Preliminary Communication

Safety Assessment of Genetically Engineered Food: Detection and Monitoring of Glyphosate-Tolerant Soybeans

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A detection technique for the genetically engineered food, glyphosate-tolerant soybean (GTS), was designed. Commercial soybeans imported from North America were cultured in pots and genomic DNA was isolated from their leaves. To detect the genes, promoter and terminator, involved in the expression of glyphosate tolerance, PCR was done using the genomic DNA and chemically synthesized primers specific to the genes. DNAs with predicted sizes were amplified and confirmed by DNA sequencing to be the genes responsible for the expression of glyphosate tolerance. Glyphosate-tolerant soybeans were found to form approximately 1.1% of the commercial soybeans, when commercially available soybeans were cultivated and number of soybeans resistant to glyphosate was found. This level is somewhat lower than an estimated value announced officially on the basis of the cultivation area of the glyphosate-tolerant soybeans.

Key words: genetically engineered food; glyphosate-tolerant soybeans; monitoring; PCR

Following the rational development of biotechnology, many genetically modified crops have been exploited in the world. These biotechnology-derived crops are expected to improve the agrifood industry facing the worldwide population explosion, limited natural resources, and loss of cultivation area due to deteriorated environments and unpredictable natural disasters. However, the transgenic crops must be ensured as to safety for both the environment and the health of human beings before they are released into nature and used in food. Recently several kinds of transgenic crops have been proved to be safe and some of them are commercialized. Especially, the production of glyphosate-tolerant soybeans (GTS) has rapidly increased in North America.

Glyphosate is a nonselective, wide-spectrum herbicide and has been reported to inhibit 5-enolpyruvylshikimate-3-phosphate synthase involved in the biosynthesis of aromatic amino acids in plants. The bacterial gene responsible for the active enzyme in the presence of glyphosate was introduced into commercial plants and several glyphosate-tolerant crops have been bred in North America. One of them, GTS, has been widely cultivated in North America since 1996. GTS allows farmers to cultivate these soybeans more easily because of the effective control of weeds and has been confirmed to be a safe food based on the compositional equivalence, the feeding value, and the newly digestible proteins expressed in GTS. In North America, the number of farmers cultivating GTS is increasing rapidly and the ratio of the cultivation area of GTS to the whole cultivation area of soybeans has been estimated to come up to 12% in 1997. In Japan, soybeans are a traditional important crop for high-protein foods, although more than 86% is imported from North America.

The monitoring of GTS is thought to be of importance to understand the stability of the bacterial gene introduced into GTS during the serial cultivation period and the influence of the artificially created crops on the environment. Although considerable effort is being focused on the detection of GTS, no actual or potential detection techniques have been developed so far. The detection techniques will fall into three major groups: immunological methods, nucleic acid probing, and molecular markers. Among them, nucleic acid probing is highly specific and allows tracking DNA. In this article, we have tried to establish detection techniques for GTS by nucleic acid probing and to measure the content of GTS in soybeans imported from North America, 1996. The technique may provide significant advantages for environmental assessment of GTS and could be applied to monitoring of other biotechnology-derived crops. In fact, by the same procedure presented in this article, we have succeeded in the monitoring of genetically engineered potatoes with soybean genes (Momma, K., et al. unpublished results).

Soybeans (IOM soybeans) produced in North America in 1996 were used as imported soybeans. Soybeans produced in Japan in 1996 were obtained from a supermarket in Kyoto, Japan. Synthetic oligonucleotides for PCR primers were from Biologica Co., Nagoya, Japan (Table). Restriction endonucleases and other DNA modifying enzymes were from Takara Shuzo Co., Kyoto, or Toyobo Co., Tokyo, Japan. The glyphosate herbicide “Roundup” was obtained from Japan Monsanto Co., Tokyo, Japan.

Isolation of genomic DNA from soybean leaves was

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Abbreviations: GTS, glyphosate-tolerant soybeans; PCR, polymerase chain reaction.
Table Oligonucleotides Used

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Source</th>
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<tbody>
<tr>
<td>Primer 1</td>
<td>5'-CAAATTTGAATTTTA-ATGGTTGTTGTAAG-3'</td>
<td>Soybean conglycinin α subunit gene</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5'-CATAGTATATCTTAA-ATTTTTAATCCCGGCG-3'</td>
<td>Soybean conglycinin α subunit gene</td>
</tr>
<tr>
<td>Primer 3</td>
<td>5'-TGCGATAAAAGGAAA-GGCTATCG-3'</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
<tr>
<td>Primer 4</td>
<td>5'-AGACTGGTGATTTCC-AGGGTTGTC-3'</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
<tr>
<td>Primer 5</td>
<td>5'-TTAAGATTGAATCC-TGTGGCCG-3'</td>
<td>Nopaline synthase terminator</td>
</tr>
<tr>
<td>Primer 6</td>
<td>5'-TAATTTATCTTAG-TTTGCGGCG-3'</td>
<td>Nopaline synthase terminator</td>
</tr>
</tbody>
</table>

done using an Isolplan Kit, Nippon Gene Co., Tokyo, Japan, according to the protocol recommended by the vendors. DNA was manipulated as described previously.  

PCR was done in a mixture (50 µl) consisting of 0.1 µg of genomic DNA, 20 pmol of specific primers, 0.3 µmol of (NH₄)₂SO₄, 0.5 µmol of KCl, 6 µmol of Tris-HCl, pH 8.0, 50 µg of Triton X-100, 0.5 µg of bovine serum albumin, 0.05 µmol of MgCl₂, 2.5 nmol of dNTPs, and 2.5 units of KOD DNA polymerase, Toyobo Co., Tokyo, Japan. The PCR program consisting of 3 steps was designed as follows: step 1, 15 cycles of 98°C for 20 sec; 58°C for 4 sec; 72°C for 15 sec; step 2, 15 cycles of 98°C for 20 sec; 54°C for 4 sec; 72°C for 15 sec; step 3, 15 cycles of 98°C for 20 sec; 50°C for 4 sec, 72°C for 15 sec. PCR was repeated twice and in the second PCR, a part of the reaction mixture of the first PCR was used as a template.

DNA sequences of PCR products were analyzed by the dyeoxy-chain termination method using automated DNA sequencer model 377 (Applied Biosystems Division of Perkin-Elmer, Foster City, Calif., USA).

About one thousand soybeans produced in North America and Japan were planted in pots at 25°C. Within a week after seeding, germination occurred and was followed by emergence of cotyledons. In the three-leaf stage (Fig. 1A), glyphosate herbicide (Roundup) was sprayed on the leaves. All of the soybeans produced in Japan were blased (data not shown). On the other hand, in the case of soybeans from North America, a few soybeans were resistant to the herbicide and grew well (Fig. 1B). The percentage of the herbicide-tolerant soybean appearance was approximately 1.1%, when commercially available soybeans were cultivated and the soybeans resistant to glyphosate were counted. This value was somewhat lower than that announced officially, which was predicted on the basis of the cultivation area of soybeans.

To identify the herbicide-tolerant soybeans bearing GTS, detection of genes concerned with the glyphosate tolerance and/or its expression in the soybeans was attempted. Although the gene of the enzyme, 5-enol-pyruvylshikimate-3-phosphate synthase from Agrobacterium sp. CP4, is reported to have been introduced into GTS, the DNA sequence of the synthase was not found in any of the literature and patents we searched. Judging from the reported construction procedure of GTS, the synthase gene is inserted between the cauliflower mosaic virus 35S promoter and the 3' nontranslated region of the nopaline synthase gene as a terminator (Fig. 2A).

Therefore, the promoter and terminator genes involved in the expression of the glyphosate-tolerant gene were targeted for the detection of GTS. The promoter and terminator DNA sequences were obtained from a DNA bank and specific primers for PCR were designed (Table, Fig. 2A).

At first, the availability for PCR of the obtained genomic DNA from leaves was checked by the detection of the conglycinin gene in soybean. By using the genomic DNA from various soybeans and specific primers for conglycinin gene, DNA with predicted size was efficiently amplified by PCR and was confirmed to be the conglycinin gene by DNA sequencing (data not shown).

Succeeding to this preliminary experiment, PCR conditions for the detection of the promoter and terminator involved in the expression of the glyphosate-tolerance
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A. Structure of glyphosate-tolerance gene (4). 3SS, cauliflower mosaic virus 3SS promoter; CTP, chloroplast transit peptide gene; CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase gene; NOS, nopaline synthase terminator. Primers 3~6 are indicated in Table. B. Electrophoretic profile of PCR products. Lane 1, molecular weight markers; lane 2, PCR product using the herbicide-tolerant soybean genomic DNA and specific primers for cauliflower mosaic virus 3SS promoter; lane 3, PCR product using the herbicide-sensitive soybean genomic DNA and specific primers for cauliflower mosaic virus 3SS promoter; lane 4, molecular weight markers; lane 5, PCR product using the herbicide-tolerant soybean genomic DNA and specific primers for nopaline synthase terminator; lane 6, PCR product using the herbicide-sensitive soybean genomic DNA and specific primers for nopaline synthase terminator.

**Fig. 2.** DNA Sequences of PCR Products and Identification of the Products.

A. DNA sequence of PCR product using the herbicide-tolerant soybean genomic DNA and specific primers for cauliflower mosaic virus 3SS promoter (Primers 3 and 4); B, DNA sequence of PCR product using the herbicide-tolerant soybean genomic DNA and specific primers for nopaline synthase terminator (Primers 5 and 6). The DNA sequence of the PCR product around Primer 5 was not analyzed. 3SS promoter, predicted amplified cauliflower mosaic virus 3SS promoter; NOS terminator, predicted amplified nopaline synthase terminator.
gene were investigated, in detail, with respect to temperature, time, and cycle of reaction. As a result, DNAs with predicted sizes were amplified from the herbicide-tolerant soybean genomic DNA by using a program consisting of 3 steps (Fig. 2B). On the other hand, no DNAs were amplified from the genomic DNAs of herbicide-sensitive soybeans or soybeans produced in Japan. Both of the amplified DNAs by PCR using specific primers for the promoter and terminator were sequenced. Nucleotide sequences of amplified DNAs almost completely matched those of the promoter and terminator (Fig. 3).

Thus, we have, for the first time, succeeded in the detection of GTS in commercially circulating soybean samples. The success makes it possible to estimate the ratio of GTS contained in soybeans, and may facilitate the discrimination of GTS from traditional counterparts, which is an important issue to be resolved for public acceptance in Japan.

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References