academicJournals

Vol. 5(7), pp. 99-103, December 2013
DOI: 10.5897/IJGMB2013.0078
ISSN 2006-9863 © 2013 Academic Journals
http://www.academicjournals.org/IJGMB

International Journal of Genetics and Molecular Biology

Full Length Research Paper

Application of multiple displacement amplification in detection of five events of genetically modified organisms

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Accepted 24 October, 2013

In this study, multiple displacement amplification (MDA) was used for transgene detection, and proved to effectively improve the sensitivity of most fluorescence polymerase chain (PCR). Five events of genetically modified plants were chosen for detection in different plant species by MDA and real-time PCRs. Individual primer/probe systems based on each transgene target were used. The extracted DNA of each sample was diluted and used as a template for MDA. All extracted DNA and diluted mdaDNA were detected by real-time PCR. The results were evaluated with respect to DNA quantity, DNA purity and difference of Ct values of the isogenetic DNA extract before and after MDA (D-values). The MDA amplifying effects were largely different among genes and plants. Organelle DNA has more copies than genomic DNA by MDA.

Key words: Multiple displacement amplification, genetically modified organisms, detection, events.

INTRODUCTION

Multiple displacement amplification (MDA) which is widely used for whole genome amplification employs a bacteriophage φ29 DNA polymerase together with exonuclease-resistant degenerate primers to amplify DNA isothermally at 30°C (Dean et al., 2002; Lage et al., 2003). The φ29 DNA polymerase extends random hexamer primers to make a DNA template copied repeatedly and displace previous copies (Roger et al., 2009). MDA has many advantages such as less biased amplification, higher DNA yield and higher fidelity to the template DNA (Pinard et al., 2006). MDA has specific advantages as compared to e.g. primer extension pre-amplification-polymerase chain reaction (PEP-PCR) and degenerate oligonucleotide-primed PCR (DOP-PCR). Real-time PCR (RT-PCR), Southern blot analysis, conventional PCR and real-time PCR and transgene detection studies have been used to describe the utility of mdaDNA for generation of representative DNA (Hosono et al., 2003; Tengs et al., 2007).

MDA has been used for accurate whole genome amplification from single cells and spores (Woyke et al., 2011). UV irradiation of MDA reagents effectively eliminates the exogenous DNA from whole genome amplification reagents for single cell sequencing and analysis (Woyke et al., 2011). The MDA of single cells for preimplantation genetic diagnosis was successfully used for analyzing Duchenne muscular dystrophy (Ren et al., 2009). MDA of gDNA from single spores of a rust fungus *Puccinia striiformis* can be used in molecular genetic analysis of the wheat yellow rust fungus (Wang et al., 2009). Moreover, MDA can convert complex mixtures of DNA fragments to long linear and circular concatamers,

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which can be further used in high throughput genetic analysis (Muhammad et al, 2008). Sufficient viral DNA fragments were produced and sequenced starting from a handful of virus-infected beetles by using MDA (Wang et al., 2008). MDA followed by standard PCR facilitated the detection of pathogen from as low as 1 CFU ml^{-1} of potato brown rot bacteria (Grover et al., 2009).

In addition, MDA was used to pre-amplify spices DNA to improve the ratio between amplifiable DNA from spices and inhibit substances, and then enable the identification of spices (Focke et al., 2011). MDA can improve the DNA template quantification by abating the inhibiting factor's concentration of PCR (Zhang et al., 2013). A automated platform was used to generate genomic DNA by MDA technique from hundreds of single cells in parallel. In this platform, MDA reactions were screened and classified by 16S rRNA gene PCR sequence (McLean et al., 2013). Also, generation of reference material by using MDA for detection of GMOs was studied. In their study, the performance of mdaDNA in RT-PCR and the relative sequence representation on mdaDNA were compared with gDNA, also, the effects of the amount of input gDNA on MDA yield were tested. The results showed that mdaDNA was highly suitable for use as a positive PCR control in qualitative GMO analysis (Lillian et al., 2008). mdaDNA could be re-amplified only once and could be kept for five months at 4°C and more than a year at -20°C without losing integrity of genome. (Kaewmanee et al., 2013). However, the reproducibility of genes from different GM plants and events after MDA has not been tested.

The objective of this study was to assess the usefulness of MDA for amplification of genes in different GM plants and events. These genes were selected from ribosomal and genomic DNA in GM cotton MON88913, GM soybeans GTS40-3-2 and MON89788, and GM maizes Bt11 and MON863.

MATERIALS AND METHODS

Samples and DNA extraction

Cotton seed MON88913 (*Gossypium hirsutum*), positive test samples were provided by Shandong Entry-Exit Inspection and Quarantine Bureau of PRC. Soybeans (*Glycine max*) GTS40-3-2 (ERM-BF410gk, Belgium) and MON89788 (AOCS 0906-B, USA), as well as maizes (*Zea mays*) Bt11 (ERM-BF412f, Belgium) and MON863 (ERM-BF416d, Belgium) were Certified Reference Materials (CRM).

Each sample was prepared into 1 wt.% test samples by GM negative cotton seed powder, soybean powder and maize powder. DNA templates were extracted from test samples using a GenoDNA Plant Mini Kit (Ambiogen, China) according to the manufacturer's instructions. Briefly, 100 mg of a ground sample was lysed with a lysis buffer until no tissue was visible. Then phenol/chloroform extraction was carried out. After centrifugation, the upper aqueous phase was transferred into a new tube, and then the buffer that was provided with the kit was added to precipitate DNA. The resulting DNA pellet was dissolved and applied to a silica column. After the column was washed twice with a washing buffer, the DNA bound to the column was eluted with an elution buffer. The eluted DNA was quantified by a spectrophotometer (BioPhotometer plus, Eppendorf, Germany).

MDA

The DNA extracts of cotton seed, soybean and maize were diluted into 30 and 0.3 ng/µL. Then 2.5 µL of each 0.3 ng/µL DNA was amplified using REPLI-g Mini kits (Qiagen, Chatsworth, USA) within 50 µL reaction volumes. The reaction systems were subsequently incubated at 30°C for 16 h and then terminated at 65°C for 3 min according to the manufacturer's instructions. The mdaDNA was diluted into 30 ng/µL with water and used in subsequent applications without further purification. The yield of double-stranded DNA (dsDNA) was quantified by the spectrophotometer at 260 nm.

RT-PCR analysis

As targets, the different crops were chosen: an 18S rDNA, AdhC endogenous gene-specific and event-specific sequence of GM MON88913 cotton; an *18S rDNA*, *lectin* endogenous gene-specificand event-specific sequence from GM soybeans GTS40-3-2 and MON89788; an *18S rDNA*, *zein* endogenous gene-specific, and event-specific sequence from GM Bt11 and MON863 maizes. All targets were amplified separatey. Amplification programmes and reaction mixtures were in accordance with some published protocols (Table 1).

All primers and probes were synthesized by Shenggong (Shanghai, China). 18S *rDNA* genes were a multi-copy gene in ribosome of each plant. The genes of *AdhC, Lectin, Zein* were indivudully used as taxon-specific genes in genome for cotton, soy bean and maize. MON88913, GTS40-3-2, MON89788, Bt11 and MON863 were event-specific boundary sequence of GM cotton, GM soybean and GM maize genome. The primers and the probes are shown in Table 1.

PCR was performed using an RT-PCR instrument (Master Cycle Realplex4, Eppendorf, Germany). Each DNA sample was amplified in a final volume of 25 µL in a 0.2-mL tube containing 200 nM each of primer F and primer R; 10×Taq Buffer (Tiangen, Beijing China), 2 mM magnesium chloride, 200 nM each of dATP, dCTP, dGTP and dTTP; 1.5 units of Taq DNA polymerase (Tiangen, Beijing China), and 60 ng of extracted DNA template or mdaDNA template. After 120 s of initial denaturation at 95°C, PCR condition was optimized as follows: 45 cycles of amplification (95°C for 15 s, 60°C for 60 s). The PCR assay was performed using the RT-PCR instrument. In each RT-PCR setup, reaction systems containing purified water (NTC) and mdaDNA from mdaNTC reactions were included as negative controls.

The threshold cycle (Ct) value was used with the same baseline to compare the RT-PCR performances of the extracted DNA and mdaDNA of GM cotton seed, GM soybean and GM maize. Each event of GMOs was conducted in triplicate and each triplicate had 8 repeats, then parallel data were averaged.

Comparison of gene representations

In fluorescence PCR amplification, the same concentration of DNA extracts and mdaDNA templates from GM cotton, GM soybean and GM maize were used. The *18S rDNA* genes from the 5 events were amplified; while the respective species-specific genes and event-specific genes from cotton, soybean and maize were amplified. These Ct D-values were used to draw a curve in Figure 1.

Table 1. Primers and probes of each gene.

Gene	Type	Primers and probe	Reference
18S rDNA	Endogenous gene in plant ribosome	F: 5'-CCTGAGAAACGGCTACCAT-3' R: 5'-CGTGTCAGGATTGGGTAAT-3' P: FAM5'-TGCGCGCCTGCTGCCTTCCT-3'TAMRA	SN/T 1204-2003
AdhC	Endogenous gene in cotton genome	F: 5'-CACATGACTTAGCCCATCTTTGC-3' R: 5'CCCACCCTTTTTTGGTTTAGC-3' P:6-FAM5'-TGCAGGTTTTGGTGCCACTGTGAATG-3'TAMRA	Joint Research Centre (2008b)
Lectin	Endogenous gene in soybean genome	F: 5'-CCAGCTTCGCCGCTTCCTTC-3' R: 5'-GAAGGCAAGCCCATCTGCAAGCC-3' P:6-FAM5'-CTTCACCTTCTATGCCCCTGACAC-3'TAMRA	SN/T 1204-2003
Zein	Endogenous gene of maize genome	F: 5'-TGAACCCATGCATGCAGT-3' R: 5'-GGCAAGACCATTGGTGA-3' P: FAM5'-TGGCGTGTCCGTCCCTGATGC-3'TAMRA	SN/T 1204-2003
MON88913	Event-specific boundary sequence of GM cotton seed genome	F: 5'-TCCCATTCGAGTTTCTCACGT-5' R: 5'-AACCAATGCCACCCCACTGA-3' P: FAM5'-TTGTCCCTCCACTTCTTCTC-3'TAMRA	Joint Research Centre (2008b)
GTS40-3-2	Event-specific boundary sequence of GM soybean genome	F: 5'-TTCATTCAAAATAAGATCATACATACAGGT-3' R: 5'-GGCATTTGTAGGAGCCACCTT-3' P:6-FAM5'-CCTTTTCCATTTGGG-3'MGBNFQ	Joint Research Centre (2009)
MON89788	Event-specific boundary sequence of GM soybean genome	F: 5'-TCCCGCTCTAGCGCTTCAAT-3' R: 5'-TCGAGCAGGACCTGCAGA-3' P:6-FAM5'-CTGAAGGCGGGAAACGACAATCTG-3'TAMRA	Joint Research Centre (2008c)
Bt11	Event-specific boundary sequence of GM maize genome	F: 5'-GCGGAACCCCTATTTGTTTA-3' R: 5'-TCCAAGAATCCCTCC-3' P: FAM5'-AAATACATTCAAATATGTATCCGCTCA-3'TAMRA	Joint Research Centre (2008d)
MON863	Event-specific boundary sequence of GM maize genome	F: 5'-GTAGGATCGGAAAGCTTGGTAC-3' R: 5'-TGTTACGGCCTAAATGCTGAACT-3' P:6-FAM5'-TGAACACCCATCCGAACAAGTAGGGTCA-3'TAMRA	Joint Research Centre (2008a)

RESULTS AND DISCUSSION

MDA

The dsDNA concentration and $OD_{260/280}$ values of DNA (cotton, soybean and maize) from mdaDNA without dilution are: each MDA reaction yielded 300-370 ng/μL of dsDNA; $OD_{260/280}$ ranged from 1.80-1.87, while 1.70-1.90 before MDA; after non-specific amplification by MDA random primers, the DNA concentration was magnified by 10^3 fold as compared to DNA templates before MDA.

The results of MDA using extracted DNA from the plant products of the five events show that MDA can increase not only the quantity but also the purity of DNA. The mdaDNA concentration was ~1000-fold that of the extracted DNA. The results for concentration of DNA templates are consistent with the data from Lillian et al. (2008). Statistics indicate that amplification rates range from 30-fold (input of 100 ng of gDNA) to 23000-fold (input of 0.1 ng of gDNA). Whole genome amplification by MDA has been proved to improve the ratio between DNA without further purification and inhibiting substances, so higher-purity DNA can be obtained (Dean et al., 2002; Hughes et al., 2005).

Comparison of gene representations

A smaller Ct indicates more DNA templates are involved in fluorescence PCR. The DNA templates from the same patch of nucleic acid extraction, were indirectly (undergoing

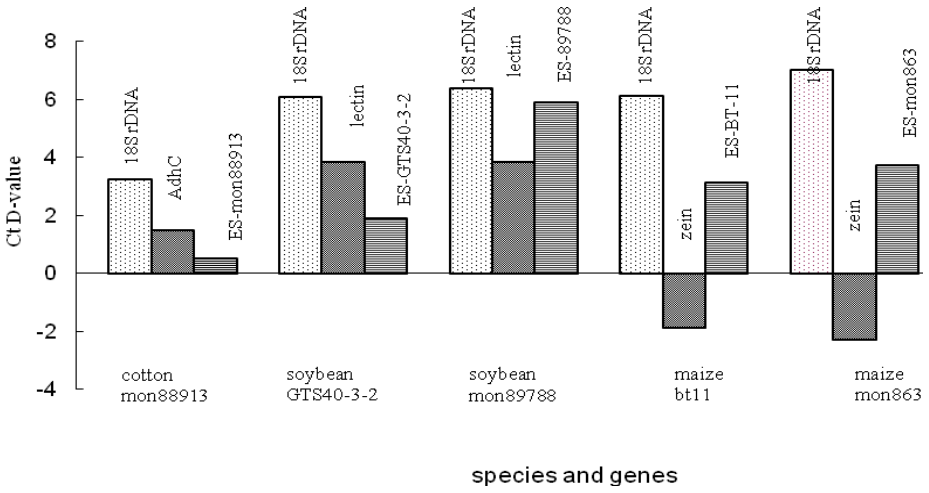

Figure 1. Ct values of pre-mda DNA and mdaDNA at the same concentration with real-time PCR. The abscissa is the difference of Ct values between pre-mda DNA and mdaDNA from real-time PCR. Ct D-value is difference of Ct values of the isogenetic DNA extract before and after MDA. A larger Ct D-value after MDA means a larger increase in number of templates.

MDA) or directly used in real time PCR. The DNA template undergoing MDA was amplified by 10^3-fold, resulting in a smaller Ct in real time PCR. Figure 1 shows that after MDA, the internal control gene *AdhC* has larger Ct D-value than the boundary sequence of MON88913, indicating that MDA amplified more *AdhC* gene templates than the boundary sequences of MON88913 in GM cotton MON88913. By mdaDNA, Ct D-value templates of *zein* gene is smaller than those of other genes. It can be inferred that the amplification efficiencies of MDA on *zein* genes are significantly lower than on other genes.

It can be seen that the amplification performances were different among the events of the same species. *18S rDNA* genes from soybeans GTS40-3-2 and MON89788 have similar performances. Both *lectin* and event-specific gene in GM soybeans GTS40-3-2 and MON89788 performed differently, and the performances of *lectin* in MON89788 were better than in GTS40-3-2. Reportedly, the internal control gene *lectin* of GM soybean and the foreign gene of GTS40-3-2 are single copy genes and can be used in quantitative detection of modified genes (Katarina et al., 2006).

In comparison of the results of genes, the copy numbers of organelle DNA were larger than gDNA in all GM samples. Since there are thousands of organelles such as ribosome, mitochondria and chloroplastid in one cell, and the copy number of the organelle genome is huge, the original base of amplification is large. So *18S rDNA* in ribosome had better amplification (larger Ct D-value) in MDA than nucleus genome genes, such as *AdhC, lectin* and *zein*. Pang et al. (2013) reported that MDA effects of single- or double-strand cDNA templates were limited, while the fold increases of double-strand cDNA templates treated with ligation could be hunched high up. Thereby, we can deduce that small fragment or lysed small fragment DNA in cell organs is more suitable for MDA when compared with DNA. We speculated that if primers and probes are designed with nucleus genomic gene and put into related PCR experiments, MDA can improve the detection sensitivity to a certain extent. However, the preamplification of MDA shows varying amplifying effects on the genes originating from genome DNA or organelle DNA, and even on genes with the same origin.

So, When MDA was applied in GMO detection, pre-amplification experiments are needed to determine the amplification efficiencies of MDA on genes. The genes with high efficiencies were selected and used in GMO detection. While, if primers and probes are designed on organelle genome and used for PCR for species identification, the pre-amplification of DNA by MDA can significantly improve the detection sensitivity. Because the amplification effects of MDA on specific genes in the genome differ, MDA cannot be used in quantitative detection of transgenes, but only in qualitative PCR detection. Lillian et al. (2008) used qualitative PCR forscreening detection of huge transgenes, and MDA helped to improve the qualitative detection rate of trangenes.

ACKNOWLEDGEMENTS

We acknowledge the financial support of Public Service Sectors (Agriculture) Research Projects of Ministry of Science and Technology: 200903055; research project of the General Administration of Quality Supervision, Inspection and Quarantine of P. R. China: 2012IK175;

Important National Science and Technology Specific Projects (2011ZX08012-001).

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