GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Highly specific detection of genetic modification events using an enzyme-linked probe hybridization chip

M.Z. Zhang, X.F. Zhang, X.M. Chen, X. Chen, S. Wu and L.L. Xu

Zhejiang Academy of Science and Technology for Inspection and Quarantine, Hangzhou, China

Corresponding author: M.Z. Zhang
E-mail: mzzhang429@163.com

Genet. Mol. Res. 14 (3): 9298-9305 (2015)
Received November 17, 2014
Accepted April 6, 2015
Published August 10, 2015
DOI http://dx.doi.org/10.4238/2015.August.10.10

ABSTRACT. The enzyme-linked probe hybridization chip utilizes a method based on ligase-hybridizing probe chip technology, with the principle of using thio-primers for protection against enzyme digestion, and using lambda DNA exonuclease to cut multiple PCR products obtained from the sample being tested into single-strand chains for hybridization. The 5'-end amino-labeled probe was fixed onto the aldehyde chip, and hybridized with the single-stranded PCR product, followed by addition of a fluorescent-modified probe that was then enzymatically linked with the adjacent, substrate-bound probe in order to achieve highly specific, parallel, and high-throughput detection. Specificity and sensitivity testing demonstrated that enzyme-linked probe hybridization technology could be applied to the specific detection of eight genetic modification events at the same time, with a sensitivity reaching 0.1% and the achievement of accurate, efficient, and stable results.

Key words: Enzyme-linked probe hybridization chip; Detection limit; Genetic modification events

INTRODUCTION

In recent years, biotechnology has been widely utilized within modern agriculture and related industries, and hundreds of genetically modified organisms (GMOs) have been approved for commercialization worldwide. A record 170.3 million hectares of biotech crops were grown globally in 2012, representing an annual growth rate of 6%, with an increase of 10.3 million over the 160 million hectares grown in 2011 (James, 2012). However, since the first genetically modified (GM) crop was generated in 1993, the question of the safety of GM has been continuously debated. In order to protect the health of humans and that of the living environment, many countries have formulated regulations toward GM organisms and implemented strict management protocols relating to the import of GM foods, requiring detection reports and a system of labeling of GM components and GM-derived food.

To execute the labeling requirements, polymerase chain reaction (PCR) amplification has become the primary technique for GMO detection. The PCR detection strategies, which discriminate between GM- and non-GM derived DNA varieties, are divided into four levels: screening, and gene-, construct-, and event-specific PCR detection. The screening method is associated with a particular risk of obtaining false positives (Xu et al., 2007). To date, a limited variety of event-specific quantitative PCR methods have been developed for a select number of GM crop events, as compared with the total number of commercialized GM events; these include detection of the GTS 40-3-2 soybean (Berdal and Holst-Jensen, 2001; Terry and Harris, 2001; Huang and Pan, 2005), MON531 and MON1445 cotton (Yang et al., 2005a), GT73 canola (Taverniers et al., 2005), MON810 maize (Hernández et al., 2003; Holck et al., 2002), Bt11 maize (Zimmermann et al., 2001; Rønning et al., 2003), GA21 maize (Hernández et al., 2004; Taverniers et al., 2005), MON863 maize (Yang et al., 2005b; Pan et al., 2006), Bt176 maize (Taverniers et al., 2005), T25 maize (Collonnier et al., 2005), NK603 maize (Nielsen et al., 2004), and CBH351 maize (Windels et al., 2003). However, these methods have the obvious limit of being only able to detect a single gene at a time.

In this study, we have developed a new method to identify multiple GMO events using an enzyme-linked probe hybridization chip. This method is based on ligase-hybridizing probe chip technology (Zhang et al., 2014), with the principle of using thio-primers for protection against enzyme digestion, and using lambda DNA exonuclease to cut multiple PCR products obtained from the sample being tested into single strand chains for hybridization. The 5'-end amino labeled probe was fixed onto the aldehyde chip, and hybridized with the single-stranded PCR product, followed by addition of a fluorescent-modified probe that was then enzymatically-linked with the adjacent, substrate-bound probe, in order to achieve highly specific, parallel, and high-throughput detection.

MATERIAL AND METHODS

Material

Certified Reference Material (CRM) of GM maize lines Bt11, TC1507, Bt176, MON863, and NK603; and GM rapeseed lines MS8, T45, and GT73 were purchased from Institute for Reference Materials and Measurements-European Reference Materials (IRMM-ERM). Conventional maize and rapeseed were purchased from a local market in Hangzhou, China. A grinding instrument (Philips Cucina Blender HR2860, Philips, Amsterdam, The

Netherlands) was used to grind the maize and rapeseed grains into powders (0.2 mm) which were then mixed according to the ratios indicated in Table 1 to form the GM and non-GM test materials, resulting in GM samples of 5, 1, 0.1, and 0.01%, respectively.

Table 1. Sample preparation of different contents.

Samples	Preparation of samples (GM components content %)
5%	100 mg A
1%	100 mg from fully mixed powder of 400 mg B and 100 mg A
0.1%	100 mg from fully mixed powder of 4900 mg B and 100 mg A
0.01%	100 mg from fully mixed powder of 49900 mg B and 100 mg A

A: CRM of GM sample powder; B: non-GM sample powder. Add 100 mg GM sample powder into Eppendorf tube for DNA extraction.

Methods

DNA extraction

The Qiagen DNeasy Plant Maxi kit (Qiagen, Venlo, The Netherlands) was used for genomic DNA extraction. Double distilled H_2O was added to dissolve the DNA. The DNA concentration was measured by absorbance at 260 nm and the DNA was stored at -20°C for further use.

Primer selection and probe design

The primers and probes used in these experiments were designed using the primer premier 5.0 Software (PREMIER Biosoft, USA) and synthesized by TaKaRa Bio (Otsu, Shiga, Japan). The synthetic primer and probe sequences and their modifications are not listed in this article for the purpose of maintaining intellectual property protection rights (IPRs).

Multiplex-PCR amplification

Target genes were amplified from the isolated genomic DNA samples and thio-labeled using a multiplex-PCR system. Amplification reactions were carried out in 25 μL total volumes on an S1000 Thermo Cycler (BioRad, Hercules, CA, USA). After considerable experimentation to optimize the PCR reactions, the optimized end concentrations of PCR components used were as follows: 1X PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.3 μM of each primer, 2.5 U Taq enzyme, and 0.5 U uracil DNA glycosylase (UNG) enzyme. PCRs were performed using 40-100 ng DNA. PCR cycling conditions were as follows: 5 min at 95°C; 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and 7 min at 72°C.

Enzyme-linked probe hybridization chip

Point-spotting and template fixation

Preparation of the aldehyde chip was performed as follows: point-spotting and fixation were carried out on the aldehyde chip using the positive probe (15 μM) and the amino-

modified probe (30 μM). The chip was hydrated for 8 h, dried for 2 h at 70°C, washed twice for 5 min each with lotion [2X saline sodium citrate (SSC), 0.5% sodium dodecyl sulphate (SDS)], and washed twice with water for 5 min each. The clean chip was blown dry and stored at 4°C.

Exonuclease digestion of the PCR products

A sample of each PCR product was taken for exonuclease digestion to generate a single-stranded product. The digestion system comprised 8 μg PCR product, 1 μL Lambda E enzyme, and 1 μL 10X buffer, and was brought to 50 μL final volume with water. The control group was generated using the same process with the exception of enzyme addition. PCR products were digested for 3 h at 37°C, and run on an agarose gel to determine whether the digestion was successful. The reaction was inactivated by incubation at 72°C for 30 min, and the products were purified with isopropanol for the determination of single-chain product concentration.

Hybridization of the single-strand product to the probe array

The single-stranded PCR product was mixed with 3X hybridization solution at a 3:1 ratio, and 1 μL mixture was used for hybridization with the probe array. The chip was incubated at 48°C for 30 min, and left to stand at room temperature for 10 min. Each array was only hybridized with one single-stranded PCR product. The hybridized arrays were washed with lotion (2X SSC, 0.5% SDS) for 5 min and with water for an additional 5 min, and were then dried and prepared for further use.

Hybridization of the fluorescent-modified probes

A Cy3-labeled probe at 2.5 μM final concentration was mixed with 3X hybridization solution at a 3:1 ratio, and 1 μL mixture was hybridized with the probe array containing the bound thio-probes annealed with the single-strand target PCR products. The chip was incubated at 48°C for 30 min, and left to stand at room temperature for 10 min. The probe utilized corresponded (was complementary) to the hybridized single-strand product. The array was washed with lotion (2X SSC, 0.5% SDS) for 5 min, then with water for another 5 min, dried, and prepared for further use.

Enzyme linkage and NaOH treatment

The enzyme reaction system constituted 1 μL 0.05 U/μL T4 ligase reaction solution and 2 μL T4 10X buffer supplemented to 20 μL final volume with water. The array was incubated at room temperature for 60 min, and washed with 0.3 M NaOH at room temperature for 5 min, followed by a 5 min wash with lotion (2X SSC, 0.5% SDS), and a final wash with water for another 5 min. The fully processed array was then dried and scanned.

RESULTS

Design of control samples

To validate the whole detection process, the following control samples were included

in addition to the samples tested in each detection: positive quality control (known GMO samples), and negative quality control (conventional samples).

Threshold determination of positive and negative results

Theoretically, a positive or negative result could be inferred according to the presence or absence of fluorescent signals from the target gene probes. In practice, the signal intensities of the negative quality control probes can differ substantially as can surface conditions. In the present study, we took the average signal intensity of the negative quality control probes as the background signal intensity and calculated the ratio of the signal intensity of the target sequence probes to the negative control probe. After considerable experimental repetition with known positive and negative samples, we set a ratio of 5:1 as the threshold for positive results, and a ratio of 3.5:1 as the threshold for negative results. So, when the average ratio was above 5, the sample was defined as positive, whereas when the average ratio was below 3.5, the sample was defined as negative (Xu et al., 2007). A sample with a ratio between 3.5 and 5 was considered to be ambiguous and was reprocessed. Each experiment was repeated three times. Using this standard, all of the positive and negative samples could be correctly identified (Figure 1).

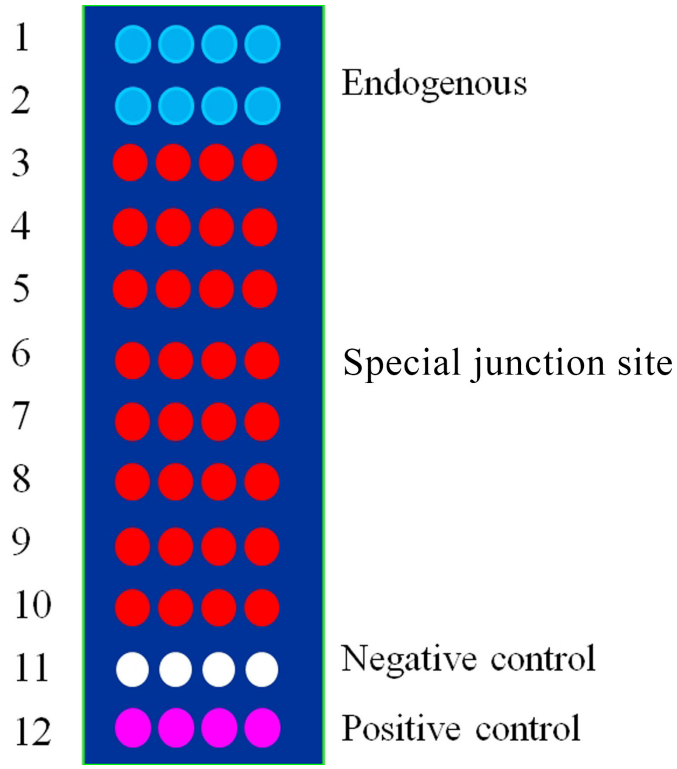

Figure 1. Oligonucleotide microarray format. Blue spots mean probes of edogenesis gene. Red spots mean probes of the special junction site sequences. White spots mean negative control and pink spots mean positive control.

Validation of the method

An enzyme-linked probe hybridization chip was used for the verification test of the endogenous and special junction site sequences of eight strains of GM corn and rapeseed. Figure 2A-D illustrates the respective test results of the positive transgenic samples Bt11, TC150, MON863, and T45. The maize endogenous gene *Zein* (James, 2012), positive control (Hernández et al., 2004), and MON863 event (Yang et al., 2005b) displayed positive hybridization signals while the other samples were detected as negative. The results from testing other GM events indicated that positive hybridization signals could only be observed from the corresponding event sites and were absent from non-target event sites and negative controls. The results showed that the enzyme-linked probe chip could accurately detect both the endogenous and special event sequences corresponding to the eight GM events.

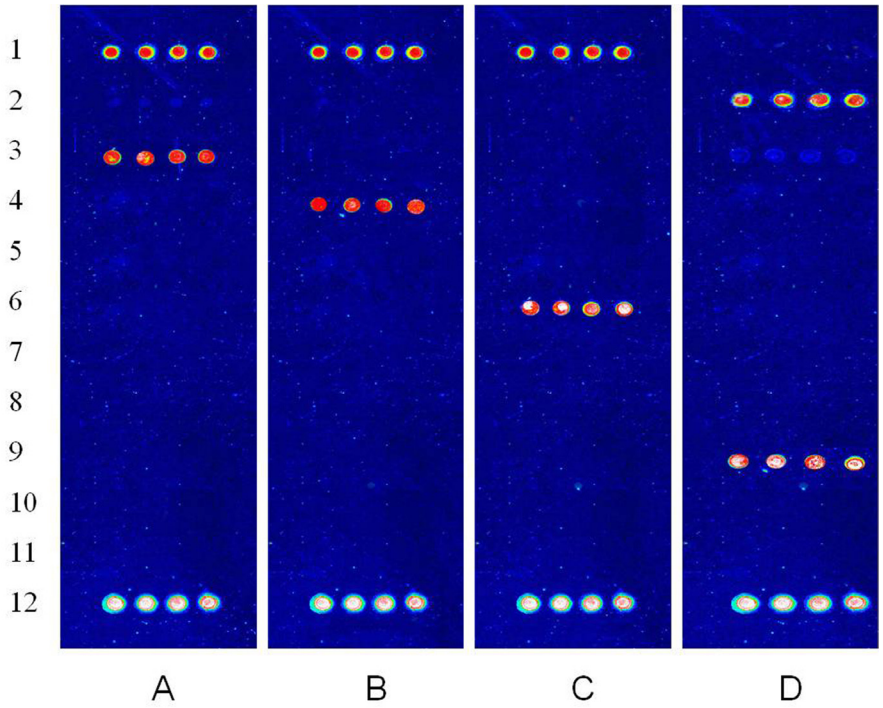

Figure 2. Detection of specificity for enzyme-linked probe hybridization chip. 1-10 mean the probe of endogenesis gene of Zein, Hmg, GM maize lines Bt11, TC1507, Bt176, MON863 and NK603, GM rapeseed lines MS8, T45 and GT73, 11 means Negative control, 12 means Positive control. **A.** Image of the microarray hybridization of 1%Bt11. **B.** Image of the microarray hybridization of 1%TC1507. **C.** Image of the microarray hybridization of 1% MON863. **D.** Image of the microarray hybridization of 1% T45.

Assessment of sensitivity and detection limits

After grinding the GM soybeans, rapeseed, corn, rice, and non-GM soybeans into powder (0.2 mm), the materials were mixed according to the guidelines shown in Table 1, resulting in sample mixtures containing GM maize and rapeseed at 5, 1, 0.1, and 0.01%. These

samples were then utilized for DNA extraction, multiplex-PCR amplification, and enzyme-linked probe hybridization chip detection. The results showed that the enzyme-linked probe hybridization chip could detect the endogenous and GM events at the level of 0.1% maize and rapeseed content with strong positive hybridization signals, indicating that the detection sensitivity of this method could reach 0.1% in practical use (Figure 3).

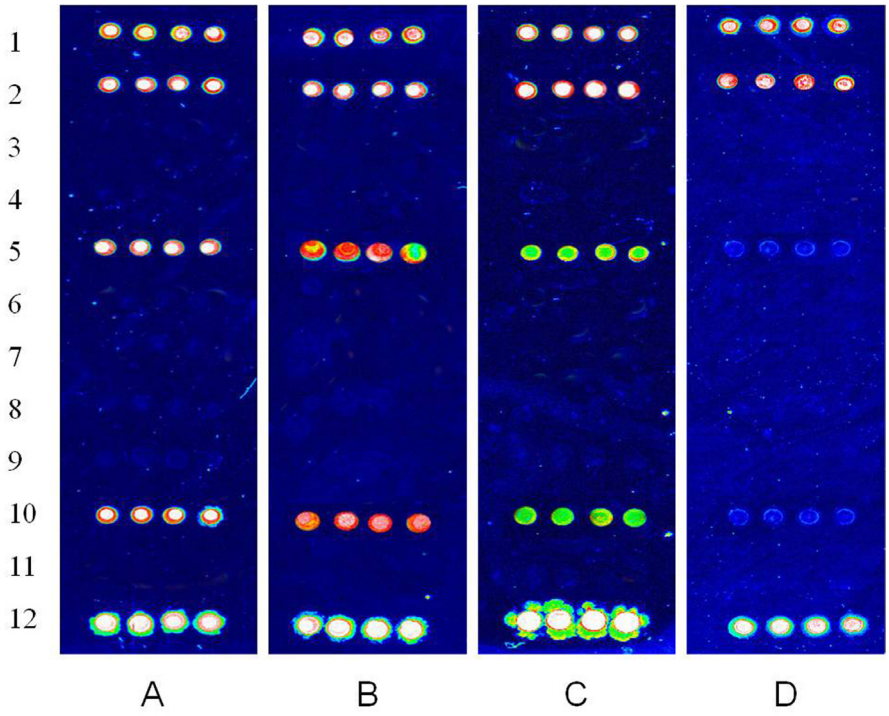

Figure 3. Limits of detection of maize MON810 and T45. **A.** Image of the microarray hybridization of 5% GMO content. **B.** Image of the microarray hybridization of 1% GMO content. **C.** Image of the microarray hybridization of 0.1% GMO content. **D.** Image of the microarray hybridization of 0.01% GMO content.

DISCUSSION

In this study, we have established an enzyme-linked probe hybridization chip system, and demonstrated the successful detection of positive hybridization signals from eight GM events. The advantage of this method is its high specificity: only when both probes exhibit complementarity with the DNA templates would the ligation reaction occur allowing detection; otherwise, the templates and secondary probes would all be eluted without generating signals, so that false positive results would effectively be avoided. The results of specificity and sensitivity testing showed that the enzyme-linked probe hybridization technology could be applied to the specific detection of eight GM events of two different kinds of crops, with the sensitivity reaching 0.1% and the generation of accurate, efficient, and stable results.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Program for Science and Technology from Zhejiang Province (#2011C12023, #2011C22065) and a grant of the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) (#2011IK249).

REFERENCES

Berdal KG and Holst-Jensen A (2001). Roundup Ready soybean event specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analysis. *Eur. Food Res. Technol.* 213: 432-438.

Collonnier C, Schattner A, Berthier G, Boyer F, et al. (2005). Characterization and event specific-detection by quantitative real-time PCR of T25 maize insert. *J. AOAC Int.* 88: 536-546.

Hernández M, Pla M, Esteve T, Prat S, et al. (2003). A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard R based on the 3'-transgene integration sequence. *Transgenic Res.* 12: 179-189.

Hernández M, Esteve T, Prat S and Pla M (2004). Development of real-time PCR systems based on SYBR® Green I, Amplifluor™ and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21. *J. Cereal Sci.* 39: 99-107.

Holck A, Vaïtilingom M, Didierjean L and Rudi K (2002). 5'-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGard maize. *Eur. Food Res. Technol.* 214: 449-453.

Huang CC and Pan TM (2005). Event-specific real-time detection and quantification of genetically modified Roundup Ready soybean. *J. Agric. Food Chem.* 53: 3833-3839.

James C (2012). Global Status of Commercialized Biotech/GM Crops: 2012. ISAAA Brief 44-2012. ISAAA, Ithaca.

Nielsen CR, Berdal KG and Holst-Jensen A (2004). Characterisation of the 5'-integration site and development of an event-specific real-time PCR assay for NK603 maize from a low starting copy number. *Eur. Food Res. Technol.* 219: 421-427.

Pan AH, Yang LT, Xu SC, Yin CS, et al. (2006). Event-specific qualitative and quantitative PCR detection of MON863 maize based upon the 3'-transgene integration sequence. *J. Cereal Sci.* 43: 250-257.

Rønning SB, Vaïtilingom M, Berdal KG and Holst-Jensen A (2003). Event specific real-time PCR for genetically modified Bt11 maize (*Zea mays*). *Eur. Food Res. Technol.* 216: 347-354.

Taverniers I, Windels P, Vaïtilingom M, Milcamps A, et al. (2005). Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 maize and transgenic GT73 canola. *J. Agric. Food Chem.* 53: 3041-3052.

Terry CF and Harris N (2001). Event-specific detection of Roundup Ready Soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol.* 213: 425-431.

Windels P, Bertrand S, Depicker A, Moens W, et al. (2003). Qualitative and event-specific PCR real time detection methods for StarLink maize. *Eur. Food Res. Technol.* 216: 259-263.

Xu J, Zhu S, Miao H, Huang W, et al. (2007). Event-specific detection of seven genetically modified soybean and maizes using multiplex-PCR coupled with oligonucleotide microarray. *J. Agric. Food Chem.* 55: 5575-5579.

Yang LT, Pan AH, Zhang KW, Yin CS, et al. (2005a). Qualitative and quantitative PCR methods for event-specific detection of genetically modified cotton Mon1445 and Mon531. *Transgenic Res.* 14: 817-831.

Yang L, Xu S, Pan A, Yin C, et al. (2005b). Event-specific qualitative and quantitative PCR detection of genetically modified MON863 maize based on the 5'-transgene integration sequence. *J. Agric. Food Chem.* 53: 9312-9318.

Zhang MZ, Chen WJ, Zhang XF, Chen XM, et al. (2014). Event-specific detection of genetically modified rice using enzyme-linked probe hybridization chip. *Food Sci.* 35:222-225.

Zimmermann A, Lüthy J and Pauli U (2000). Event specific transgene detection in Bt11 corn by quantitative PCR at the integration site. *Lebensm.-Wiss. Technol.* 33: 210-216.