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Determination of the presence of transgenic soybean in Indonesian commercial soybean and tempeh using 35S promoter primer by real-time polymerase chain reaction

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Abstract

The present of genetically modified organism (GMO) in food materials such as soybeans regulated by Indonesian Food and Drug Administration. To impose the law, reliable methods to detect transgenic component are needed. Validation of the real-time polymerase chain reaction (real time-PCR) to detect the presence of transgenic in soybeans and tempeh has been accomplished. The method used a specific primer to amplify promoter 35S. The method could correctly distinguish GMO soybeans from non-transgenic one. The precision of the method is high as represented by CV value of Ct, which is 7.1% (acceptance criteria < 25%). The real-time PCR still give precise fluorescent signal up to 5pg of DNA template, as a representation of limit of detection of the methods. By the method, one commercial soybean labeled as non-GMO has been detected as GMO soybean, while most imported soybeans as well as the commercial tempeh are GMO.

Introduction

Genetically modified crops (transgenic or GMO crops) has been developed as an agricultural crop in the United States, Canada, China, Argentina and several other developing countries. In 2008 more than 130 transgenic crops were consisting of 22 species are distributed in the world market (Ramessar *et al*., 2008). Data in January 2011 showed that there were 31 varieties of agricultural plant transgenic consisting of six different species listed in the European Union, including 17 varieties of corn, six varieties of cotton, three varieties of canola, three soybean varieties, one variety of potato and one varieties of sugar (The European Parliament and The Council of The European Union, 2003).

Many studies have concluded that genetically modified food has been consumed by millions of people around the world for more than 15 years without any reported adverse effects of the disease (Aumaitre, 2004; Key *et al.*, 2008). Several other studies give the opposite result where the consumption of transgenic plants provides results that are harmful to health (Tudisco *et al.*, 2006; Malatesta *et al*., 2008).

Concerning to their potential health effects of GMO crops makes labeling is required to provide information to consumers about GMOs. Currently, the Europe Union has a limit on the labeling of GMO products by 0.9%, Australia, and New Zealand have a maximum limit of 1%, and Japan has a maximum limit of 5%. Indonesia is currently evaluating four varieties of genetically modified seeds to be commercialized to the market and according to Indonesian FDA the labeling of GMO required for the content of minimum 5% GMO (Regulation of the Head Food and Drug, Republic of Indonesia No. HK.03.1.23.03.12.1563., 2012). The amount of imported agricultural products into the Indonesian market, mainly soybean does not rule out the possibility that there is a GMO product in foodstuffs sold in the market.

Transgenic plants are physically not much different from the conventional crop counterpart. The detection method directly against genetically modified organisms could, in theory, can be carried out with DNA through PCR as the detection of other species (Raharjo *et al*., 2012, Maryam *et al.*, 2016; Rahmawati *et al*., 2016). Several PCR methods using the single target as well as multiplex has been performed for the analysis of GMO (Foti *et al*.; 2006; Quirasco *et al*., 2008; Waiblinger *et al*., 2008; Berdichevets *et al*., 2010; Tian *et al*., 2011). The method using real-time PCR has also been widely reported for the same purpose (Quirasco *et al*., 2008; Waiblinger *et al*., 2008; Grohmann *et al*., 2009; Pansiot *et al*., 2011; Tian *et al*., 2011; Gerdes *et al.*, 2012). Real-time PCR is a proven as most accurate, sensitive method.

The real-time PCR include probes TaqMan to

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detect endogenous species genes (zeins and lectin), synthetic genes (CryIA and CP4-epsps), specific varieties genes (e.g MON 810, Bt11, GA21), terminator transgenes (T-NOS) and transgenic promoter (Waiblinger *et al*., 2008; Querci *et al*., 2009; Tian *et al*., 2011). Those gene or promoter are also used as amplification target using SYBR Green real time-PCR (Chaouachi *et al*., 2008; Koppel *et al.*, 2010; Gerdes *et al*., 2012).

This study reports an efforts to validate the methods of detection soybeans GMO using SYBR Green real-time PCR targeted amplification of 35S promoter using common primers that designed by Lipp *et al*. (1999) which has been used as a standard method (International Standard Organisation, 2005). The primers were designed to amplify 195 bp DNA fragment which is common for PCR but not for real time-PCR. Therefore, a validation required to demonstrate the primers possibility to be used in real time-PCR. The validation parameters cover specificity, precision, as well as the limit of detection of the method. At the same time, an attempt to determine the existence of GMO both in soybeans and processed products traditional Indonesian food of Tempeh are also performed.

Materials and Methods

Materials

Primer F35S: 5'-GCT CCT ACA AAT GCC ATC A-3' and primer R35S: 5'-GAT AGT GGG ATT GTG CGT CA-3' was synthesized by Genetika Science Indonesia. SsoFast EvaGreen Supermix was from BioRad. The CRM (Certified Reference Material) of soybean GMO was kindly provided by Quarantine Office Semarang Indonesia. Commercial soybeans and tempeh were purchased at the traditional market, supermarket, covers three soybeans of the traditional market, three imported soybeans, four supermarket soybeans and three tempeh samples from traditional market. All other chemicals are analytical grade.

Soybean DNA isolation and real-time PCR

Isolation of soybean DNA was performed using DNA isolation method according to Sambrook with slight modifications (Septianingtyas, 2011; Green and Sambrook, 2012). For Real-time PCR, 50 ng of DNA was mixed with ten µL EvaGreen, F35S 1 µL, one µL R35S, as well as DNAse -free water to a volume of 20 µL. Real time-PCR was performed with machine Biorad CFX-96 with conditions used were pre-denaturated at temperatures of 95°C for 30 seconds, denaturation at a temperature of 95°C for 2 seconds, annealing and extension temperature optimum outcome for 5 seconds. Running real-time PCR performed 35 times cycle continues melt curve analysis. Temperature is tested for optimization of annealing and extension i.e. 50.1; 50.8; 52.1; 54; 56.4; 58.4; 59.5; 60.1°C (Hasyyati, 2012).

Method validation

Specificity test

Isolated DNA from two kinds of soybeans: the positive control (transgenic soybean CRM) and negative control (non-transgenic soybeans) were subjected as real-time PCR sample under the optimized condition. The method is said to be specific if it gives positive results for CRM transgenic soybean DNA and negative for no- transgenic soybeans DNA.

Precision test

Precision was determined using repeatability test which was done through repetition method ten times using the same sample of positive and negative soybeans controls. The repetition included the isolation of DNA as well as the real-time PCR. Precision is determined by calculating the CV of Ct value with acceptance criteria refers to the provisions of the Codex Guidelines.

Limit of detection determination (LOD)

Determination of the limits of detection performed by making serial dilution standard curve DNA of CRM transgenic soybean 50000, 5000, 500, and five pg. LOD value is the lowest concentration that still shows fluorescent on real-time PCR.

Detection of GMO in commercial soybean and tempeh

Ten commercial soybean samples was randomly taken from traditional markets, supermarkets, and imported soybean imports as well as three samples of tempeh. DNA of all ten samples of soybeans was isolated using the validated methods while tempeh DNA was isolated without powdering step. The isolated DNA was analyzed using real-time PCR analysis at the optimized condition.

Results and Discussion

The annealing temperature (Ta) optimization is a crucial factor in the development of methods of PCR or real-time PCR. The Ta associated with the annealing PCR primers to target which related to the specificity of a PCR or real-time PCR method. Optimization of Ta for 35S promoter primer in transgenic soybean analysis performed at a temperature range of 50.1 up

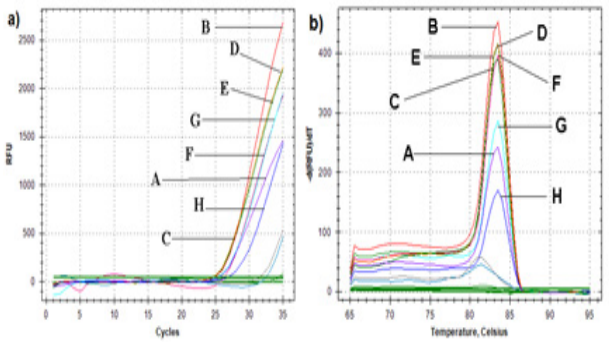

Figure 1. Real-time PCR data at various tested annealing temperature (A, 60,1; B, 59,5; C, 58,4 D, 56,4 E, 54 F, 52,1 G, 50,8; H, 50,1 °C) (a) Amplification curve; (b) Melt Curve Analysis

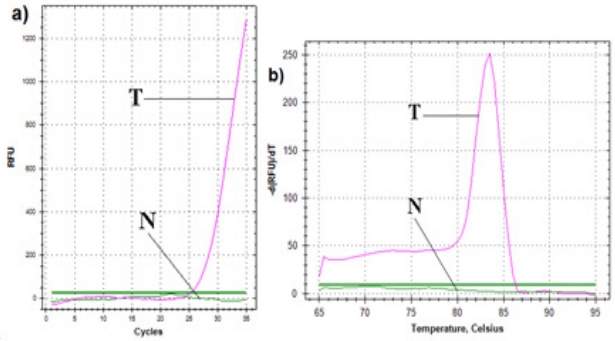

Figure 2. Real-time PCR data of specificity test (T: CRM transgenic, N: Negative control) (a) Amplification curve; (b) Melting curve analysis

to 60.1°C.

Figure 1b shows that based on the melt curve analysis (MCA) it appears that at all Ta obtained the value of the same temperature of melting of 83.5°C represent that the primers only attach to the DNA template at one position on the DNA template generating a single PCR product. At the same time Figure 1a and 1b also show Ta, 59.5°C has the highest fluorescent intensity meaning the optimum annealing temperature is 59.5°C (Hasyyati, 2012). Further investigation using Ta 60.1°C produces a fluorescent signal lower show that primers interaction is not as strong as at temperatures of 59.5°C.

Specificity of the method

The specificity is one critical parameter in the validation of a method. In this real-time PCR, it performed to determine the specificity primer P35S for the identification of transgenic soybeans. Figure 2a shows the result of real-time PCR of positive control (CRM of transgenic soybean) and negative controls. Transgenic DNA was amplified to the value of RFU (Relative Fluorescent Units) reached more than 1200 and Ct (threshold cycles) of 26 with no fluorescent signal appear of the negative control. Regarding the requirement for validation, specificity

Table 1. Ct value of repeatability of real time-PCR

Replicate number	Ct value
1	28.0
2	24.0
3	27.5
4	24.0
5	24.0
6	23.0
7	25.7
8	27.6
9	25.3
10	25.0
Average	**24.7**
STDEV	**1.75**
CV	**7.1%**

demands no false positive or false negative result (Broeders *et al*., 2014). The result show consistent result for both negative and positive control. Melting curve analysis (Figure 2b) shows that transgenic DNA amplicons that have a consistent melting point or Tm of 83.5°C, the same as the value of Tm is a typical value of the real-time PCR product of promoter 35S of genetically modified organisms. From this graph, it can be concluded that the primer 35S promoter has excellent specificity in detection of transgenic soybeans.

Precision of the method

Precision test conducted by repeatability test covering a method of DNA isolation to real-time PCR with ten times replication. Table 1 shows the Ct value of each replication. Ct value is ranging between 23-28 with an average value of 24.7, STDEV 1.75 and CV (RSD) 7.1%. CV value is far below the 25% which is the limit of acceptance of Ct value for real -time PCR according to Codex (Codex Guidelines, 2010). The results also meet the criteria for real-time PCR validation for precision using repeatability method (Broeders *et al.*, 2014). This data conclude the performance of the laboratory in running method of real-time PCR test with transgenic soybean using P35S primer has excellent precision. Although the techniques reported here only real-time PCR using specific primers no less specificity generated by real -time PCR using a probe (Chaouachi *et al.*, 2008; Querci *et al*., 2009).

Limit of detection of the methods

Figure 3a shows that all variants of the mass of transgenic DNA in the sample amplified with the Ct value continue to expand as the reduced mass of DNA in the sample. The entire amplicon of four variations of mass DNA had Tm of 83.5°C by the data of GMO amplicon Tm for primers P35S (Figure 3b). As little as five pg of DNA in the sample (in a 20 mL reaction mixture) appeared to be still detected using real-time PCR, it concludes that real-time PCR method is sensitive to 5 pg of DNA in a sample or DNA equal

Table 2. Test result of commercial soybean and tempeh

No	Sample source	Sample code	GMO label	Test result
1	Imported	I_1	No label	+
2	Imported	I_2	No label	+
3	Imported	I_3	No label	+
4	Traditional market	P_1	No label	+
5	Traditional market	P_2	No label	-
6	Traditional market	P_3	No label	-
7	Supermarket	S_1	Labeled as NonGMO	+
8	Supermarket	S_2	No label	+
9	Supermarket	S_3	No label	+
10	Supermarket	S_4	No label	+
11	Tempeh of Traditional market	T_1	No label	+
12	Tempeh of Traditional market	T_2	No label	+
13	Tempeh of Traditional market	T_3	No label	+

to 0.25 pg/mL in concentration. Thus, the validation results showed a laboratory capable of detecting a small amount of up to 5 pg of DNA in a sample of real-time PCR or DNA concentration was 0.25 pg/mL. This value is excellent because it is very low compared to the standard value of the amount of DNA that could be detected by PCR were an average of 50 ng. This value is far more than enough to confirm if a sample of soybeans categorized as transgenic or non-transgenic since the limit criteria set by Indonesian FDA is 5%. The limit of detection reported here seems to be much higher compared to other reports which able to detect up to less than 0.1% (Chaouachi *et al.,* 2008; Querci *et al.*, 2009; Rosa *et al*., 2016). Regarding the use of P35S as amplification target, low detection limit as low as 0.1% has been reported (Barbau-Piednoir *et al*., 2014).

Detection of GMO in commercial soybean and tempeh

Table 2 shows the results of testing ten samples of commercial soybean consists of three samples of imported soybean, three samples of soybeans from traditional markets, four samples of soybeans from the supermarket and three tempeh samples obtained from traditional markets. Eight of the ten soybean samples tested using real-time PCR method proved to be a transgenic soybean including one of which has a non-GMO label. This fact demonstrates the importance of the presence of transgenic soybean trials conducted on a commercial sample. Non-GMO labeling on one of the test samples turned out to be a transgenic soybeans need to be reconfirmed and marked by public institutions. Two samples of soy that are proven not genetically modified soy and not labeled are commercial samples from traditional markets. It is estimated that soybeans from traditional

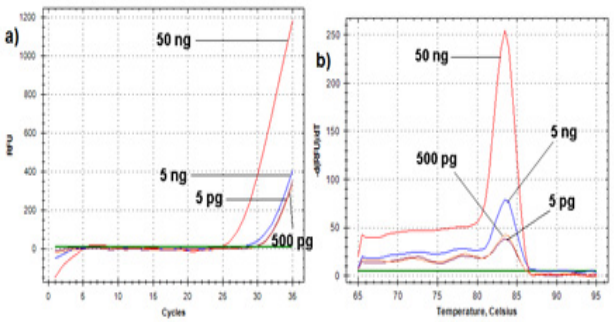

Figure 3. Real time-PCR data of LOD determination (a) Amplification curve (b) MCA curve

markets are the kind of local soybean breed from conventional soybean seeds. Table 2 also shows that three samples of tempeh contain the transgenic soybeans component. Considering tempeh producers prefer using imported soybean as raw material which by this study are proven as transgenic soybean.

Conclusion

Real-time PCR using primers to amplify 35S promoter capable of detecting specific transgenic soybean with optimum Ta conditions at 59.5°C. The reaction produces amplicon with a value of Tm, 83.5°C. The method has a good performance with precision up to 7.1% CV value of Ct and the detection limit of 5 pg that meets the acceptance requirement of methods. The Methods also successfully applied to detect the presence of transgenic soybean on commercial soybean including those labeled as non-transgenic or GMO, or proving tempeh on the market made from transgenic soybeans.

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