Ligation-dependent probe amplification for the simultaneous event-specific detection and relative quantification of DNA from different genetically modified organisms

F. Moreano [1], A. Ehlert[1], U. Busch [2] und K.-H. Engel [1]

[1] Technical University Munich, Chair of General Food Technology , Am Forum 2, D-85350 Freising-Weihenstephan
[2] Bavarian Health and Food Safety Authority, Veterinärstr. 2, D-85764 Oberschleißheim

Center of Food and Life Sciences Weihenstephan

Introduction

The application of a ligation-dependent probe amplification (LPA) technique for the event-specific detection and relative quantification of DNA from different GMO in a single reaction is described. Originally, ligation-dependent PCR has been applied to the relative quantification of DNA in the field of medical diagnostics [1]. The present approach was conceived to support the implementation of regulatory provisions regarding the use of GMO derived products in foods and feeds [2, 3]. Further, this particular LPA-system was designed to use exclusively synthetic oligonucleotides, which avoids complex cloning and preparation steps required for the isolation of single stranded DNA probes. Four pairs of synthetic oligonucleotides were designed to allow the detection of reference genes in the genomes from maize and soya (*HMGa*- and *Le1*-genes, respectively), as well as event-specific regions of the transgenic maize line MON810 and Roundup Ready soya. Specificity and sensitivity of the system and parameters related to the relative quantification of DNA were assayed using mixtures of isolates extracted from certified reference maize and soybean flours. The application of this novel analytical strategy results in a flexible, modular system that can be complemented with further probes to broaden the range of target sequences.

1

Ligation-dependent probe amplification reaction

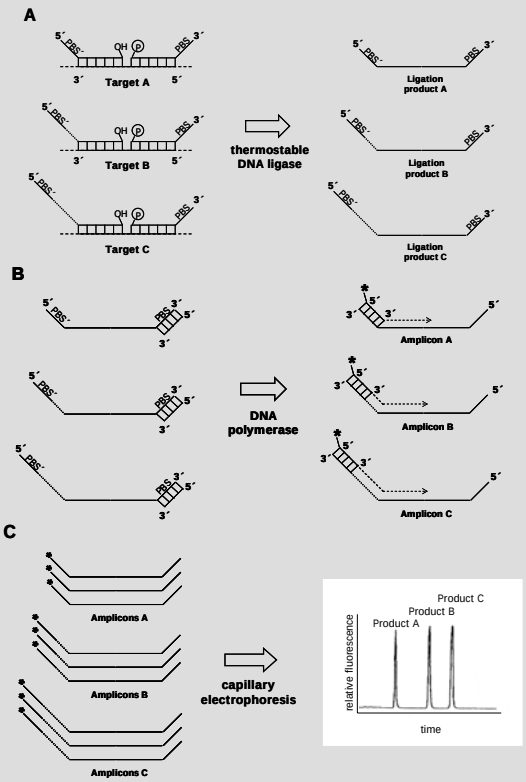

Figure 1. Ligation-dependent amplification reaction.

As illustrated in Figure 1A, LPA requires the design of two DNA probes for the detection of each target sequence. Probes consist of synthetic oligonucleotides, containing a target-specific hybridization site and identical primer binding sites (PBS) at their 5´- or 3´-ends, respectively. In the case of successful hybridization, probes are ligated by the action of a thermostable ligase. This enzyme catalyzes exclusively the linkage of adjacent 3´-hydroxylated and 5´-phosphorylated termini in duplex DNA structures that are stable at high temperatures. The use of spacer sequences between PBS and hybridization sites renders ligation products with a characteristic length for each target DNA. In the second step of the reaction (Figure 1B), ligation products are amplified using one common pair of primers. In the case of successful ligation, synthesis of complementary strands results in the formation of PBS for the labeled primers. The amplification process is competitive, i.e. ratios between the amounts of ligation products are preserved throughout the PCR reaction. Labeled PCR amplicons are finally separated and detected via capillary electrophoresis (Figure 1C).

Sequences of LPA probes and primers are listed in Table 1. Primer and spacer sequences were derived from the bacteriophage λ genome in order to obtain sequences without affinity to the targeted regions. Criteria for the selection of the targeted hybridization sites were (*i*) similarity in thermodynamic properties and (*ii*) nearly constant lengths. The lengths of the targeted hybridization sites were kept nearly constant (±3 bp) to avoid analytical pitfalls emerging especially when working with processed products, where a degradation of DNA may take place [4].

3

The event-specific detection of maize MON810 and Roundup Ready soya was performed by targeting the junction regions between the 35S promoter and the plant DNA in each GM line [5, 6]. The targeted regions of the reference genes used in this study (*HMGa*- and *Le1*-gene, for maize and soya, respectively) were previously shown to be suitable for quantification purposes [7].

Table 1. LPA probes and primers

Target / GenBank accession nr	Left probe	Right probe	Ligation products [bp]
Maize Mon810 / AF434709	5´ – **CAGGCGGCGCATTTTTATTGC**taattcttctatttctga CTAACGTTTAACATCCTTTG*CCATTGC* - 3´	P – 5´ – *CCAGCTATCTGTCACTTTATTGTGAAGA* **CTACGGCAAATGTCATCGACG** - 3´	115
Roundup Ready soya / AJ308514	5´ – **CAGGCGGCGCATTTTTATTGC** *TCGCAATGATGGCATTTGTAGGAGC* - 3´	P – 5´ – *CACCTTCCTTTTCCA*TTTGGGTTCCCT taattcttctattte**CTACGGCAAATGTCATCGACG** - 3´	109
Maize *HMGa*-gene / AJ131373	5´ – **CAGGCGGCGCATTTTTATTGC**taattcttctattt CGTTTGTGTGGATTGTAGGACAAGG - 3´	P – 5´ – CTCCCTATGTAGCCAAGGCTAACA **CTACGGCAAATGTCATCGACG** - 3´	105
Soya *Le1*-gene / K00821	5´ – **CAGGCGGCGCATTTTTATTGC** TTCCTTCAACTTCACCTTCTATGCCC - 3´	P – 5´ – CTGACACAAAAAGGCTTGCAGATGG taattct**CTACGGCAAATGTCATCGACG** - 3´	100
Sanprime - unlabeled	5´- **CGTCGATGACATTTGCCGTAG** - 3´		
Sanprime - labeled	**FAM** – 5´-**CAGGCGGCGCATTTTTATTGC** - 3´		

Uppercase bold: **primer binding sites** Uppercase: plant DNA
Lowercase: spacer DNA *Uppercase italics:* *insert DNA*

4

Detection and relative quantification of DNA targets

The performance of the LPA system was assayed using reference standards from maize and soya with defined GMO contents. DNA isolates from these standards were mixed in different regimes to simulate DNA extracts from composed food products containing equal or different GMO fractions. As illustrated in Figure 2, the applied technique allowed the detection of the respective target sequences in a single reaction tube, depending on the composition of the template DNA used in each experiment. Target specificity remained unaffected either by the presence of DNA from different sources, or by the simultaneous use of multiple pairs of probes.

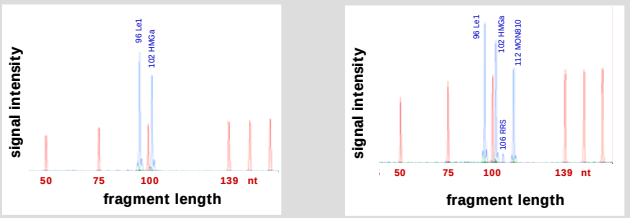

Figure 2. Simultaneous detection of event-specific junction regions and reference genes (*HMGa*- and *Le1*-gene, respectively) in the genomes of conventional and transgenic maize and soya. Figure 2A shows an electropherogram obtained by the analysis of a mixture of DNA isolates from conventional maize and soya (75 ng each). Figure 2B corresponds to the analysis of a mixture containing respectively 75 ng DNA from maize (5% GMO) and soya (0.1% GMO).

The suitability of this system for the relative quantification of GMO target sequences was tested exemplary analyzing DNA from maize standards with GMO proportions of 0.1, 0.5, 1, 2 and 5%. As illustrated in Figure 3, a calibration curve was created by determining the ratios between peak heights from the transgenic and the corresponding reference target, and plotting these against the defined GMO-content of each sample. The calibration curve showed a good linear correlation between the normalized peak heights and the increasing GMO contents in the reference standards (R^2 > 0.98). Results obtained by sixfold sample analysis exhibited standard deviations comparable to those obtained in real-time PCR assays.

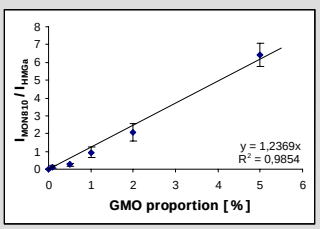

Figure 3. Calibration curve for the relative quantification of DNA from transgenic maize MON810.

5

Summary

- Criteria for the development of a modular ligation-dependent amplification system, as well as for the design of synthetic primers and probes suitable for the detection of DNA in food samples have been described.
- The novel approach was successfully applied for the detection of event-specific junction regions and reference genes in the genomes of transgenic and conventional maize and soya in a single reaction.
- The potential of this analytical strategy to be applied for the relative quantification of DNA sequences, as required for the surveillance of GMO-derived products in foods and feeds, was demonstrated on the basis of commercially available maize standards.

6

References

[1] Schouten J. P., McElgunn C. J., Waaijer R., Zwijnenburg D., Diepvens F., Pals G. (2002): Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucl. Acids. Res. 30 (12); e57
[2] Regulation (EC) No 1829/2003. Official Journal of the European Union, L 268/1
[3] Regulation (EC) No 1830/2003. Official Journal of the European Union, L 268/24
[4] Moreano F., Busch, U., Engel K.-H. Distortion of GMO Quantification Results: Influence of Heat Induced DNA Degradation as Assayed on Different Maize Milling Fractions. J. Agri. Food Chem. Submitted
[5] Windels P., Taverniers I., Depicker A., van Bockstaele E., de Loose M. (2001): Characterisation of the Roundup Ready soybean insert. Eur. Food Res. Tech. 213; 107-112
[6] Holck A., Vaitilingom M., Didierjean L., Rudi K. (2002): 5´-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGuard maize. Eur. Food Res. Tech. 214; 449-453
[7] European Committee for Standardization. Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods (ISO/DIS 21570:2003). Project Reference: prEN ISO 21570. Status: Under Approval

7