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Detecting *cry1Ac* by loop mediated isothermal amplification by SYBR green-I

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Abstract
Detection of Genetically Modified Organisms (GMOs) is an important part of GMO labelling, as without detection methods the traceability of GMOs would rely solely on documentation. Efficient detection strategies for GM crops need to be in compliance with regulatory frameworks and address consumer concerns. The DNA based techniques are currently the major detection methods that are widely used due to their ease and accuracy Loop mediated isothermal amplification (LAMP) is a simple, rapid, specific and cost effective nucleic acid amplification method. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. A set of six specific primers was designed to recognize six distinct sequences on the target *cry1Ac* gene, including a pair of inner primers, a pair of outer primers, and a pair of loop primers. The optimum reaction temperature and time were optimised to be 65°C for 60 min respectively and stained with SYBR Green –I for visual detection of GMO.

Keywords: LAMP, GMO, detection

Introduction
Management of insect pests through synthetic insecticides is being practiced and was a boon in the advent era of green revolution. Sole reliance on insecticides has caused an imbalance in the agro-ecosystem creating resistance and resurgence problems warranting alternate control measures. As an alternative to the insecticides inserting of foreign gene through genetic engineering and evolving transgenic cotton is considered to be an important strategy for the management of major pests of cotton, particularly bollworms without inimical effects on ecosystem. *Bt* cotton was among the first GM crops to be commercialised during the 1900s at the global level and was officially approved for sale in India in 2002. It consists of a gene from the soil bacterium *Bacillus thuringensis (Bt*), which provides resistance to different bollworm species, a major pest in cotton and helps cotton growers benefit through efficient pest control. India is the second largest producer and consumer of cotton accounting 33%of the global cotton production in 2015 - 16 (Anon) and is cultivated in an area of about 11.8mha. However consumer concerns related to development and marketing of GMO and derived food products have resulted in increased awareness regarding the food labelling all over the world. The development and commercialisation of GM crops is increasing at a faster pace, to develop qualitative and quantitative methods for detection of GM crops has become even more challenging. Polymerase Chain Reaction (PCR) being a reliable, robust and sensitive technique, has broad application in GM detection. However, high-precision equipment and procedures associated with PCR analysis are some of the constraints, which limit their use for on-site detection. Moreover, the qPCR technique is often sensitive to inhibitors present in plant extracts. [Boonham *et al*] [2]

Loop-mediated isothermal amplification (LAMP), an isothermal nucleic acid amplification technique, is less sensitive to inhibitors, does not require sophisticated equipment, and has the potential to be deployed on site. [Notomi *et al*., 2002] [3] LAMP is gaining importance because of its sensitivity and specificity, being superior to PCR and usually comparable to qPCR. In addition, LAMP provides results on site in a significantly shorter time than conventional PCR-based techniques. LAMP is characterized by the use of four different primers, specifically designed to recognize six distinct regions on the target DNA template. An inner primer containing sequences of sense and antisense strands of the target DNA initiates LAMP reaction, which proceeds at a constant temperature, followed by strand displacement DNA synthesis primed by an outer primer set. [Futuka *et al*., 2004, Tomita *et al*., 2008] [4-5] The addition of two "loop" primers or two "stem" primers further increases the speed of

amplification of the LAMP assay [Nagamine *et al*, 2002] [6]. Amplification and detection of target genes can be completed in a single step at a constant temperature, by incubating the DNA template, primers and a strand displacement DNA polymerase. The significant advantage of LAMP is that it can amplify DNA isothermally (60−65°C) with a simple isothermal instrument, based on strand displacement synthesis of DNA by *Bst* DNA polymerase. It provides a high amplification efficiency, with replication of the original template copy 10^9 −10^{10} times during a 15−60 min reaction. LAMP products show a ladder-like pattern on an agarose gel or can be monitored in real time using turbidometry [Mori *et al*., The amplicons specific for DNA can alternatively be visualized after completion of the LAMP reactions using nucleic acid staining or fluorescent dyes such as SYBR Green Iand hydroxyl naphthol blue [Chen *et al.,* In this study, LAMP-based visual detection have been employed as cost-efficient, rapid and reliable screening tools for checking the GM status of the sample. The developed LAMP assays targeted *cry1Ac* gene of *Bt* cotton and further four visualisation approaches *i.e.*, gel electrophoresis, visual detection using intercalating dye (SYBR-Green)

Material and Methods

Study comprised of seven hybrids which includes four *Bt* hybrids *viz*., ACH-155, Laxmi-Gold, Jadoo, Bahubali and three non - *Bt* hybrids *viz*., RAHH-455,SHH-818 and Suvin obtained from, AICCIP Cotton, Main Agricultural Research Station, Raichur.

DNA Isolation and Quantification-Genomic DNA from transgenics and non transgenics cotton leaves were extracted using CTAB method [9].

Leaf samples of young seedlings (two grams) were weighed and homogenized using autoclaved mortar and pestle. The homogenized tissue was transferred to 1.5ml centrifuge tube containing1.2ml of preheated extraction buffer. Tubes containing ground tissue were placed in water bath (with gentle shaking) for 60 minutes at 65ºC with periodical shaking at an interval of five minutes. Later, the tubes with leaf tissue extract were incubated at room temperature for 15 minutes. Ten ml of Chloroform: Isoamyl alcohol (CIA) mixture (24:1) was added to tissue extract and the contents were mixed by shaking then the tubes were centrifuged for 10 minutes at 10,000 rpm at room temperature. Equal quantity of chilled isopropanol was added to each tube and mixed by inverting and incubated at -20ºC for overnight. The content was centrifuged for ten minutes at 10,000 rpm at 40ºC. The supernatant was discarded. The DNA pellet obtained was washed with 70 per cent ethanol and the tubes were inverted on blotter paper to dry the pellet. The DNA was dissolved in 100µl T_{10} E_1 buffer and stored at -20ºC for further study.

Primer design- The outer primers F3, B3, inner primers FIP, BIP, loop primers LF and LB were designed using Primer explorer version software (http://primerexplorer.jp/e/).
Primers were synthesized by Sigma Aldrich.

Optimization of LAMP assay detection for cry1Ac in Bt cotton

Reaction conditions were optimized to establish fast and effective amplification. Different parameters were tested including amplification temperatures (58, 60 and 65ºC), incubation time (60, 75 and 90min.), $MgSO_4$ concentrations (10mM, 12mM, 14mM, 16mM, 18mM), varying concentrations of *Bst* polymerase (0.8U, 2U, 4U, 6U, 8U), betaine concentration (0.8M to 1M) and outer: inner primer ratio(1:2 to 1:20).

LAMP was carried out in 25µl reaction mixture in a 0.2 ml tube with 10XThermoPol Reaction Buffer [(20mM Tris-HCl, 10mM KCl, 10mM $(NH_4)2SO_4$, 2mM $MgSO_4$, 0.1%Triton X100) (pH 8.8)], 0.2mM each of F3 and B3, and 0.8mM each of FIP and BIP, 0.4mM each of LF and LB primers,1mM dNTP 8 units of *Bst* DNA polymerase large fragment (New England Biolabs, Hitchin, UK),0.8M betaine (Sigma, Northbrook, USA) and 2 µl of template DNA (Table.1) LAMP was performed in Eppendorf thermal cycler according to the following program and reactions were incubated at 65°C for 1 h, followed by 82°C for 10 min to inactivate the enzyme. The LAMP assay was carried out for each template DNA, and non-transgenic cotton was used as negative control (Table.2).

Analysis of LAMP products

Intercalating dye- Lamp-amplified products were directly observed by the naked eyes by adding 2.0ul of 1000X SYBR Green dye.

Gel electrophoresis- The specificity of LAMP amplified products were further confirmed by checking the ladder profile using gel electrophoresis on 1.2% agarose (Xcelris) in 1X TAE stained with ethidium bromide.

Results

DNA Quantification- The quality and quantity of DNA were estimated using a spectrophotometer based on the 260/280-nm and 260/230-nm UV absorption ratios and analysed by 0.8% agarose gel electrophoresis.

1. **Optimisation of LAMP assay for detection of cry1Ac in Bt cotton:** A series of reactions were performed to determine the optimum conditions for LAMP Different parameters were tested including amplification temperatures (58, 60 and 65ºC)
2. **Temperature titration:** Incubation time (60, 75 and 90min.) $MgSO_4$ concentrations (10mM, 12mM, 14mM, 16mM, 18mM) varying concentrations of *Bst* polymerase (0.8U, 2U, 4U, 6U and 8U), betaine (0.8M to 1M) and different ratio of outer primers to inner primers. The optimum incubation temperature for LAMP assay with the *cry1Ac* primer set was established using a range of temperatures (58, 60 and 65ºC) for different incubation time of 60, 75 and 90min. to optimize the reaction conditions and then heated at 80°C for 5 min to terminate the reaction. The LAMP assay was successful at temperatures of 60°C and 65°C with the incubation time of 60min. However, better results on agarose gels were obtained when using 65°C. Thus, the optimum temperature and time for LAMP was 65°C at 60 min. The concentration of added Mg^{2+} was 10 mM-18 mM and 12.0 mM was optimum.
3. **Optimisation of BST polymerase:** Among the variable concentrations of Bst polymerase used the amplification was optimum at 8U of *Bst* polymerase. Addition of freshly prepared 0.8M of betaine was found effective but it was not consistent. In addition, outer primers (F3 and B3) were used in excess, compared to outer primers (FIP and BIP). The ratio of outer primers to inner primers was optimized from 1:2 to 1:20. The result showed that the optimal ratio of outer primers to inner primers was 1:4. (Fig.1) To enhance the reliability of this technique, a

positive control containing the genomic DNA from *Bt* hybrids, a negative control with the genomic DNA from non-transgenic varieties and a blank control of water was introduced. Based on the optimized reaction conditions described above, the LAMP assays for detecting *cry1Ac* was established. The LAMP reaction used in further experimentation was carried out in a 25 µl reaction mixture system containing 10X Thermopol buffer [10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH_4SO_4), 4mM $MgSO_4$, 0.1% Triton X-100] 0.2 µM each F3 and B3, 0.8 µM each FIP and BIP, 8U *Bst* DNA polymerase large fragment and 1.4 mM dNTPs and 2µl of template.

Primer standardisation

The standardization of LAMP primers was carried out with two sets of primers with an annealing temperature of 65ºC and all the optimized concentration of reagents (*i.e.*, 10X Thermopol buffer(10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM$(NH)_4$ SO_4), 4mM $MgSO_4$ 8U *Bst* DNA polymerase large fragment and 1.4 mM dNTPs). The primer set two gave the specified LAMP product with ladder like profile on 1.2% agarose gel electrophoresis and was visually detected by SYBR Green Dye-1 staining.

Standardisation for BST polymerase

The standardization of *Bst* polymerase was carried out with an annealing temperature of 65ºC, various concentrations (0.8U, 2U, 4U, 6U, 8U) of *Bst* polymerase were tested for amplification by keeping all the other components constant. (i.e., 10XThermoplol buffer [10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH)_4$ SO_4], 4mM $MgSO_4$, 0.2µM each F3 and B3, 0.8 µM each FIP and BIP, 1.4 mM dNTPs). The amplification was obtained at the concentration of 1µl (8U) and gave the ladder like profile on 1.2% agarose gel stained with ethidium bromide (Fig.2)

Results

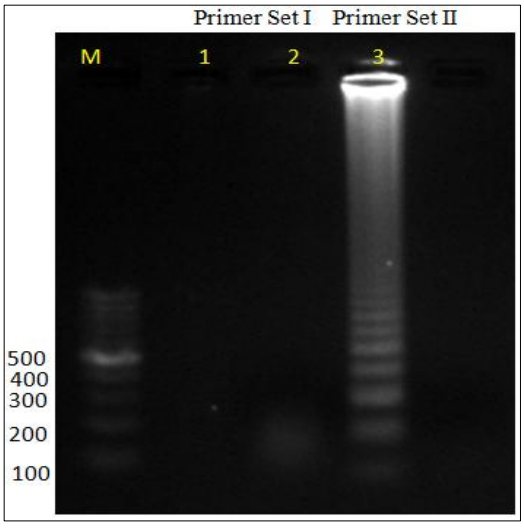

Fig 1: Optimisation of LAMP Primers

Analysis of LAMP product

Gel electrophoresis - A LAMP reaction results in products with stem-loop structures and several inverted repeats of the target DNA. The specificity of amplified products were further confirmed by checking the ladder profile using gel electrophoresis on 1.2% agarose in 1X TAE stained with ethidium bromide. The ladder profile of LAMP product for *Bt* transgenics is depicted in Fig.3

Visual detection of LAMP products- Intercalating dyes-Following amplification by the LAMP method, the products were detected by the addition of 2.0µl 1000X SYBR Green (molecular probe) Samples that turned yellowish green were positive, while those that remained orange were negative. *Bt* hybrids showed the green colour confirming the presence of *cry1Ac* while non *Bt* varieties showed orange colour for SYBR Green. The optimized LAMP reaction was used for screening *Bt* hybrids (Jadoo, Laxmi-Gold, ACH-155) and the results obtained by staining with intercalating dye SYBR Green Dye-1 at 1000X dilution. The SYBR Green clearly differentiated the amplified product with green colour and the non amplified products with orange colour and thus helpful in visual detection of LAMP products. (Fig.4)

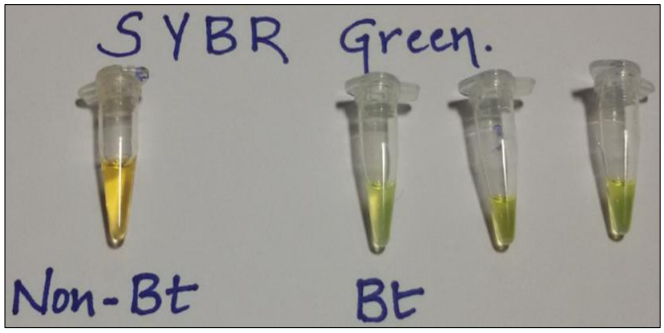

Fig 2: Visual detection of LAMP products using intercalating dye SYBR Green-I

Discussion

As a novel nucleic acid amplification method, LAMP has already been applied for the detection of several GM crops and their components. Futuka *et al* (2004) established a LAMP method for the detection of CaMV35S promoter in transgenic tomato and its products. Similarly Lee *et al* (2009) detected CaMV35S promoter, nopaline synthase gene promoter and terminator in transgenic rapeseed by LAMP.

Optimization of LAMP assay detection for *cry1Ac*

In the present study, a LAMP assay was optimized with a set of six specifically designed primers capable of recognizing a total of six distinct regions on the target *cry1Ac* gene. The effects of Mg^{2+} concentration, *Bst* DNA polymerase amount, concentration ratio between inner and outer primers, and the effect of the addition of 0.80 M betaine on the LAMP reaction were tested.

Effect of Mg^{2+} concentration

Because free Mg^{2+} availability affects primer annealing and DNA polymerase activity (Saiki *et al.*, 1998), the effect of Mg^{2+} concentrations ranging from 0 to 10 mM on the LAMP reaction was determined. The Mg^{2+} concentration at 8 mM gave the optimal amplification This concentration falls in the published ranges between 4 mM and 8 mM (Kuboki *et al*., 2003; Notomi *et al*., 2000) [11].

Effect of deoxynucleotide triphosphate concentration

It is known that the deoxynucleotide triphosphate concentration affects the specificity of DNA polymerase amplification (Innis *et al*., 1988). The LAMP reaction in the presence of t 1.0 mM gave the maximal reaction product. This concentration is much lower than those reported for bacteria by others (Enosawa *et al.*, 2003; Iwamoto *et al* 2003; Maruyama *et al*., 2003; Savan *et al.*, 2004) [13-16]

Effect of primer ratio

Nagamine *et al*. accelerated the LAMP reaction by using loop primers, which suggested that the LAMP reaction times would be shorter than the original method when using loop primers

Effect of betaine concentration

Previous studies revealed that 0.80 M betaine resulted in elevated sensitivity and increased effectiveness of the LAMP assay. Betaine is assumed to be capable of promoting GC-rich DNA amplification and preventing secondary structure formation in GC-rich regions, due to reduction of base stacking (Chen *et al*, 2011) [18]. However in this study addition of freshly prepared 0.8M of betaine was found effective but it was not consistent which may be due to the non-GC-rich target sequences, indicating that betaine is not an essential requirement for amplifying non-GC-rich target sequences.

Effect of temperature

Although the *Bst* DNA polymerase has the optimal activity at 65ºC, several reports showed this enzyme can amplify DNA templates at lower temperatures in the LAMP reaction (Endo *et al*., 2004; Iwamoto *et al*., 2003; Parida *et al*., 2004; Poon *et al*., 2004; Yoshikawa *et al*., 2004) [19-22]. The effect of temperature on the LAMP reaction was determined, LAMP reaction temperature at 65^0C generated ladder-like pattern products, but no such typical pattern product was detected at 58 and 60 ^{0}C.

Effect of reaction length

Several reports (Iwamoto *et al*., 2003; Savan *et al*., 2004; Yoshikawa *et al*., 2004) have demonstrated that amplified products can be detected less than 60 min in the LAMP assay. We observed maximal amplification at 60min.

Analysis of LAMP products by *Gel electrophoresis*

The gel electrophoresis for LAMP showed typical ladder like profile on 1.2% gel stained with ethidium bromide. the ladder like profile is expected because we used 2 different set of primers, Thus it confirms the amplification.

Visual detection of LAMP products- Intercalating dyes

Frequently used detection methods, includes precipitation observation, intercalating dyes such as SYBR Green I, Calcein, HNB etc, Mn complex, and detection under ultraviolet light at a wavelength of 365 nm. Earlier studies revealed the feasibility of SYBR Green I, as well as the calcein and Mn complex method. Since SYBR Green I, HNB, calcein and Mn complex are based on a color reaction, both are easier ways to detect LAMP products than that of precipitation observation (Zhou *et al*, 2014) [23].

In the present study we used SYBR Green 1to visualise the LAMP amplified products. the concentration of SYBR Green 1 was optimised so that there was clear distinguishing difference between amplified and non amplified products. Amplified products showed Green colour whereas non amplified and water blank showed orange colour. Since SYBR Green I detection was based on the amplified fluorescence emitted from its binding to the minor groove of double-strand DNA, a higher sensitivity can be expected compared to the formation of the white precipitate. Previous studies revealed that it would reduce product amplification if SYBR Green I was added before the reaction, while aerosol contamination would often lead to false positives when the dye was added after the reaction (Wang *et al*, 2004).

Conclusion

The LAMP assays can also be employed on a simple heating block or a thermal cycler, In that case, the LAMP amplification is visually verified by adding SYBR Green I after the reaction, for further confirmation, gel electrophoresis analysis can also be performed. The flexibility of the LAMP assays can facilitate its applicability for reliable GMO detection in the laboratory and also on site, if it is combined with simple and fast DNA extraction methods like those recently applied for the other LAMP assays, using either a portable isothermal heating block, which would be further useful for GMO screening by customs authorities to check the unauthorized imports at ports of entry or by the field inspectors or farmers in the field.

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