



Application of loop-mediated isothermal amplification (LAMP) in the detection of genetically modified RT 73 rapeseed: a qualitative fast inspection

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ABSTRACT

The study intended to establish a rapid, simple, efficient and sensitive method for detecting transgenic RT 73 rapeseed using the loop-mediated isothermal amplification (LAMP) technology. The transgenic RT 73 rapeseed sequences locating at the interface of endogenous gene to exogenous gene was taken as the target sequences, and four LAMP primers, inner primers FIP, BIP, and outer primers F3, B3 were designed, with specific identification to six independent regions of target genes. The LAMP reaction was performed. The LAMP primers specifically amplified the exogenous DNA of transgenic RT 73 rapeseed and the detection limit was 0.01ng DNA. The results could be determined with the help of real-time turbidimeter or directly observed by eyes for the qualitative determination. LAMP was specific to amplify exogenous genes of genetically modified RT 73 rapeseed, and the detection limit of 0.1 ng DNA was similar to that of real-time PCR. The LAMP operation was simple to greatly improve the efficiency of transgenic detection and have a good application prospect.

Key words: Genetically modified rapeseed, Isothermal condition, Loop-mediated isothermal amplification, Rapid detection

Transgenic rapeseed is one of the most studied transgenic plants. Currently, there have been 11 varieties of transgenic rapeseeds to put into commercial production in Canada. Since 1995, transgenic rapeseed has passed the assessment of environmental safety and the approval of its planting in succession in Canada, Japan, and the United States. Several products of the rapeseed have passed the assessment of environmental safety in China, for example, Monsanto's Roundup Ready (glufosinate), GT 73/RT 73 and 6 Bayer's herbicide resistants (OXY 235, T 45, Ms8Rf3, Ms1Rf1, Ms1Rf2, TOPAS 19/2). The Company obtained the import licensing of the processed raw materials in China in March 2004 (Lu 2005). From 1996 to 2015, the cumulative planting area of transgenic crops reached an unprecedented 2 billion hectares throughout the world, in which the transgenic rapeseed was a total of 100 million hectares (James 2009). When they bring huge economic benefits and social benefits to people, transgenic plants also bring some controversy, such as the food safety and ecological safety. In January 2001, European Union (EU)

revised the transgenic food law and regulations. According to the new law and regulations, any genetically modified crops or foods containing more than 0.9% transgenic DNA or protein must be labeled (Bonfini *et al.* 2002).

At present, real-time fluorescent quantitative PCR is a commonly used technology for the transgenic detection, and has a high sensitivity and accuracy. However, it takes a long time, is costly and needs professional technicians to operate (Qiu *et al.* 2007). The loop-mediated isothermal amplification (LAMP) is a new type of nucleic acid amplification method developed by Notomi *et al.* (2000), in which 4 primers are designed, a large fragment of Bst polymerase is used to directly amplify the double-stranded DNA, the amplified product is kept at 60~65°C for 30~60 min to complete the amplification reaction, and the amplification efficiency is up to 10⁹~10¹⁰ times. In the reaction process of DNA amplification, the pyrophosphate ions in DNA triphosphate matrix are replaced out to bind to magnesium ions in solution, forming magnesium pyrophosphate white precipitates. As the amplification reaction progresses, magnesium pyrophosphate white precipitates are increased, so that the reaction results can be directly observed with the naked eyes. This method removes the traditional electrophoresis and ultraviolet lamp for outcome confirmation. Alternatively, after the reaction, SYBR Green nucleic acid dye can be added to determine whether the reaction happens (Notimi *et al.* 2000).

The LAMP method is not only similar to real-time

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PCR in the sensitivity but also shows some advantages to save both time and labor, taking only 30–40 min constant temperature at 60°C is the sole requirement for the reaction equipment. The reaction results could be determined by directly observing the white turbidity with naked eyes, or collecting the turbidity signals using a real-time turbidimeter once every 6 sec, for the real-time monitoring of the reaction process (Mori *et al.* 2004).

The purpose of this study was to detect the edge sequence of genetically modified RT 73 rapeseed using LAMP technology, and establish a LAMP method for detecting transgenic RT 73 rapeseed.

MATERIALS AND METHODS

Genetically modified (GM) rapeseed RT 73 was purchased from American Oil Chemists' Society (AOCS, Urbana, IL). Fresh materials of peas, lentils, green beans, lupins, potatoes, corn, soybeans, chickpeas, mung beans, walnuts, carrots, and wheat were purchased from supermarkets (Carrefour, Changchun, China).

The experimental materials were ground into powder with a grinding mill. The genomic DNA was extracted and purified using DNA extraction kit. The extracted genomic DNA was dissolved in 100 µl AE solution. The concentration of the purified DNA samples was measured by an ultraviolet-visible spectrometer, and its quality was checked by 1% (w/v) agarose gel electrophoresis.

The specificity of LAMP is mainly embodied in the 4 primers targeting 6 regions in the primer sequences, two inner primers (FIP, BIP) and two outer primers (F3, B3), respectively. Based on the literature review, the sequence (Accession no. AX685147) at the junction part of the exogenous gene and endogenous gene of RT73 rapeseed was selected as the target sequence for primer design. The AX685147 sequence of NCBI Gen Bank was downloaded, and the online software Primer Explorer 3.0 (<http://primerexplorer.jp/lamp3.0.0/index.html>) was used to design the primers for LAMP reaction. Then, based on the principles of the primer design, two pairs of primers were selected manually, the primer sequences were F3-GGTAATTACT CTTTCTTTT CTCC, B3-GTGGAATGTT CAATACCTTG A, FIP-GGAGGATGAT CTTCAATGCC GG-ATATTGACCATCATACTCATTGC, BIP-GCCTTTCCTT CCTTTTCTTG CC-TGTTTCT GAGTAATTCT TCAGC. All the primers were synthesized by Sangon Co. Ltd (Shanghai, China). The length of the amplified fragment was 207 bp. The synthesized primers were dissolved and diluted, in which they were centrifuged at 12000 rpm for 5 min and the corresponding volume of ddH₂O was added to them, so that the final concentration of FIP (BIP) was 20mM and the final concentration of F3(B3) was 10 µM. The LAMP reactions require two pairs of primers, the outer primers F3(B3) and the inner primers FIB(BIP), which play different role in the LAMP reaction. The inner primers' concentration was more than the outer one. After the LAMP reaction, the results were judged mainly based on the change of turbidity, or the chromogenic reagents are

Table 1 Samples used in the specificity experiment

Sample	Transgenic characteristics
Rice Bt-63 standard (5%)	Bt-63
Rice KMD standard (100%)	KMD
Rice Bt-63 sample	Bt-63
Rice KF-8 sample	KF-8
Corn Bt-176 standard (5%)	Bt-176
Corn MON810 standard (10%)	MON 810
Potato sample	EH92-527-1
Soybean MON 89788 (100%)	MON 89788
Wheat sample	Transgene negative
Cotton sample	Rt-Cry
Corn sample	GA 21
Oilseed rape standard 1	T 45
Oilseed rape standard 2	Oxy 235
Oilseed rape standard 3	Topas 19/ 2
Oilseed rape standard RT 73	RT 73

added to determine the results, rather than the length of the nucleotide fragment.

The LAMP reaction system included 1× ThermoPol buffer, 0.2 µM outer primer F3 and B3, 1.6 µM inner primer FIP and BIP, 1.5 mM dNTPs, 5 mM MgCl₂, 0.16 U/µl Bst DNA polymerase, 8 ng/µl DNA templates, up to a total volume of 25 µl. The mixture was placed in the reaction hole of turbidimeter at constant temperature of 61°C for 60 min, and then inactivated at 80°C for 5 min to complete the reaction.

Rapeseed plants were sampled. In order to ensure the uniformity in the quality of nucleic acid extracted, the QIAGEN nucleic acid automatic extraction system was used for the extraction of nucleic acids. The LAMP reaction of the genomic DNA from the rapeseed of different plants and from different species plants was conducted, in which the LAMP reaction was carried out according to the optimized LAMP reaction conditions, in order to verify the specificity of the primers. The samples used in the specificity experiment are shown in Table 1.

Six copies of DNA solution of non-transgenic rapeseed samples (blank sample 0%) were taken, and the 6 copies of the blank sample DNA were mixed with 100% transgenic rapeseed standard DNA, respectively, to prepare RT 73 simulated samples DNA at 6 concentration levels, forming the transgenic proportion of 5%, 2%, 0.5%, 0.1%, 0.01%, and 0.001%, respectively. LAMP reactions were carried out in accordance with the optimized LAMP conditions, to detect the sensitivity of LAMP primers. Non-transgenic DNA was used as negative control. Deionised water was used for blank control.

RESULTS AND DISCUSSION

Specificity of LAMP assay

Four transgenic rapeseed samples and 12 other

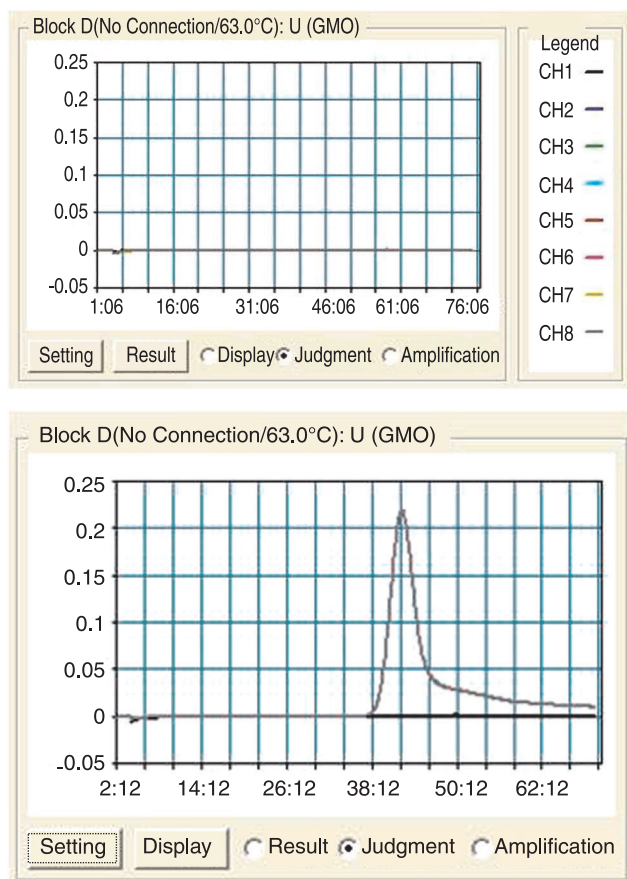


Fig 1 Specificity of the turbidity-based real-time LAMP assay. CH1-CH8: sample 1-8.

transgenic plant samples were selected for the LAMP detection according to the optimized conditions, and the results are shown in Table 2 and Fig 1. As shown in Fig 1, the primers could generate only the amplification of gene samples containing RT 73 rapeseed gene, but not that of other plant genes and those from other transgenic rapeseed, indicating that the LAMP primers designed in this experiment did not generate any nonspecific amplification products, and can be used as the specific primer for the detection of transgenic RT 73 rapeseed.

Sensitivity of LAMP assay

LAMP reactions of simulated samples DNA with a transgenic ratio of 5%, 2%, 0.5%, 0.1%, 0.01% and 0.001% were carried out in accordance with the optimized LAMP conditions. Fig 2 shows the real-time turbidity amplification curve for the sensitivity detection. As shown in the figure, with the decrease of transgenic content, the peak time was gradually extended, the transgenic content detected before 60 min was 0.1%, and the detection limit was 0.01%, equivalent to the amount of 0.01 ng transgenic DNA.

LAMP detection technology has been used for the detection of several kinds of transgenic crops, such as transgenic corn and soybean (Xu *et al.* 2013, Huang *et al.* 2014, Zahradnik *et al.* 2014). The first thing for LAMP amplification is to design 4 primers targeting the 6 regions,

Table 2 Results of specificity determination

Sample	Transgenic characteristics	Result
Rice Bt-63 standard (5%)	Bt-63	Negative
Rice KMD standard (100%)	KMD	Negative
Rice Bt-63 sample	Bt-63	Negative
Rice KF-8 sample	KF-8	Negative
Corn Bt-176 standard (5%)	Bt-176	Negative
Corn MON 810 standard (10%)	MON 810	Negative
Potato sample	EH 92-527-1	Negative
Soybean MON 89788 (100%)	MON 89788	Negative
Wheat sample	Transgene negative	Negative
Cotton sample	Rt-Cry	Negative
Corn sample 1	GA 21	Negative
Corn sample 2	NK 603	Negative
Oilseed rape sample 1	T 45	Negative
Oilseed rape sample 2	Oxy 235	Negative
Oilseed rape sample 3	Topas 19/ 2	Negative
Oilseed rape standard RT 73	RT 73	Positive

because inner primers can participate in the cycle reaction to generate a large number of target products only when they are successfully amplified to become the dumbbell-shaped structure. With the typical feature, Bst DNA polymerase used in LAMP can directly react with the double-stranded DNA, called chain replacement DNA polymerase (Notomi *et al.* 2000). Natural Bst DNA polymerase is derived from the high temperature resistant *Bacillus stearothermophilus* DNA polymerase, with the activity of a 5'→3' DNA polymerase, but not the activity of a 5'-3' exonuclease (Mori *et al.* 2001, Huang *et al.* 1999). The principle and process for LAMP is described in supporting information.

In the current study, the sensitivity of LAMP real-time turbidimetric method for the detection of transgenic crops was 0.01%, equivalent to about 0.01ng RT 73 rapeseed genomic DNA. This was consistent with other results to

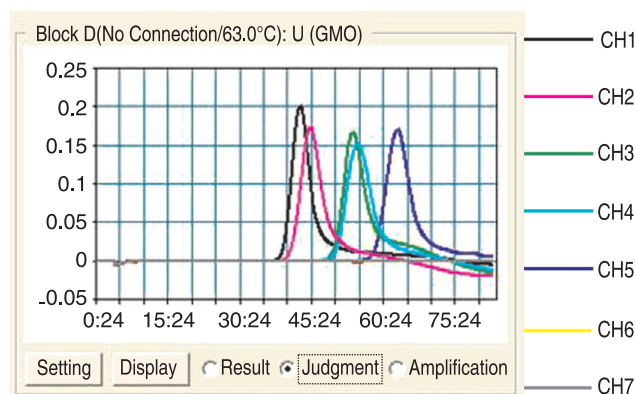


Fig 2 Sensitivity of the turbidity-based real-time LAMP assay. CH1: 5%, CH2: 2%, CH3: 0.5%, CH4: 0.1%, CH5: 0.01%, CH6: 0.001%, CH7: negative control.

detect RT 73 rapeseed using real-time fluorescent PCR and to detect transgenic soybean using LAMP (Zhang 2006, Guan 2010).

In conclusion, LAMP was specific to amplify exogenous genes of genetically modified RT 73 rapeseed, and the detection limit of 0.1 ng DNA was similar to that of real-time PCR. The LAMP operation was simple to greatly improve the efficiency of transgenic detection and have a good application prospect.

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