

Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of Rf1 Event in Transgenic Rapeseed (*Brassica napus* L.)

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Abstract: Rapeseed (*Brassica napus* L.) is one of the most studied crops for genetic engineering. Up to now, at least 11 GM rapeseed varieties have been approved for commercial production. During this study, a simple and rapid LAMP method was established for detection of Rf1 event in transgenic rapeseed. Position 143-353 in the right border junction sequence of *B. napus* transgenic line Rf1 (accession number: EU090199.1) was chosen as the target region for primer design and LAMP detection. Reaction mixture at the volume of 25 μ L contained 12.5 μ L 2 \times buffer mix, 1.28 μ M FIP, 1.28 μ M BIP, 0.16 μ M F3, 0.16 μ M B3, 0.64 μ M LF, 480 U/mL Bst DNA polymerase, 2.0 μ L DNA template and 4.6 μ L ddH₂O. SYBR Green I was added to the tube lid. After incubation at 60°C for 60 min, the reaction was terminated by heating at 80°C for 10 min. Then the tube was centrifuged for 60 s at 8,000 r/min to mix dye with reaction mixture. Results indicate that our LAMP assay is highly specific and sensitive (9.2×10^3 copies/ μ L, 0.001%). This LAMP assay for Rf1 event in rapeseed, which is simple, time effective, specific, sensitive and result visible without expensive instruments, is suitable for application in wide fields.

Keywords: Rf1 Event, Loop-Mediated Isothermal Amplification, Genetically Modified Organism, Sensitivity, Specificity

1. Introduction

Gene related modern technologies is potentially a shortcut towards improving domesticated plants, as they bypass biological barriers for recombination and achieve genetic exchange among unrelated species. The first genetically modified (GM) plant (the Flavr-Savr tomato) authorized on sale was in 1994 [1]. The number of genetically modified organisms (GMOs) planted for marketing has been increasing at the speed of an average of almost 10% per year among the last decade [2]. During 2007, the planting area for GMOs reached more than 143 million ha in 23 countries, with soybean, maize, cotton and rapeseed as the four dominant crops [3].

Rape (*Brassica napus* L.) is one of those crops that suffer severe damage from weeds. Weeds have become an important factor restricting the improvement of rapeseed production in China. To achieve the desired control effects,

rape varieties resistant to broad-spectrum non-selective herbicides have been bred. There are mainly two types of herbicide-resistant rape varieties cultivated. One is the mutant rape obtained via chemical or physical mutagenesis. The other are those rape varieties obtained via genetic engineering technology, among which the most widely used is glyphosate-resistant variety [4]. It is reported that GM soybeans and rapeseed crops have increased farmers' income by nearly 5% [5]. Until now, at least 11 GM rapeseed varieties have been approved for commercial production [6].

With regard to safety, many countries or regions have market-specific labeling laws stipulating that products containing approved GMO traits to a certain degree must be labeled as such [7, 8]. To enforce the labeling laws, sensitive, reliable, standardized, specific, and quantitative GMO detection methods are in strong needs. Qualitative testing methods can be used to discriminate between authorized and

unauthorized materials or ingredients, to identify safe or potentially unsafe materials. Quantitative testing approaches can be deployed to monitor compliance with legal or contractually agreed thresholds [2]. Loop-mediated isothermal amplification (LAMP) is simple, rapid, and specific. It has abilities of strand-displacement synthesis and auto cycling to amplify targeted sequence under isothermal conditions between 60 and 65°C [9]. So far, LAMP assay has been applied in many fields, such as detection of pathogenic microorganisms [10-12] and GM ingredients [13-14].

In this study, a rapid, sensitive and specific LAMP method was developed for screening of the Rfl event in transgenic

rapeseed.

2. Materials & Methods

2.1. Sample Collection

Transgenic rapeseed line Rfl was used along with other eighteen plant materials (listed in Table 1), including 1 conventional rapeseed variety (Huayou8) as negative control, 4 other transgenic rapeseed lines (Ms8, RF3, GT73, T45), 13 other transgenic plant materials (soybean, maize, cotton, alfalfa, pawpaw).

Table 1. Plant materials used in this study.

No.	Samples	Sources
1	Transgenic rapeseed line Rfl	Reserved sample
2	Transgenic rapeseed line Ms8	Reserved sample
3	Transgenic rapeseed line RF3	Reserved sample
4	Transgenic rapeseed line GT73	Putiantongchuang Biological Technology Co., LTD
5	Transgenic rapeseed line T45	Putiantongchuang Biological Technology Co., LTD
6	Rapeseed Huayou8 (conventional variety)	Reserved sample
7	Transgenic soybean line TS40-3-2	Reserved sample
8	Transgenic soybean line A2704-12	Reserved sample
9	Transgenic soybean line MON89788	Reserved sample
10	Transgenic soybean line A5547-127	Reserved sample
11	Transgenic soybean line MON87708	Reserved sample
12	Transgenic rice line Bt63	Donated by Chinese Academy of Inspection and Quarantine
13	Transgenic maize line BT11	Donated by Chinese Academy of Inspection and Quarantine
14	Transgenic maize line MON810	Donated by Chinese Academy of Inspection and Quarantine
15	Transgenic maize line TC1507	Reserved sample
16	Transgenic maize line NK603	Reserved sample
17	Transgenic alfalfa line J101	Reserved sample
18	Transgenic cotton line Zhongmiansuo38	Reserved sample
19	Transgenic pawpaw line GMYK	Reserved sample

Based on the right border junction sequence of *B. napus* transgenic line Rfl from GenBank (accession number: EU090199.1) [6], position 1-594 was selected, synthesized and inserted into plasmid pMV vector as the positive control (carried out by BGI Co., Ltd., Guangdong).

2.2. DNA Extraction

A total of 100 mg plant material from each sample was ground with the crushing apparatus (MM 400, Retsch, Germany). Corresponding genomic DNA was extracted using Dneasy Plant Mini Kit 50 (QIAGEN, Germany) according to the manufacturer's instruction. The purity

and concentration were measured by Nanodrop 2000 (Thermo, USA). The genomic DNA (in TE Buffer) was frozen at -20°C.

2.3. Primer Design

Position 143-353 from the right border junction sequence of *B. napus* transgenic line Rfl (accession number: EU090199.1) [6] was selected as target region for primer design and LAMP detection. Primer design was carried out via the online tool Primer Explorer v4.0 (<http://primerexplorer.jp/elamp4.0.0/index.html>). Primers were synthesized and purified by BGI (Guangdong) Co., Ltd.

Table 2. Primers designed in this study for LAMP.

Name	Sequence (from 5' to 3')
Rfl-F3	GAAACTGGTAGCTGTTGTG
Rfl-B3	CGCGACTCATCATCTCA
Rfl-FIP	ACAATATTACTCACCGGTGCGATTTTTGATGGATCATTAAATTTCCACCTTC
Rfl-BIP	TAAGAGCGAATTTGGCCTGTAGTTTATCATCACACCAAAAGTTAGG
Rfl-LF	ACCTCAATTGCGAGCTTCTAA

2.4. Establishment of LAMP Assay

LAMP reaction was performed using Loop-mediated isothermal DNA amplification Kit (Double Helix Biotechnology

Co., LTD, Guangzhou, China) according to the manufacturer's instruction. Reaction mixture at the volume of 25 µL contained 12.5 µL 2×buffer mix, 1.28 µM FIP, 1.28 µM BIP, 0.16 µM F3, 0.16 µM B3, 0.64 µM LF, 480 U/mL Bst DNA polymerase, 2.0 µL DNA template and 4.6 µL ddH₂O. Dye (0.1 µL

10000×SYBR Green I, INVITROGEN) was added to the tube lid separated from the former mixture. After incubation at 60 °C for 60 min in Gene Explorer (BIOER, China), the reaction was terminated by heating at 80°C for 10 min. Then the tube was centrifuged for 60 s at 8,000 r/min to mix SYBR Green I with reaction mixture. Results were judged positive in two ways: 1) the appearance of white precipitation; 2) the color change from light orange to green under 310 nm UV light.

2.5. Analytical Specificity and Sensitivity of LAMP Assay

To assess the sensitivity of LAMP method, the recombinant pMV plasmid DNA (9.2×10^8 copies/ μ L) was used as the original solution (10^0), which was ten fold serially diluted with TE buffer from 10^{-1} to 10^{-8} . Those 9 concentrations were used as templates in sensitivity test.

The specificity of LAMP was analyzed via specificity test based on plant materials in table 1.

3. Result and Analysis

3.1. Primer Design

LAMP primers designed in this study were listed in table 2, of which the amplicon was 211 bp long. Specificity test of primers was carried out via Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The result showed that selected primer pair (Rf1-F3 & Rf1-B3) is specific to input template (EU090199.1) as no other targets were found in selected database (Figure 1).

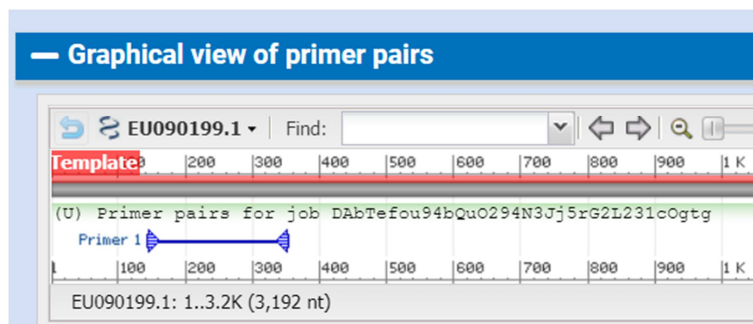


Figure 1. Primer-BLAST result of LAMP primer pair F3 and B3.

3.2. Specificity Tests

To confirm specificity of the LAMP assay, genomic DNA from transgenic rapeseed line Rf1 and 18 different transgenic and conventional plant varieties (rice, soybean, rapeseed, maize, cotton, alfalfa and pawpaw, listed in table 1) were

isolated and tested, making sure the purity ranging from 1.8 to 2.0. Recombinant plasmid pMV and TE buffer were used as the positive and negative control, respectively. Color change and white precipitation could only be monitored in tubes containing plasmid pMV and transgenic rapeseed line Rf1 (Figure 2), indicating the high specificity of designed LAMP.

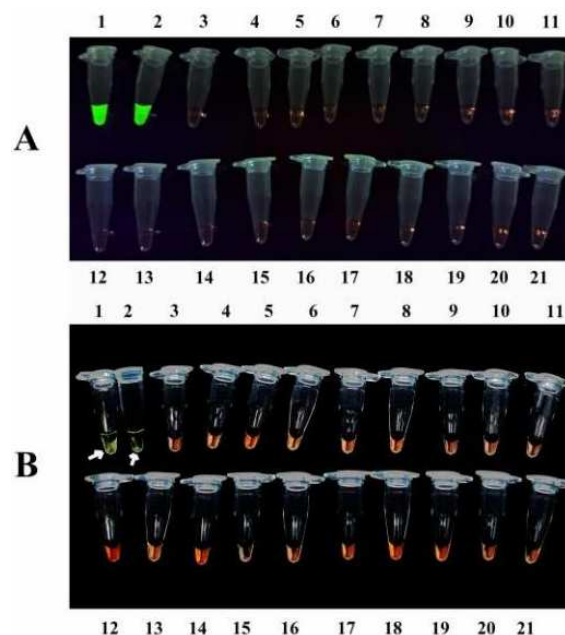


Figure 2. Results of specificity test for LAMP under UV light (A) and natural light (B). 1: Recombinant plasmid pMV; 2: transgenic rapeseed line Rf1; 3-20: other 18 plant materials listed as 2-19 in table 1; 21: TE buffer. White arrows: white precipitation.

3.3. Sensitivity Tests

To evaluate the sensitivity of LAMP, positive plasmid pMV solution (9.2×10^8 copies/ μL , as 10^0) was used and diluted in ten-fold series. Totally 9 solutions were used (from 10^0 to 10^{-7} , and TE buffer as negative control). According to the result, solution 10^{-5} (9.2×10^3 copies/ μL) was near the detection limit. Thus, sensitivity of this LAMP method was 9.2×10^3 copies/ μL (0.001%) (Figure 3).

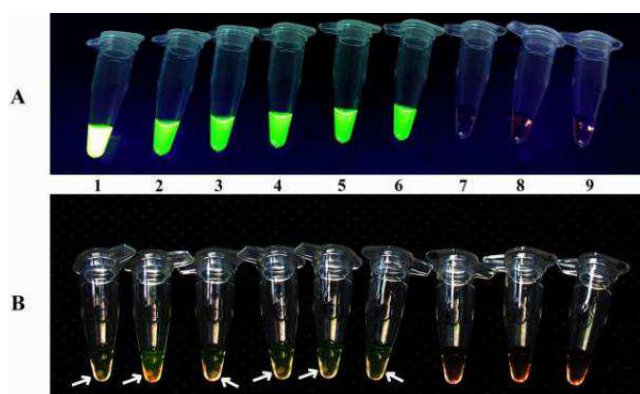


Figure 3. Results of sensitivity test for LAMP under UV light (A) and natural light (B). 1-8: Solutions 10^0 to 10^{-7} ; 9: TE buffer. White arrows: white precipitation.

4. Discussion

During this study, a simple and rapid LAMP method was established for detection of Rfl event in transgenic rapeseed. Results indicate that this LAMP assay is highly specific and sensitive (9.2×10^3 copies/ μL , 0.001%). Currently, real-time PCR has been widely applied in GMO trait identification and discriminating between authorized and unauthorized materials. For Rfl event detection in rapeseed, there is already the real-time PCR method with a sensitivity at 0.01% [15]. The LAMP assay designed in this study is ten times more sensitive than real-time PCR method. Real-time PCR assay has been famous for its out-standing performance in sensitivity, false positive rate and quantitative analysis compared to conventional PCR, however relying on expensive reagents and instruments limits its wide application, especially in fields. LAMP technology which is simple, time effective, specific, sensitive and result visible without expensive instruments has been rapidly applied in wild fields.

Choosing a suitable analytical method can sometimes be challenging, especially when multiple options are at hand. The most proper one for this situation may not necessarily fit for another. In many cases, cost and time is prioritized which often leads to the decline of reliability. Specificity and sensitivity can be the most remarkable advantages of nucleic acid based methods, while cost and competence requirements are main drawbacks. Generally speaking, real-time PCR is not cost efficient and unsuitable for wild applications. Yet conventional PCR is not specific enough and fails to quantify the GMOs. As for LAMP, it still has two aspects to be improved. Firstly, it could only achieve qualitative analysis,

not quantitative analysis. Secondly, it detects just one target in one reaction which is not efficient. Future methodology is expected to further boost test speed, cost and focus on multiplexing, quantitation, and independence on complex equipment.

5. Conclusion

During this study, a simple and rapid LAMP method was established for detection of Rfl event in transgenic rapeseed. Position 143-353 in the right border junction sequence of *B. napus* transgenic line Rfl (accession number: EU090199.1) was chosen as targeted region for primer design and LAMP detection. Reaction mixture at the volume of 25 μL contained 12.5 μL 2 \times buffer mix, 1.28 μM FIP, 1.28 μM BIP, 0.16 μM F3, 0.16 μM B3, 0.64 μM LF, 480 U/mL Bst DNA polymerase, 2.0 μL DNA template and 4.6 μL ddH₂O. SYBR Green I as the dye was added to the tube lid. After incubation at 60°C for 60 min, the reaction was terminated by heating at 80°C for 10 min. Then the tube was centrifuged for 60 s at 8,000 r/min to mix dye with reaction mixture. Results indicate that this LAMP assay is highly specific and sensitive (9.2×10^3 copies/ μL , 0.001%). This LAMP assay for monitoring Rfl event in rapeseed, which is simple, time effective, specific, sensitive and result visible without expensive instruments, is quite suitable for application in wide fields.

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