Detection of Roundup Ready Soybean by Dot Blot

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ABSTRACT

The determination of the presence of the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein in Roundup Ready soybean (RRS) using Dot blot was evaluated in comparison with the PCR. Experiments were done at the laboratory of GMO detection, the Department of Agriculture, during May 2011-July 2012. Initially, twenty four samples of RRS were collected, screened and verified for real RRS by PCR. After that one sample of verified RRS was ground and protein was extracted using different buffers in comparison with clean water. A suitable ratio of sample and buffer was also determined. The same lot of twenty four RRS samples were then examined for the presence of CP4 *EPSPS* by the Dot blot using the suitable buffer and sample : buffer ratio condition and self-made specific antibody. Finally, analysis of RRS by PCR and Dot blot was compared. Detection of twenty four RRS samples by PCR revealed that only 11 out of 24 samples (ca 45.3%) were the real Roundup Ready soybean, while detection those by Dot blot showed that 9 out of 24 (ca 37.5%) samples were the real Roundup Ready soybean when extracted by guanidine buffer. This result demonstrated that both PCR and Dot blot could be complementary. However, sample by sample Dot blot and PCR analysis of the RRS occurred 62.5% only. This could be due to the nature and protocol of detection method, sample size, heterogeneity and concentration of GM materials.

Key-words: detection, Roundup Ready soybean (RRS) and Dot blot

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Introduction

Roundup Ready soybean (RRS), is normally known as and glyphosate-tolerant soybean (GTS), has been genetically modified by transferring the 5-enolpyruvylshikimate 3-phosphate synthase (CP4 *EPSPS*) gene obtained from the soil bacterium namely *Agrobacterium* sp. Strain CP4 into the soybean genome (Padgette *et al.*, 1995). It was approved for environmental food and animal feed use in the United States since 1994 (Shehata, 2005). The expression of the CP4 EPSPS gene makes the RRS tolerant to glyphosate. The RRS is equivalent to a traditional soybean with the exception of the presence of the CP4 *EPSPS* gene and gene product.

The RRS is generally identified by either the inserted CP4 *EPSPS* gene at the DNA level or the resulting protein *EPSPS*. Currently, several immunological-based detection methods such as western blotting (van Duijn *et al.*,1999) enzyme-linked immunosorbent assay (ELISA) (Rogan *et al.*, 1999) and lateral flow strip are available. The use of which methods is dependent on the particular purpose. Some of these methods, for example, western blotting and ELISA require several hours, laboratory equipment, while some methods such as lateral flow strip is expensive.

Dot blot is a simple method and routinely used in research and diagnostic laboratory. This method works by fixing protein samples onto nitrocellulose membrane, probing with labelled antibody specific to protein of interest and detecting hybridization by colorimetric method. Detection is not only fast and but also not too expensive. However, the amount of the target protein present in crude extract is variable. It depends on several factors such as extraction buffer (Sussulini *et al.*, 2007), sample : buffer ratio (Rogan *et al.*, 1992) and etc. The goal of research is to a) examine types of buffer used for *EPSPS* protein extraction from soybean powder, b) test the suitable sample : buffer ratio for *EPSPS* protein extraction and c) evaluate the Dot blot method for detection of RRS in comparison with PCR.

Materials and Methods

Soybean Enzymes and Reagents

Roundup Ready® raw soybeans were obtained from the Department of

Agriculture, Ministry of Agriculture and Cooperatives, Thailand (Table 1). Testing samples were coded and used. The texture of the samples was solid. Fast Red TR/Naphthol AS-MX phosphate was used as substrate for the detection of alkaline phosphatase activity. This substrate system for enzyme detection was purchased from (Sigma). Reagent and enzyme used in PCR were purchased from (Promega).

Table 1. Roundup Ready raw samples used in PCR and Dot blot analysis

Sample code	Source
16144	Imported from USA
16145	Imported from Canada
16152	Unknown
16157	Unknown
16158	Unknown
16159	Unknown
16161	Unknown
16187	Imported from USA
16188	Imported from Canada
16189	Imported from USA
18809	Imported from USA
18816	Imported from USA
18857	Imported from Canada
18897	Unknown (Daryplus Ltd.)
18901	Unknown (Daryplus Ltd.)
18902	Unknown (Daryplus Ltd.)
18903	Unknown (Daryplus Ltd.)
18904	Unknown (Daryplus Ltd.)
18905	Unknown (Daryplus Ltd.)
18907	Unknown (Daryplus Ltd.)
18908	Unknown (Daryplus Ltd.)
20558	Domestic
22683	Imported from USA
24079	Imported from Indonesia

Protein and antibody

The purified CP4 EPSPS protein used for the primary antibody production was kindly provided by the Office of Biotechnology Research and Development, Department of Agriculture. The CP4 *EPSPS* protein was purified from the culture of *Escherichia coli* strain BL21 under the expression of pET200 with CP4 *EPSPS* insert. The purified *EPSPS* protein was prepared by loading the cleared lysate onto the column containing nickel nitrilotriacetic acid (Ni-NTA) resins and eluted with elution buffer. The flow-through fraction was collected, dialysed and kept at 4 °C before use (Wongwathanarat *et al.*, 2010). Anti-Rabbit IgG produced in goat and conjugated to alkaline phosphatase (GAR-AP) was used as secondary antibody. The GAR-AP was purchased from (Sigma).

Preparation of test samples

Roundup Ready soy grain and

non GMO soy grain were ground as fine powder with a blender. Each sample of Roundup Ready soy powder and non GMO soy powder were separately kept in a plastic bag and stored at 4 °C before used.

Experiments were done at the Laboratory of GMO Detection, the Department of Agriculture, during May 2011- July 2012.

Verification of Roundup Ready soybean by PCR

Each ground sample was subjected to DNA extraction using DNeasy Plant Mini Kit (QIAGEN) and genomic DNA concentration was adjusted to 50 ng/mL before PCR. Prior to verification of Roundup Ready samples, the quality of extracted DNA of each sample and PCR condition were examined by the amplification of the 181 bp DNA fragments of the soybean lectin gene using the primers Lec1 (5'-GACGCTATTGTG AGCTCCTC-3') and Lec2 (5'-TGTCAGGGC CATAGAAGGTG-3').

Verification of the Roundup Ready soybean was then performed using the primers RD-F (5'-TGATGTGATATCTCCACT GACG-3') and RD-R (5'TGTATCCCTTGAGC CATGTTGT-3') to amplify the 171 bp DNA fragments of CP4 *EPSPS* and genomic DNA of each sample as template. All PCR cycle was set up (Figure 1) with varying annealing temperatures according to the primers used. The PCR products were analyzed by electrophoresis on a 1.0 % agarose gel.

Production of polyclonal antibody specific to CP4 *EPSPS* protein

New Zealand white rabbits were immunized with purified recombinant CP4 *EPSPS* protein. The rabbits received the first injection of antigen in complete Freund's adjuvant intramuscularly and subcutaneously. All subsequent injections were in incomplete Freund's adjuvant. All injections were 7 days apart. The primary immunization injection and each subsequent booster injections contained 500 mg CP4 *EPSPS*. The rabbits were bled on days 14, 21, 28 and 35 (14 days

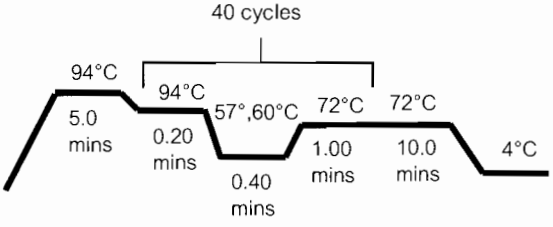


Figure 1. Schematic of PCR cycle with varying annealing temperatures

after incomplete adjuvant injection). Thirty mls of blood was collected each booster injection. The sera were separated from blood cells by centrifugation at 3,000 rpm for 10 mins and added 0.2% NaN_3 (v/v). The antibody was tittered with spectrophotometer. The antibody with high titer was pooled and kept at 4 °C before used.

Determination of suitable buffer and sample:buffer ratio

Extraction of CP4 *EPSPS* protein from the verified Roundup Ready soybean was performed with 3 different buffers (Table 2) and clean water as a control. Zero point one g. of the verified Roundup Ready soybean powder was put in 2 ml vial and 0.5 ml of each buffer (Table 2) was added into each vial separately. All vials were vortexed for 5 mins and centrifuged at 12,000 rpm for 10 mins. Supernatant was transferred to each new vial and subjected to Dot blot analysis (Figure 3). Non-GMO soybean was also included as a negative control.

The same verified Roundup Ready soy bean powder (same code) and the suitable buffer (as the previously experiment) were used to determine the suitable ratio of samples to volume buffer. The ratio of soybean powder to buffer (w/v) was examined in different ratios as following; 1:1.25, 1:5, 1:10, 1:15, 1:20 and 1:25 respectively. The purified CP4 *EPSPS* protein and blank suitable buffer were also included as positive and negative control, respectively (Figure 4).

Table 2. Buffer used for protein extraction and its composition

Type of buffer	Buffer composition
Water	-
PBS	Phosphate buffer saline (PBS) pH 7.4 (composed of 1.37 M NaCl: 26.8 mM KCl: 78.1 mM Na_2HPO_4:14.7 mM KH_2PO_4, protease inhibitor cocktail)
Tris-HCl	Tris-HCl buffer (composed of 100 mM Tris-HCl, pH 7.5: protease inhibitor cocktail)
Guanidine buffer	Guanidine-HCl buffer (composed of 10 mM Tris-HCl: 150 mM NaCl: 2mM EDTA: 1%SDS: 5M Guanidine-HCl: protease inhibitor cocktail)

Determination of suitable buffer and sample:buffer ratio was repeated four times independently.

Detection of Roundup Ready soybean by Dot blot

Samples of the Roundup Ready soybean (Table 1) from various sources were assayed using the optimal buffer and the sample : buffer ratio and the antibody as previously experiments by Dot blot for detection the presence of CP4 *EPSPS* protein.

Dot blot assay

Ten µl of each extracts, purified CP4 *EPSPS* protein solution and blank buffer were spotted on a nitrocellulose membrane (Figure 6) presoaked in 1X TBS buffer and air dried for 15 mins. The air dry membrane was blocked in blocking buffer (containing 2% skim milk in 1X TBS buffer and 4% TritonX-100) for 1 hr. The blocking buffer was discarded and the membrane was incubated with the primary antibody at room temperature for 1 hr with gentle shaking. After being washed with TBS-T buffer three times for 3 mins each. The GAR-AP was added to the the membrane (1:3,000 dilution) and incubated at room temperature for 1 hr with gentle shaking. After being washed with TBS-T buffer again, the membrane was incubated with Fast Red TR/Naphthol AS-MX substrate solution for 1 min with gentle shaking. The reaction was stopped by discarding the substrate solution and washing the membrane with sterile distilled water.

Results and Discussion

Verification of Roundup Ready soybean by PCR

Although the 24 test samples were Roundup Ready soybean, all of the test samples needed to be verified by PCR which was more sensitive, precise and accurate method. The PCR method was reproducible since the DNA fragment of 181 bp of the host specific internal target (the gene of lectin) was amplified in all assays (Figure 2a) as control to evaluate DNA quality and PCR efficacy, reducing the risk of false negatives, thereby increasing reliability. Once the specific primers (RD-F and RD-R) for CP4 *EPSPS* were used, it was found that only 11 out of 24 test samples (ca.45.3%) were positive for the CP4 *EPSPS* (Figure 2b). A PCR result indicated that sample codes

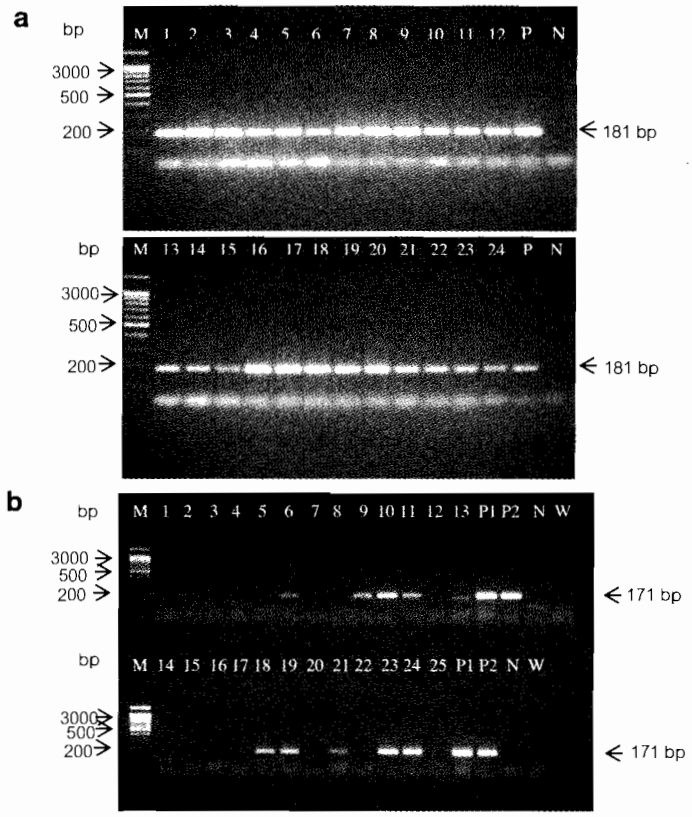


Figure 2. Agarose gel electrophoresis of PCR amplification of a) lectin and b) CP4 *EPSPS* gene from Roundup Ready soybean (Note: Lane 25 of Figure 2b is lane N as resulted from error loading)

M = EZ standard marker;

P = positive control (DNA of Roundup Ready soybean)

P1 and P2 = certified reference materials standards consisting of dried soybean powder with 100 and 5% (w/w) Roundup Ready soybean.

N = negative control (Non GMO soybean)

W = negative control (sterilized water)

Lane 1 = sample code 16144

Lane 2 = sample code 16145

Lane 3 = sample code 16152

Lane 4 = sample code 16157

Lane 5 = sample code 16158

Lane 6 = sample code 16159

Lane 7 = sample code 16161

Lane 8 = sample code 16187

Lane 9 = sample code 16188

Lane 10 = sample code 16189

Lane 11 = sample code 18809

Lane 12 = sample code 18816

Lane 13 = sample code 18857

Lane 14 = sample code 18897

Lane 15 = sample code 18901

Lane 16 = sample code 18902

Lane 17 = sample code 18903

Lane 18 = sample code 18904

Lane 19 = sample code 118905

Lane 20 = sample code 18907

Lane 21 = sample code 18908

Lane 22 = sample code 20588

Lane 23 = sample code 28433

Lane 24 = sample code 22683

no. 16159, 16188, 16189, 18809, 18857, 18903, 18904, 18905, 18908, 28433 and 22683 were real Roundup Ready soybean.

Quantity of polyclonal antibodies production

Antibodies specific for CP4 *EPSPS* protein were produced within the first week after the second immunization of the rabbits with *EPSPS* antisera. The antisera titer peaked from the fourth week (Figure 3). The antisera titer of the third and the fourth was not different at 1:10 to 1:1,000 dilution (antibody : buffer), while the antisera titer of the fourth week was higher than that of the third week for 9.4% at 1:10,000 dilution (Figure 3). The antibodies of the third and fourth were then pooled and the concentration of the pooled antibodies was measured. The antibody concentration was 3 mg/ml. This antibody was used for further experiments.

Optimal buffer and buffer ratio

In Dot blot assay, a suitable buffer and buffer ratio of sample to volume buffer was required to obtain high amount of solubilized the CP4 *EPSPS* protein with a simple and rapid extraction method.

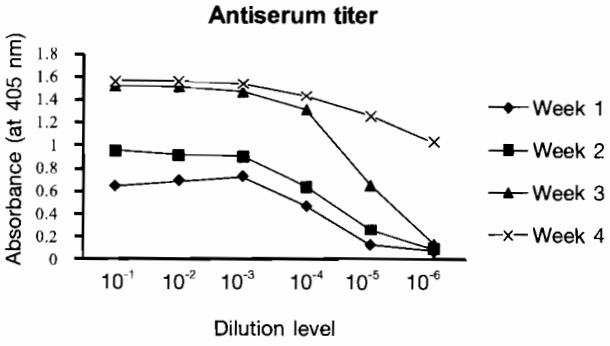


Figure 3. Tendency of polyclonal antibody production in the New Zealand white rabbit

Three type of buffers were examined and compared with clean water and various ratio of samples : buffers were also tested. It was shown that guanidine-HCl buffer gave the best result as indicated by an intense red stain resulting from the activity of alkaline phosphatase on Fast Red TR/Naphthol AS-MX substrate (Figure 4). It could be that guanidine-HCl had chaotropic properties and was generally used to denature protein. Thus, using guanidine-HCl in combination with neutral detergent might denature unspecific or membrane protein and affect the charge properties of solubilized protein (Hjelmeland, 1980). This result was also in accordance with (Rogan *et al.*, 1999; Wongwathanarat *et al.* 2010). Rogan *et al.* (1992) also performed the

experiment to determine the optimal ratio of sample : buffer between 1:10 to 1:320 for extraction of neomycin phosphotransferrase (NaTII) from cotton seed and was found that the ratio between 1: 40 and 1: 160 gave nearly equivalent amounts of extracted NPTII and concentration of NPTII above 1:160 were greatly decreased but this result showed that the ratio of samples : buffer at 1:1.25 and 1:5 were not different and the intense of red stain tended to decrease with the higher ratio (Figure 5). According to Rogan *et al.* (1992), decrease in amount of extracted protein at the higher ratio might due to degradation of the protein or nonspecific binding of target protein during extraction.

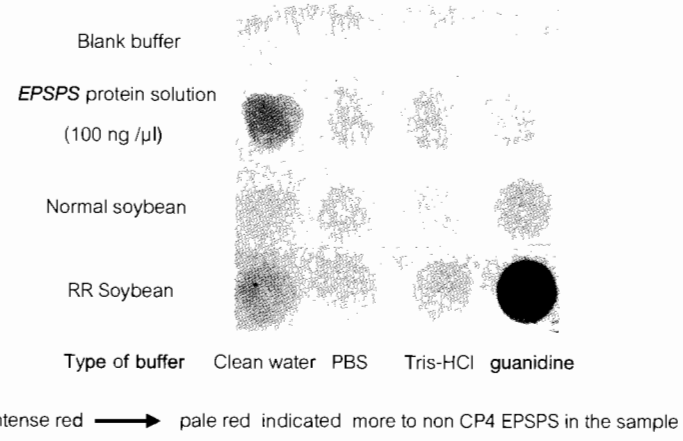


Figure 4. Dot blot analysis of Roundup Ready soybean code no 22683 using different buffer for extraction

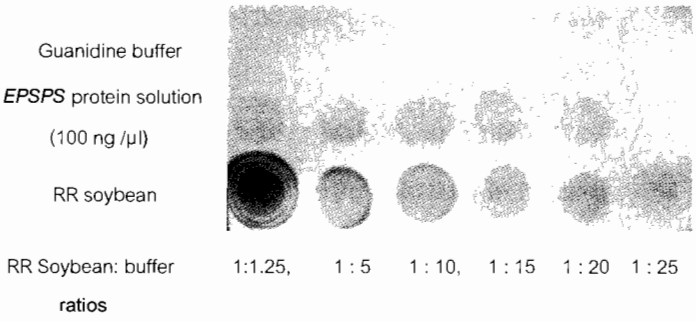


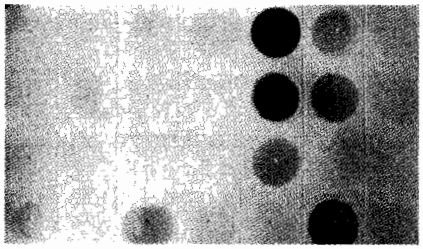
Figure 5. Dot blot analysis of Roundup Ready soybean code no. 22683 using different ratios of RR soybean: guanidine-HCl buffer for extraction

Detection of Roundup Ready soybean by Dot blot

Twenty four Roundup Ready soy bean samples were examined by Dot blot using guanidine-HCl as extraction buffer and 1:5 (sample: buffer) ratio. It revealed that only 9 out of 24 test samples (ca 37.5%) were positive for CP4 *EPSPS* protein but the amount of *EPSPS* protein in each sample varied as indicated by the different intensity of red stain (Figure 6). These nine positive samples were the code no 16152, 16157, 16159, 16189, 18816, 18903, 18904, 22683 and 24079.

Roundup Ready soybean analysis: a comparison PCR versus Dot blot

Results of Roundup Ready soybean detection by PCR and Dot blot have been compared a sample by sample. Only 15 out of 24 samples (ca



Pure *EPSPS* solution	Sample code no					
Blank guanidine	16144	16145	16152	16157	16158	16159
EPSPS (50ng/ml)	16161	16187	16188	16189	18809	18816
EPSPS (100 ng/ml)	18857	18897	18901	18902	18903	18904
EPSPS (200 ng/ml)	18905	18907	18908	20558	22683	24079

Figure 6. Dot blot assay of 24 Roundup Ready soybean. a) sample position on b) nitrocellulose membrane

62.5%) analyzed by Dot blot were in line with the results obtained by PCR analysis, but 6 out of 15 corresponding results including both positive and negative samples were still in doubt (Table 3). Furthermore, some of uncorresponding results obtained by both methods, for example sample code no 16157, showed very contrast. Remund *et al.*, (2001) stated that uncorresponding result of detection by two different based methods could result from nature and protocol of detection method, the sample size, heterogeneity and concentration of GM materials.

Table 3. Comparison results of PCR and Dot blot assay on the sample by sample

Sample code	PCR	Dot blot	Corres-ponding
16144	-	-	/
16145	-	-	/
16152	-	+ (?)	x (?)
16157	-	+++	x (?)
16158	-	-	/
16159	+ (?)	+ (?)	/ (?)
16161	-	- (?)	/ (?)
16187	-	-	/
16188	+ (?)	- (?)	x (?)
16189	+	+++	/
18809	+ (?)	- (?)	x (?)
18816	-	+ (?)	x (?)
18857	+ (?)	- (?)	x (?)
18897	-	-	/
18901	-	-	/
18902	-	-	x (?)
18903	+ (??)	+ (?)	/ (?)
18904	+ (?)	+ (?)	/ (?)
18905	+ (?)	- (?)	x (?)
18907	-	-	/
18908	+ (?)	- (?)	x (?)
20558	-	-	/ (?)
22683 (P)	+	+++	/
24079	+	+ (?)	/ (?)

\+ Means positive result for Roundup Ready® soybean (*EPSPS* protein, + <200 ng/µl, ++ ≈ 200 ng/µl and +++ > 200 ng/µl)

\- Means negative result for Roundup Ready® soybean

/ Means PCR and Dot blot results are matched

x Means PCR and Dot blot results are not matched

(?) Means undoubted results

Conclusion

Efficiency of detection of Roundup Ready soybean by PCR was similar to that by Dot blot (ca 40%). Detection results obtained by PCR and Dot blot analysis was corresponded about 15 out of 24 (ca 62.5%) including positive and negative samples.

In Dot blot assay, titer of antibody was peaked at the fourth week after the second immunization injection. Gunidine-HCl and 1: 5 ratio was the optimal extraction buffer and ratio of sample: buffer in this study.

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