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Analytical Methods

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

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ABSTRACT

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 6×10^1 to 6×10^7 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 (2002) and Notomi et al. (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) strands 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. Taibei 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 14,500*g* at room temperature and 700 μl chloroform was added to the supernatant. The mixture was centrifuged for 10 min at 14,500*g*, 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 14,500*g*, the supernatant was treated with isopropanol (0.8 equivalent volume) and centrifuged for 10 min at 14,500*g*. 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′-TCAGGGGTATCAATGCTTCT CGG-3′) and reverse primer (5′-CTAAGCAAAACACTCCGCCCAAT-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 GENO MAX, 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 $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 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 ddH_2O 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

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