Detection of promoter designed for transgenic plant in local soybean

Genesiska* and R C Suratmi
Department of Agrotechnology, Faculty of Agriculture, Universitas Muhammadiyah
Yogyakarta, Bantul 55183, Yogyakarta - Indonesia

*E-mail: genesiska@umy.ac.id

Abstract. Potential risk of allergenic, toxic, and dietary risks from Genetically Modified Organism (GMO) has become critical issues. The transgenic soybean of United States has commonly been exported to countries around the world, including Indonesia. Unfortunately, there has not yet been any label given to packaging product of Genetically Modified Organism (GMO) including soybean grain sold in Indonesia. The aim of the study was to detect promoter of Cauliflower Mozaic Virus 35 S which potentially indicate the transgenic plants. Samples used in this research were 12 different brands of soybean sold in the 4 local markets. Screening of CaMV 35 S genes was done by Polymerase Chain Reaction (PCR) methods using specific primers. The result indicated that positive signals detection refers to transgenic plants in the local soybean grains with the amplification area of 123 basepairs.

1. Introduction

According Imbalance of production and consumption of soybean in Indonesia has lead to increase high import rate of soybean every year. The implication of the condition was Indonesia has imported around 50 % of soybean needs from United States [1]. However, more than 90 percents of soybean production from that country is transgenic plant. The transgenic soybean of United States has been usually exported to countries around the world [2]. Unfortunately, there has not yet been any labels given to packaging product of Genetically Modified Organism (GMO) including soybean grain sold in Indonesia. Recognition of GMO and non-GMO products is one of problems in relation to fulfill consumer rights.

Potential risk of allergenic, toxic, and dietary risks from Genetically Modified Organism (GMO) has become critical issues. All of the products derived from genetically modified technology called transgenic products need to pass an evaluation and assessment before entering the markets in order to ensure food safety controls. In Indonesia, the requirement of products released in the market is based on regulation of No. 69/1999 about Label and Food Advertisement.

According to the genetically engineered plants, promoter of 35S CaMV is one of important genes used to carry out DNA recombinant to almost GMO crops. The function of this promoter is to induce strong and long term expression of genes. This promoter is derived from a virus namely Cauliflower Mozaic Virus (CaMV). The presence of this gene could be used as a signal of transgenic gene [3]. This promoter is reported as the first category targets to detect GMO beside T-Nos (Nopaline Synthase
Terminator) region which all of gene encodes resistance to ampicilin (bla), and kanamycin (nptII) antibiotics [4].

Virus *Cauliflower Mozaic Virus* (CAMV) was first found in 1921 infected Chinese cabbage which caused abnormal phenotype likely mosaic on the leaves surface. This disease was commonly found in crucifer family (Cruciferae or Brassicaceae) such as cauliflower, broccoli, cabbage, and chinese cabbage. The virus vector could be found in three different aphids. Although this mosaic disease could infect many plants, this virus was given a special name of *Cauliflower Mozaic Virus* (CaMV). In 1960, this identified virus consisted of double stranded of Deoxyribonucleic acid (DNA). It indicates that the DNA might be transcripted in plant cells. In 1985, add or deletion expression in plants was impossible to study the specific gene until the identification of 35S promoter of CaMV appeared. Upstream sequences of 35S promoter which consisted of 46 base pairs revealed an expression, and its downstream sequences of 343 base pairs could strongly be expressed in plants. Thus, eighty percent of genetically modified organism harbouring 35S promoter of CaMV such Roundup Ready soybean, Bt corn and cotton, also papaya “sunset” could be resistant to ringspot virus [5].

In plant transformation process, a vector construction requires DNA sequences inserted into target organism [6]. Commonly, such insertion is done to border of T DNA including promoter and terminator sequences. The sequences are known to enable the desired expression of the gene interest. One of promoters is CaMV. This promoter has double strand DNA virus which could infect Solanaceae, and Cruciferae. Commonly, the gene is used to design Genetically Engineered crops for commercial production such as maize, soy, canola and papaya [7]. The benefit of this promoter insertion is functional, well characterized and constitutively expressed promoter [8]. Promoter size of CaMV 35S is 342 basepairs including region of CAAT and TATA box. Nucleotide sequence of promoter and position of the region is presented in Figure 1.

![Figure 1. Nucleotide sequence of promoter of CaMV 35S. The position of CAAT and TATA box is marked with black colour [8]](image)

The size of DNA bands which detects the promoter of 35S of CaMV will be around 100-150 base pairs. This appearance was positive signals as Wardani et al. [1] explained that primer pairs of 35S detection has amplification area about 123 basepairs. The investigation of presence of 35S CaMV promoter has been commonly used for detection of Genetically Engineered plant material [7]. A number of methods were developed to detect genetically modified organism. Polymerase Chain Reaction (PCR) is one of the approaches for detecting transgenic gene [9]. The method was chosen because of its high specificity, efficiency and validity [10] Designing a primer based on regulatory sequence is used to detect gene target [11].

The purpose of this research was to detect promoter of 35S CaMV which potentially indicates transgenic gen of soybean. This detection results presumably could help Indonesia in implementing the government regulation on labelling of GMO and non-GMO products on market packaging of soybean grain. Moreover, the results might investigate the contamination among local and imported soybean grain sold in Indonesia.
2. Materials and Methods

2.1. Observation and Preparation of Samples
Soybean grains were used in this research were all brand of soybean sold totally 12 brands from 4 central markets in Special Region of Yogyakarta called Beringharjo Market, Sentral Market, Prawirotaman Market and Gamping Market. The interview was also conducted to support identification of phenotype among imported and local soybean grain sold in the market.

2.2. Extraction of DNA
Fifty milligrams of leaves of 14 days old seedlings were used for extraction of Genomic DNA mini kit according to Gene-aid Biotech Ltd. The DNA pellet was dried and re-suspended in 100 milliliters of de-ionized water (ddH2O). The product of extracted DNA was stored at 4ºC.

2.3. Yield and Quantity of DNA
Quantity and purity of isolated DNA samples were measured by Optical Densities (OD) of 260 nm and 280 nm of genequant 1300 spectrophotometer. The expect value of DNA purification was around ~1.8 ratios of A260/280 nm. Then, the DNA purity value was around 2.0-2.2 ratio of A260/230 nm [12]. The extracted DNA was adjusted by dilution to 200-400 ng/μl to PCR total volume.

2.4. Optimization of Primer Annealing Temperature and Detection of CaMV (Cauliflower Mozaic Virus) 35S Promoter
In this research, screening of promoter of CaMV 35 S was conducted by Polymerase Chain Reaction (PCR) methods using specific primers. Polymerase Chain Reaction (PCR) is a method to amplify specific DNA by using two primers of oligonucleotides which is helped by polymerase enzyme [13]. Primer used and its target in this research are listed in Table 1.

| Oligonucleotide primer pair sequence and its targets [4] |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Amplicon (basepairs) |
| Gene specificity | Primer name | Sequences | Amplicon (basepairs) |
| Promoter of CaMV 35 S | P35S-CF3 (f) | 5’-CCA CGT CTT CAA AGC AAG TGG-3’ | 123 |
| | P35S-CR4(r) | 5’-TCC TCT CCA AAT GAA ATG AAC TTC C-3’ |

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 Gotag®Green Master Mix, Cat.9PIM712. The temperature profiles used for optimization of primers was presented in Table 2.

| Time and temperature profiles for optimal primers annealing [4] |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Primer pairs | Initial denaturatio n | Denaturation | Annealing | Elongation | Cycles | Final Elongation |
| RRS0t-5(f)/RRS01-3 (r) | 4 min at 95ºC | 1 min at 95ºC | 1 min at 55-60ºC | 1.5 min at 72ºC | 34 cycles | 7 min at 72ºC |
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. The 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. DNA band in the gel was visualized by UV transilluminator.

3. Results and Discussion
3.1. Percentage of Local and Imported Soybean Based on Observation and Grain Phenotype
Addition Percentage of local soybean grains collected from the markets was about 30, 77 %. Meanwhile, the percentage of imported brand of soybean and phenotype-like imported soybean which collected from the market was about 69, 23 %.

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 has some representative brand name such as Lokal, Wonosari, and Galunggung, also Anjasmoro. Meanwhile, imported and phenotype like-imported soybean could be recognized with white color and oval grains. The white color grain is the phenotype representative of brand name, America No. 1, America No. 3, America, No Name no. 1 and No Name No. 2 (Figure 2).

![Figure 2](image)

Figure 2. Two phenotypic characters of observed soybean grain from the market: (a) local soybean with the phenotype of yellow doff grains and (b) imported soybean with white pale color grains

Although the traders could mention the types of the local and imported soybeans, verification method is also required by detection of transgenic gene target. One of the ways was to verify the presence of CaMV 35 S promoter, isolation of DNA and genotyping analysis by using Polymerase Chain Reaction.

3.2. Yield and Quantity of DNA
Extraction 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 is 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 [14]. In this research, DNA concentration was around 200 to 400 ng/μl which was qualified for DNA template of Polymerase Chain Reaction (PCR) methods.
Table 3. Quantitative test of isolated DNA of soybean (Glycine max L.) collected from central markets

| Code | Market Brand of Soybean | Location (market) | Absorbance 260/230 | Absorbance 260/280 | DNA concentration (ng/µl) |
|------|-------------------------|-------------------|---------------------|--------------------|--------------------------|
| 1    | America No. 1           | Beringharjo       | 3,00                | 3,00               | 300                      |
| 2    | America No. 2           | Beringharjo       | 2,00                | 1,33               | 400                      |
| 3    | America                 | Gamping           | 2,00                | 1,00               | 200                      |
| 4    | America                 | Sentral           | 2,00                | 2,00               | 200                      |
| 5    | No name 1               | Prawirotaman      | 2,00                | 2,00               | 400                      |
| 6    | No name 2               | Prawirotaman      | 1,50                | 3,00               | 300                      |
| 7    | Lokal                   | Prawirotaman      | 1,00                | 1,00               | 200                      |
| 8    | Galunggung              | Beringharjo       | 1,00                | 2,00               | 200                      |
| 9    | Wonosari                | Gamping           | 2,00                | 1,00               | 400                      |
| 10   | Anjasmoro               | Java Timur        | 0,80                | 1,33               | 400                      |

3.3. Optimization of Primer Annealing Temperature and Detection of CaMV 35 S Promoter by using Polymerase Chain Reaction (PCR)

Optimization of annealing steps for PCR was conducted to maximize the process of complementary primers to the target nucleotide sequences. Primers of P35S-CF3 (f) and P35S-cr4 (r) used to detect Promoter of CaMV 35 S was successful in the temperature range of 55, 56, 58, and 59 ºC. The optimal annealing temperature was 56 ºC by signal of the white thickest band 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 ºC was used in the annealing temperature of primers for PCR program setting.

Figure 3. Visualization of primer optimization of CaMV 35 S promoter which was optimal in annealing temperature of 56 ºC 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.
Figure 4 visualized of DNA amplification of soybean (*Glycine max* L.) detected by Polymerase Chain Reaction (PCR) by using specific primers revealed positive signals that the bands appeared for all samples. The size of DNA bands which detected the promoter of CaMV 35 gene promoter was around 100-150 basepairs. The signals explained that primer pairs of CaMV 35 S promoter could be detected with the amplicon products was about 123 base pairs, the yield similar results from the previous research for detection of this promoter [4]. Meanwhile, another appearance in the gel electrophoresis result was white band under every band of DNA appearance called Primer Dimmers (PDs) which commonly present in the result of amplification. A possible reason was caused of template independent primers interaction could take a position that increases production of non-specific products called PDs. These primers appear in high concentration, and weak interactions. Also, the complement of a nucleotide between amplimer of 3’ends could increase to primer dimers after 30 cycles [1].

In details, the results of the band appearance with the amplification area of 123 basepairs also revealed in Lane 7, 8, 9 and 10 which indicated that the local soybean namely *Lokal*, *Galunggung*, and *Wonosari* also contained Genetically Engineered (GE) herbicide-tolerant gene called CaMV 35 S promoter gene of transgenic soybean. Considering the result of identification of transgenic soya products in previous research, the findings of this study also revealed similar results shown by positive signals of CaMV 35 S promoter gene with the amplification area around 123 basepairs [15].

![Figure 4. Detection of CaMV 35 S Promoter of soybean (*Glycine max* L.) using primer P35S-CF3 (f) and P35S-cr4 (r) on electrophoresis gel concentration of 1.5 %. Lane L, molecular marker of 50 bp DNA ladder. Lane B, negative control, no DNA template added. Lane 1, market brand of imported soybean, America No 1. Lane 2, 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 Sentral Market. 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*.

Although the CaMV 35 S promoter is not the only gene which could indicate the transgenic soybean, the presence of this gene could be detected accurately to identify of major genetically modified crop species, Roundup Ready soybean [16]. In this research, the local soybean of Indonesia might be contaminated because seed producers cultivated during seed production together with imported soybean. A possible reason is regular farmers of soybean has main responsibility for the contamination of GM and non-GM soybean by their cultivation without considering distance isolation. Another explanation is that this promoter which has double strand DNA virus might be inserted because of contamination from another family such Cruciferaceae or other. Commonly, this fragment of DNA is used to design Genetically Engineered crops for commercial production such as maize, soy, canola and papaya [7]. The previous report explained that such contamination also results
in similar cases in Europe where seed producers might be 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 the process of cleaning, sizing and packing [9]. One of most frequent factors affects contamination was isolation distances. The distance could vary a couple of meters to 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 [17]. The result of this study would be a factor to develop government policy in assisting farmer to study of cultivation system among GMO and non-GMO plants. It also recommended to government in the role of assist and implement the labelling system of the market products in Indonesia, especially soybean.

On the other hand, Indonesia is one of the countries which is classified to receive the mandatory of labelling for many GE foods and the labelling threshold is higher than 1% or undefined. This includes laws with a threshold of 1% for the entire food item [18]. Indonesian farmers are also available to use biotechnology products. The technology rapidly adopted by farmers following commercialization. Meanwhile, the information and general knowledge about biotechnology might not be sufficient to the farmer in the field [19]. Although legal provisions related to genetic engineering of food crop, it could not approve the guarantees to the community including farmers [20]. Presumably, the transgenic imported soybean or another family is contaminated extensively to the local soybean by cultivation without compromising the isolation and any factors.

4. Conclusion

Samples of local soybeans (Glycine max L.) revealed positive signals of promoter of CaMV (Cauliflower Mosaic Virus) 35 S of transgenic soybean.

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References

[1] Wardani A K, Alirsyah A and Fauziah A 2018 Agritech 37 237.
[2] James C 2011 ISAAA Brief 43 338.
[3] Raharjo T J and Surajiman S 2017 Indones. J. Chem. 17 415-21
[4] Meriç S, Çakır Ö, Turgut-Kara N and Arı Ş 2014 Genet. Mol. Res. 13 1160-8.
[5] Somssich M A 2019 short history of the CaMV 35S promoter peerj.preprints.27096v3
[6] Lee L Y and Gelvin S B 2007 Plant Physiol. 146 325-32.
[7] Holden M J, Levine M, Scholdberg T, Haynes R J and Jenkins G R 2010 Anal. Bioanal. Chem. 396 2175-87.
[8] Benfey P N and Chua N H 1990 Science 250 959–66.
[9] Wu H, Zhang Y, Zhu C, Xiao X, Zhou X, Xu S, et al 2012 Int. J. Mol. Sci. 13 1919-32.
[10] Yusuf Z K 2010 Saintek 5.
[11] Randhawa G J and Firke P K 2006 Detection of transgenes in genetically modified soybean and maize using polymerase chain reaction Indian J. Biotechnol. 5 510-3.
[12] Wilfinger W W, Mackey K and Chomczyński P 1997 Biotechniques 22 474–81.
[13] Ilhak O I, Arslan A 2007 Turk. J. Vet. Anim. Sci 31 159-63.
[14] Scientific, Thermo 2012 T123 Technical Bulletin (Wilmington, DE: Thermoscientific).
[15] Brownie J 1997 Nucleic Acids Res. 25 3235-41.
[16] Nguyen T and Wong C M 2018 Int. Food Res. J. 15 156-7.
[17] Franke A C, Greco F M, Kleter G, Noordam M Y, Roza P and Eaton D J F 2009 The institutional and legal environment for GM soy in Brazil (Wageningen, The Netherlands: Plant Research International B.V.).
[18] Verrière P, Gall E, Carrasco A G, Metera D, Payton L and Oehen B 2003 Preventing GMO Contamination: An Overview of National CoExistence Measure in the EU (Brussels: IFOAM EU Group).

[19] Rahayu T 2015 Agricultural Biological Annual USDA Foreign Agricultural Service 1526.

[20] Prianto Y and Yusahasmita S 2017 The Genetically Modified Organism (GMO) of Plant and Their Legal Perspective in Indonesia J. Biol. 10 (2).