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Detection methods for genetically modified crops

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Abstract

Due to the market introduction of genetically modified crops (GMOs) as the Roundup Ready (RR) soya and Bt corn, the European food industry came face to face with the question of the use and labeling requirements on GMO crops and its derivatives. Although even today, no defined European legislation is available, a definitive need for detection methods exists. Both DNA and protein based methods have been developed and applied for the detection of RR soya beans and its derivatives. For the CP4 synthase, synthetic peptides corresponding with the antigenic and non-homologous parts of the CP4 synthase were synthesized and mono-specific anti-CP4 synthase monoclonal antibodies were prepared by hybridoma technology. The monoclonal antibodies were able to detect the CP4 synthase in the RR soya using Western blotting analysis. Detection limits were found between 0.5% and 1%. The method is currently validated for half-and final products. The applied DNA methodology was making use of polymerase chain reactions (PCR) using sets of primers along the gene encoding the Agrobacterium CP4 synthase. DNA extraction and purification conditions were examined on a case-by-case approach for a scala of soya products (lecithin, oil, soybean meal, soy protein isolates etc.), half-products and final consumer products. Detection limits were found between 0.01% and 0.1%. In this paper a comparison will be made between the two types of methods in relation to sample preparation, sensitivity, validation and the use for half-products and final consumer products.

1. Introduction

Biotechnology has enabled the modification of agricultural materials in a very precise way thereby improving productivity and yields. The first transgenic food crops like tomatoes, squash, potatoes, canola and also cotton are now appearing on the market. In the coming years other transgenic crops such as sugar beets and sunflower will follow, whereas transgenic cereal crops such as rice and wheat will probably enter the market after the year 2000.

When gene technology has been applied in order to obtain improved functional properties – such as starch potatoes with amylopectin and without amylose, and tomatoes without pectolytic enzymes – transgenic and non-transgenic crops are being sold separated. However, when only agronomic properties such as yield or pest resistance, have been improved – as is the case with the Roundup Ready (RR) soy (Monsanto) and the Bt resistant maize (Novartis) – transgenic and non-transgenic crops are not kept apart after harvesting. In 1997, about 8% of the soy plants grown in the USA has been genetically modified. In 1998 this percentage will probably increase to values between 15% and 20%. In this so-called RR soy strain as developed by Monsanto, a glyphosate-tolerance derived from the microorganism Agrobacterium CP4 has been introduced. In Europe the introduction of this genetically modified soy beans falls under the forthcoming Novel Food Regulation which means that provisions containing non-equivalent soy ingredients have to be labelled.

In order to possibly discriminate between genetically modified and non-genetically modified soy, a series of analytical tools is a prerequisite. At TNO analytical methods have been developed for the identification of materials of transgenic origin in food products and of raw materials for food products. For soy, TNO has developed methods for the detection of DNA of transgenic origin, as well as methods for the detection of protein of transgenic origin.

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In this paper both types of methods will be discussed in relation to specificity and sensitivity. Next, a comparison will be made between the two types of methods in relation to the use for detection in half-products and final consumer products. Finally, results will be presented from a comparitive validation study using both types of detection methods.

2. Materials and methods

2.1. DNA isolation

Prior to the DNA isolation from various types of material, homogeneous samples are prepared by milling in a whole food machine. Depending the type of material between 100 and 1000 g of starting material is milled to a fine powder. Homogeneous samples of 100 mg are transferred into a 2 ml centrifuge tube adding 1 ml extraction reagent and the samples are rotated at 70°C for 1 h. Next, the samples are centrifuged for 10 min at 14,000 *g*. From the supernatant 0.75 ml is transferred to a 2 ml microfuge tube and an equal volume of chloroform is added.

Following mixing for 10 min., phase separation is enhanced by centrifugation. 0.5 ml of the aqueous phase is transferred to a new microfuge tube and 1 ml of Wizard™ resin is added in order to further purify the isolated DNA.

The quality and concentration of DNA are determined spectrophotometrically at 260 nm.

2.2. Oligonucleotides

Since the RR soy contains a variety of transgenic DNA like the Agro-bacterium CP4 synthase gene for obtaining resistance towards glyphosate, and cauliflower mosaic virus- and petunia-genes for regulatory purposes, different primers have been synthesised for the polymerase chain reaction (PCR). The oligonucleotides TN1A and TN1B are complementary to the NOS 3′ terminator region. The primer TN23 was designed for the specific detection of the EPSPS gene of Agrobacterium sp. strain CP4. This primer is used in combination with the oligonucleotide TN-NOS3 in order to amplify in the PCR reaction a unique DNA construct comprised of parts of the EPSPS gene from Agrobacterium and the NOS 3′ terminator from Petunia. As a positive control for the soy DNA PCR reaction, specific primers for the soy lectin or heat-shock protein gene were designed.

2.3. Polymerase chain reaction

About 1 mg of the isolated DNA (5 ml) is added to 95 ml reaction mixture for the PCR. These amplifications are carried out in 100 ml reaction tubes containing PCR reaction buffer, 1.75 mm magnesium chloride, 1 mm of the primers, 0.2 mm each of dATP, dCTP, dGTP, and dTTP, and 2.5 units of Taq polymerase. In order to cover the reaction mixture, 30 ml of mineral oil is added. Amplification is performed during 40 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 60 s) following a first denaturation at 95°C for 10 min.

In the case that the PCR reaction is performed using the oligonucleotides TN23 and TN-NOS3, 2 ml of the final product is used for a nested PCR experiment. Such a nested PCR reaction is performed under identical circumstances as the regular PCR reaction with the only exceptions that only 20 amplification are performed and the primerset TN1A and TN1B is used only.

Amplification products are submitted to electrophoresis on 4% non-denaturing polyacrylamide gel electrophoresis and made visible by staining with ethidium bromide at UV (254 nm) transillumination. Registration of data is performed by video scanning. The expected size of amplified fragments is estimated by comparison with DNA fragments of known sizes.

2.4. Monoclonal antibodies

Based on the principles of Western blotting analysis we developed a detection and quantification method for the enzyme which provides the glyphosate tolerance in RR soya plants, CP4 synthase-in full: the bacterial (*Agrobacterium* sp. Strain CP4) form of 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS). Since this protein was not available, we synthesized three peptides corresponding with three parts of CP4 synthase. Using Swiss Protein Database analysis amino acid sequences were derived which were highly specific for the enzyme from this microbial source.

After conjugation of these peptides to a carrier protein like bovine serum albumin, the conjugates were used for the immunisation of mice in order to raise antibodies. Using hybridoma technology, monoclonal antibody producing cell lines were generated. In order to produce larger amounts of these antibodies, the cell lines were cultured ‘‘in vitro’’ in protein-free growth medium. Purification of monoclonal antibodies was performed using immunoaffinity chromatography (Protein G).

2.5. Validation of the tests for RR soy

Both the protein-and the DNA-based method were developed using batches of RR soybeans and non-transgenic soybeans. In order to check the quality of these batches new soy plants were grown from these beans; all plants were given a treatment with the herbicide RR. According to our expectations only the plants of RR soybeans survived this treatment.

3. Results and discussion

3.1. PCR analysis

A first prerequisite for using a test for the analysis of the presence of transgenic DNA in food ingredients or food samples, is its specificity. Therefore, in a first series of experiments, standard solutions of RR DNA, diluted in several ratios with non-genetically modified soy DNA, were tested in PCR amplification experiments using the different available primer sets. In a typical experiment the percentages of RR soy DNA in mixtures with soy DNA were 0.1%, 0.01%, 0.001% and 0%. Further, as a positive control PCR experiment, the amplification reaction was performed using the primers which specifically anneal with the soy heat-shock protein gene. As can be seen in Fig. 1, for all four different DNA preparations a positive signal can be observed with an expected size of 586 bp. Since in these 4 reactions the total amount of soy DNA is comparable no significant differences in signal intensities are expected.

More interestingly, using the primer combination TN23 and TN-NOS3 in a nested PCR experiment, a very specific PCR amplification can be performed for the analysis for the presence of the RR DNA construct comprised of the CP4-EPSPS and a Petunia terminator sequence. As represented in Fig. 2, for this primer combination specific RR DNA amplifications can be seen with a sensitivity of 0.01%. In the absence of RR DNA, no signals are found using these primers which is indicative for the specificity of this method.

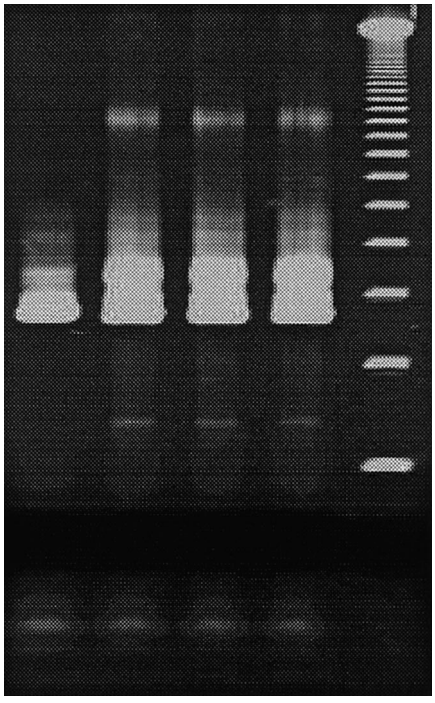

Fig. 1. Lanes: 1–5 Video scan of control soy PCR experiment on four dilutions of RR soy DNA in non-genetically modified soy DNA (0%, 0.001%, 0.01% and 0.1% RR, in lanes 1, 2, 3 and 4, respectively). Lane 5: mol. Size markers.

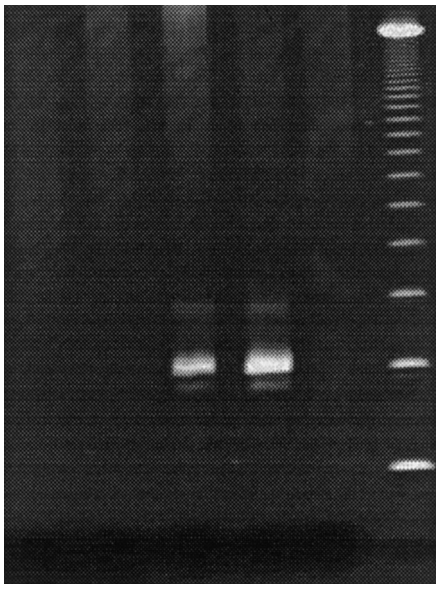

Fig. 2. Lanes: 1–5 Video scan of nested PCR experiment for the specific detection of RR soy DNA in mixtures with DNA from non-genetically modified soy. (0%, 0.001%, 0.01% and 0.1% RR, in lanes 1, 2, 3 and 4, respectively). Lane 5: mol. Size markers.

These results indicate that this PCR analysis is a very sensitive and accurate method for the detection of transgenic crops, however, the use of a good combination of different primer sets and the performance of adequate control experiments is a prerequisite.

3.2. Application of the PCR test for DNA into practice

Samples are given to TNO predominantly by producers of food products and food ingredients, mainly

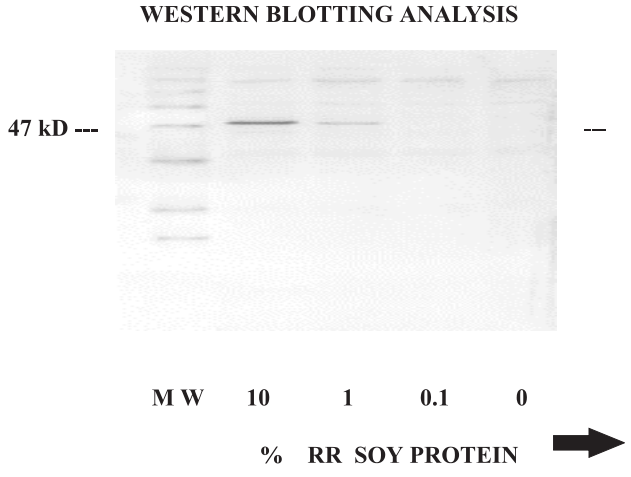

Fig. 3. M.W. 10 1 0.1 0% RR Soy protein Western blotting analysis of the RR specific soy protein.

from European countries, in order to check the presence of transgenic components in raw materials.

We have been able to demonstrate the presence of transgenic soybean DNA in soybeans, soybean protein-concentrates and – isolates, and in biscuits and sauces. We have also been able to demonstrate the presence of DNA in samples of lecithin and soy oil, which, apparently were not well purified.

3.3. Protein analysis

Using the highly specific monoclonal antibodies, the transgenic soy protein EPSPS can be visualized using Western blotting analysis. As can be seen in Fig. 3 at the level of 47 kD proteins, the sensitivity of this protein method is around 1%. A validation study has revealed that this protein analysis system is applicable for the detection of transgenic RR soy protein in raw materials and soy protein fractions. However, further processing of the soy ingredients results in loss of immunochemical recognition.

3.4. Final conclusions

A highly specific and sensitive (0.01%) PCR method has been developed for the detection of transgenic RR DNA. This method is applicable in a wide range of samples starting from beans to soybean meal, protein, lecithin and oil to half-and final products.

Besides a method for the detection of transgenic DNA, also a specific method is available for the quantification of RR protein.