Analytical Methods

Rapid visual detection of phytase gene in genetically modified maize using loop-mediated isothermal amplification method

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A B S T R A C T

Transgenic maize plant expressing high phytase activity has been reported and approved by Chinese government in 2009. Here, we report a highly specific loop-mediated isothermal amplification (LAMP) method to detect the phytase gene in the GMO maize. The LAMP reaction takes less than 20 min and the amplification is visible without gel electrophoresis. The detection sensitivity of the LAMP method is about 30 copies of phytase genomic DNA, which is 33.3 times greater than the conventional PCR method with gel electrophoresis. The quantitative detection results showed that the LAMP method has a good linear correlation between the DNA copy number and the associated Tt values over a large dynamic range of template concentration from 6 × 10⁶ to 6 × 10⁷ copies, with a quantification limit of 60 copies. Therefore, the LAMP method is visual, faster, and more sensitive, and does not need special equipment compared to traditional PCR technique, which is very useful for field tests and fast screening of GMO feeds.

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1. Introduction

Cereal grains, especially maize seeds are the major ingredients of animals feed (Wenk, 2000). Phosphorus is one of the most important minerals in animal nutrition (Weremko et al., 1997). However, phosphorus in maize seeds exists predominately in the form of phytate, which is not available to monogastric animals such as pigs, poultry, and fish (Vohra & Satyanarayana, 2003; Wodzinski & Ullah, 1996). Therefore, inorganic phosphates or microbial phytases have been added to animal rations to increase phosphorus availability and reduce phosphorus excretion in manure (Brinch-Pedersen, Sørensen, & Holm, 2002; Verwoerd et al., 1995). Recently, alternative transgenic maize plants expressing high phytase activities have been reported and have obtained the approval on 27 November, 2009 in China (Chen et al., 2008). Phytase activity in these transgenic maize seeds is about 50-fold higher compared to non-transgenic maize seed. It can simplify the feeding process using this type of green and efficient transgenic maize for there is no need to add microbial phytase to the feed. Therefore, this high phytase containing transgenic maize has good prospects of commercialization. On the other hand, consumers have become increasingly concerned about the safety of GMO products. A number of national governments have their compulsory labeling policy. The ability to detect the presence of GMO is critical for consumers to exercise their lifestyle choice of whether to consume food/feed containing GMOs. Reliable and sensitive methods are needed for detection and identification of the phytase gene in genetically modified maize.

Currently, the most commonly used DNA-based methods involve amplification of a specific DNA by the PCR technique for GMO detection (Gryson, 2010; Huang et al., 2011). Jiajian Xie and Peng (2010) have developed a PCR-based event-specific detection method for the GMO maize BVLA430101. However, PCR-based method is expensive, time-consuming and not applicable for field tests (Lauri & Mariani, 2009). A novel nucleic acid amplification method designated loop-mediated isothermal amplification (LAMP) was developed by Nagamine, Hase, and Notomi (2000). This method can amplify DNA with high specificity, efficiency and rapidity under isothermal conditions. A set of four specially designed primers were required due to recognition of target sequence by six distinct sequences. Auto cycling strand displacement DNA synthesis was involved to perform the reaction by the large fragment of Bst DNA polymerase with high strand displacement activity. The LAMP reaction can be conducted under isothermal conditions ranging from 60 to 65 °C. Continuous amplification synthesizes extremely large amounts of the target DNA as well as large amounts of a by-product, pyrophosphate.

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ion, which yields white precipitate of magnesium pyrophosphate in the reaction mixture. Judging the presence or absence of this white precipitate allows easy distinction of whether nucleic acid was amplified by the LAMP method (Mori, Nagamine, Tomita, & Notomi, 2001). Furthermore, this method enables simple visual (naked-eye) judgment of the reaction by a color change of a mixture with SYBR Green I (Iwamoto, Sonobe, & Hayashi, 2003). An increase in the turbidity of the reaction mixture due to the increase of the amplification production can be measured as a real-time monitoring of the LAMP process (Mori, Kitao, Tomita, & Notomi, 2004). To perform the LAMP reaction more efficiently and sensitively, two additional loop primers can be added to the reaction, which reduces the LAMP reaction time to less than half of the original LAMP method. The LAMP assay has been used to identify and detect several viral (Imai et al., 2006; Poon et al., 2004) and bacterial (Hara-Kudo, Yoshino, Kojima, & Ikedo, 2005; Seki et al., 2005) strains in clinical laboratories. In this study, we aimed to develop a sensitive, specific and rapid method for the detection of the phytase gene in genetically modified maize using the LAMP method.

2. Materials and methods

2.1. Plant materials

Transgenic maize with phytase (BVLA 430101) was provided by Biotechnology Research Institute, CAAS. Certified Reference Materials (CRMs) including Mon810 maize, Mon863 maize, Mon88017 maize, TC1507 maize (Fluka Co.) were used as negative controls. Tahei 309 rice, 72KD wheat, canola and CPTI cotton used as negative controls were standard samples stored in the Institute of Plant Quarantine, Chinese Academy of Inspection and Quarantine (CAIQ).

2.2. DNA extraction

Plant genomic DNA was extracted from starting amounts of 0.1 g grounded samples using the cetyltrimethylammonium bromide (CTAB) method (Dellaporta, Wood, & Hicks, 1983). Briefly, 1.5 ml of CTAB extraction buffer supplemented with Ribonuclease A (at a final concentration of 10 µg/ml) was added, and the samples were mixed and incubated for 30 min at 65 °C with occasional stirring. The suspension was then centrifuged for 10 min at 14,500g at room temperature and 700 µl chloroform was added to the supernatant. The mixture was centrifuged for 10 min at 14,500g, and the supernatant was precipitated using the CTAB precipitation buffer. The supernatant was discarded and the pellet was dissolved in 1.2 M of NaCl and extracted with chloroform (1 equivalent volume). After centrifugation for 10 min at 14,500g, the supernatant was discarded, and the pellet was treated with isopropanol (0.8 equivalent volume) and centrifuged for 10 min at 14,500g. The pellet was washed with 70% ethanol, vacuum-dried, and dissolved in 50 µl TE (10 mM Tris–HCl pH 8.0; 1 mM EDTA).

The DNA concentration was initially determined by measuring the absorption at 260 nm on a UV–Vis spectrophotometer (Evolution 3000, Thermo company), and the DNA purity was evaluated from absorption ratio of 260/280 nm and 1% (W/V) agarose gel electrophoresis.

2.3. Preparation of plasmid DNA

In order to construct the standard curve of the transgenic maize with phytase using LAMP method, a 1300 bp DNA fragment of phytase gene was amplified by PCR from transgenic maize BVLA430101 using forward primer (5'-TCAGGGGTATCATGCTCTCGG-3') and reverse primer (5'-CTAAGCAAAACACTCCGCCCAT-3'). The amplified product was cloned into pMD18-T Simple Vector (Takara Biotechnology, Dalian, China) and transformed into trans-5-α chemically competent cell (TransGen Biotech, Beijing, China) according to the manufacturer’s instructions. Clones containing the correct inserts were confirmed by sequencing (SINO GENOMAX, Beijing, China).

2.4. Primer design for LAMP

The primers were designed according to the sequence of phytase gene obtained from the GenBank database (GenBank accession No. HQ233651.1). The LAMP reaction required a set of four primers: a forward inner primer (FIP), a backward inner primer (BIP), and two outer primers (F3 and B3). They were designed using the online PrimerExplorer V3 software (http://primerexplorer.jp/e/) and Primer Premier 5.0 software. Two additional loop primers (loopF and loopB) were also designed to accelerate the LAMP reaction, as shown in Fig. 1. To confirm the specificity of primers, the sequences were checked in GenBank by online BLAST. The size of target region was 270 bp.

2.5. LAMP reaction

The LAMP reaction was carried out in a total of 25 µl solution containing 2.4 µM of each inner primer (FIP and BIP), 0.2 µM of each outer primer (F3 and B3), 1.2 µM of each loop primer (loopF and loopB), 2.8 mM dNTPs, 0.6 M betaine (Sigma, St. Louis, USA), 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 5 mM MgSO4, 0.1% Tween 20, 12 units of the Bst DNA Polymerase large fragment (New England Biolabs, Ipswich, MA), and 2 µl of the target DNA. After the denaturation step at 95 °C for 5 min, the mixture was incubated at 65 °C for 60 min and heated at 80 °C for an additional 5 min to terminate the reaction. The reaction was performed using a Loopamp real-time turbidimeter (LA-320c, EIKEN CHEMICAL CO., Ltd., Tokyo, Japan). The LAMP assay was carried out in triplicate and the no template control (NTC) contained ddH2O instead of the template.

2.6. Detection of LAMP products

The turbidity of the LAMP reaction mixture was measured by using a real-time turbidimeter. The apparatus could continuously detect the turbidity of the reaction mixture. Therefore, real-time turbidity measurements of LAMP reactions were conducted for several dilutions of available DNA of known concentrations containing phytase gene. In order to watch LAMP amplification directly by naked eye, 10 µl of 1/10-diluted original SYBR Green I (Molecular Probes Inc.) was added to the reaction tube to observe the change in color. In addition, the variation of the turbidity was visually detected between the positive and the negative reaction products. For further detection, the LAMP products could also be subjected to 3% agarose gel electrophoresis, stained with SYBR Green I and assessed photographically under UV light.

2.7. Conventional PCR reaction

The conventional PCR method was established to compare the detection limit of the LAMP method. Template DNA containing the phytase gene was used at the same concentrations for the LAMP and conventional PCR assays. The sequence used to design the PCR primers was the same as the LAMP’s, and the primers used for PCR were the outer primers of LAMP (F3 and B3). The expected product size was 270 bp.

The PCR mixture (25 µl total volume) contained 10 × Ex Taq Buffer (Takara Biotechnology, Dalian, China), 0.3 mM of each dNTPs, 0.4 µM of each primer, 2.5 U of Ex Taq DNA polymerase (Takara Biotechnology, Dalian, China) and 2 µl of target DNA. The
PCR was performed on a Veriti 96 Well Thermal Cycler (Applied Biosystems, USA). The thermal cycling condition was as follows: denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s, and terminal elongation at 72 °C for 7 min. The PCR products were electrophoresed in a 1% agarose gel and stained with SYBR Green I for visualization under UV light.

2.8. Specificity of LAMP

The transgenic maize with phytase genomic DNA and other non-phytase gene DNA, including Mon810 maize, Mon863 maize, Mon88017 maize, TC1507 maize, Taibei309 rice, 72KD wheat, canola and CPTI cotton, were used to study the specificity of the LAMP and PCR assay.

To confirm the structure of the LAMP product, the amplicon was digested with restriction enzyme Hinf I and Hind II (Takara Biotechnology, Dalian, China), and the size of digested products were analyzed by using gel electrophoresis. The Hinf I recognition site was located within B2 primer, and the Hind II site was between B1 and B2 primers. Furthermore, the product of Hind II digestion was amplified by PCR using forward primer (5'-GGAGAACGACTT GTCTGGCG-3') and reverse primer (5'-GACGAATGGAAGGGACGA AC-3'), and cloned into pMD18-T vector (Takara Biotechnology, Dalian, China) and sequenced.

2.9. Calibration curve

To quantify the phytase gene of unknown concentration using the real-time LAMP assay, a calibration curve was established. The constructed plasmid containing phytase gene prepared above was used as the template DNA.

Quantification of the constructed plasmid was achieved using a Qubit fluorometer, and a series of 10-fold dilutions (10^1–10^7 copies) were made to evaluate the real-time LAMP. Each experiment was repeated three times. The copy numbers of the plasmid DNA were calculated based on the molecular weight and Avogadro's number, and a calibration curve was established. The reaction setup was the same as that of the LAMP reaction above, and the reactions were carried out by using the Loopamp real-time turbidimeter.

3. Results

3.1. DNA extraction

Total DNAs were successfully extracted from maize, soybean and rice. The purities of the extracted DNA solutions are summarized in Supplementary Table S1 and the values of A260/A280 were between 1.8 and 2.0. The DNA solutions were also applied to agarose gel electrophoresis. The results revealed that the DNAs in all samples were not degraded as shown in Fig. S1. Therefore, it was believed that the DNA solutions obtained by using the CTAB method were of adequate purity for applying LAMP analysis.

3.2. Detection of LAMP products

In order to realize real-time detection, the turbidity of by-products generated during DNA synthesis was measured by using the Loopamp real-time turbidimeter. In addition, the amplification curve could be displayed on the computer connected with the turbidimeter and the raw data could also be exported for subsequent analysis. The LAMP-positive reaction mixture became turbid while the negative reaction without amplified products remained clear (Fig. 2A). After adding SYBR Green I to the reaction tubes, the positive reaction mixture turned green whereas the negative reaction remained orange (Fig. 2B). Due to the characteristic of LAMP reactions, amplicons exhibited a ladder-like pattern when analyzed by using gel electrophoresis as a result of the formation of stem-loop DNAs with several inverted repeats of the target (Fig. S2).

3.3. Specificity of LAMP

The specificity assay showed that positive amplification was obtained from the transgenic maize with phytase genomic DNA after 26 min of incubation (Fig. 3). In contrast, no DNA amplification was observed in the other samples even after 60 min of incubation. To confirm the amplification was specific, the product was digested with restriction endonuclease Hinf I and Hind II, and the predicted fragment sizes were 221, 252 and 283 bp for Hinf I as well as 222, 252 and 282 bp for Hind II. The size of digestion products were in good agreement with the prediction (Fig. S2). The structures of the amplified products were cloned into pMD18-T simple vector as mentioned in the Section 2, and confirmed by sequencing. The results matched well with the expected nucleotide sequences perfectly (data not shown).

3.4. Detection limit of the LAMP assay

The detection limits of the LAMP and PCR method were evaluated using genomic DNA extracted from transgenic maize with phytase. A series of 10-fold dilutions of genomic DNA from 3 × 10^6 to 6 × 10^8 copies per reaction were used as DNA templates for the real-time LAMP assay under the optimized conditions. The detection limit was the concentration of which the test could no longer detect all three samples of lower concentration. The sensitivity test showed that the LAMP method can be used to detect the target phytase genomic DNA at 30 copies present in the reaction as shown in Fig. 4. As a comparison, the detection limit of the conventional PCR with agarose gel electrophoresis was 1000 copies (Fig. 5). The sensitivity of the LAMP assay was estimated 33.3 times greater than that of the conventional PCR method with...
gel electrophoresis. Therefore, the developed LAMP assay is a much sensitive and reliable detection method for GMO identification.

3.5. Quantification limit of the LAMP assay

Fig. 6A shows the LAMP turbidity graphs when a variety of diluted plasmid DNA solutions were used. The turbidity time (Tt) was defined as the time at which the turbidity exceeded the level of 0.1 (threshold). The calibration curve was generated using a plot of the turbidity time (Tt) versus the log of the initial copy number of template DNA from $6 \times 10^1$ to $6 \times 10^7$ copies, with a quantification limit of 60 copies (Fig. 6B). The Tt values were obtained the average value of three repeats and the error bars represent the standard errors. The slope of the calibration curve was $-1.1518$ and the square of the regression coefficient ($R^2$) after the linear regression was 0.995, indicating excellent linear relationship between the log of DNA copy number and the Tt value. The above three repetitive measurements of LAMP products at each DNA concentration yield a good reproducible signal with an estimated relative repeatability standard deviation (RSDr) between 2.1% and 5.6%. These results demonstrated the acceptable measurement-to-measurement repeatability of the LAMP assay for phytase gene.

4. Discussion

With the ever-increasing global diffusion of genetically modified organisms (GMOs) and the resulting socio-economical impact, foods or feeds containing GMOs are subject to compulsory labeling in several countries, e.g., European Union (EU) (EC 1829/2003) (“On genetically modified food and feed,” 2003), Japan (Notification No. 1775, 2000) (“Guidelines for Labeling of Genetically Modified Agricultural Products,” 2000) and Korea (Notification No. 2000-31) (2000). Therefore, analytical methods to accurately quantify GMO contents are required.

In this study, we have developed a visual and rapid LAMP assay for the detection of phytase gene contained GM maize. The LAMP assay reported here is advantageous due to its simple operation, rapid reaction, and sensitive detection. Owing to the fact that the LAMP is operated under isothermal conditions at 65°C, no time is lost as a result of changes in temperature. The LAMP could be completed within 20 min in this study under the optimized conditions (Fig. 4) whereas conventional PCR required 2–3 h. Moreover, the LAMP can be performed by using simple reaction equipment such as a regular laboratory bath or heat block that provides a constant temperature of 65°C. It is possible to determine the reaction directly by naked eye with the LAMP, without electrophoretic analysis, unlike PCR, which cannot.

It was reported that non-specific amplification of LAMP can be easily distinguished on a gel because of its characteristic for each assay (Lee, La Mura, Allnutt, & Powell, 2009). The gel electrophoresis requires an open-tube procedure after amplification, which may act as a significant source of cross-contamination, so close-tube
endpoint detection has been suggested as an alternative. Therefore, it would be more practical to use the LAMP detection by naked eye as a quick primary on-site screening.

In this study, we tried to use the LAMP assay to quantify template DNA of transgenic maize with phytase in real time by a Loopamp real-time turbidimeter. The curve obtained using the real-time turbidity measurements had a good linear correlation ($R^2 = 0.995$) between the logarithmic value of initial copy number of template DNA in the LAMP reaction and the associated $T_t$ values over a wide dynamic range of template concentrations (6 × 10^2–6 × 10^7 copies/reaction). The $R^2$ value and the repeatability standard deviation were evaluated to fulfill the minimum performance requirements as reported by Meyer et al. (2012). This indicated the quantitative capability of the real-time LAMP assays when detecting this transgenic maize with phytase. In addition, compared with conventional PCR, the sensitivity of LAMP reaction are more than 30 times greater. This is because of the use of three pairs of primers that recognize eight distinct regions of the target DNA. Therefore, using the real-time turbidity monitoring system, we will be able to estimate the concentration of phytase DNA in the transgenic maize.

5. Conclusion

In this study, we developed a visual and rapid assay for phytase gene in genetically modified maize using the LAMP method. The LAMP method was specific for phytase gene and takes only 20 min, as compared with 2–3 h for traditional PCR. The LAMP assay sensitivity is about 30 copies, which is 33.3-fold more sensitive than that of conventional PCR method. LAMP amplicons can directly be detected by eye inspection after adding SYBR Green I. Eye inspection is much simpler and faster. Furthermore, the LAMP method has a good linear correlation between the DNA copy number and the associated $T_t$ values over a large dynamic range of template concentration from 6 × 10^1 to 6 × 10^7 copies. In summary, the LAMP method is visual, faster, and more sensitive and does not need special equipment compare to the traditional PCR technique, which making it a very attractive method for field tests and fast screening of GMO feeds. Since the reactions are performed at a single temperature, the LAMP assays of GMO products can be performed very quickly and used as a convenient tool for on-site fast detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.01.102.

Reference


