

A powerless on-the-spot detection protocol for transgenic crops within 30 min, from leaf sampling up to results

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Received: 19 August 2015 / Revised: 13 October 2015 / Accepted: 16 October 2015 / Published online: 29 October 2015
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Abstract The requirement of power-dependent instruments or excessive operation time usually restricts current nucleic acid amplification methods from being used for detection of transgenic crops in the field. In this paper, an easy and rapid detection method which requires no electricity supply has been developed. The time-consuming process of nucleic acid purification is omitted in this method. DNA solution obtained from leaves with 0.5 M sodium hydroxide (NaOH) can be used for loop-mediated isothermal amplification (LAMP) only after simple dilution. Traditional instruments like a polymerase chain reaction (PCR) amplifier and water bath used for DNA amplification are abandoned. Three kinds of dewar flasks were tested and it turned out that the common dewar flask was the best. Combined with visual detection of LAMP amplicons by phosphate (Pi)-induced coloration reaction, the whole process of detection of transgenic crops via genetically pure material (leaf material of one plant) could be accomplished within 30 min. The feasibility of this method was also verified by analysis of practical samples.

Keywords Crude DNA extract · Unpowered device · Loop-mediated isothermal amplification · Visual detection · Transgenic crop detection

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-9128-x) contains supplementary material, which is available to authorized users.

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Introduction

In 2014, around 181.5 million ha was planted with genetically modified (GM) crops throughout the world. The area has been increased more than 100-fold since 1996 [1]. However, consumers are still concerned about the safety of transgenic ingredients in foods or feeds. Therefore, strict regulations on restricting commercial plantation of transgenic crops are implemented in many countries. In Australia, only some transgenic canola (*Brassica napus* L.) crops are approved for commercial release [2]. In China, only transgenic papaya (*Carica papaya* L.) is allowed to be planted on a commercial scale [3]. For Europe, only transgenic maize (*Zea mays* L.) MON810 listed in the European permission catalogue is planted, i.e., in Spain [4]. Accordingly, effective detection methods are required to monitor the plantation of transgenic crops.

Polymerase chain reaction (PCR) is the gold standard for detection of transgenic ingredients. Nevertheless, it is not practical for use in the field due to the long operation time and expensive, non-portable instruments. The emergence of isothermal amplifications like loop-mediated isothermal amplification (LAMP), cross primer amplification (CPA), rolling circle amplification (RCA), and helicase-dependent amplification (HDA) abandons the use of the thermal cyclers. However, a device for temperature controlling such as a water bath or heat block is still necessary.

Another obstacle for rapid detection in the field is DNA extraction. Though various standard extraction methods have been reported, they are not convenient to be used in the field because of their long operation time and cumbersome steps [5, 6]. Some commercial kits are available, but they are probably unaffordable to some less developed areas owing to their high cost.

To overcome the weakness of the methods mentioned above, we presented a method which could discriminate

transgenic crops from non-transgenic ones via analysis of genetically pure material (leaves of a single plant) within 30 min (Fig. 1). The method is based on LAMP amplification and visual detection recently developed in our lab [7]. It is characterized by two advantages: (i) A very short (~4 min) DNA extraction step was performed on genetically pure material (leaf material of a single plant) and (ii) no power-dependent instrument is needed during the whole detection process, with incubation under controlled temperature obtained in a dewar flask.

Materials and methods

Materials

Rice (*Oryza sativa* L.) leaves of non-transgenic Minghui 63 and homozygous diploid transgenic TT51-1 and Kemingdao1 (KMD1) were obtained from Zhejiang Academy of Agricultural Science (Hangzhou, China).

Simple screen of lysis agent for crude extraction of DNA

A piece of fresh leaf (around 1×1 cm) from a single plant (tillering stage) was placed in a mortar and mixed with 200 μ L of different lysis agents [6, 8–11]. (The composition of lysis agent is listed in Table S1.) After 1 min of grinding and 9 min of standing, the slurries were serially diluted (1:1, 1:10, 1:100, and 1:1000) with Tris-EDTA (TE) buffer and amplified by LAMP. The durations of lysis were studied by crushing the leaves in 0.5 M NaOH for 1 min and then letting the slurry stand for 3, 6, and 9 min, respectively. Crude extraction was performed in triplicate.

LAMP assay

LAMP assays were carried out with Loopamp DNA amplification Kit (Deaou Biotechnology Co., Ltd, Guangzhou,

China) in total reaction volumes of 20 μ L containing 17.4 μ L reaction mix, 1 μ L Bst DNA polymerase, and 1.6 μ L template. For no-template controls (NTCs), 1.6- μ L templates were replaced with 1.6- μ L TE buffer. Primers for LAMP assay of *Agrobacterium tumefaciens* nopaline synthase terminator (T-nos; Product No. PA101S/L), *Cauliflower mosaic virus* 35S promoter (CaMV 35S; Product No. PD104S/L), and phospholipase D gene (PLD; Product No. PD104S/L) were provided in these kits. The real-time LAMP was performed in triplicate on a MyiQ2 Two-Color Real Time PCR Detection System with collection of fluorescence signal by iQTM5 Optical System Software at the end of every minute. The threshold time (T_t) values were automatically set by the software as well. For amplifications performed in the dewar flasks, preheated water was mixed with cool water or cooled to around 63 °C (monitored by thermometer) and used for incubation of amplification tubes.

Pi-induced visual detection

Phosphate (Pi)-induced visual detections were performed according to Zhang et al. [7]. Briefly, 0.1 U thermostable inorganic pyrophosphatase (New England Biolabs, Ipswich, MA) was added to the 20- μ L total LAMP reaction mixtures before amplification. After amplification, 4 μ L of Mo-Sb solution (containing 21 mM ammonium molybdate, 2 mM potassium antimonyl-tartrate, and 5.4 M sulfuric acid) and 2 μ L ascorbic acid (10 %) were added to the reaction mixture and mixed with 174 μ L distilled water. The volume of distilled water can also be doubled or halved to make the products 20-fold or fivefold diluted. Reactions were kept at room temperature and results read visually after 5 min. Visual detections of all samples were repeated twice.

Detection of practical samples

DNA was extracted from a piece of fresh leaf (1×1 cm) with 0.5 M NaOH for 4 min (including 1 min of grinding and 3 min

Fig. 1 Schematic of screening strategy for transgenic crops in the field without DNA purification steps or any powered instruments

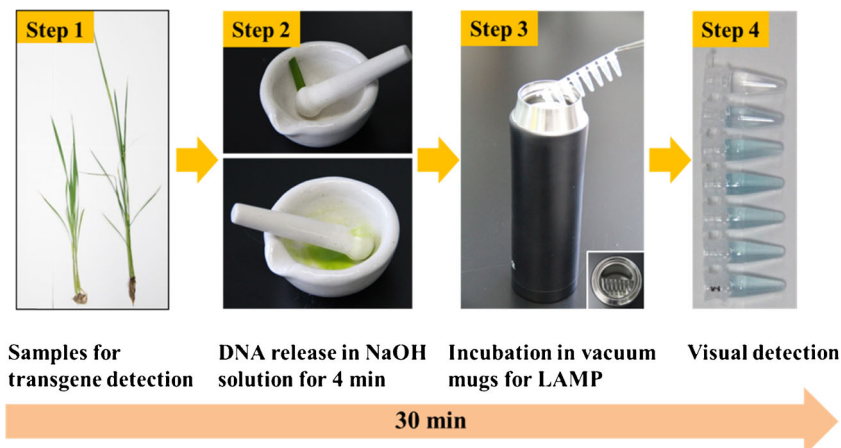


Table 1 Detailed estimation of lysis agent for crude extraction of DNA by real-time LAMP analyses

Lysis agent	Time for lysis (min)	Repeat	T_i values (min) ^a			Mean of T_i values (min)	SD	RSD (%)	Mean of all T_i values (min)	SD ^f	RSD ^f (%)
			1	2	3						
1 % SLS	10	1	14.1	14.0	13.7	13.9	0.2	1.5	14.2 a	0.3	2.2
		2	14.6	14.1	14.1	14.3	0.3	2.2			
		3	13.9	14.4	14.6	14.3	0.4	2.7			
3 M CH ₅ N ₃ ·HCl	10	1	12.2	12.3	11.7	12.1	0.3	2.8	12.0 b, c	0.3	2.6
		2	11.7	11.5	12.0	11.7	0.2	2.1			
		3	11.9	12.3	12.2	12.1	0.2	1.8			
2.5 M GITC	10	1	11.8	11.9	11.8	11.8	0.1	0.5	11.9 c	0.2	2.1
		2	12.1	12.2	11.7	12.0	0.3	2.3			
		3	11.4	11.7	12.0	11.7	0.3	2.7			
0.5 M NaOH	10	1	11.8	12.0	12.1	12.0	0.1	1.0	12.0 b, c	0.3	2.5
		2	12.6	12.3	11.9	12.3	0.3	2.8			
		3	11.6	11.8	11.8	11.7	0.1	1.0			
0.5 M NaOH	7	1	12.1	11.9	11.7	11.9	0.2	1.7	11.9 c	0.2	2.0
		2	11.9	12.1	12.1	12.1	0.2	1.3			
		3	11.6	11.5	11.9	11.7	0.2	1.9			
0.5 M NaOH	4	1	12.2	12.0	12.4	12.2	0.2	1.6	12.3 b	0.3	2.1
		2	12.3	11.9	12.5	12.3	0.3	2.1			
		3	12.4	12.0	12.8	12.4	0.4	3.0			

a, b, c The level of significant difference ($p < 0.05$). The data marked with the same letter indicates there was no significant difference between the groups

^a T_i values for tenfold-diluted DNA obtained from TT51-1 leaves targeting T-nos; T_i values were automatically determined by the iQTM 5 Optical System Software which collected fluorescence signal at the end of every minute

^f Calculations based on all T_i values.

of standing), diluting it tenfold in TE buffer before amplification. 0.1 U thermostable inorganic pyrophosphatase was added to 20- μ L LAMP reaction mixtures and incubated in a common dewar flask with an initial water temperature of 63 °C (as mentioned above) for 20 min. The products were fivefold diluted with chromogenic reagents as described above and photographed after standing ~5 min.

Results

DNA extraction without purification

To simplify the extraction process and shorten operation time, purification steps were omitted. Existing in almost the whole growth stage and very easy to be picked, leaves were used to screen transgenic crops in our study. Eight kinds of lysis reagents (read Electronic Supplementary Material for details), including surfactant, salts, or chaotropic salts and base (Table S1), were employed to release DNA from transgenic rice leaves (1 \times 1 cm pieces), TT51-1, for 10 min. PCR (read Electronic Supplementary Material for the performing method) and LAMP were both applied to determine the amplification ability of the extracted DNA (Table S3). Positive signals were obtained with all lysates when they

were sufficiently diluted as both the amount of DNA as well as that of the PCR-inhibitory compound arising from the cell or from the lysis buffer might influence the amplification results [12]. With appropriate dilution, some samples such as those obtained with 1 % sodium lauryl sulfate (SLS), 3 M guanidine hydrochloride (CH₅N₃·HCl), 2.5 M guanidinium thiocyanate (GITC), and 0.5 M NaOH could achieve similar threshold time (T_i) values as that purified by standard cetyltrimethyl ammonium bromide (CTAB) extraction method. Thus, we propose that an extra process of DNA purification is not necessary, making detection in the field more efficient and convenient. Differences between PCR and LAMP may reflect their different tolerances to different inhibitors due to their distinct amplification mechanisms and polymerases, as previously discovered [13].

To figure out which lysis agent works best, 1 % SLS, 3 M CH₅N₃·HCl, 2.5 M GITC, and 0.5 M NaOH were further investigated by LAMP and results were analyzed by analysis of variance (ANOVA). As shown in Table 1, 1 % SLS ($T_i = 14.2$; $p < 0.05$) was less effective than the other three agents for DNA extraction. However, there was no significant difference ($p > 0.05$) among 3 M CH₅N₃·HCl ($T_i = 12.0$), 2.5 M GITC ($T_i = 11.9$), and 0.5 M NaOH ($T_i = 12.0$). As the cheapest and most available chemical reagent among these lysis agents, NaOH was chosen for DNA extraction in our further study.

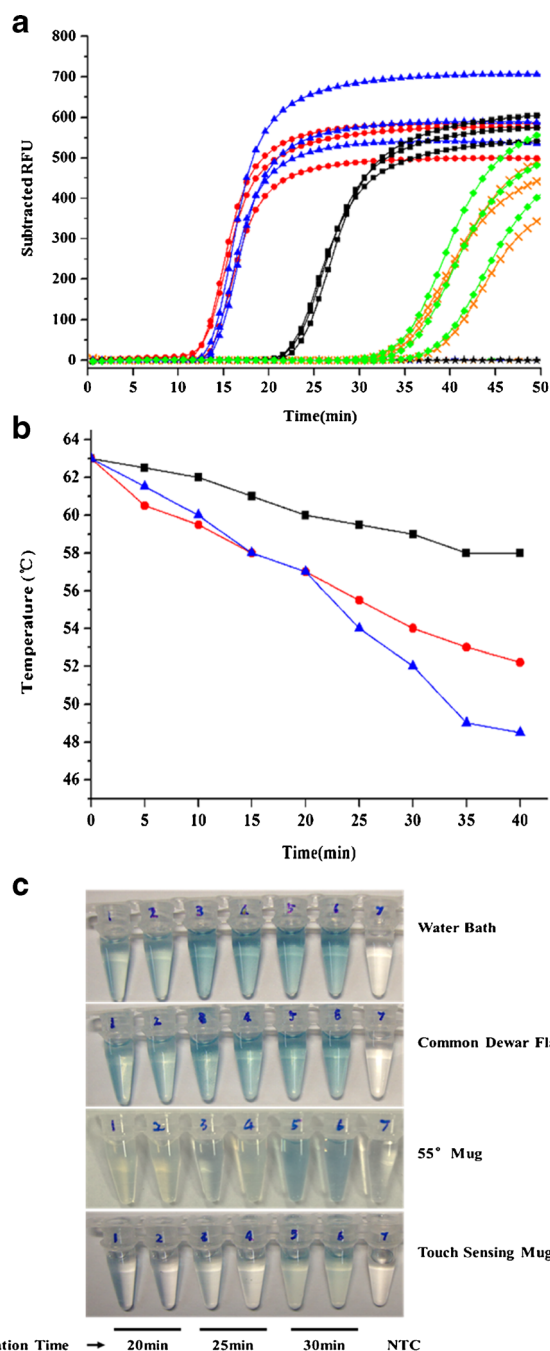


Fig. 2 **a** Amplification curves of gradient LAMP at 66.6 °C (orange cross), 63.0 °C (red circle), 58.0 °C (blue triangle), 54.4 °C (black square), and 51.9 °C (green diamond) with T-nos (from transgenic rice leaves, TT51-1) as target. NTCs at any temperature are marked with stars. **b** Temperature variation curves of 63 °C water in common dewar flask (black square), touch-sensing mug (blue triangle), and 55° mug (red circle). **c** Visual detection of transgenic rice TT51-1 after 20, 25, and 30 min of LAMP reaction incubated in four kinds of heater with T-nos as target

To shorten the whole detection time, different durations of lysis were also studied (Table 1). No significant difference ($p=0.109$) was obtained when 4 min ($T_l=12.3$) and 10 min ($T_l=12.0$) were applied for extraction. Though 7 min ($T_l=$

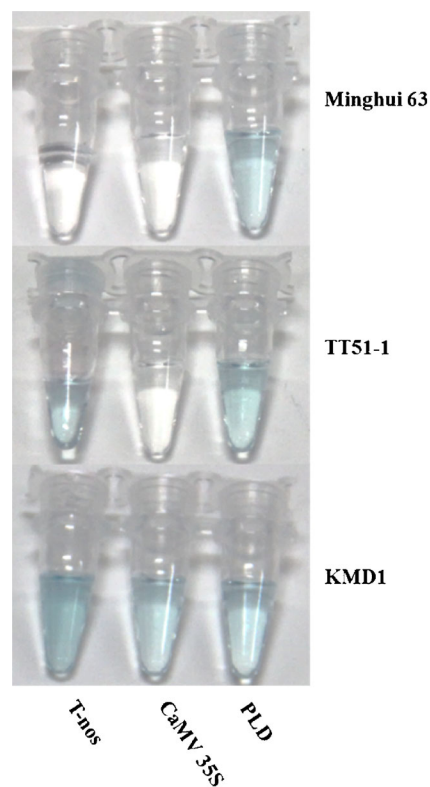


Fig. 3 Detection of practical samples of *Minghui 63*, *TT51-1*, and *KMD1* targeting *T-nos* and *CaMV 35S*. *PLD* was used as endogenous reference gene of rice

11.9; $p<0.05$) could save several seconds than 4 min during amplification, it took longer to extract DNA. Hence, tenfold-diluted lysate obtained with 0.5 M NaOH within 4 min (including 1 min for crushing of leaves) was used for detection in further study.

What needs to be mentioned is that the high copy number of target DNA in transgenic pure materials might ensure the success of amplification when the template was not purified. However, for some samples such as those mingled with a low presence of transgenic ingredients or the ones that have been processed, the low abundance of target DNA and the existence of inhibitor in the non-purified templates could lead to false-negative results. That is, whether the extraction method might apply to these samples needs further study.

Feasibility of amplification in dewar flasks and visual detection

During isothermal amplification, constant temperature is usually provided by some special instrument, heat block or water bath. For detection in the field, amplifications which require no electricity supply would be ideal. The cheap and portable dewar flasks were examined as an alternative to maintaining water temperature above a certain value for hours or days without additional energy

supply. But absolute constant temperature is hard to be maintained in the dewar flasks. Hence, the range of temperature which is suitable for reaction was studied by real-time LAMP between 51.9 and 66.6 °C targeting T-nos (Fig. 2a). Though the lowest T_t values were obtained at 63.0 °C, specific amplification could be obtained within 40 min at all studied temperatures (Fig. S1). Therefore, successful amplification would be obtained if dewar flasks could keep water temperature between 51.9 and 66.6 °C for 40 min when used as incubator. Thereafter, three kinds of dewar flasks, namely common dewar flask, 55° mug, and touch-sensing mug, were studied. (Read Electronic Supplementary Material for further details.) Temperature changes of water with initial temperature of 63 °C were measured in these dewar flasks (Fig. 2b). Even if their thermal insulation properties differed, they all could keep water temperature above 58 °C for 15 min and 52 °C for 30 min.

It is important to discriminate positive samples from negative ones with effective methods after isothermal amplification. Chromogenic reactions have some advantages in terms of the operation time and equipment. Some existing visual detection methods have difficulties to discriminate positive samples from negative ones because the colorimetric change is from one color to another one [14, 15]. In view of this problem, a visual detection method based on the accumulation of phosphate ions during amplification has been developed in our lab recently [7]. Using Pi, the color of positive amplification products turns blue soon after the addition of chromogenic reagents so that it is easy to distinguish positive amplification products from the colorless negative ones. Moreover, amplicon contamination could be avoided by the use of a simple strip which contains three interlinked tubes (Fig. S4). Because of these advantages, the phosphate (Pi)-induced coloration reaction was employed in this method.

Considering the suitable temperature for LAMP and thermal insulation properties of these dewar flasks, 20, 25, and 30 min for DNA amplification (targeting T-nos in transgenic rice TT51-1) in these dewar flasks were studied. Amplifications performed at constant 63.0 °C in a water bath served as controls. The amplification products were visually detected 5 min after they have been mixed with chromogenic reagents. As shown in Fig. 2c, all NTCs were colorless, which indicated that there were no false-positive results. Obvious color changes were observed in all positive products amplified for 30 min no matter which dewar flask was used. For amplifications performed in the common dewar flask and water bath, 20 or 25 min was also enough to have positive samples detected. Yet target DNA could not be detected when the reaction was performed in the 55° mug

or touch-sensing mug for the same time. The chromogenic reaction results indicated that all these dewar flasks could serve as temperature controller during LAMP if enough time was provided but the common dewar flask was the best with the shortest time required. Thus, LAMP was performed in the common dewar flask for 20 min in our further study.

Detection of practical samples

The feasibility of DNA amplification in dewar flasks without template purification for detection of transgenic crops needs to be further verified by practical samples. Therefore, some rice leaf samples like non-transgenic rice Minghui 63, transgenic rice TT51-1, and KMD1 were analyzed (Fig. 3). As the most common markers of transgenic crops, T-nos and CaMV 35S were used as targets. PLD gene was used as endogenous reference gene of rice. Results based on the chromogenic reactions showed that endogenous gene PLD was detected in all samples. For Minghui63, neither T-nos nor CaMV 35S showed a positive signal. For TT51-1, T-nos was positive, but CaMV 35S was negative, while both T-nos and CaMV 35S were detected in KMD1. These detection results of samples with the expected composition were consistent with that reported in papers [16, 17]. The developed method was also used for detection of some blind samples of rice and canola leaves (Fig. S5). The results were consistent with that obtained with the gold standard real-time PCR method (using standard CTAB method for DNA extraction), indicating that the developed method could be used for the rapid detection of transgenic crops in the field.

Conclusion

Without using any power-dependent instruments, a rapid method for detection of transgenic crops in the field has been developed. With 4 min for DNA extraction (including 1 min for crushing of leaves and 3 min for standing), around 20 min for LAMP amplification, and 5 min for visual detection, the whole detection process could be completed within 30 min. After release from leaves with 0.5 M NaOH, tenfold dilution of non-purified DNA lysates could be amplified directly. Traditional instruments used for thermal controlling during isothermal amplification are replaced by a common dewar flask. The feasibility of this method has been verified by analysis of real samples. In addition, there is no requirement of highly trained operators because of the convenience and practicability of this approach, which is a great benefit for resource-limited areas.

Acknowledgments This work is supported by the Synergistic Innovation Center of Modern Agricultural Equipment and Technology (NZXT01201402) and National Natural Science Foundation of China (31271617). We thank the reviewers' elaborate advice on improving the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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