

Detection of Genetic Modification 'ac2' in Potato Foodstuffs

Radka Pribylova^{1*}, Petr Kralik¹, Ivo Pavlik¹ and Milan Bartos²

¹Veterinary Research Institute, Hudcova 70, CZ-621 00 Brno, Czech Republic

²Genex CZ, s.r.o., Vinicni 235, CZ-615 00 Brno, Czech Republic

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Summary

The genetic modification 'ac2' is based on the insertion and expression of *ac2* gene, originally found in seeds of amaranth (*Amaranthus caudatus*), into the genome of potatoes (*Solanum tuberosum*). The purpose of the present study is to develop a PCR method for the detection of the mentioned genetically modified potatoes in various foodstuffs. The method was used to test twenty different potato-based products; none of them was positive for the genetic modification 'ac2'. The European Union legislation requires labelling of products made of or containing more than 0.9 % of genetically modified organisms. The genetic modification 'ac2' is not allowed on the European Union market. For that reason it is suitable to have detection methods, not only for the approved genetic modifications, but also for the 'unknown' ones, which could still occur in foodstuffs.

Key words: GMO, *Solanum tuberosum*, PCR, antimicrobial peptide, food safety

Introduction

The basis of genetic modification (GM) 'ac2' is the insertion of the *ac2* gene, originally found in seeds of *Amaranthus caudatus*, into the genome of potato, *Solanum tuberosum* (1) and its expression into the fungicidal peptide Ac-AMP2 (2,3). The Ac-AMP2 peptide belongs to a group of hevein-like peptides (4). The hevein-like peptides are members of chitin-binding proteins as they can bind to chitin in fungal cell walls, change its polarity and thus inhibit the fungal growth. Ac-AMP2 from *A. caudatus*, as well as other chitin-binding proteins, plays an important role in the protection of plants or their seeds, mainly against whole range of fungi and Gram-positive bacteria (4–6). This antimicrobial feature of Ac-AMP2 was used to construct GM potatoes (carrying the *A. caudatus ac2* gene) with increased resistance against fungal and bacterial infections (1).

According to the Regulation of European Commission (EC) No 1829/2003 on Genetically Modified Food and Feed, a register of GM food and feed containing GM products, which may be legally used on the market of the European Union, was made. At present, twenty-

-seven modifications of GM crops, bacterial and yeast biomass are authorised or their authorisation process is being renewed (7). The above-mentioned modification of potatoes is not authorised in the European Union and therefore no detection method exists. However, because of the possibility of illegal production, we have developed the polymerase chain reaction (PCR) method for the detection of GM 'ac2' in potato tubers (8,9) and in this paper in foodstuffs made of potatoes.

The first aim of the study is the development of a detection method based on the identification of the *ac2* gene in foodstuffs. Besides the *ac2* gene, the *StTS1* gene was also detected. The *StTS1* gene (putative trehalose synthase gene, GenBank AF483209) was found in all potato tissues (oral communication) and it is closely related to other plant trehalose syntase genes (10,11). The sequence of *StTS1* gene served as an endogenous potato gene. The reason for using another target sequence, which originated from the plant used for genetic transformation, lies in revealing the false negative results. These false negative results can occur as a consequence of PCR inhibitors present in the examined samples (12). The

*Corresponding author; Phone: ++420 5 3333 1611; Fax: ++420 5 4121 1229; E-mail: pribylova@vri.cz

presence of false positive results as a consequence of contamination of foodstuffs by amaranth was prevented by the detection of the cauliflower mosaic virus 35S promoter (CaMV 35S), which was used for the above mentioned GM potato development (1). The second aim of the study is the examination of twenty various potato-based foodstuffs from the Czech Republic market for the presence of the 'ac2' genetic modification.

Materials and Methods

Biological material and DNA isolation

Tubers of *S. tuberosum* (cultivar Impala) and seeds of *A. caudatus* were kept for 7 to 10 days and 3 to 4 days at room temperature, respectively. A total of 100 mg of fresh potato sprouts and 20 mg of *A. caudatus* germinated seeds were used for DNA isolation using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the supplier's instructions. DNA extraction was preceded by homogenization of tissues under liquid nitrogen by mortar and pestle.

Potato-based food

Twenty various foodstuffs made of potatoes were purchased from grocery stores (Table 1). DNA was isolated using the NucleoSpin® Food Kit (Macherey-Nagel, Germany). After dilution in 550 µL of lysate buffer and 10 µL of proteinase K (NucleoSpin® Food Kit, Macherey-Nagel, Germany), each sample was disrupted using 500 mg of 1.0-mm zirconia beads and fifteen 2.5-mm zirconia/silica beads (BioSpec Products, Inc., USA). The homogenisation was performed using the MagNA Lyser Instrument (Roche Molecular Diagnostic, Germany) at 3300×g per 7 min, and the DNA was then isolated according to the manufacturer's instructions. Each sample of food was prepared in duplicates and both duplicates of one sample were concentrated on one column of the

isolation kit to obtain a sufficient amount of DNA. The isolation of a negative control was also prepared to verify that contamination did not occur during the process of isolation.

Potato product spiked with amaranth flour

Each of the five test tubes contained a sample of 200 mg of potato product No. 1 (Table 1). Subsequently, amaranth flour was added to each tube to reach the resulting concentrations of flour as follows: 5, 2, 1, 0.5 and 0 %. Each sample was prepared in duplicates. Both duplicates of one sample were concentrated on one membrane of the NucleoSpin® Food Kit (Macherey-Nagel, Germany) column to obtain sufficient amounts of DNA. Besides the series of amaranth flour dilutions, an isolation of negative control without any DNA matrix was prepared.

Polymerase chain reaction

Generally, all PCRs were carried out in a final volume of 20 µL. The reaction mixtures consisted of 10 µL of HotStartTaq Master Mix (Qiagen, Germany), 10 pmol of each primer (AcUNI-F, AcUNI-R, StUNI-F and StUNI-R) and 2 µL of isolated genomic DNA (approx. 100 ng). The PCRs were conducted in a gradient thermocycler PTC-200 (MJ Research, USA) according to the following PCR protocol: preincubation at 96 °C for 15 min, denaturation at 96 °C for 10 s, annealing at 60 °C for 20 s and extension at 72 °C for 40 s. The cycle was repeated 40 times followed by a final extension at 72 °C for 2 min. PCR products were visualised by electrophoresis (voltage: 5 V/cm, time: 40 to 50 min) using 2.5 % agarose gel (Amresco, USA) stained with ethidium bromide (Amresco, USA) dissolved in 0.5×TBE buffer. The length of PCR product was compared to 100 bp (Malamite, Czech Republic) or 50 bp (New England Biolab, USA) ladder.

PCR positive controls were prepared by TA-cloning using pCR2.1 vector (Invitrogen, the Netherlands). PCR amplification product for the *ac2* gene was obtained using AcUNI-F (5' GTG GGA TGT GTT GCA GTC AG-3') and AcUNI-R (5' CCA GCA CCA GCA AGT TTA GC-3') primers. The size of the resulting PCR product was 145 bp. Part of *StT51* gene was amplified by means of StUNI-F (5' CTT CAC AGT CCA TTT CCT TCA TC-3') and StUNI-R (5' GCA TAG TCG AAA GTG TGG AAT C-3') primers, which amplified a product of 113 bp in length. Both primer sets were proposed by the Primer3 software. The presence of the correct sequence was confirmed by sequencing (MWG-Biotech, Germany). The resulting recombinant plasmids pAcUNI (for the 145-bp amplicon) and pStUNI (for the 113-bp amplicon) were diluted in elution buffer (EB buffer; QIAprep Spin Miniprep Kit, Qiagen, Germany) to obtain 10⁵ copies of each plasmid. The mix of diluted plasmids (1:1) was used as a positive control for PCR.

PCR used for the sensitivity test with spiked samples followed the conditions described in the first paragraph of PCR chapter with three modifications: (i) the concentration of AcUNI-F/R primers was 4 pmol instead of 10 pM (the most appropriate concentration after experimental testing of various combinations of primer

Table 1. List of tested potato-based food

Category	No.	Potato-based product
Powdery mixtures: solid consistency dishes	1	Traditional potato pancakes
	2	Potato pancakes
	3	Potato pasta balls
	4	Potato powdered dumplings
	5	Potato dumplings
Powdery mixtures: liquid and semi- liquid consistency dishes	6	Potato soup
	7	Potato soup with mushrooms
	8–10	Mashed potatoes (dehydrated product)
Potato crisps	11–13	Potato crisps – salted
	14	Potato crisps – with pepper flavour
	15	Potato crisps – salted
Potato salads	16–20	Potato salad with mayonnaise

concentrations), (ii) 4 μ L of DNA template instead of 2 μ L, and (iii) 45 PCR cycles instead of 40.

The specificity of AcUNI-F/R and StUNI-F/R primers was verified on a set of various genetically unmodified plants: barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*), oilseed rape (*Brassica napus* ssp. *napus*), soybean (*Glycine max* L.) and two representatives of the *Solanaceae* family: tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum annum* L.). Amaranth and potato were examined as positive controls. The specificity test followed the conditions described in the first paragraph of this chapter. In the case of unspecific products, these were cloned into plasmids (Invitrogen, The Netherlands) and sent for sequencing (MWG-Biotech, Germany).

Twenty potato-based foodstuffs were tested for the presence of GM 'ac2'. Preparations of the PCR reaction and PCR protocol were identical as in the first paragraph of the *Polymerase chain reaction* chapter, except for two differences. As in the case of sensitivity test, the amount of DNA template was increased to 4 μ L and the number of PCR cycles to 45. Each of the twenty food samples was tested twice.

For the detection of the CaMV 35S promoter, CaMV35-F (5' TCC ACT GAC GTA AGG GAT GAC-3') and CaMV35-R (5' CTG GTG ATT TCA GCG TGT CC-3') primers, amplifying a gene fragment with the size of 105 bp, were proposed by the Primer3 software. The PCR was performed with a volume of 20 μ L. The reaction mixture contained 10 μ L of HotStartTaq Master Mix (Qiagen, Germany), 5 pM of each primer and 4 μ L of DNA. The PCR ran according to the following program: preincubation at 96 °C for 10 min, denaturation at 96 °C for 20 s, annealing at 56 °C for 20 s and extension at 72 °C for 20 s. The cycle was repeated 40 times followed by a final extension at 72 °C for 3 min. DNA from reference maize material (13) with 1 % of GM maize line NK603 was used as a positive control. The method was applied to test twenty foodstuffs made of potatoes. Each of the samples was examined twice.

Results

Before testing a set of spiked samples (potato product spiked with amaranth flour), the optimisation of primer concentrations had to be performed. Experimental proofing of the various combinations of primer concentrations showed that the concentrations of 10 and 4 pM were the most suitable for StUNI-F/R and AcUNI-F/R primers, respectively (data not shown). Using the mentioned concentrations, the method enabled us to detect the *ac2* gene to a level of 0.5 % (amaranth flour in the potato product). In the 0 % sample, which was not spiked with amaranth flour, the presence of *ac2* gene was not detected, which is in agreement with our expectations (Fig. 1).

Fig. 2 shows the high specificity of AcUNI-F/R primers that amplified the specific PCR product corresponding to the *ac2* gene only in the case of amaranth seeds (lane 1). On the other hand, the StUNI-F/R primers amplified the PCR fragments of soybean (line 7) and representatives of the *Solanaceae* family – sweet pepper

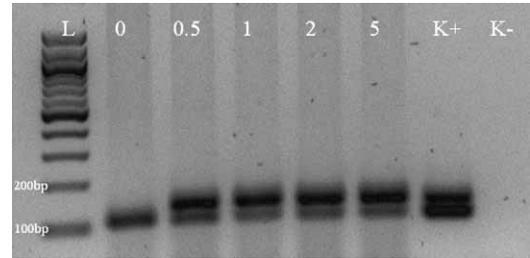


Fig. 1. Detection of the *ac2* and *StTS1* genes using a set of spiked samples
From the left: 100 bp ladder (lane L), 0 % sample (lane 0), 0.5 % sample (lane 0.5), 1 % sample (lane 1), 2 % sample (lane 2), 5 % sample (lane 5), positive PCR control (lane K+) and negative PCR control (lane K-)

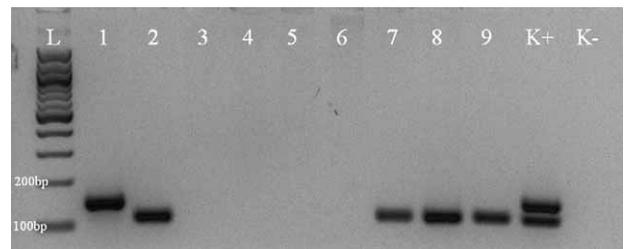


Fig. 2. Specificity of AcUNI-F/R and StUNI-F/R primers
From the left: 100 bp ladder (lane L), amaranth (lane 1), potato (lane 2), maize (lane 3), barley (lane 4), wheat (lane 5), oilseed rape (lane 6), soybean (lane 7), sweet pepper (lane 8), tomato (lane 9), positive PCR control (lane K+) and negative PCR control (lane K-)

(line 8) and tomato (line 9). All three PCR products were sequenced and their sequences are shown in Fig. 3. Sequencing confirmed the high level of similarity of all these sequences with specific part of *StTS1* potato gene. In the case of sweet pepper and tomato, one nucleotide substitution occurred (purine base was changed to pyrimidine). Concerning the soybean, one deletion, one insertion and 22 nucleotide substitutions occurred (Fig. 3).

Twenty foodstuffs made of potato were tested for the presence of the GM 'ac2'. Amplification of potato *StTS1* gene with the size of 113 bp occurred in all sam-

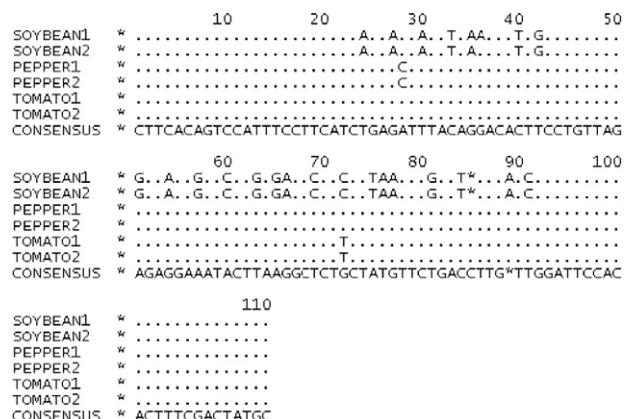


Fig. 3. Sequencing of unspecific products from non-GM plants (soybean, sweet pepper and tomato) using StUNI-F/R primers. Each sample was sequenced twice

ples. However, the intensity of bands in the case of samples 17, 18 and 19 was weak. The PCR fragment with the size of 145 bp corresponding to the *ac2* gene was not amplified in any of the tested products (Fig. 4). At the same time, the set of foodstuffs was examined for the presence of CaMV 35S promoter. Specific PCR product corresponding to 105 bp amplification fragment of CaMV 35S promoter did not occur in any of the samples, except in the positive control (Fig. 5).

Discussion

DNA isolation is a crucial step for PCR and must yield an adequate purity and quantity. Due to the damage of DNA during food production (the effect of high temperature, pressure, *etc.*), the material from each potato product was collected twice for DNA isolation. Both duplicates were processed on one column membrane with the aim of obtaining a higher DNA concentration in the sample and consequently higher PCR sensitivity. Chen *et al.* (14) tested degradability of selected genes in the Roundup Ready soybean throughout the process of foodstuff production. The influence of milling, mixing, homogenisation, sterilisation, boiling and spray drying on the DNA fragment length were investigated. Results showed a noticeable degradation of the investigated genes, up to a quintuple DNA fragment length decrease compared with the original length. Similar results were also found by Rizzi *et al.* (15), who proved the close relationship between foodstuff processing and the DNA ex-

traction method on the one hand and PCR detection of a chosen gene on the other.

DNA fragmentation and degradation are closely related to the expected length of PCR products. While the length of PCR products is not so much significant in the case of fresh plant tissues, it is a crucial factor in foodstuff analysis. These results were confirmed by our experiments. Applying the primers for the detection of GM 'ac2' in potato tubers used in our previous study (8) to foodstuffs made of potato, no amplification product occurred. Therefore, PCR sequences of the *ac2* and *StTS1* genes (306 and 554 bp, respectively) amplified in our previous study were used to design new sets of primers (AcUNI-F/R and StUNI-F/R), which subsequently amplified shorter sequences (145 and 113 bp, respectively) within the mentioned PCR products. For the reason of higher PCR sensitivity, 4 μ L of DNA were pipetted in the case of processed food samples.

Using primers that amplify shorter sequences within chosen genes, PCR product corresponding to amplification product of *StTS1* gene occurred in all twenty food samples. Nevertheless, in some samples (17, 18 and 19) the bands were very weak (Fig. 4). This could be connected with DNA isolation, as it was mentioned in the first paragraph. Thus, two explanations are possible: (i) it was mentioned above that various physical and chemical factors affect raw material during its processing, such as high pressure, temperature or acidity. This leads to fragmentation or to complete degradation of DNA, which cannot then be isolated from the samples, and (ii)

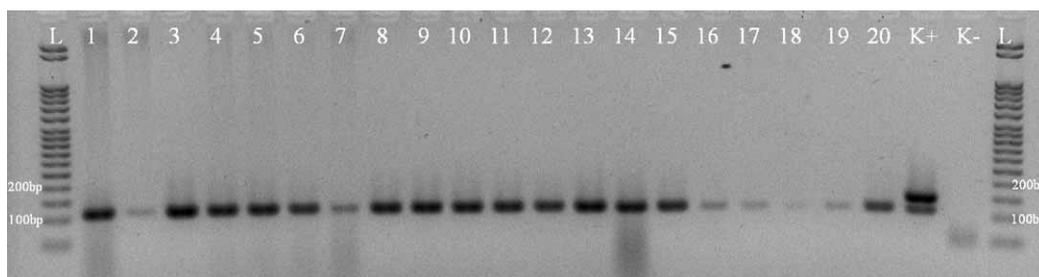


Fig. 4. Detection of the 'ac2' genetic modification in the potato-based foodstuffs
From the left: 50 bp ladder (lane L), tested foodstuffs (lanes 1–20), positive PCR control (lane K+), negative PCR control (lane K-) and 50 bp ladder (lane L)

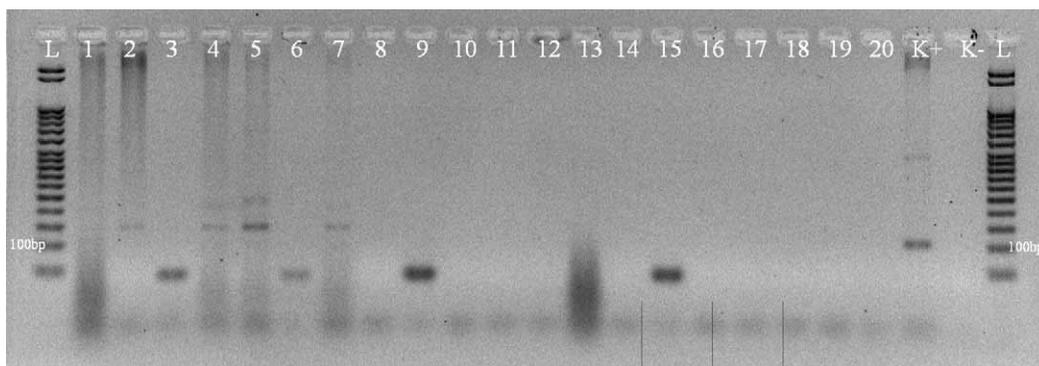


Fig. 5. Detection of the CaMV 35S promoter in various potato-based foodstuffs
From the left: 50 bp ladder (lane L), tested foodstuffs (lanes 1–20), positive PCR control (lane K+), negative PCR control (lane K-) and 50 bp ladder (lane L)

besides the major compound, finished products and oven-ready food often contain other ingredients like dyes, stabilisers, antioxidants, emulsifiers, *etc.*, which may function as PCR inhibitors (for more information about PCR inhibitors see 12). Due to the fact that samples 17, 18 and 19 originated from delicate potato salads, these might have been affected by PCR inhibitors rather than physical factors.

Avoiding the possibility of false positive results due to either food contamination by amaranth flour or by simultaneous use of amaranth as one of the food components, the *ac2* gene detection was accompanied by CaMV 35S promoter gene identification (Fig. 5). The 35S promoter from the cauliflower mosaic virus is one of the most commonly used promoters in plant biotechnology. Together with neomycin phosphotransferase II gene (*npt II*) and nopaline synthase terminator (*nos3'*), it is frequently used for production of genetically modified organisms and could be used for their detection (16,17). Moreover, these commonly used sequences are often the only possibility for detection of unauthorised GMOs (18). Since CaMV 35S gene was used for the development of GM 'ac2', it could also be used for its detection. In view of the fact that neither *ac2* nor CaMV 35S gene was found, the presence of GM 'ac2' was excluded from all of the twenty tested products.

Testing the primers specificity, three unspecific bands for sweet pepper, tomato and soybean occurred. Although the previous search for unspecific sequences using GenBank did not find any similarity with sweet pepper, tomato or soybean, all sequences showed 96 % similarity with the *S. tuberosum StTS1* gene (GenBank AF483209.1). Also, in the official protocol of the European Commission describing the detection method for amylopectin potato, detectable unspecific PCR products were found in the case of *Solanaceae* species (tomato and sweet pepper) during the primer specificity trial (19).

GM reference materials have been prepared for GM plant that are authorised for commercial usage. Nevertheless, no reference materials exist for other, unauthorised modifications (13). In this case, spiking of foodstuffs with another food or raw material is possible. Therefore, a series of potato product samples spiked with known amounts of amaranth flour were prepared, which enabled the determination of the detection limit of the system (Fig. 1). Due to the higher level of processing in potato product rather than in amaranth flour, the concentration of AcUNI-F/R primers had to be decreased to amplify both fragments in one reaction. Using these conditions, the amaranth flour in potato product at a level of 0.5 % was detected.

Conclusion

PCR method for the detection of genetically modified potatoes 'ac2' in foodstuffs was developed. The method was based on the detection of the *ac2* gene from amaranth as well as the *StTS1* gene from potatoes in one reaction. The crucial steps of the described method were the isolation of DNA from processed foodstuffs and consequently the design of primers amplifying shorter sequences of the target genes. In the case of foodstuff contamination by amaranth, the PCR method for the detec-

tion of CaMV 35S promoter was adopted. The method was used to test twenty potato-based foodstuffs from the Czech market. According to our expectations, none of them were positive for GM 'ac2'.

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