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Visual Detection of Rf2 Event in Transgenic Rapeseed (*Brassica napus* L.) Using Loop-Mediated Isothermal Amplification

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Abstract: Specific methods have been developed in order to efficiently detect GMOs (genetically modified organisms) in both feed and food. Current approaches often rely on thermal cycling devices such as PCR amplifier, which makes it difficult for applications in the wild. Therefore a visual LAMP (Loop-Mediated Isothermal Amplification) method for rapid screening Rf2 event in transgenic rapeseed (*Brassica napus* L.) is established in this paper. For primer design, position 167-366 in the right border junction sequence of *B. napus* trangenic line Rf2 (accession number: EU090197.1) was chosen as the amplicon. Reaction mixture (at the volume of 25 μL) consisted of 12.5 μL 2×buffer mix, 1.28 μM FIP, 1.28 μM BIP, 0.16 μM F3, 0.16 μM B3, 480 U/mL Bst DNA polymerase, 2.0 μL DNA template and 5.0 μL ddH_2O. Dye (SYBR Green I) was pre-added onto the tube lid. The reaction tube was incubated at 60°C for 60 min, followed by heating at 80°C for 10 min to end it. To mix dye with reaction mixture, the tube was centrifuged for 60 s at 8,000 r/min. Corresponding analysis results indicate that this LAMP assay is highly specific and sensitive (1.15×10^3 copies/μL). In one word, the visual LAMP method specific for Rf2 event in rapeseed, which turns out to be simple, time-effective, sensitive without relying on expensive instruments, is suitable for quick screening in ports.

Keywords: Brassica Napus, Rf2 Event, LAMP, Quick Screening, Ports

1. Introduction

Rape (*Brassica napus* L.) is an important oil crop in the world. In the early 21st century, both production and consumption of rapeseed increased significantly worldwide, with the planting area expanding by 17% from 23.31 million ha to 27.35 million ha. The total output increased from 36.03 million tons to 48.55 million tons, accounting for 34.7%. Rapeseed oil consumption rose 29 percent from 13.2 million tons to 17.05 million tons [1].

Genetic modification as a technology is widely regarded as a shortcut to efficiently improving plants and animals, because it is able to realize genetic exchange across unrelated species in spite of biological barriers [2]. Gene technology was firstly applied on rape plants with *Agrobacterium tumefaciens* in 1987 [3]. Since then, the speed of experimental and commercial release of transgenic rape has accelerated. In 2004, six GM (genetically modified) rapeseed varieties (MS1×RF1, MS1×RF2, MS8×RF3, OXY235, TOPAS19/2 and T45) developed by Bayer and one variety RT73 (GT73) by Monsanto were allowed for importation into China as raw materials [4]. It has been reported that 92.6% of total 27 lots of rapeseed samples from Canada and Ukraine were tested positive for genetic modification in Shanghai port. Among those events, hybrids MS1×RF1 and MS1×RF2 accounted for 40% [5].

Detection and timely monitoring of transgenic rapeseed and its products are vital to safe management measures.

Commonly used approaches to screen or identify GM plants are mostly based on thermal cycling devices, such as amplifiers for PCR. No detection method has been established specifically for event Rf2 in rapeseed so far. Therefore, development of a rapid visual detection assays for Rf2 event in rapeseed is in urgent need. Loop-mediated isothermal amplification (LAMP), a relatively new amplification method, integrates result visualization, rapidity, simplicity, and high specificity. It is capable of auto-cycling amplification at a fixed temperature. Thus, LAMP is supposed to have a wide range of applications, like point-of-care detecting, genetic screening in resource-poor areas, and rapid testing of food and feed samples [6].

In this research, a rapid, highly sensitive and specific LAMP approach was designed and characterized for Rf2 event in transgenic rapeseed.

2. Materials & Methods

2.1. Sample Preparation

Twenty plant samples (listed in Table 1) including one transgenic rapeseed line Rf2 were used in specificity test along with one positive and one negative controls. The other 19 samples consisted of 5 GM rapeseed lines (Rf1, Ms8, Rf3, GT73, T45) and 14 transgenic plant materials related to soybean, maize, cotton, alfalfa and pawpaw. Based on the right border junction sequence of *B. napus* trangenic line Rf2 from GenBank (accession number: EU090197.1) [7], position 1-688 was selected, synthesized and inserted into plasmid pMV vector as the positive control (carried out by BGI Co., Ltd., Guangdong). One conventional rapeseed variety (Huayou8) was used as negative control.

Table 1. *Plant samples used in specificity test.*

No.	Samples	Sources
1	Transgenic rapeseed line Rf2	Reserved sample
2	Transgenic rapeseed line Rf1	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	Transgenic rapeseed line Ms8	Reserved sample
7	Transgenic soybean line DAS-44406-6	Reserved sample
8	Transgenic soybean line TS40-3-2	Reserved sample
9	Transgenic soybean line A2704-12	Reserved sample
10	Transgenic soybean line MON89788	Reserved sample
11	Transgenic soybean line A5547-127	Reserved sample
12	Transgenic soybean line MON87708	Reserved sample
13	Transgenic rice line Bt63	Donated by Chinese Academy of Inspection and Quarantine
14	Transgenic maize line BT11	Donated by Chinese Academy of Inspection and Quarantine
15	Transgenic maize line MON810	Donated by Chinese Academy of Inspection and Quarantine
16	Transgenic maize line TC1507	Reserved sample
17	Transgenic maize line NK603	Reserved sample
18	Transgenic alfalfa line J101	Reserved sample
19	Transgenic cotton line Zhongmiansuo38	Reserved sample
20	Transgenic pawpaw line GMYK	Reserved sample
21	Rapeseed Huayou8 (conventional variety, as negative control)	Reserved sample
22	Positive clone (as positive control)	Reserved sample

2.2. DNA Extraction

Nearly 100 mg of plant tissue from each sample was prepared for grinding with crushing apparatus (MM 400, Retsch, Germany). Dneasy Plant Mini Kit 50 (QIAGEN, Germany) was then used for genomic DNA extraction according to the Kit User Manual. DNA purity and concentration were conformed by Nanodrop 2000 (Thermo, USA). The extracted DNA solutions (in TE Buffer) were stored at -20°C for further use.

2.3. Primer Design

Primer design and further LAMP assay development were based on the position 167-366 (200 bp in length) in the right border junction sequence of *B. napus* trangenic line Rf2 (accession number: EU090197.1) [7]. On-line tool Primer Explorer v4.0 (http://primerexplorer.jp/elamp4.0.0/index.html) was employed and the chosen primers were listed in Table 2. Primer synthesis and purification were performed by BGI (Guangdong) Co., Ltd.

Table 2. *Primers designed for event Rf2.*

Name	Sequence (from 5' to 3')
RF2-2-F3	GTGGTCTCAAGATGGATCA
RF2-2-B3	GCCCAGTTCAATATTATAGATCA
RF2-2-FIP	GCTCTTAGCCGTACAATATTACTCATTTTTTAATTTCCACCTTCACCTACG
RF2-2-BIP	ACTGGCACAGCTATATATACGTCGTTTTCTAGAGCCCAGTTCTTCC

2.4. Establishment of LAMP Method

Loop-mediated isothermal DNA amplification Kit (Double Helix Biotechnology Co., LTD, Guangzhou, China) was adopted for LAMP reaction according to the manufacturer's instruction. Reaction mixture (25 μL) consisted of 12.5 μL 2×buffer mix, 1.28 μM FIP, 1.28 μM BIP, 0.16 μM F3, 0.16 μM B3, 480 U/mL Bst DNA polymerase, 2.0 μL DNA template and 5.0 μL ddH_2O. SYBR Green I (0.1 μL 10000×, INVITROGEN) was pre-attached to the tube lid separated from the main reaction mixture. Reaction termination was performed via heating at 80°C for 10 min, after incubation for 60 min in Gene Explorer (BIOER, China). Then centrifugation at 8,000 r/min for 60 s was done to mix dye with main solution. Results were interpreted positive with naked eyes in either way: 1) appearance of white precipitation; 2) color change from orange to bright green under UV light at the wavelength of 310 nm.

2.5. Reaction Temperature, Specificity and Sensitivity of LAMP Detection

Six LAMP reactions were performed at temperatures from 60°C to 65°C respectively, to determine which temperature is the most practical and economical.

To analyze the sensitivity of LAMP assay, a recombinant pMV plasmid containing target Rf2 sequence was used as the parent solution (1.15×10^9 copies/μL, 10^0). It was then serially diluted at a 10-fold gradient with TE buffer, resulting in 10 concentrations from 10^0 to 10^{-9}. TE buffer served as the negative control.

The specificity of LAMP method was assessed via specificity test based on 22 plant samples listed in table 1.

3. Result and Analysis

3.1. Primer Design

LAMP primers were designed and chosen accordingly via Primer Explorer v4.0 (table 2), with an amplicon of 200 bp in length. Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) was used to do specificity test. The outcome showed that targeted primer pair (Rf2-2-F3 & Rf2-2-B3) is specific to template as no other matching was found in selected database: Refseq mRNA (Organism limited to *B. napus*) (Figure 1).

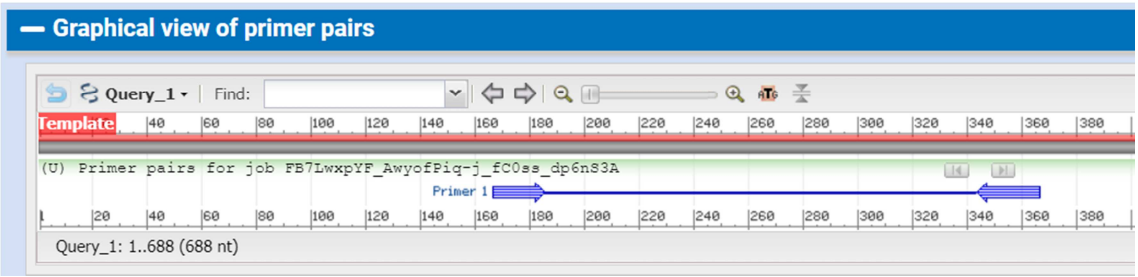

Figure 1. *Primer-BLAST outcome of designed primer pair F3 and B3.*

3.2. Amplification Temperature Optimization

Totally six amplification were performed at temperatures from 60°C to 65°C, respectively. The optimal setting was decided according to electrophoresis results of amplified products (Figure 2). Since all six settings showed no differences in outcome, 60°C was chosen on the purpose of energy saving.

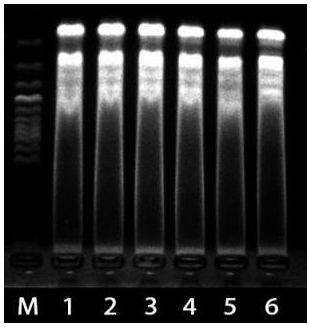

Figure 2. *Electrophoresis result of temperature optimization test. M: 100 bp Plus DNA Ladder (TransGen Biotech Co., Ltd.); 1-6: 60°C -65°C, respectively.*

3.3. Specificity Tests

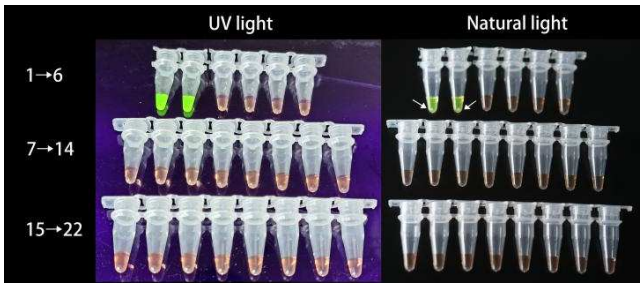

Figure 3. *Results of specificity test for designed assay. 1: Recombinant plasmid pMV (positive control); 2: transgenic rapeseed line Rf2; 3-22: other 20 plant materials listed as 2-21 in table 1 including the negative control; White arrows: white precipitation.*

To further analyze amplification specificity, 21 different transgenic and conventional plant samples (rice, soybean, rapeseed, maize, cotton, alfalfa and pawpaw, listed in table 1) were prepared. Genomic DNA was extracted and tested, to ensure the purity varying from 1.8 to 2.0. Recombinant plasmid pMV and rapeseed Huayou8 (conventional variety) were adopted as positive and negative controls, respectively.

White precipitation and color change were detected only in two reactions containing positive control and transgenic rapeseed line Rf2 (Figure 3), demonstrating enough specificity for this assay.

3.4. Sensitivity Tests

To assess the sensitivity of LAMP assay, positive plasmid pMV (1.15×10^9 copies/μL, as 10^0) was prepared as the parent solution and diluted in ten-fold series accordingly, resulting in 10 solutions (from 10^0 to 10^{-9}) in total. Solution 10^{-6} (1.15×10^3 copies/μL) was proved to approach the detection limit (Figure 4). Thus, sensitivity of LAMP assay designed in this study was 1.15×10^3 copies/μL.

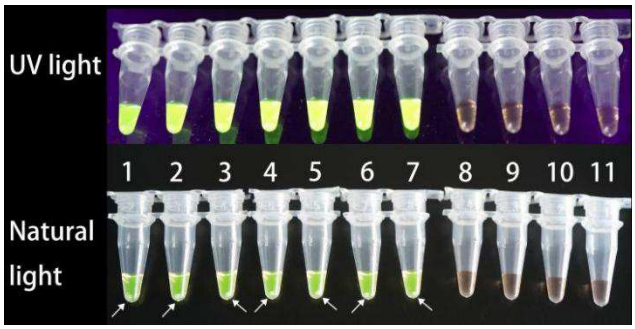

Figure 4. *Results of sensitivity analysis for this method. 1-10: Solutions 10^0 to 10^{-9}; 11: TE buffer. White arrows: white precipitation.*

4. Discussion

Genetically engineered crops have been cultivated commercially for over 20 years. They can express one or more beneficial traits like biotic or abiotic stress tolerance, and nutritional enhancement. Impact studies have showed that genetic engineering technologies have been so far beneficial to both farmers and consumers, with large aggregate welfare gains and positive influence on the ecosystem and human health [8-10]. Yet controversies never stop. Uncertainty about the impacts, including potential toxicity or allergenicity to human health, latent environmental risks, such as risks of random gene flow, adverse effects on non-targets, resistance in weeds and insects etc, is regarded as one main reason for widespread public reservation. It has been proved that transgenic technology adoption has reduced pesticide use by 37%, increased yields by 22%, and improved farmer income by 68% on average [11]. On the other hand, alternative tools, such as cisgenesis and genome-editing free from introducing any foreign gene into crops, may address this issue and diversify the toolbox to develop GM crop varieties [12].

Up to now, almost 525 transgenic events have been approved for commercial use in 32 crops all over the world. Given the huge planting scale, there is no query of acceptance or outright need for GM crop varieties. With precise, efficient, yet affordable genetic engineering tools, new GM crop varieties are entering country regulatory schemes for commercialization [13]. The question is how we regulate them properly. Detection methods are technical supports of GMO safety management. Nowadays, new methods, such as portable electrochemical immunosensor [14] and digital PCR [15] keep promoting the reform and innovation of qualitation and quantitation. With the revolution trend to be simple, rapid and instrument-free, it is essential to develop visual detection, which means the outcome is visible and can be distinguished by naked eyes [16]. Nevertheless, organisms generated via new technologies like genome editing can be indistinguishable from naturally occurring or conventionally bred counterparts with current analytical methods [17].

Transgenic rapeseed, one of the four major transgenic crops, is an important target of GMO safety supervision in China. Considering several characteristics of GM rapeseed, including the ability to achieve feral populations and perform as small seeded weeds, and high potential of hybridization with relatives, effective management methods are needed [18]. So far, there has been no report on the detection method of transgenic rape event Rf2, thus it is necessary to establish corresponding qualitative and quantitative assays. In the past two decades, nucleic acid based isothermal amplification has emerged as an essential diagnostic technology in clinical applications, food quality control and environmental monitoring [19]. LAMP, famous for its robust sensitivity and specificity, excels through energy efficient amplification and independence of expensive instruments, rendering it a prime candidate for on-site diagnostics [20].

5. Conclusion

In this paper, a visual LAMP method for quick screening of Rf2 event in transgenic rapeseed was established and characterized. Position 167-366 in the right border junction sequence of *B. napus* trangenic line Rf2 (accession number: EU090197.1) was chosen as the amplicon for LAMP primer design. Reaction mixture (at the volume of 25 μL) consisted of 12.5 μL 2×buffer mix, 1.28 μM FIP, 1.28 μM BIP, 0.16 μM F3, 0.16 μM B3, 480 U/mL Bst DNA polymerase, 2.0 μL DNA template and 5.0 μL ddH_2O. Dye (SYBR Green Ⅰ) was added to the tube lid. Reaction tube was incubated at 60°C for 60 min, followed by heating at 80°C for 10 min to end it. To mix dye with reaction mixture, the tube was centrifuged for 60 s at 8,000 r/min. Corresponding analysis results indicate that this LAMP assay is highly specific and sensitive (1.15×10^3 copies/μL, 0.001%). In one word, the visual LAMP method specific for Rf2 event in rapeseed, which turns out to be simple, time-effective and sensitive without relying on expensive instruments, is suitable for on-site screening in ports.

6. Recommendations

This study may pave way for quick screening of Rf2 event in transgenic rapeseed in ports, or even in the field. On the other hand, this LAMP method can be further developed onto a chip and carried out with other assays (for analysing other events, for example) at the same time.

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