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Short communication

Real-time and visual loop-mediated isothermal amplification: Efficient GMO screening targeting *pat* and *pmi* marker genes

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ABSTRACT

Rapid and efficient screening assays based on loop-mediated isothermal amplification (LAMP) were developed, targeting two marker genes, namely, phosphinothricin-N-acetyltransferase *(pat)* and phosphomannose isomerase (*pmi*). These marker genes are being employed in more than 35% of GM events approved globally. Specificity of developed visual and real-time LAMP assays was confirmed using seven GM events of two crops, *viz*., maize (3272, 59122, *Bt*11, *Bt*176, MIR604, TC1507), and cotton (Widestrike™). In visual LAMP, positive amplification can be directly analyzed by the colorimetric change from orange to green, whereas real-time LAMP is based on the monitoring of fluorescence signals as amplification and annealing curves. Visual LAMP was found sensitive enough to detect up to 0.05% GM content for *pat* and 0.1% for *pmi* within 40 min. Real-time LAMP efficiently detected up to 0.01% GM content within 30 min. Practical applicability of developed assays was confirmed using proficiency test samples of maize. LAMP assays for *pmi* gene have been reported for the first time. Due to portability of systems, the developed LAMP assays when combined with a fast DNA extraction method could facilitate on-site GMO screening.

1. Introduction

For stable expression of a transgene, along with the use of regulatory sequences as essential components, selectable or scorable marker genes are also integrated in the transgenic construct to ensure its constitutive expression throughout the plant. Marker genes may encode for antibiotic resistance, herbicide tolerance, a drug/metabolite analogue or a carbon supply or phytohormone precursor. The *pat* encoding phosphinothricin-N-acetyltransferase, derived from *Streptomyces viridochromogenes*, is herbicide tolerant gene, which has been employed as the marker gene in 26% of globally commercialized GM events (http://www.isaaa.org/gmapprovaldatabase). To address biosafety issues related to the use of antibiotic resistance or herbicide tolerance genes, different selection strategies, including non-toxic enzymes for conditional positive selection, or co-transformation with marker genes have been emerged. The *pmi*, encoding phosphomannose isomerase enzyme catalyzing the conversion of mannose-6-phosphate into fructose-6-phosphate is employed as selectable marker. Mannose is added to the culture medium for selection of transformed cells, which would be able to utilize mannose. Non-transformed cells are unable to utilize mannose and plant growth is restricted due to accumulation of mannose-6-phospahte. The *pmi* has been employed as marker gene in 13% of GM events commercialized globally (http://www.isaaa.org/gmapprovaldatabase/). GM detection assays targeting *pat* and *pmi* marker genes could facilitate screening for more than 35% of globally approved GM events (Fig. 1).

GM detection assays targeting most commonly employed marker gene, *nptII*, have been reported using polymerase chain reaction (PCR), real-time PCR, loop-mediated isothermal amplification (LAMP) (Randhawa, Chhabra, & Singh, 2009a; Randhawa, Singh, & Sharma, 2009b; Randhawa, Singh, Morisset, Sood, & Žel, 2013; Reiting, 2010). Real-time PCR assays for *pat* and *pmi* marker genes have been reported (Ingham, Beer, Money, & Hansen, 2001; Weighardt et al., 2004).

Though real-time PCR assays are being routinely used for GM detection and quantification due to their efficiency, sensitivity and reproducibility, but use of expensive, non-portable and sophisticated equipments for thermal cycling, limits their on-site applicability. This limitation can be overcome by real-time LAMP assays, in

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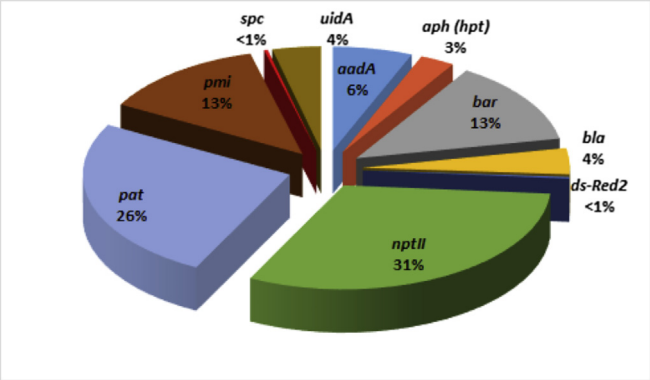

Fig. 1. Distribution pattern of marker genes being employed in the GM events approved globally. Source: Based on data available at http://www.isaaa.org/gmapprovaldatabase.

Table 1
GM events used for testing specificity of LAMP assays.

Event	*pat*		*pmi*	
	S	R	S	R
3272	−	−	+	+
59122	+	+	−	−
*Bt*11	+	+	−	−
*Bt*176	−	−	−	−
MIR604	−	−	+	+
TC1507	+	+	−	−
Widestrike™	+	+	−	−
Non-GM maize (*Z. mays* L.)	−	−	−	−

(+) and (−) show presence and absence of selected target in particular event, respectively.
S: Theoretical data based on the information available in GM databases, *viz.*, www.igmoris.nic.in, www.cera-gmc.org, http://www.isaaa.org/gmapprovaldatabase/
R: Verified experimentally with PCR assays, further specificity was checked using LAMP assays.

which fluorometric detection is achieved using a portable real-time isothermal system, such as Genie®II. Unlike conventional PCR, LAMP assays do not require time-consuming gel electrophoretic analysis of products and *Taq* DNA polymerase that may be inactivated by inhibitors present in crude biological samples and thus, may be applicable for on-site GM detection (De Franchis, Cross, Foulkes, & Cox, 1988). In LAMP assays, detection of specific target is completed at constant temperature, employing four primers specifically recognizing six distinct regions on the template and additional loop primers further increase speed and specificity of the assays (Nagamine, Hase, & Notomi, 2002; Notomi et al., 2000). LAMP products can be visualized as change in colour using nucleic acid staining or fluorescent dyes, or real-time monitored by measuring fluorescence (Guan, Guo, Shen, Yang, & Zhang, 2010; Randhawa et al., 2013).

In recent past, LAMP has been employed in GM detection due to ease-of-use and time-efficiency. LAMP-based visual and real-time assays targeting commonly employed promoters, namely, *P-35S*, *P-FMV* and marker genes, *viz.*, *aadA*, *nptII* and *uidA* have been developed (Randhawa et al., 2013). Gene-specific LAMP assays for detection of *cry1Ac, cry1Ab*, *cry2Ab2, cp4-epsps, phytase* have been reported (Huang et al., 2014; Li et al., 2013; Singh, Randhawa, Sood, & Bhoge, 2015; Zhou et al., 2014). Event-specific LAMP assays have also been developed for GM cotton, maize, rice and soybean (Bhoge, Chhabra, Randhawa, Sathiyabama, & Singh, 2015; Chen, Guo, Wang, Kai, & Yang, 2011; Guan et al., 2010; Kiddle et al., 2012; Randhawa, Chhabra, Bhoge, & Singh, 2015; Wang, Teng, Guana, Tiana, & Wang, 2013; Xu et al., 2013).

In the present study, visual and real-time LAMP screening assays targeting *pat* and *pmi* marker genes have been reported, which are specific and sensitive to check the GM status of samples in food and supply chain.

2. Materials and methods

2.1. Test samples and DNA extraction

Six GM events of maize, namely, 3272 (SYN-E3272-5), 59122 (DAS-59122-7), *Bt*11 (SYN-BTØ11-1), *Bt*176 (SYN-EV176-9), MIR604 (SYN-IR6Ø4-5), TC1507 (DAS-Ø15Ø7-1), and one of cotton, *viz.*, Widestrike™ (DAS-21Ø23-5 x DAS-24236-5) were used as positive test sample(s) for respective target(s) for testing specificty (Table 1). Certified reference materials (CRMs) of GM maize events, *viz.*, 3272 (ERM-BF420c), 59122 (ERM-BF424d), *Bt*11 (ERM-BF412f), *Bt*176 (ERM-BF411f) and MIR604 (ERM-BF423d), were procured from the Institute for Reference Materials and Measurements (IRMM), European Commission-Joint Research Centre, through Sigma Aldrich. DNA samples of GM maize event TC1507 and GM cotton event Widestrike™ imported for research purposes through ICAR-National Bureau of Plant Genetic Resources, were used. Non-GM maize (*Zea mays* L.) seeds procured from the National Seeds Corporation, New Delhi were used as negative control. For checking the practical applicability of developed assays, proficiency test samples with the specific GM events with target genes (*pat/pmi*) were used: C1 (59122, TC1507 and MIR162), C2 (59122, *Bt*11), C3 (no specific GM event), and C4 (TC1507 and MIR162) (Proficiency Testing organized by the United States Department of Agriculture and the Grain Inspection, Packers and Stockyards Administration (USDA-GIPSA) in April 2015).

Genomic DNA from 100 mg of homogenized seed powder of each CRM was extracted using sodium dodecyl sulphate (SDS) method (Dellaporta, Wood, & Hicks, 1983) with minor modifications. Quantity and quality of purified DNA samples were measured and evaluated using a UV Spectrophotometer (Eppendorf, Hamburg, Germany). DNA samples were diluted to a concentration of 40 ng/μl. For on-site application, method for DNA isolation as optimized earlier using the RED Extract-N-Amp™ Plant PCR Kit (Sigma Aldrich, MO, USA), was used, as the protocol involves few simple steps without centrifugation (Randhawa et al., 2015).

Prior to conducting LAMP experiments, DNA samples were checked for specific GM event with respective event-specific real-time PCR assays using validated protocols (http://gmo-crl.jrc.ec.europa.eu/gmomethods/).

2.2. Designing of LAMP primers

LAMP primers including forward and reverse primers (F3, B3), forward inner and backward inner primers (FIP, BIP) and loop-F and loop-B primers were designed using LAMP Designing Software "PrimerExplorer V4" (https://primerexplorer.jp/e/). Parameters for primer designing included melting temperature (Tm) range from 55 to 58 °C for outer primers, and 60–63 °C for inner primers and % GC range from 30 to 65%. To increase the specificity of LAMP assays, loop primers were designed using default set conditions. Primers for *pat* and *pmi* genes were designed using the sequences of GenBank Accession numbers DQ156557.1 and CP012127.1, respectively. Specificity of designed primers was confirmed using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi by comparing with available sequences. Table 2 shows the sequence and target details of the designed primers. Primers were synthesized by Sigma Aldrich (New Delhi, India).