and visualized under UV transilluminator after staining with 0.5 g/ml of ethidium bromide.

RESULTS

DNA Extraction and Amplification

Most of the DNA extracted by CTAB methods in this study showed a high molecular weight and high purity with A_{260}/A_{280} ratios ranging from 1.8 to 2.0. The purity of DNA extracted from samples was confirmed by PCR amplification using soybean-specific (*lectin* gene) and maize-specific (*zein* gene) primers for samples derived from soybean and maize, respectively. The amplicon of *lectin* gene (Vollenhofer *et al.*, 1999) was 164 bp, while the amplicon of *zein* gene (Pauli *et al.*, 2000) was 227 bp which corresponded with the reported sizes. These tests also indicated whether the AF products contained either soybean or maize materials. Overall, all the DNA stocks from soy, maize and AF samples extracted by the CTAB protocol (Tinker *et al.*, 1993) were good enough as templates for PCR amplification using *lectin* and *zein* specific primers. AF samples 6, 8, 15, 16, 19 and 20 did not contain soybean or maize (Table 3).

Table 2: Amplification condition for PCR

Step	LEC1/LEC2			ZE03/ZE04			P35S 1-5' / P35S 2-3' EPSPS 1-5' / EPSPS 3-3'*			HA-nos118-F/ HA-nos118-R		
Pre-denaturation	12 min, 95°C			4 min 30 sec, 95°C			10 min, 95°C			10 min, 95°C		
Denaturation	1 min, 95°C			1 min 45sec, 96°C			1 min, 95°C			25 sec, 95°C		
Annealing	30 sec, 72°C	50		2 min, 60°C	40		2 min, 55°C	45		30 sec, 62°C	50	
Extension	30 sec, 72°C	cycles		1 min 50 sec, 72°C	cycles		2 min, 72°C	cycles		45 sec, 72°C	cycles	
Final extension	10 min, 72°C			4 min 50 sec, 72°C			7 min, 72°C			7 min, 72°C		

Table 3: Summary of sample status with three basic assays in GMO detection

Samples	Lectin gene	Zein gene	P35S Promoter	NOS Terminator	EPSPS gene	Conclusion GM (+) or non-GM (-)
S1-S12	12+/12		-	-		-
M1-M24		24+/24	-	-		-
AF1	+	+	+	+	+	+
AF2	+	+	+	+	+	+
AF3	+	+	+	+	+	+
AF4	+	+	-	+	-	+
AF5	+	+	+	+	+	+
AF6	-	+	+	+	+	+
AF7	+	+	+	+	+	+
AF8	-	+	-	+	-	+
AF9	+	+	-	+	+	+
AF10	+	+	+	+	+	+
AF11	+	+	+	+	-	+
AF12	+	+	+	+	+	+
AF13	+	+	+	+	+	+
AF14	+	+	+	+	+	+
AF15	-	-	-	+	-	+
AF16	-	-	+	+	-	+
AF17	+	+	+	+	+	+
AF18	+	+	+	+	+	+
AF19	+	-	-	-	-	-
AF20	+	-	-	-	-	-
AF21	+	+	+	+	+	+
AF22	+	+	+	+	+	+
AF23	+	+	-	-	-	-
AF24	+	+	-	-	-	-

Screening for the P35S Promoter and NOS Terminator

After PCR amplifications of the *lectin* and *zein* genes, all the DNA stocks were subjected to PCR amplification of the 35S promoter and terminator. Two primers, P35S (Matsuoka *et al.*, 2002) and HA-NOS 118 (Lipp *et al.*, 2001), which are specific to the 35S promoter originating from CaMV virus and NOS terminator originating from *Agrobacterium tumefaciens*, respectively, were selected for PCR analysis. Screening methods using the 35S promoter and NOS terminator sequences evidently are the most favorable candidates for broad method applicability. Most of the currently available GMOs worldwide contain any of three genetic elements: the cauliflower mosaic virus (CaMV) 35S promoter, the nonpalin synthase (nos) terminator or the kanamycin-resistance marker gene (*npt*II). For instance, 22 and 16 out of 28 commercially available GM crops contained the 35S promoter and NOS terminator, respectively (Hemmer, 1997). In addition, a suitable PCR product for GMO detection should be small in size ranging from 150 bp to 300 bp especially when the samples were processed foods.

The results in Table 3 show that none of the soy and maize samples was positive for the 35S promoter amplification. Similarly, no positive result was obtained from the NOS terminator assay on both soy and maize samples. It can be concluded that both the soy and maize samples were not contaminated by GM materials containing 35S promoter and NOS terminator. In contrast, 20 out of 24 AF samples were positive for the P35S promoter (Figure 1). Among these 20 AF samples, eight were from Vietnam and twelve from Malaysia. In the NOS terminator assay, 20 AF samples showed positive signals. Interestingly, all 16 AF samples from Malaysia and four from Vietnam were contaminated by the presence of NOS terminator. The results are summarized in Table 3.

Screening for Glyphosate-Tolerant Trait by the Presence of EPSPS Gene

After the initial screening steps, specific detection was done to determine the structural genes of the introduced traits. Two main traits of interest mostly used in the construction of transgenic plants are herbicide tolerance and insect resistance. Herbicide tolerance is the leading trait in

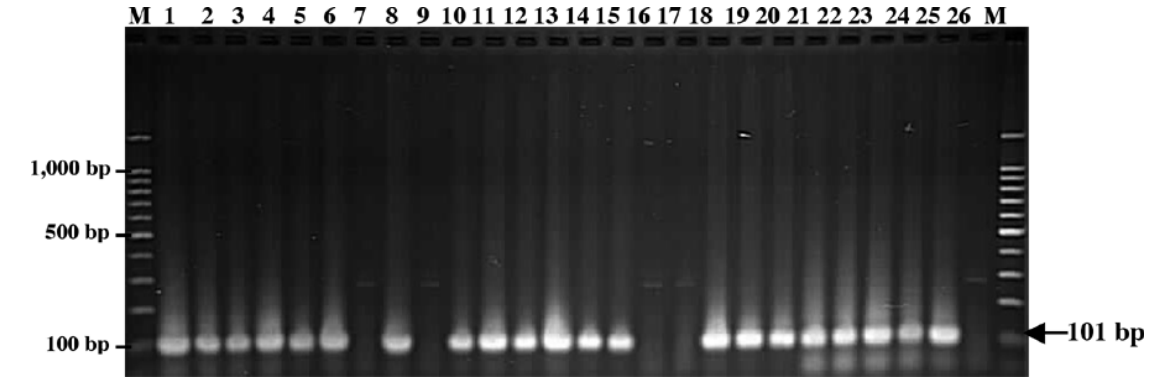

Figure 1: Agarose gel electrophoresis (1.8%) of amplification products of 35S promoter from AF samples
Lane M: 100 bp DNA ladder (New England Biolabs); Lane 1: positive control; Lane 26: negative control; Lanes 2 to 25: AF1 to AF24

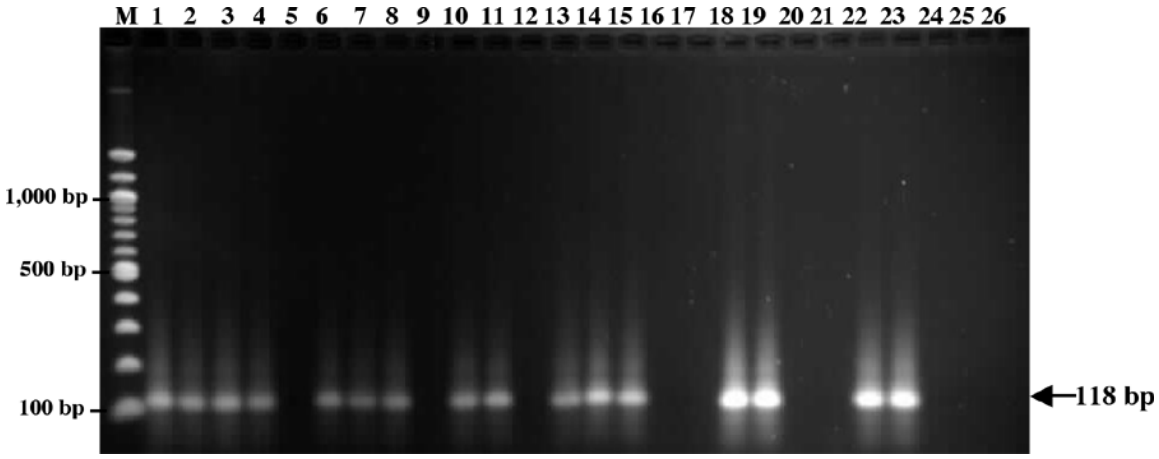

Figure 2: Agarose gel electrophoresis (1.8%) of amplification product of EPSPS gene from AF samples
Lane M: 100 bp DNA ladder (New England Biolabs); Lane 1: positive control; Lane 26: negative control; Lanes 2 to 25: AF1 to AF24

commercialized GM plants with 23 lines having been approved for cultivation and/or food and feed use worldwide (http://www.agbios.com/dbase.php; Stirn and Lorz, 2003). The results showed that 15 out of 24 AF samples were positive when they were tested for the presence of the EPSPS gene. Among the 15 EPSPS positive samples, four of them came from Vietnam with clear DNA bands and the rest of the 15 EPSPS positive AF samples were from Malaysia. The positive samples from Vietnam originated from Taiwan and Korea while the sources of positive samples from Malaysia were not definitive. The results for the 60 samples analyzed are shown in Table 3.

Confirmatory Assay of PCR Products Using DNA Sequencing

DNA sequencing appeared to be the method of choice to verify the PCR products in order to rule out false-positive or false-negative results in GMO detection procedure (Gachet *et al.*, 1999; Anklam *et al.*, 2002). Therefore, DNA sequencing was chosen for the verification of PCR products in this study to get unambiguous and unbiased conclusion. Purified PCR products using the QIAquick gel extraction kit (Qiagen, Germany) were sequenced. The sequencing results of P35S promoter, EPSPS and NOS terminator were compared to those in the Genbank database managed by the National Center for Biotechnology Information (NCBI) by using BLASTN program (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.*,

1997). According to Pearson (1991), BLAST score greater than 80, or expected (E) value less than 1 x 10^{-4}, are considered as significant matches. The expected values of P35S promoter, EPSPS and NOS terminator were 1 x 10^{-42} (score = 178), 4 x 10^{-40} (score = 170) and $3e^{-50}$ (score = 204), respectively. In other words, these E values fulfilled the requirements of significant matches.

Alignment of P35S Sequence

The P35S sequence result in Figure 3 showed high similarity of 97% identities to construct vectors, pGA1611, pRE1, pAMPAT-MCS, pgR106 and pgR107, and the CaMV genome. Those vectors contained genes under the control of 35S promoter from CaMV (Lu *et al.*, 2003). Therefore, CaMV genome would consist of the sequence for 35S promoter of transgenic plants (Wolf *et al.*, 2000). However, there were some minor changes in single stranded DNA of two directions (forward and reverse) sequencing results.

There were 15 mismatch points of the P35S promoter sequence in comparison with pGA1611, pRE1, pAMPAT-MCS, pgR106 and pgR107 construct vectors and CaMV genome in which three of them were located in forward strand and the rest were detected in reverse strand. In addition, a single nucleotide insert occurred on the forward strand at the 57th nucleotide (Figure 3). According to NCBI information (http://www.ncbi.nlm.nih.gov/About/primer/est.html), the 3'-DNA sequence is less conserved in comparison with 5'-DNA sequence. Another possibility of the mismatches were due to the mismatches of *Taq* polymerase during PCR amplification and troubleshooting in DNA sequencing; for example gel electrophoresis, the appearance of the sequencing ladder, anomalous spacing of band (Surzycki, 2000) rather than mutations because of the location of nucleotides changed on single stranded sequence only. However, the final sequence of 35S promoter could be synthesized by the assembly of forward and reverse sequence. Based on the final sequence, there was complete similarity in comparison with the original sequences of 35S promoter in database.

Alignment of EPSPS Sequence

Similar to the 35S promoter, the comparison between the final EPSPS gene sequence and synthetic construct CP4EPSPS protein and *Glycine max* CP4EPSPS genes were almost identical with 95% identities. Based on the available database in Agbios (http://www.agbios.com/dbase.php), the CP4EPSPS coding sequence isolated from *Agrobacterium sp.* strain CP4 was constructed in the transformation vector to introduce glyphosate-tolerant trait into transgenic plants particularly soybean, Roundup Ready soybean (Windels *et al.*, 2001) and maize, Mon802 maize (Matsuoka *et al.*, 2002). In Figure 4, there were two insertions on the double-stranded DNA at positions 69 and 78 in the displayed sequence. Other insertions located in the single-stranded sequence were at positions 43, 56 and 96 on the forward strand and positions 71 and 80 on the reverse strand. The insertions resulted in the change of whole amino acid sequences of EPSPS gene from the inserted points. If those insertions were permanent in the final sequence of EPSPS gene, they would have an impact on the structure of EPSPS proteins. As a result, the glyphosate tolerance trait in transgenic plants would be affected.

Alignment of NOS Terminator Sequence

Like EPSPS structural gene, there were three single nucleotide insertions within the NOS terminator in both forward and reverse strands at position 35 ('G' inserted in forward strand), 66 ('T' inserted in reverse strand) and 87 ('A' inserted in reverse strand) in the displayed sequences in Figure 5. In addition, three single nucleotide changes in reverse primer sequence were found at positions 98 (change from 'C' to 'T' in reverse strand), 100 (change from 'A' to 'T' in reverse strand) and 101 (change from 'G' to 'A' in reverse strand) in the displayed sequences. However, the final sequence of the NOS terminator could be produced with minor changes except at insertion points with 97% identities.

In conclusion, those mutations caused by single nucleotide insertions or single nucleotide changes might have resulted from the mismatches of *Taq* polymerase during PCR amplification to get the amplicons and the troubleshooting of DNA sequencing rather than the permanent changes themselves because of the location of nucleotide changes on single stranded sequence only. They can also occur in transformation processes or natural selections to transgenic plant and should be taken into consideration because of their unpredictable impact on either human or animal health. In general, all of the achieved sequences (35S promoter, EPSPS coding sequence and NOS terminator) matched tightly to the respective sequences in database with identities over 95% and confirmed the accurateness of primers (P35S 1-5'/P35S 2-3'; EPSPS 1-5'/P35S 2-3' and HA-nos118-F/ HA-nos118-R) in PCR amplification.

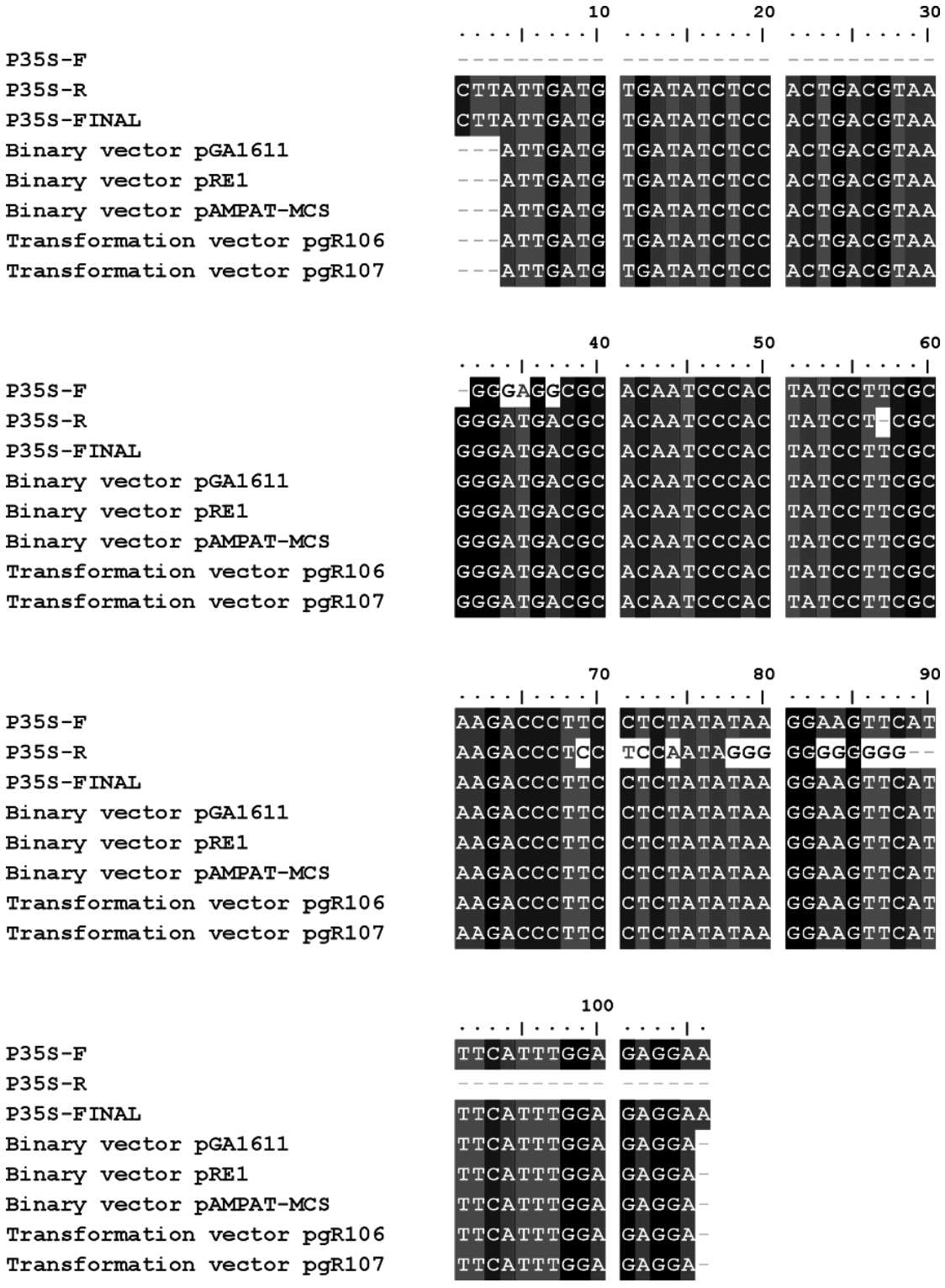

Figure 3: The comparison of partial sequence of P35S promoter from Cauliflower mosaic virus with the most five-respective sequences in database through BLASTN program

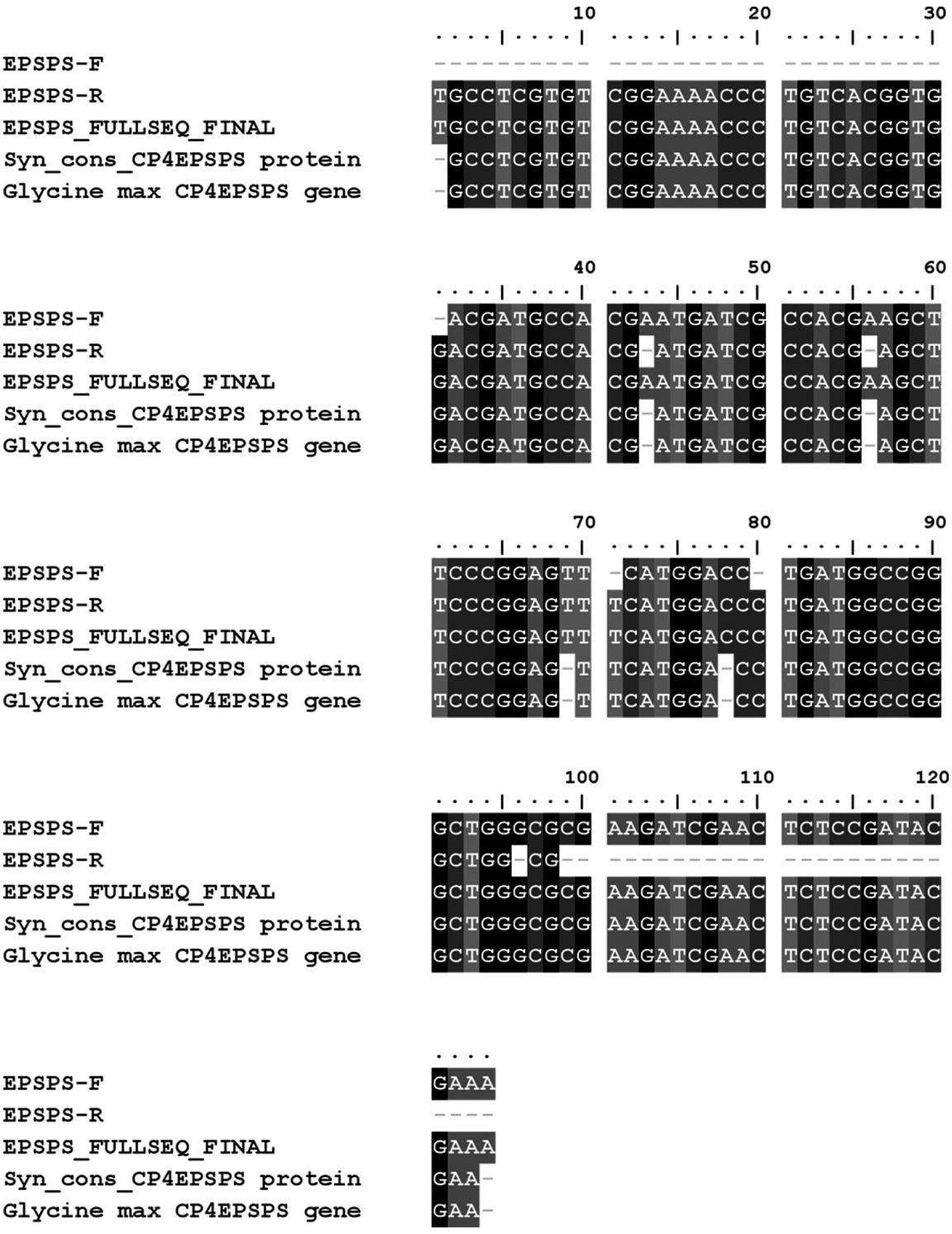

Figure 4: The comparison of partial sequence of EPSPS gene referring to glyphosate tolerance with the most two-respective sequences in database through BLASTN program

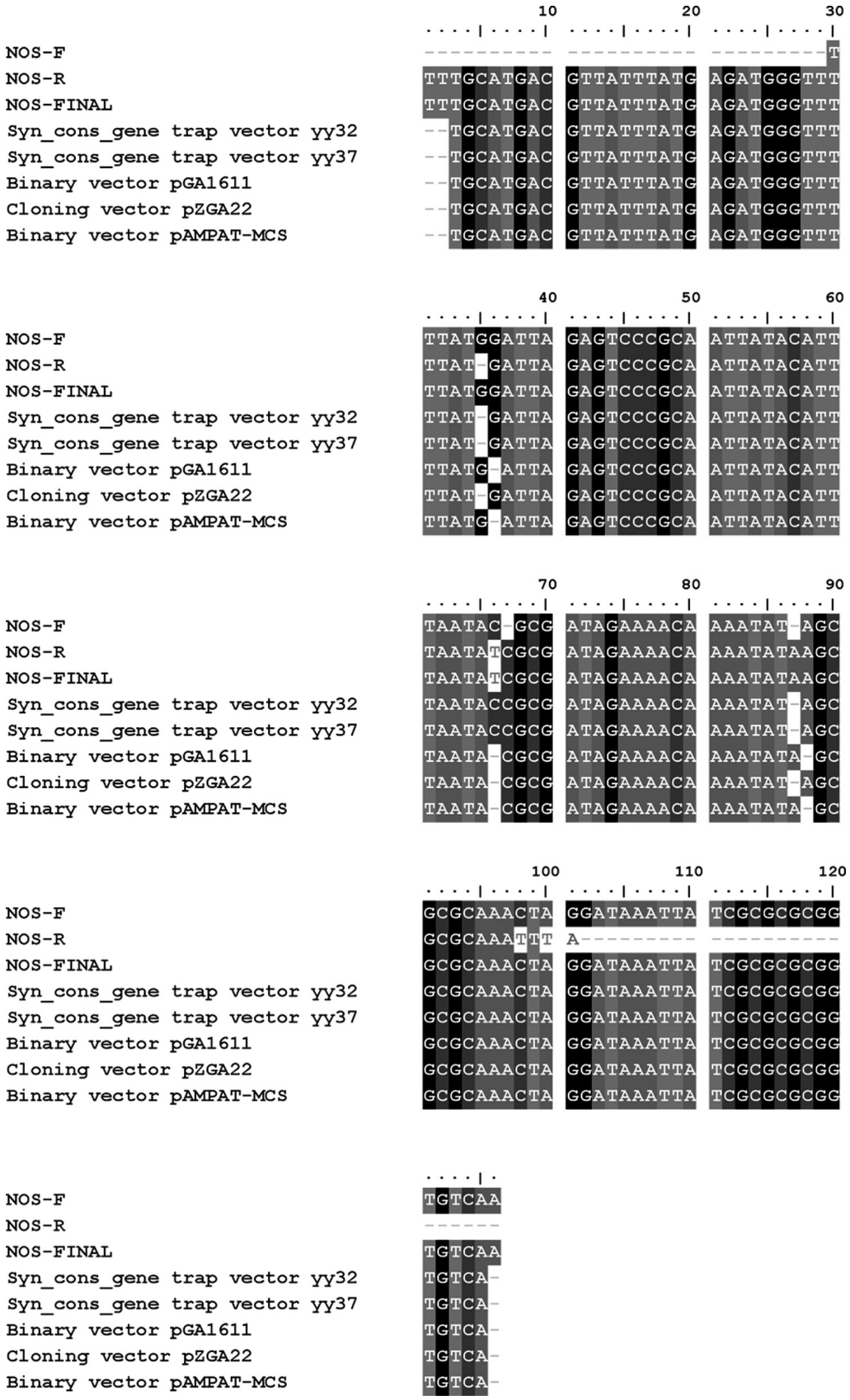

Figure 5: The comparison of partial sequence of NOS terminator from *A. tumefaciens* with the most five-respective sequences in database through BLASTN program

CONCLUSION

The results in this study demonstrated the presence of glyphosate-resistant GM plants under the control of P35S promoter and NOS terminator in animal feed products with relatively high frequency, 20 out of 24 analyzed samples from Malaysia and Vietnam. It was interesting to note that all the tested animal feed samples from Malaysia contained GM-contaminants. Malaysia and Vietnam do not produce GM plants but the results of this study indicated that the distributions of GMO are quite significant. The possible explanation for this case may be that the GM materials originated from contaminated or ambiguous imported sources. These results serve as the first document marking the presence of GMO in Malaysian and Vietnam markets. These results also indicate that there is a need to carry out more and thorough studies to determine the distribution of other GM materials in the two countries. In Japan, more than 70% of feedstuffs were imported from foreign countries especially the United States of America (Yonemchi *et al.*, 2003). Therefore, they have good analytical laboratories to monitor the presence of GMO in imported food. Malaysia and Vietnam are expected to carry out the same tasks when the Biosafety Law or GMO labeling guidelines of the two countries are fully implemented in the future.

This study proposed a complete procedure of GMO detection in food and feed products with the key advantages of high sensitivity, and robust and rapid operation in both the detection and confirmation steps. Five primer pairs of LEC1/LEC2; ZE03/ZE04; P35S 1-5'/P35S 2-3'; HA-NOS118-F/HA-NOS118-R and EPSPS 1-5'/EPSPS 3-3' chosen in this study fulfilled the product-size requirement and completed the whole GM event detection procedure for raw soybean and raw maize as well as the animal feed samples. In addition, PCR amplification and DNA sequencing protocols presented in this study could provide a very useful tool for routine detection of GM events in foods and feeds with regard to false-negative and/or false positive results. Finally, the original CTAB protocol was the most suitable protocol to extract genomic DNA from raw soybean, raw maize and AF samples. This study serves as an initial indicator of the distributions of some of the GM material in Malaysia and Vietnam. We hope that some of the methods described in this paper are useful to laboratories intending to conduct GM detection.

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