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Cloned-DNA Detection in Raw and Processed Food and Feed Derived from Genetically Modified Plants

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Abstract
With the increasing availability of genetically modified plant products, it has become necessary to develop easy routine techniques for identification of such products in order to follow labeling requirements and other EU and National regulations. Widely applicable PCR-based screening methods might be most adequate and effective. Sequences commonly found in many transgenic crops, such as the CaMV-35S promoter, the *nptII* antibiotic resistance gene, or the *nos*-terminator may provide a primary target for screening. Alternatively, PCR-based identification may be directed to the modification-specific gene. In this study, we developed specific primer pairs and tested commercial kits for detection of cloned DNA in fresh and processed material.

Introduction
Advances in plant biotechnology and their application in plant breeding permitted the creation of new transgenic plant varieties and their wide use in modern agriculture. Introduction of foreign genes into plant genomes presents potential safety risks for man and the environment, and commercial use of Genetically Modified Organisms (GMO) and their products is regulated by relevant legislation in European Union and consequently in our country. Deliberate release of GMOs to the environment is regulated according to EU directive 220/90 and labeling of raw material, food and feed derived from GMOs, is enforced by directives 1813/97 and 1139/98. This creates the need for development of easy routine methods for detection of GMOs in raw material, and processed food and feed.
Detection of the modification in Genetically Modified Plants (GMPs) can be targeted to the DNA construct integrated into the host genome, the products of the incorporated genes at the mRNA and protein level, or the identification of the novel phenotype conferred by the modification. Although in fresh plant material detection can be efficient for all the above targets, in processed material the only available target is (in most of the cases) the inserted DNA. DNA-sequences introduced into the plants by genetic engineering can be amplified and detected using the polymerase chain reaction (PCR) (Saiki et al., 1985).
Target sequences available for screening may be common in many transformed varieties. Such sequences are present in genetic elements most commonly found in transgenic crops, including the neomycine-3'-phosphotransferase (*npt*II) marker gene, the cauliflower mosaic virus 35S promoter (CaMV-35S) or the nos-terminator (*nos*-3'). The modification-specific gene, which is unique for each transgenic variety could be conclusively identified through a gene-specific primer pair. The accessibility to comprehensive databases containing relevant genetic information on genetically engineered products may be an important factor in promoting and coordinating the development of identification techniques (Hemmer, 1997).
In this study we present our results on the effectiveness of PCR in detection of cloned DNA in fresh and processed material using commercial kits and specific primer pairs designed from sequences commonly used in transgenic plants.

Materials and Methods
Tested Material: DNA used as template in PCR reactions derived from fresh plant tissue or processed plant material. More specifically, DNA from fresh material was extracted from maize leaves, dry or imbibed corn seeds and soy beans. DNA from processed material was extracted from corn starch, soy meal and soy flakes. In addition, plasmid DNA harboring specific gene constructs was used as positive control to test specificity of the designed primer pairs.

DNA isolation: DNA from fresh or processed plant material was extracted using the Gene Check Kit (Hanse Analytic, Bremen, Germany) according to the instructions. The DNAeasy kit (Qiagen, Valencia, CA) was also used for DNA extraction or further DNA purification.
PCR Primers: Primers used in this study were designed from sequence data available in the GenBank or were purchased as commercially available detection kits. The specific primers designed for PCR detection of common genetic modifications are shown in Table 1.

Table 1. Primers designed to detect sequences commonly used in Genetically Modified Plants. For details on the PCR program see PCR conditions.

CLASS	GENE	GENBANK ACCESSION NUMBER	PRIMER PAIRS	PCR-PRODUCT SIZE/PROGRAM
Promoter	CaMV 35S	A09031	5'-AGGAGCATCGTGGAAAAAGAAGAC-3' 5-TTGCGAAGGATAGTGGGATTGTG-3'	111bp - B
			5'-GTAAGGGATGACGCACAA-3' 5'-CTCTCCAAATGAAATGAACTT-3'	77bp - A
Herbicide Resistanse Gene	*Bar*	A02465	5'-CCGCAGGAACCGCAGGCGTG-3' 5'-AGCAGGTGGGTGTAGAGCGTGGAG-3'	215bp - B
			5'-GCACGCAACGCCTACGACT-3' 5'-CAGCCCGATGACAGCGACCAC-3'	150bp - C
Marker Genesς	*Gus*	U12639	5'-GGGCAGGCCAGCGTATCGTG-3' 5'-GTCCCGCTAGTGCCTTGTCCAGTT-3'	463bp - B
	Npt II	U12639	5'-GGGCGCCCGGTTCTTTTTGTCA-3' 5'-GCCAGTCCCTTCCCGCTTCAGTG-3'	148bp - B
	Hyg	K01193	5'-ATTGACTGGAGCGAGGCGATGTT-3' 5'-GCTTCTGCGGGCGATTTGTGTA-3'	297bp - B
			5'-AGCTGCGCCGATGGTTTCTACAA-3' 5'-ACAGCGGGCAGTTCGGTTTCA-3'	179bp - B

Genes and modifications that can be detected with commercial kits tested in this study are shown in Table2. The exact sequence of the primers included in the kits was not disclosed.

Table 2. Sequences detected in PCR using commercial Kits (Hanse Analytic GmbH, Bremen, Germany). For details on the PCR program see PCR conditions.

CLASS	SEQUENCE	KIT NUMBER	PCR PRODUCT SIZE /PROGRAM
Promoter	CaMV 35S	8021018	195 bp - B
Terminator	*Nos* 3'UTR	8021018	180 bp - B
Internal genes	Invertase - maize	8025101	226 bp - C
	Lectin - soy	8021010	145 bp - C
Specific Sequences	CaMV 35S - Petunia transit peptide for detection of Roundup Ready soy	8021010	172 bp - C
	Unspecified sequence of the modification of Maximizer Bt-maize	8025101	211 bp - C

PCR conditions: PCR was performed in a thermocycler UNO II (Biometra, Tampa, FL). Three PCR programmes were employed. The program steps were: denaturation at 94^0C for 10 min, 50 cycles of amplification in three steps, denaturation at 94^0C for 25 sec, annealing at 50^0C (program A) 55^0C (program B) or 62^0C (program C) for 30 sec, extension at 72^0C for 45 sec, followed by extension at 72^0C for 5 min. PCR bands were separated on 2% agarose gels in TBE buffer after electrophoresis at 60V for 2h.

Results and Discussion

The aim of this study was to develop and test specific primers and commercial detection kits in order to identify the presence of cloned DNA in raw and processed material derived from transgenic plants. Although the detection of every possible specific modification in

commercially released GMPs is a very difficult task, the majority of such plants carry common elements. One example is the presence of the CaMV-35S promoter in many GMPs. In addition, a few gene constructs have been used for transformation of several major crops. Thus, most of the modifications concerning insect resistance utilize the Bt gene, for herbicide resistance the EPSP-synthase gene which confers resistance to the herbicide Roundup, or the bar and Pat genes conferring resistance to the herbicide basta, in several plant species including maize, cotton, soy and oilseed rape. Development of efficient primers to detect these most abundant modifications may provide a start point for detection of GMPs for certain plant species and enforcement of the relative legislation.

While isolation of high quality template DNA for PCR is relatively easy in fresh material, it may present a major obstacle in processed material. Usually DNA from processed material is highly degraded and only low MW fragments can be isolated (Fig.1A).

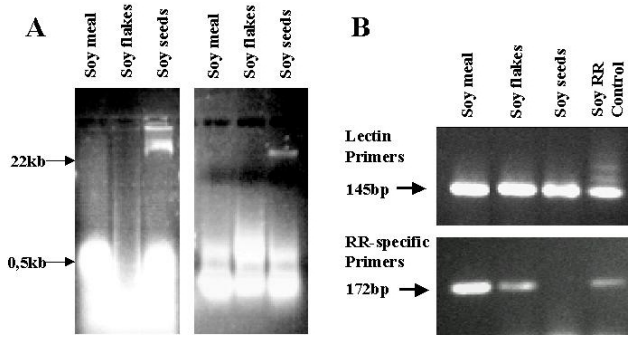

Fig.1. A. Isolation of genomic DNA from soy meal, flakes, and beans before (left gel) and after (right gel) purification with the Dneasy kit (Qiagen). B. PCR using the purified DNA from soy meal, flakes, and beans as template. PCR in the upper gel was with lectin-specific primers and a band is present in all the templates. At the lower gel primers specific for the Roundup Ready (RR) soy were used and did not detect presence of RR in the beans. PCR using unpurified DNA was unsuccessful

Also other contamination due to processing may affect PCR and further purification may be necessary to amplify the specific gene parts. Using the commercial kit for detection of Roundup Ready soy Gene Check kit (Hanse Analytic GmbH, Bremen, Germany) we were able to detect the presence of such material in soy flakes and soy meal while no amplification could be detected using template from non-transgenic soy beans (Fig.1B lower). The quality of the template was confirmed in a control reaction for amplification of the soy lectin gene (Fig.1B upper).

We also used the RR-soy DNA template along with positive and negative controls to detect the presence of the kanamycin resistance gene and the CaMV-35S promoter. As shown in Fig.2 the expected bands were observed. In the same figure the effectiveness of the specific primers designed to detect the hygromycin resistance (Hyg) and the glucuronidase (Gus) marker genes was also shown.

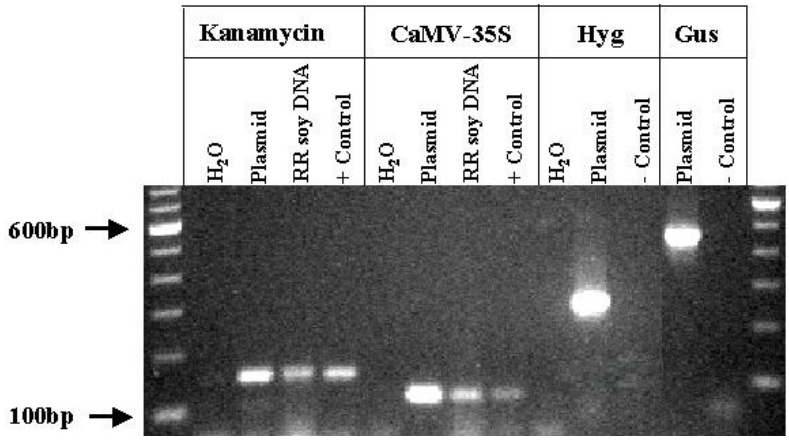

Fig. 2. PCR with primers shown in Table 1 specific for the marker genes *NptII*, *Gus* and *Hyg*^{R} and the CaMV-35S promoter. Plasmid template in each reaction was positive control. Additional positive controls where indicated were from the Gene Check kit (Hanse Analytic GmbH, Bremen, Germany). Negative controls were also included. The specific band of the expected MW was observed in the positive reactions while no amplification occurred in the negative reactions

The two *Hyg* primer pairs as well as the two *bar* primer pairs (shown in Table 1) were examined with positive plasmid templates and negative controls (Fig.3) and amplification of

the expected band of the correct MW was observed. In this experiment, there was no available

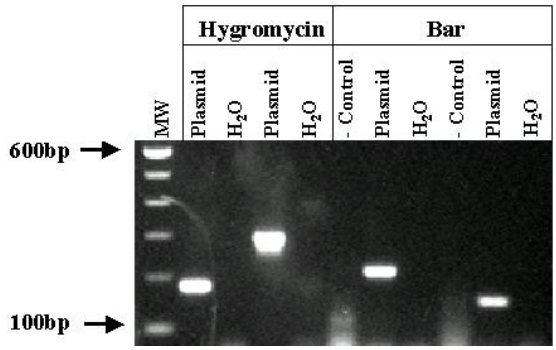

Fig. 3. PCR with primers shown in Table 1 specific for the marker gene *Hyg*^{R} and the *bar* gene for Basta resistance. Plasmid template in each reaction was positive control. Negative controls were also included. The specific band of the expected MW was observed in the positive reactions while no amplification occurred in the negative reactions

DNA from transgenic plants.

Finally, we examined several maize materials for the presence of Roundup Ready and Bt-maize. DNA was extracted from dry and germinating maize seeds, maize leaves and corn starch. The control reaction with primers included in the Gene Check kit (Hanse Analytic GmbH, Bremen, Germany) amplified part of the maize invertase gene in all the materials (Fig.4A). In most of our tests no GMP DNA for the above modifications could be detected. However, in one instance we detected the presence of Bt-maize in starch (Fig.4B)

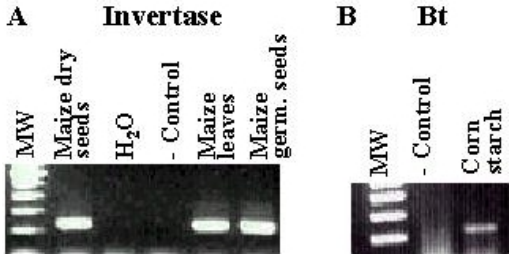

Fig.4. A. PCR detecting the maize invertase gene in dry and germinating maize seeds and maize leaves using the Gene Check kit (Hanse Analytic GmbH, Bremen, Germany). Negative controls were also included.
B. PCR detecting the presence of Bt-maize in corn starch using the same kit. Negative control was also included.

Using the primers and commercial kits tested in this study we were able to identify cloned DNA in different plant species and raw or processed materials. With data provided herein as well as utilizing other sources (Hemmer, 1997) and sequence information publicly available in the GenBank it is possible to design and use specific primers establishing routine methods to detect the presence of GMOs in raw and processed material derived from plants.

There are a few simple rules and precautions, which were taken into consideration in this study and criteria that should be met in future experiments:

Primers should be selected that are specific for genetic elements present in a large number of genetically engineered agricultural crops. The genetic elements on which the assay is based should not occur naturally in the respective plants. The assay should not rely on genetic elements that occur in organisms that may appear frequently as contaminants of the food stuff under analysis. The designed amplicon should be relatively small to warrant broad applicability of the test also to heat-treated samples or materials with low pH and/or highly degraded DNA.

References

Hemmer, W. (1977) Foods derived from genetically modified organisms and detection methods. BATS-Reports online. http://www.bats.ch/reports.htm

Saiki, R.H, Scharf, S, Faloona, F, Mullis, K.B., Horn, G.T., Ehrlich, H.A., Arnheim, N. (1985) Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230: 1350-1354.