

# Genetically Engineered (GE) Herbicide-Tolerant Gene was Detected on Local Soybean of Indonesia

Genesisiska<sup>1</sup>, Rachma Camelia Suratmi<sup>2</sup>

<sup>1,2</sup>Department of Agrotechnology, Faculty of Agriculture, Universitas Muhammadiyah Yogyakarta, Indonesia. 55183

{genesiska@umy.ac.id<sup>1</sup>, rachmacamelias@gmail.com<sup>2</sup>}

**Abstract.** Fifty percents of soybean in Indonesia has been imported from the United States. Most of 94 % imported soybean was genetically engineered soybean. In meanwhile, products of Genetically Modified Organism (GMO) of Indonesia were not labeled yet in the market packaging products. This aim of the study study was to detect EPSPS-CP4 gene which potentially indicates of transgenic gen of soybean in order to help Indonesia to implement government regulation on label and food advertisement and fulfill consumer right. The result indicated that the local soybean also contained Genetically Engineered (GE) herbicide-tolerant gene called EPSPS-CP4 gene of transgenic soybean by using Polymerase Chain Reaction (PCR) method. Those Local soybeans brand name sold in the market were *Lokal*, *Galunggung*, and *Wonosari*.

**Keywords:** *Consumer Right, Genetically Engineered, Herbicide-Tolerant Gene, Local Soybean.*

## 1 Introduction

Soybean is a raw material of food products after rice and maize. In Indonesia, productivity of the commodity only 1.3 tons/ha in meanwhile the potential of production might reach up to 2.0 - 2.5 ton/ha. Every year, production of *Témpe* required at least 1.85 million tons of soybean as well as soy and *tauco* products spent at least 8.32 tons of soybean [1]. However, production of soybean in Indonesia only reached 65.61 % for domestic consumption. Thus, national security of soybean was up to 35 % solved from import of soybean [2]. Instability of soybean production in Indonesia happened due to decreasing of harvesting area was not equal to increasing of soybean production [3].

Export rate of Indonesia decreasingly up to 5, 92 % from 1961 to 2012. Unbalance of production and consumption of soybean affects high import rate of soybean every year. Import rate increases up to 0, 05 % per year. Fifty percent of soybean import in Indonesia originated from United States cultivar. Most of 94 % of soybean production and consumption from United States is transgenic soybean [2]. Transgenic soybean called genetically modified soybean seeds for food production has been used continuously throughout the world [4].

Commonly, engineered transgenic plant is designed to have resistance to the herbicide in order to gain agricultural efficiency improvement. The broad-spectrum herbicide called Roundup Ready has the active ingredient namely Glyphosate. The herbicide ingredient known could inhibit of 5-enolpyruvylshikimate-3-phosphate (EPSP) Synthase [5]. EPSPS enzyme has the essential function toward biosynthesis of aromatic amino acid for survival of the plants. Therefore, the transgenic plant is created by carrying out the gene coding for glyphosate

insensitive form of the enzyme which obtained from *Agrobacterium* sp. Strain CP4. It confers crop resistance to glyphosate, enabling more effective weed control by allowing post-emergent herbicide application [6]. Herbicide resistance plants are created by insertion of Enolpyruvylshikimate-3-Phosphate Synthase called EPSPS gene into the target plant is dominant characters in the GMO crops [7]. Genetically Modified Soybean which tolerance of environmental effects was created by transfer of CP4-EPSPS gene from a soil bacterium *Agrobacterium tumefaciens* L. This could determine the synthesis of a glyphosate insensitive protein called EPSPS-CP4 [8]. This genetically modified soybean was popular by Monsanto [2].

Potential risk of transgenic components such as allergenicity, toxicity, and dietary risks have become important issues around the world. In order to ensure food safety control, all products derived from genetically modified technology has to undergo a comprehensive evaluation before release to the market [9]. According to Indonesian seed import procedures, a regulation based on the Minister of Agriculture Regulation No. 37 and 38/Permentan/OT.140/8/2016 issued on August 31, 2006 stated that import of transgenic seeds for non-research purposes has to pass bio-security and food security test. Food derived from genetic engineering requires labeling. This requirement based on the published guidelines food labeling to implement Government Regulation No. 69/1999 on Label and Food Advertisement [10]. This biosafety including environmental, food and feed safety is proposed to prevent potential adverse risks to biodiversity as result of utilization of GMO and to prevent negative risks to human being, animal and fish health as result of process of production, preparation, storage, distribution and utilization of GMO [11]. Meanwhile, Genetically Modified Organism (GMO) soybean sold in the market of Indonesia obviously were not labeled yet on the packaging.

A number of methods were developed to detect genetically modified organism. Polymerase Chain Reaction (PCR) is one of approaches for detecting transgenic gene [9]. PCR is a technique to amplify DNA molecules enzymatically by temperature range mechanism [12]. This method has high specificity, efficiency and validity [13]. A primer pair is designed based on regulatory sequence for detection of genetically modified plants [14]. Therefore, the aim of this study was to detect CP4-EPSPS gene which potentially indicates of transgenic gen of soybean in order to help Indonesia to implement government regulation on label and food advertisement and fulfill consumer right.

## **2 Material and Methods**

### **2.1 Sample Preparation**

Seed of soybean was collected by method of purposive sampling in four central markets, Pasar Beringharjo, Pasar Sentral, Pasar Prawirotaman and Pasar Gamping in Special Region of Yogyakarta. All local and imported brands of soybean were collected as samples. A blank sample was used as negative control, and non GMO-certified soybean samples var. *Anjasmoro* was used as positive control.

### **2.2 Extraction of DNA**

Fifty to a hundred milligrams of leaves samples of 14 days old seedlings were used for extraction of Genomic DNA mini kit according to Geneaid Biotech Ltd. The DNA pellet was dried and resuspended in 100 milliliters of deionized water (ddH<sub>2</sub>O). Product of extracted DNA was stored at 4°C.

### 2.3 Yield and Quantity of DNA

Quantity and purity of isolated DNA samples were measured by optical densities of 260 nm and 280 nm of genequant 1300 spectrophotometer. Expect value of DNA purification was around ~1.8 ratios of A260/280 nm. DNA purity value was around 2.0-2.2 ratio of A260/230 nm [15]. Extracted DNA was adjusted by dilution to 200-400 ng/μl to PCR total volume.

### 2.4 Detection of 5-enolpyruviyl-Shikimate Synthase (EPSPS)-CP4 Gene

Polymerase Chain Reaction (PCR) is a method to amplify specifically DNA by using two primers of oligonucleotides which helped by polymerase enzyme [16]. In this research, screening of EPSPS-CP4 genes was done by Polymerase Chain Reaction (PCR) methods using specific primers. Primer used and its target in this research are listed in **Table 1**.

**Table 1.** Oligonucleotide primer pair sequence and its targets [8]

Gene specificity	Primer name	Sequences	Amplicon
EPSPS CP4	RRS0t-5 (f)	5'CCT TTA GGA TTT CAG CAT CAG TG-3'	121 basepairs
	RRS01-3 (r)	5'GAC TTG TCG CCG GGA ATG-3'	

PCR conducted in thermocycler (Biorad) using prepared master mix. Ten milliliters of PCR total volume consisted of 2.6 μl of 50-100 ng extracted DNA samples, 0.25 μl of forward primer, 0.25 μl of reverse primer, 2 μl of nuclease-free water, and 5 μl of master mix gotagreen. Temperature profiles used for optimization of primers in the PCR condition explained in **Table 2**.

**Table 2.** Time and temperature profiles for optimal primers [8]

Primer pairs	Initial denaturation	Denaturation	Annealing	Elongation	Cycles	Final Elongation
RRS0t-5 (f)/ RRS01-3 (r)	4 min at 95°C	1 min at 95°C	1 min at 55-60°C	1.5 min at 72°C	34 cycles	7 min at 72°C

### 2.5 Gel Electrophoresis and DNA Visualization

Gel electrophoresis was prepared by using 1.5 % of agarose soluted in Tris/Borate/EDTA (TBE) buffer 1X. Electrophoresis was conducted in 60 Volt for 30 minutes. Total volume of every tube for DNA running was 15 μl consisted of 10 μl of DNA template and 5 μl of staining/loading dye. Preparation of the gel after electrophoresis was soaked by using EtBr for 15 minutes. Then, DNA band in the gel was visualized by UV transilluminator.

### 3 Results and Discussion

Fifty percents of soybean in Indonesia has been imported from the United States. Most of 94 % imported soybean was genetically engineered soybean. In meanwhile, soybean grain of Indonesia were obviously not labeled yet in the market packaging products.

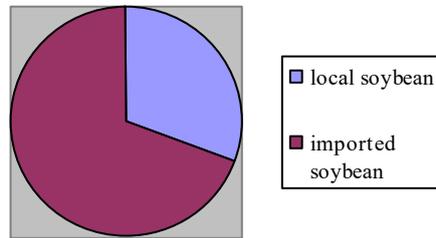
#### 3.1 Percentage of Local and Imported Soybean Based on Observation and Grain Phenotype

In this research, sample collections were conducted by purposive sampling. Based on the interview process and observation of phenotype identification, local brand of soybean has grain phenotype with yellow doff and big grains. This phenotype representative of brand name, *Lokal, Wonosari, and Galunggung*, also Anjasmoro. Meanwhile, imported and phenotype like imported soybean could be recognized with the white color and oval grains. The white color grain was the phenotype representative of brand name, Amerika No. 1, Amerika No. 3, America, No Name no. 1 and No Name No. 2, **Figure 1**.



**Fig 1.** Two phenotypic characters of observed soybean grain from the market, a. Imported and phenotype like-imported soybean could be recognized by the white pale color grains, b. Local soybean has phenotype with yellow doff grains.

Percentage of local soybean grains collected from the markets was about 30, 77 %. Meanwhile, percentage of imported brand of soybean and phenotype like imported soybean which collected from the market was about 69, 23 %, **Figure 2**.



**Fig 2.** Percentage of local and imported soybean grains sold in central markets

Although market traders could mentioned which type of the local and imported brand of soybean, verification method was also required by detection of transgenic gene target. To verify the presence of the Genetically Engineered herbicide-tolerant gene called EPSPS- CP4, isolation of DNA and genotyping analysis by using Polymerase Chain Reaction was conducted.

### 3.2 Yield and Quantity of DNA

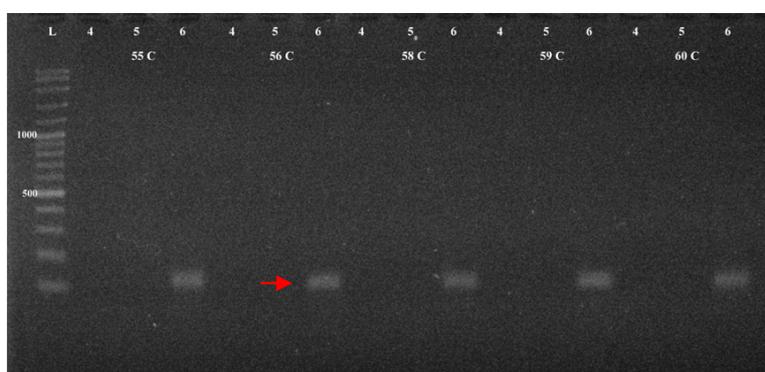
Extracted of selected 10 (ten) DNA samples from local, imported, phenotype like-imported soybean sold in the market was conducted. Then, the DNA purity was measured to ensure quality DNA template for next analysis. A quantitative test of DNA isolation was shown in **Table 3**. Good purity level of DNA is around ~ 1.8 ratio of absorbance level on 260/280 nm and around ~ 2.0-2.2 ratio of absorbance level on 260/230 nm[17]. In this research, DNA concentration was around 200 to 400 ng/  $\mu$ l which qualified for DNA template of Polymerase Chain Reaction (PCR) methods.

**Table 3.** Quantitative test of isolated DNA of soybean (*Glycine max* L.) collected from the market

Code	Market Brand of Soybean	Location	Absorbance level		DNA concentration (ng/ $\mu$ l)
			260/280	260/230	
1	America No. 1	Beringharjo	3,00	3,00	300
2	America No. 2	Beringharjo	1,33	2,00	400
3	America	Gamping	1,00	2,00	200
4	America	Sentral	2,00	2,00	200
5	No name 1	Prawirotaman	2,00	2,00	400
6	No name 2	Prawirotaman	3,00	1,50	300
7	<i>Lokal</i>	Prawirotaman	1,00	1,00	200
8	<i>Galunggung</i>	Beringharjo	2,00	1,00	200
9	<i>Wonosari</i>	Gamping	1,00	2,00	400
10	<i>Anjasmoro</i>	Jawa Timur	1,33	0,80	400

### 3.3 Optimization of Annealing Temperature of EPSPS-CP4 Gene for Polymerase Chain Reaction (PCR) Setting

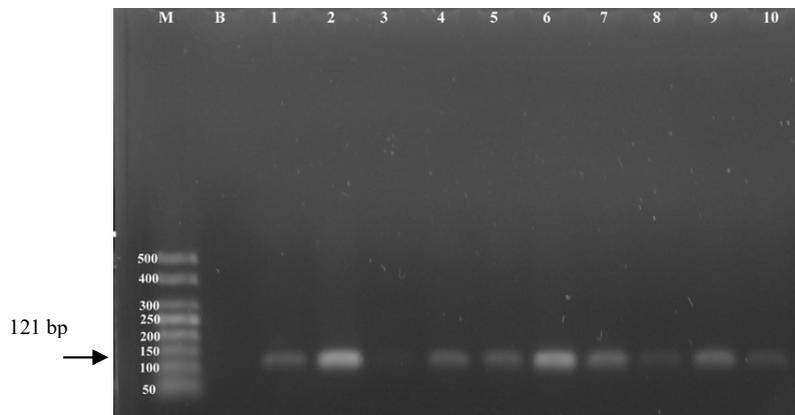
Optimization of annealing steps for PCR was conducted to maximize the process of complementary primers nucleotide sequences to the target nucleotide sequences. Primer of RRS0t-5 and RRS0t-3 (forward and reverse) used to detect EPSPS-CP4 was successfully in the temperature range of 55, 56, 58, 59, and 60 °C. The optimal annealing temperature was 56 °C by signal of the white thickest bands of DNA appearance in the electrophoresis gel, illustrated in **Figure 3**. Appearance of bands indicated optimal temperature of primer could be complement with extracted DNA of samples. Then, this optimal temperature of 56 °C was used in the annealing temperature of primers for PCR program preparation.



**Fig 3.** Visualization of primers optimization of EPSPS-CP4 gene was optimal in annealing temperature of 56 °C by signal of the white thickest bands of DNA appearance on the electrophoresis gel of 1.5 %. Lane L, molecular marker of 100 bp DNA ladder. Lane 4, market brand of soybean America. Lane 5, market brand of soybean called No Name 1. Lane 6, market brand of soybean No Name 2.

### 3.4 Detection of EPSPS (5-EnolPyruvylShikimate-3-Phosphate Synthase)-CP4 Gene

EPSPS, abbreviation of 5-EnolPyruvylShikimate-3-Phosphate (EPSP) Synthase, an enzyme catalyzes a reaction of plant cells in order to synthesize amino acids. Herbicide notably Round-Up could bind enzyme of EPSPS and block the ability. Thus, plants could not produce amino acid then growth and development were stopped. This herbicide is non-selective systemic which aggrieved and kills almost plants. Addition of Chloroplast Transit Peptide (CTP) carried out in the termination of coding region of EPSPS. The sequences were used in order to transfer EPSPS gene to chloroplast cells of plants to finish the function which *Agrobacterium* does not have this sequence, thus CTP addition is important to insert [17]. In this research, size of DNA bands which detect the promoter of EPSPS-CP4 gene were around 100-150 basepairs, visualized in **Figure 4**.



**Fig 4.** Visual detection of EPSPS-CP4 gene of soybean (*Glycine max* L.) using primer RRS0t-5 (f) and RRS01-3 (r) on electrophoresis gel concentration of 1.5 %. Lane L, molecular marker of 50 bp DNA ladder. Lane 1, negative control, no DNA template added. Lane 2, market brand of imported soybean, America No 1. Lane 3, market brand of imported soybean, America No 2. Lane 3, market brand of imported soybean, America from Pasar Gamping. Lane 4, market brand of imported soybean, America from Pasar Sentral. Lane 5, market brand of phenotype like- imported soybean, No Name 1. Lane 6, market brand of phenotype like- imported soybean, No Name 2. Lane 7, market brand of local soybean, *Lokal*. Lane 8, market brand of local soybean, *Galunggung*. Lane 9, market brand of local soybean, *Wonosari*. Lane 10, market brand of certified soybean, *Anjasmoro*.

DNA amplification of soybean (*Glycine max* L.) samples detected by Polymerase Chain Reaction (PCR) by using specific primers revealed positive signals that DNA bands appeared for all samples, except code of soybean sample 3 (three). There were strong signals of white band of DNA appearance from the samples of 3 (three) imported soybean which the Lane 1 (America no 1), Lane 2 (America No 2), and Lane 4 (America), 2 (two) phenotypic like-imported soybean which the Lane 5 (No Name 1) and Lane 6 (No Name 2), 3 (three) local soybean which the Lane 7 (Local), Lane 8 (Galunggung) and Lane 9 (Wonosari), and 1 (one) positive control of certified grain of Lane 10 (Anjasmoro). Those appearances revealed DNA fragment presence which located around 100-150 basepairs. The signals explained that primer pairs of EPSPS-CP4 could be detected with the amplicon products was about 121 basepairs, the similar the result of previous research for detection of EPSPS-CP4 gene [18]. In this research, the results of white band also appeared in Lane 7, 8, 9 and 10 which indicated that the local soybean also contained Genetically Engineered (GE) herbicide-tolerant gene called EPSPS CP4 gene of transgenic soybean. Considering the similar result of identification of transgenic soya products of previous research also revealed positive signals of EPSPS-CP4 gene with the amplification area around 121 basepairs [2].

Although the EPSPS-CP4 gene is not only the gene could indicate the transgenic soybean [18], presence of this gene could be detected accurately to identify of major genetically modified crop species, Roundup Ready soybean [19]. In this research, the local soybean namely *Lokal*, *Galunggung*, and *Wonosari* of Indonesia might be contaminated because of seed producers cultivated during seed producing together with imported soybean. A possible

reason was regular farmers of soybean had main responsibility for the contamination of GM and non-GM soybean by their cultivation without considering distance isolation.

The previous report mentioned that this contamination also happened such similar case in Europe which seed producer might responsible for segregation of Genetically Modified (GM) and non-GM. However, Food and Veterinary Office (FVO) of European Union (EU) in 2007 conducted tests during process of cleaning, sizing and packing [20]. One of most frequent factor affects contamination was isolation distances. The distance could vary a couple of meters to several kilometers, depending on the crop and sometimes on regional characteristics. This measurement can be partial or full replacement by zones between GM and non-GM crops [21].

On the other hand, Indonesia is one of country which classified received the mandatory for labeling of many GE foods and a labeling threshold higher than 1% or undefined. This includes laws with a threshold of 1% for the entire food item [22]. Indonesian farmers are also available to use biotechnology products. The technology rapidly adopted by farmers following commercialization. In meanwhile, the information and general knowledge about biotechnology might not sufficient to the farmer in the field [23]. Assumably, the transgenic imported soybean contaminated extensively to the local soybean by cultivation without compromising the distance isolation. This unpredictable result could remind government in assisting farmer in cultivation system among GMO and non-GMO plants. It also recommend government to be more concern to assist and implement the labeling system of the market products in Indonesia, especially soybean.

#### 4 Conclusion

Genetically Engineered (GE) herbicide-tolerant gene called EPSPS-CP4 gene of transgenic soybean was detected on local soybean grain *Lokal*, *Galunggung*, and *Wonosari* as the similar result of imported soybean grain.

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