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Full Length Research Paper

Detection of herbicide-resistant maize by using loop-mediated isothermal amplification of the pat selectable marker gene

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Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that reagents react to under isothermal conditions with high specificity, efficiency and rapidity. We used LAMP for detection of herbicide-resistant maize, which is a widespread genetically modified crop. Pat gene, a widely used selectable marker, was amplified by a set of four special primers that recognized six distinct sequences of the target. We studied the optimized conditions for LAMP using different Mg^{2+}, dNTPs, betaine and primers concentrations. Furthermore, we tested the sensitivity and specificity of LAMP as compared to PCR. This work shows that the LAMP method can detect a specific pat gene from herbicide-resistant maize and their test results are consistent with the results of the conventional PCR methods. However, LAMP assay results were found to be more sensitive than the conventional PCR.

Key words: Herbicide-resistant maize, loop-mediated isothermal amplification (LAMP), transgenic maize detection, pat gene.

INTRODUCTION

From the first commercialization of genetically modified crops in 1996 to 2009, the number of countries planting genetically modified crops increased to 25. Global hectarage of genetically modified crops continued to grow in 2009 and reached 134 million hectares, with the genetically modified maize having 42 million hectares at 31%. Genetically modified crops played an important role by contributing to economic, environmental and welfare benefits over the last fourteen years. In 2009, the global market value of genetically modified crops, estimated by Cropnosis, was US$10.5 billion including US$5.3 billion for genetically modified maize (James, 2009). However, since some consumers have concern for the safety of genetically modified food, reliable and sensitive methods for the detection of genetically modified organisms (GMOs) become necessary.

Currently, various molecular methodologies based on nucleic acid and protein analysis, such as Multiplex PCR (Matsuoka et al., 2001), competitive PCR (Hardegger et al., 1999; Hübner et al., 1999; Studer et al., 1998), real-time PCR (Ahmed, 1995; Hernández et al., 2004; Chaouachi et al., 2008), PCR-ELISA (Brunnert et al., 2001; Liu et al., 2004), Southern blots (Jennings et al., 2003) and Western blots (Van Duijn et al., 1999) have been developed and used for GMOs detection. Of these, the methods based on PCR are the most widely used because of their high efficiency, sensitivity, and stability (Holst-Jensen et al., 2003; Berdal and Holst-Jensen 2001). However, the requirements of either a high-precision instrument for PCR amplification or complicated procedures for PCR analysis are their main disadvantages, which limit these methods being widely used on spot detection.

Loop-mediated isothermal amplification (LAMP) is a

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Abbreviations: LAMP, Loop-mediated isothermal amplification; **PCR,** polymerase chain reaction; **FIP**, forward inner primer; **BIP**, backward inner primer; **GMOs**, genetically modified organisms; **CTAB**, cetyl trimethyl ammonium bromide; pat, phosphinothricin acetyltransferase.

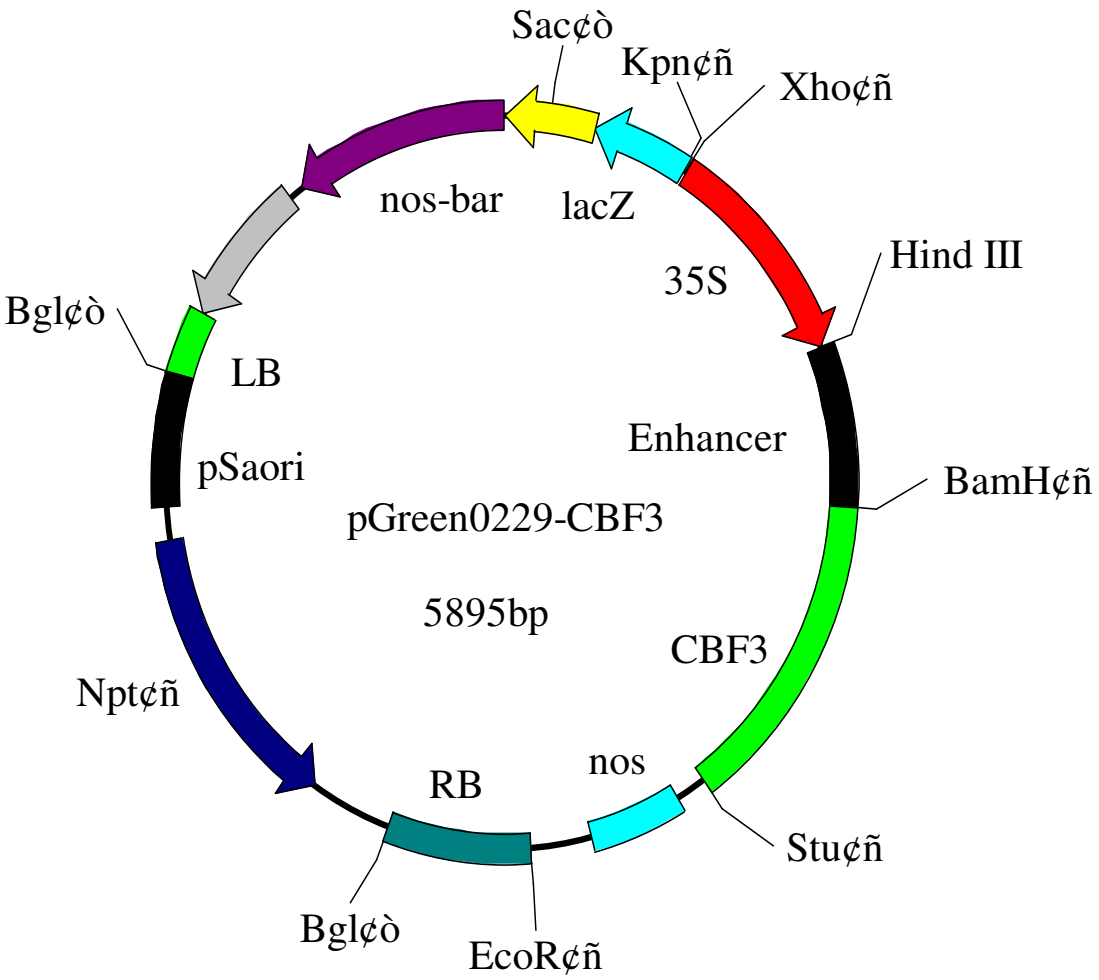

Figure 1. Transformation vector pGreen0229-CBF3.

very efficient and specific method for nucleic acids amplification developed by Notomi et al. (2000). The LAMP reaction is performed by a set of two specially-designed inner primers[forward inner primer (FIP) backward inner (BIP)] and outer primers (F3 and B3), using Bst DNA polymerase large fragment (New England Biolabs) with strand displacement activity under isothermal conditions ranging from 60 to 65°C within 1 h. The reaction continues with accumulation of $l0^9$ copies of target. The final products are a mixture of stem-loop DNAs of various lengths. It can be detected as a typical ladder-like pattern by agarose gel electrophoresis or techniques that rely on the detection of by-products of DNA synthesis, for example, the use of magnesium pyrophosphate precipitation (Mori et al., 2001) or the use of SYBR Green dye (Iwamoto et al., 2003). LAMP technique has been used successfully to detect bacterial microorganisms, pathogenic virus, parasites, etc (Yoshida et al., 2005; Parida et al., 2005; Ikadai et al., 2004). However, there are few reports on GMOs detection (Fukuta et al., 2004; Lee et al., 2009).

In this study, we developed and evaluated a LAMP method for the rapid detection of pat gene in herbicide-resistant maize.

MATERIALS AND METHODS

Plasmid DNA and maize sample

The empty vector pGreen0229 with the pat gene conferring resistance to the herbicide glufosinate is from John Innes Center, UK, and CBF3 gene from Arabidopsis (Gilmour et al., 2000) driven by CaMV35S promoter was inserted into plasmid pGreen0229 (Figure 1). The herbicide-resistant maize sample (sprayed with 200 mg/L glufosinate, Sigma 45520) was developed by our laboratory using the transformation vector as shown in Figure 1.

Primer design for LAMP

The LAMP method requires a set of four specially designed primers (F3, B3, FIP and BIP) that recognize a total of six distinct sequences (F1, F2, F3, B1, B2, and B3) in the target DNA. As shown in Figure 2, the primers were designed from the sequence of the pat gene targeting the region between 361 and 578 bp

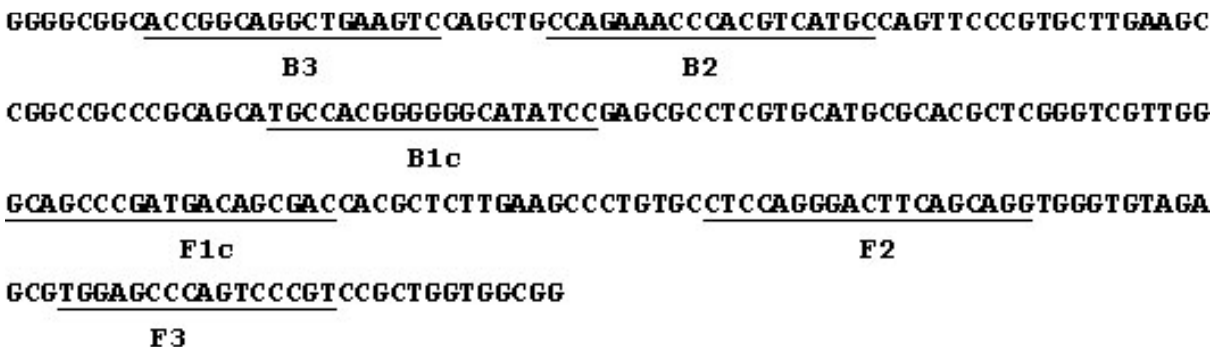

Figure 2. The DNA sequences of the LAMP primers recognition region for pat gene. The DNA sequences used for the primer design are shown by lines.

Table 1. Oligonucleotide primers for LAMP.

Primer	Sequence
F3	5'-ACGGGACTGGGCTCCA-3'
B3	5'-ACCGGCAGGCTGAAGTC-3'
FIP (F1c+F2)	5'-GCAGCCCGATGACAGCGACCCTGCTGAAGTCCCTGGAG-3'
BIP (B1c+B2)	5'-GGATATGCCCCCCGTGGCACCAGAAACCCACGTCATGC-3'

(GenBank accession NO.EU048867.1) using PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). The oligonucleotide primers used in this study are listed in Table 1.

Extraction of genomic DNA

Genomic DNA was extracted from 0.05 g leaves of maize by cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980), and about 50 ng DNA was used as the template for PCR or LAMP amplification.

LAMP analysis

The LAMP reaction was carried out in a 25 µl volume containing 0.2 ~ 2.0 µmol/L of inner primers (FIP and BIP), 0.2 µmol/L of outer (F3 and B3), 0.2 ~ 3.5 mmol/L of deoxynucleotide triphosphate (dNTPs), 0 ~ 2 mol/L of betaine (Sigma), 2 ~ 16 mmol/L $MgSO_4$, 8U of the Bst DNA polymerase large fragment (New England Biolabs), 2.5 µl of 10×Bst polymerse buffer, and 1 µl of target DNA. The mixture was incubated at 65°C for 1 h and was heated at 80°C for 2 min to terminate the reaction. A total of 10 µl of LAMP product was electrophoresed in 1% agarose gel followed by staining with ethidium bromide, and then visualized by UV transillumination. Each experiment was conducted three times, and one representatives picture is shown in the article.

PCR analysis

The PCR product was 202 bp of the pat gene using the outer primers (F3, B3) of LAMP. The reaction was carried out in a 25 µl volume, including 2.5 µl of 2.5 mmol/L dNTPs, 1 µl of 10 mmol/L for each primer, 2.5 µl of 10×buffer, 1.25 U Taq DNA polymerase (TaKaRa) and 1 µl of the target DNA. PCR was performed in gene amplification equipment EDC-810 (Eastwin) according to the following program: an initial denaturation at 94°C for 4 min; 30 thermal cycles of denaturation at 94°C for 1 min, annealing at 56°C for 40 s, extension at 72°C for 30 s; and a final extension at 72°C for 7 min. The PCR products were analyzed by 1% agarose electrophoresis with ethidium bromide staining.

Sensitivity assessment

A ten fold serial dilution of plasmid DNA was used as templates for LAMP and PCR, following optimization. A total of 10 µl of LAMP or PCR product was electrophoresed in 1% agarose gels followed by staining with ethidium bromide, and then were visualized by UV transillumination.

RESULTS

Optimized conditions for LAMP

To determine the optimal concentrations of Mg^{2+}, dNTPs, betaine and primers, the LAMP reaction was conducted with various concentrations of Mg^{2+}, dNTPs, betaine and primers, and about 6.5 ng plasmid DNA were used as template. We tested the concentrations of Mg^{2+} from 2 to 18 mmol/L and found out that no amplified LAMP product was observed under 2 mmol/L Mg^{2+}. The intensity of the typical ladder-like pattern products increased from 4 to 8 mmol/L Mg^{2+}, but decreased when the Mg^{2+} was 10 mmol/L higher (Figure 3A).

When we tested dNTP effects for LAMP, there was no amplified LAMP products observed under 0.2 mmol/L or 3.5 mmol/L dNTP. The intensity of the typical ladder-like

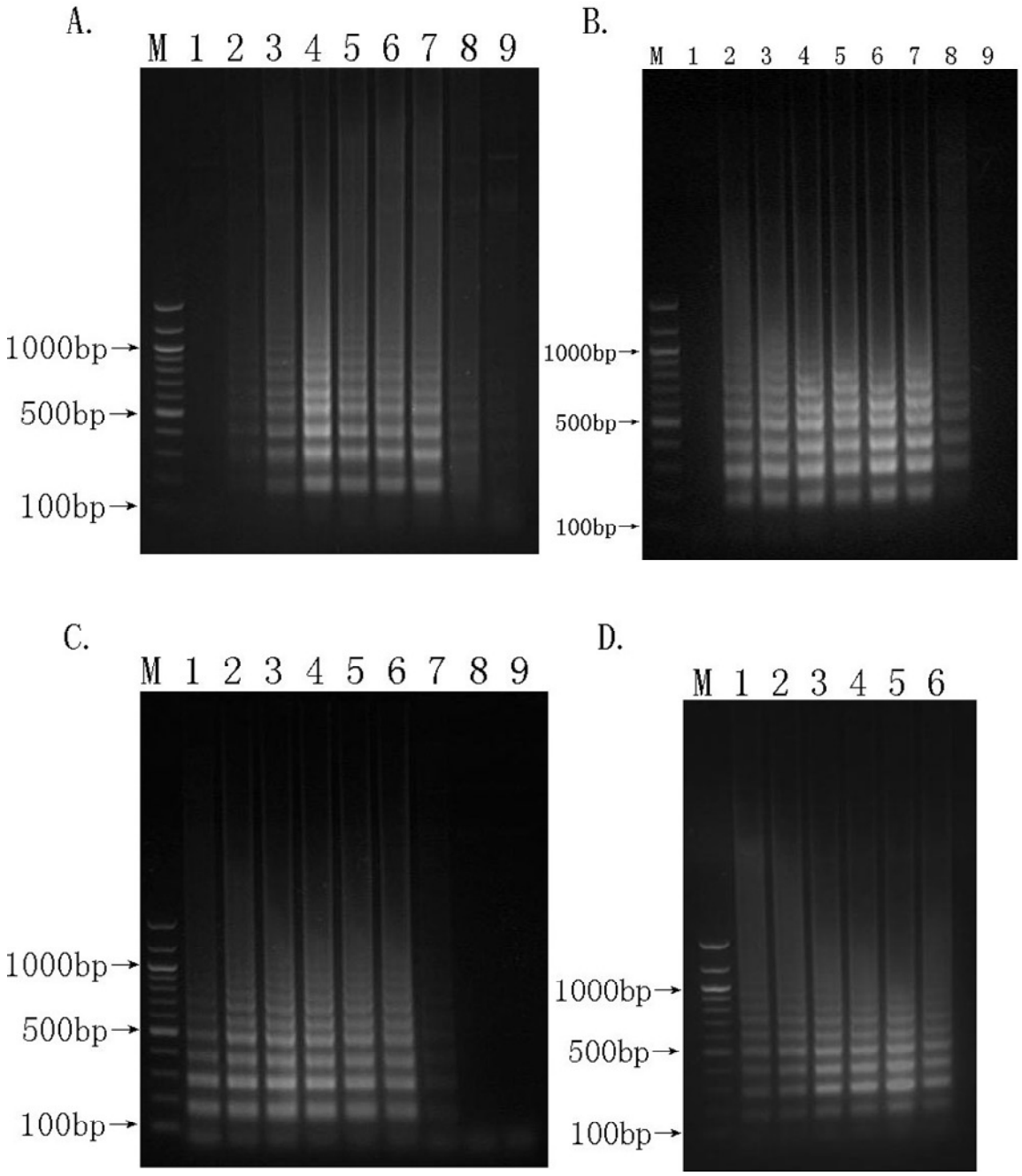

Figure 3. Determination of the optimal concentrations of Mg^{2+}, dNTPs, Betaine and primers. (A) Effects of the Mg^{2+} concentrations on the LAMP reaction. M, 100 bp ladder marker; 1, 2 mmol/L; 2, 4 mmol/L; 3, 6 mmol/L; 4, 8 mmol/L; 5, 10 mmol/L; 6, 12 mmol/L; 7, 14 mmol/L; 8, 16 mmol/L; 9, 18 mmol/L. (B) Effects of the dNTPs concentrations on the LAMP reaction. M, 100 bp ladder marker; 1, 0.2 mmol/L; 2: 0.5 mmol/L; 3, 0.8 mmol/L; 4,1 mmol/L; 5, 1.5 mmol/L; 6, 2 mmol/L 7: 2.5 mmol/L; 8, 3 mmol/L; 9, 3.5 mmol/L. (C) Effects of Betaine concentrations on the LAMP reaction. M, 100 bp ladder marker; 1, 0 mol/L; 2, 0.3 mol/L; 3, 0.5 mol/L; 4, 0.8 mol/L; 5, 1 mol/L; 6, 1.3 mol/L; 7, 1.5 mol/L; 8, 1.7 mol/L; 9, 2 mol/L. (D) Effects of the outer and inner primer concentrations ratio on LAMP reaction. M, 100 bp ladder marker; 1, 0.2:0.2 µmol/L; 2, 0.2:0.4 µmol/L; 3, 0.2:0.8 µmol/L; 4, 0.2:1.2 µmol/L; 5, 0.2:1.6 µmol/L; 6, 0.2:2 µmol/L.

pattern products increased from 0.5 to 1mmol/L dNTP, and remained the same until the concentration reached 2.5 mmol/L, but decreased when dNTP was higher than that of 2.5 mmol/L (Figure 3B). As for betaine effects, there was no amplified LAMP products observed from 1.7 to 2 mmol/L (Figure 3C). With the betaine concentration increasing from 0 to 0.5mol/L, the LAMP products increased, but decreased when the betaine was raised

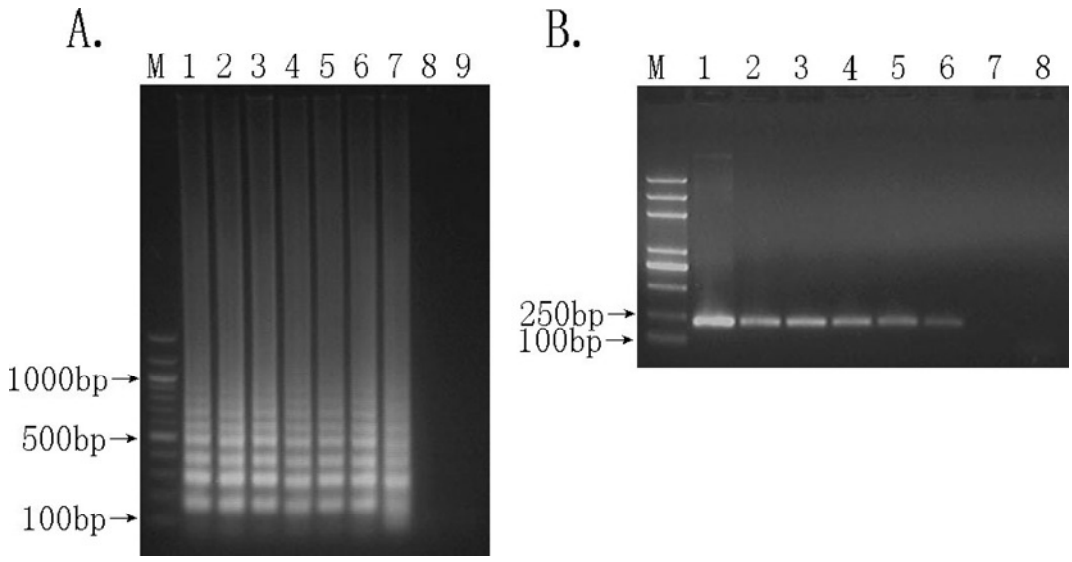

Figure 4. Detection limit of LAMP (A) and PCR (B) for pat gene using plasmid DNA as template. (A) M, 100 bp ladder marker; DNA template 1, 1×10^{-1}; 2, 1×10^{-2}; 3, 1×10^{-3}; 4, 1×10^{-4}; 5, 1×10^{-5}; 6, 1×10^{-6}; 7, 1×10^{-7}; 8, 1×10^{-8}; 9: H_2O. (B) M, trans 2k plus marker; DNA template 1, 1×10^{-1}; 2, 1×10^{-2}; 3, 1×10^{-3}; 4, 1×10^{-4}; 5, 1×10^{-5}; 6, 1×10^{-6}; 7, 1×10^{-7}; 8, H_2O.

from 0.5 to 1.5 mmol/L.

The outer primers, B3 and F3 (0.2 µmol/L), together with the inner primers FIP and BIP (0.2 ~ 2 µmol/L) were used to optimize the LAMP reaction (Figure 3D). As the inner primers concentrations increased from 0.2 to 1.6 µmol/L, the intensity of the LAMP products increased, but decreased when the inner primers concentrations increased from 1.6 to 2 µmol/L.

Sensitivity of the LAMP and PCR

After the optimization of LAMP, the sensitivity of LAMP was evaluated when compared with PCR using the same plasmid DNA. The initial concentration of template was 65 ng/µl, and a ten fold serial dilution of plasmid DNA was used to evaluate the detection limit. As shown in Figure 4, the detection limit of LAMP was 0.65 fg/tube, while the limit of PCR was 6.5 fg/tube.

Application of LAMP to maize samples

After the optimization of LAMP, the procedure was applied to detect pat gene in herbicide-resistant transgenic maize that has the selectable marker PAT which confers tolerance to the glufosinate herbicide. The pat gene was clearly amplified by LAMP in seven glufosinate herbicide resistant maize samples and plasmid DNA (Figure 5A). There was no amplified product observed in the number 4 sample, the water control and non transgenic control (Figure 5A). This result was consistent with that of PCR (Figure 5B).

DISCUSSION

It has previously been reported that the concentrations of Mg^{2+}, dNTPs, betaine and primers obviously affect the efficiency of LAMP (Notomi et al., 2000); so, we tested various concentrations of Mg^{2+}, dNTPs, betaine and primers for the LAMP of pat gene. The betaine may not be necessarily required for this LAMP reaction (Figure 3C). However, the addition of betaine slightly elevated the amplification efficiency. As a result, the concentrations of 1.6 µmol/L FIP and BIP primers, 0.2 µmol/L F3 and B3 primers, 8 mmol/L Mg^{2+}, 1 mmol/L dNTPs and 0.5 mol/L betaine were found to be the optimal concentrations of LAMP for pat gene. When compared to the conventional PCR analysis, the LAMP assay has the advantages such as time saving, cost-effectiveness, simple procedures, high specificity and sensitivity that allow this method to be used conveniently in GMOs analysis (Fukuta et al., 2004). The LAMP sensitivity was evaluated as compared to PCR. The LAMP method was 10 fold more sensitive than PCR (Figure 4). The detection limit of the plasmid DNA in LAMP for pat gene was about 100 copies/tube by calculation, and is higher than the results reported (6 copies/tube) by Lee et al. (2009). This may be due to the usage of their loop primers, which can accelerate the reaction according to a previous study by Nagamine et al. (2002).

Since LAMP has a high sensitivity, even very low levels of contamination with the target DNA will result in a positive signal. To prevent false results, the experiments for sample extraction, reaction preparation and detection have to be conducted in separate places. The appliances used in this experiment have to be sterilized with UV

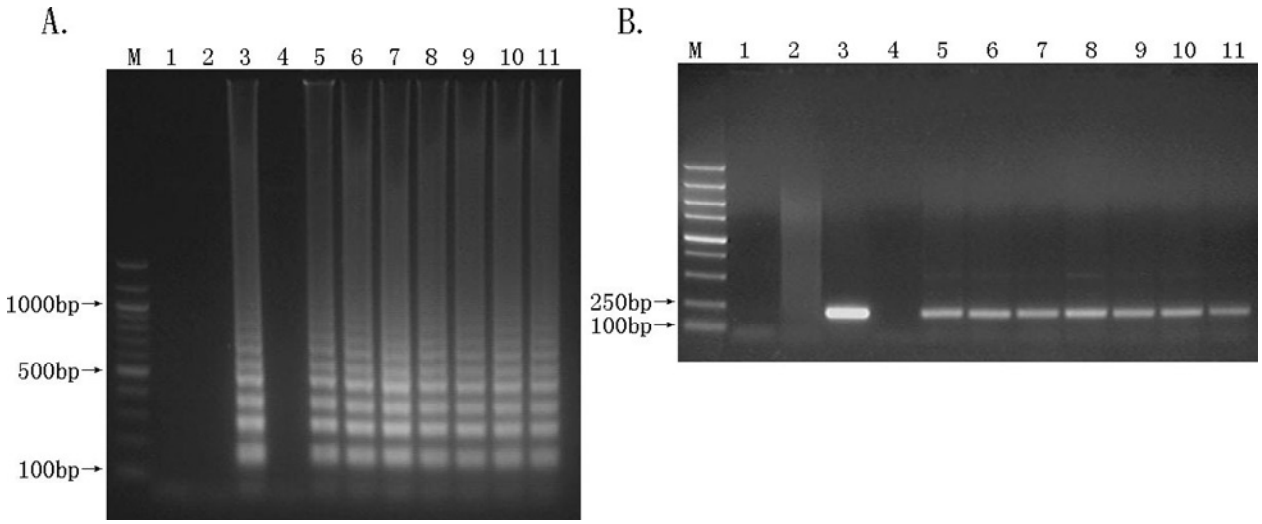

Figure 5. Results of LAMP (A) and PCR (B) amplification of transgenic maize. (A) M, 100 bp ladder marker; 1, H_2O; 2, non-transgenic maize sample; 3, plasmid DNA; 4 to 11, herbicide-resistant maize samples. (B) M, DL5000; 1, H_2O; 2, non-transgenic maize sample; 3, plasmid DNA; 4 to 11, herbicide-resistant maize samples.

light, ethanol, sodium hypochlorite, etc. However, using DNaseI to digest the contaminating DNA can be a good quality control in LAMP experiment. The procedure that was used for DNaseI treatment comprised the following: 25 µl of each of the volume reagents (excluding the sample DNA and the Bst DNA polymerase large fragment) was added to 0.5 U DNaseI. This mixture was incubated at 37°C for 30 min and was heated at 65°C for 10 min to terminate the reaction. Then the LAMP reaction was carried out by adding the sample DNA and the Bst DNA polymerase large fragment. Using these procedures, the LAMP experiments can be repeated well in our laboratory.

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