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Optimization of ISSR-PCR Reaction System in *Pinellia ternata*

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Abstract In order to establish and perfect ISSR-PCR reaction system of *Pinellia ternata*, we used *P. ternata* from Sichuan region as the test material in this research. Genomic DNA of whole plant of *P. ternata* was used as template, UBC818 primer was used in ISSR-PCR system, $L_{16}(4^5)$ orthogonal test and single factor experiment were used to optimize the system. Based on the visual analysis of orthogonal experiment, we found the following are the influences of various factors on the experimental results in turn: dNTPs, primer, Mg^{2+}, *Taq* DNA polymerase, DNA template. Based on single factor screening test, we found the best ISSR-PCR reaction system. The total reaction volume is 20.0 μL, containing dNTPs 0.2 mmol/L, primer 0.8 μmol/L, Mg^{2+} 2.0 mmol/L, *Taq* DNA polymerase 0.075 U/μL, DNA 15 ng, annealing temperature 56℃. In this study, ISSR-PCR reaction system was optimized which will be applied to detect the genetic stability of *P. ternata* tissue culture seedlings.
Keywords *Pinellia ternata*; ISSR-PCR; Optimization

Pinellia ternata (Thunb.) Breit. (Also known as *Pinellia ternata*) is perennial herb of *Pinellia* Ten. genus in the family of Araceae, which is well known as a medicinal material with high medicinal value. Its tubers can be used as medicine and has significant effects on the treatment of lung diseases, lipid-lowering, blood pressure lowering and anti-tumor. It is widely used in China's long history of traditional Chinese medicine and has broad prospects (Li, 2017, Zhejiang Sci-Tech University, pp.1). In recent years, due to excessive mining and abuse of pesticides and fertilizers, wild resources are increasingly exhausted. More and more *Pinellia ternata* are bred in a large area through artificial cultivation and plant tissue culture technology, but artificial breeding often leads to intraspecific differentiation. In order to ensure the medicinal value and people's needs, it is urgent to explore and evaluate the germplasm resources of *Pinellia ternata* at the molecular level. At present, there are a lot of reports on the physiological research of *Pinellia ternata*, including the medicinal components and pharmacological effects of *Pinellia ternata* (Huang et al., 2011; Liu et al., 2015), processing (Liu et al., 2018), artificial cultivation technology (Ma et al., 2019) and so on; Studies at the molecular level, including studies on polyploidy of *Pinellia ternata* (Jia et al., 2013) and pedigree analysis (Du et al., 2006), have been reported. The ISSR (Inter-simple sequence repeat) technology of *Pinellia ternata* has been used for genetic diversity analysis (Yang and Chen, 2010) and the construction of DNA fingerprints (Li et al., 2008), but the application of genetic stability of tissue culture seedlings has not been reported.

ISSR technology, that is, simple repeat sequence marker technology, is a marker technology developed in recent years, which combines the advantages of RAPD (Random amplified polymorphic DNA) and SSR (Simple sequence repeat) technology. It has the advantages of rich polymorphism, strong repeatability, simple operation (Gupta et al., 1994; Zhan et al., 2010), good test stability (Azevedo et al., 2011), low amount of DNA template and low experimental cost (Qian et al., 2000). It is often used to analyze the genetic relationship and genetic diversity of animals and plants, as well as the relationship between the population genetic variation and human activities and environmental changes, and plays an important role in genetic research.

As an important molecular biotechnology, ISSR technology can reveal the variation of variety genetic information at the molecular level. Because it is sensitive to reaction conditions, when ISSR-PCR is carried out, it is necessary

to explore its best reaction conditions in *Pinellia ternata* and establish an excellent reaction system with high repeatability (Zhou et al., 2019). The optimum ISSR-PCR reaction system of different species is different. By optimizing the ISSR-PCR reaction system, this experiment provides a certain reference basis for the accurate ISSR analysis of the genetic stability of tissue cultured seedlings of *Pinellia ternata*.

1 Results and Analysis

1.1 ISSR-PCR orthogonal test analysis of *Pinellia ternata*

The orthogonal test $L_{16}(4^5)$ was used for pre-experiment, each group was repeated three times, the results of each time were scored, the three repeated values were averaged, and input into the orthogonal test software assistant for analysis. The test results showed that groups 7 and 12 amplified a large number of bands, and the backgrounds were clear and bright (Figure 1), so the scores were the highest (Table 1); groups 8, 10 and 14 amplified a small number of bands, or even none, so the scores were the lowest. Group 12 scored the highest in the visual analysis results of 16 groups.

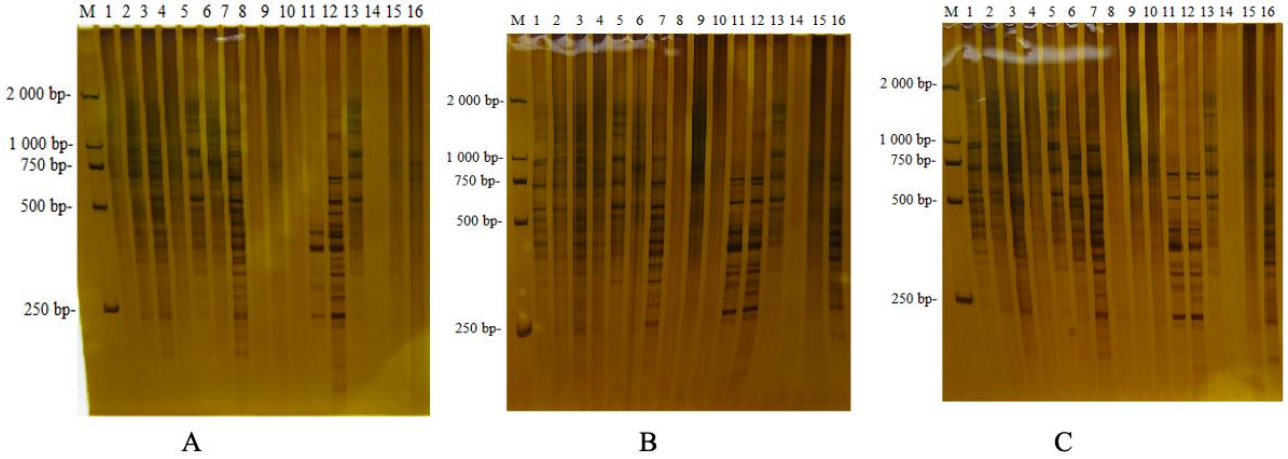

Figure 1 PAGE electrophoresis of ISSR-PCR orthogonal test reaction system of *P. ternata*

Note: A: Orthogonal test repeat 1; B: Orthogonal test repeat 2; C: Orthogonal test repeat 3; M: DL2000 DNA Marker; 1~16: 16 treatments of $L_{16}(4^5)$ orthogonal design; Primer: UBC818

Table 1 ISSR-PCR orthogonal test scores of *P. ternata*

Group	Repeat 1	Repeat 2	Repeat 3
1	3	9	9
2	10	8	7
3	14	9	11
4	4	6	5
5	11	9	10
6	6	5	10
7	16	15	15
8	1	1	1
9	2	3	3
10	1	1	2
11	8	15	15
12	16	16	16
13	10	10	11
14	1	1	1
15	2	2	2
16	4	7	8

Through the visual analysis of the orthogonal test results, the influence of each factor was ranked (Table 2). It can be seen that dNTPs has the greatest impact on the system and DNA template has the least impact. The impact of various factors on the experimental results from strong to weak was as follows: dNTPs, primers, Mg^{2+}, *Taq* DNA

polymerase and DNA template. The optimum 20 μL reaction system obtained by orthogonal test was as follows: 0.3 mmol/L dNTPs, 0.6 μmol/L primer, 1.0 mmol/L Mg^{2+}, 0.075 U/μL *Taq* DNA polymerase, 10 ng DNA template, and annealing temperature 53.3℃. Based on the optimum system obtained by orthogonal pre-experiment, the single factor test method was used to further optimize each factor.

Table 2 Visual analysis of orthogonal experiment of *P. ternata*

No.	DNA template (ng)	Primer (μmol/L)	*Taq* DNA polymerase concentration (U/μL)	Concentration of $MgCl_2$ (mmol/L)	Concentration of dNTPs (mmol/L)
T1	31.67	30.00	33.00	39.33	11.33
T2	33.33	17.67	36.33	32.33	32.67
T3	32.67	41.33	16.00	29.00	44.67
T4	19.67	28.33	32.00	16.67	28.67
X1	7.92	7.50	8.25	9.83	2.84
X2	8.33	4.42	9.08	8.08	8.17
X3	8.17	10.33	4.00	7.25	11.17
X4	4.92	7.08	8.00	4.17	7.17
R	3.42	5.92	5.08	5.67	8.33
Ranking	5	2	4	3	1

1.2 ISSR-PCR single factor test analysis of *Pinellia ternata*

1.2.1 Impact of dNTPs on ISSR-PCR amplification

As the substrate in the PCR reaction system, the concentration of dNTPs has a great impact on the amplification results. If the concentration is too low, the amplification effect will not be achieved, while if the concentration is too high, it will combine with Mg^{2+}, so as to inhibit the activity of *Taq* DNA polymerase (Luo et al., 2010). In this experiment, the concentration of dNTPs was 0.1 mmol/L, 0.2 mmol/L, 0.3 mmol/L and 0.4 mmol/L. From the reaction results, the amplification effect of low concentration dNTPs was better, the polymorphism was better and the band was clear. However, with the increase of concentration, high concentration dNTPs did not amplify the band (Figure 2A). The No. 2 amplified the best bands, so 0.2 mmol/L was selected as the optimum concentration of dNTPs.

1.2.2 Impact of primer concentration on ISSR-PCR amplification

Primer concentration is one of the key factors for PCR specific reaction of *Pinellia ternata*. If the primer concentration is too low, there will be few amplification bands. If the primer concentration is too high, it is easy to form dimers between primers, resulting in nonspecific amplification bands. In this experiment, when the primer concentration was 0.2 μmol/L~0.8 μmol/L, the bands can be amplified, and when the primer concentration was 0.2 μmol/L~0.4 μmol/L, the number of bands was small and the bands were light, and when primer concentration was added to 0.6 μmol/L, the amount of amplified products increased significantly, and the bands were clearer, and when the primer concentration was 0.8 μmol/L, the bands were the clearest and the polymorphism was the best, so the optimum concentration of primer was 0.8 μmol/L (Figure 2B).

1.2.3 Impact of Mg^{2+} on ISSR-PCR amplification

Taq DNA polymerase is an Mg^{2+} dependent enzyme in PCR system. Mg^{2+} has a great impact on the activity of *Taq* DNA polymerase. Appropriate concentration of Mg^{2+} can improve the activity of *Taq* DNA polymerase and obtain clear bands. Too high concentration will competitively combine with dNTPs, and affect the reaction efficiency of PCR. The results of this study showed that when other conditions remain unchanged, the effect was the best when the dosage of Mg^{2+} was 2.0 2.0 mmol/L (Figure 2C).

1.2.4 Impact of *Taq* DNA polymerase on ISSR-PCR amplification

Taq DNA polymerase activity is the key factor affecting PCR amplification. Too low concentration can easily lead to insufficient enzyme activity, incomplete extension, and low amplification efficiency, while too high concentration is prone to nonspecific fragments. Under the condition that other conditions remain unchanged, 0.05

U/μL, 0.075 U/μL, 0.1 U/μL and 0.125 U/μL were studied in this experiment (Figure 2D), and it was found that when the dosage of *Taq* polymerase was 0.125 U/μL, the amplification was not obvious, and when the dosage of *Taq* polymerase was at 0.05 U/μL and 0.1 U/μL, the bands were not abundant, so the optimum dosage of *Taq* polymerase was 0.075 U/μL.

1.2.5 Impact of template on ISSR-PCR amplification

The results showed that ISSR polymorphism amplification is sensitive to template DNA, and its purity and concentration can directly affect the polymorphism of PCR product bands. Therefore, high-quality genomic DNA is the key to the success of ISSR-PCR. In this study, the whole genomic DNA of wild *Pinellia ternata* was extracted by kit, and its quality met the requirements of ISSR-PCR after preliminary inspection. In 20 μL system, the impact of four concentration gradients of 5 ng, 10 ng, 15 ng and 20 ng on PCR amplification were studied under the same other conditions (Figure 2E). The results showed that the bands were relatively clear, the polymorphic effects of groups 1, 3 and 4 were relatively good, and there were no bands in group 2. There were great differences among groups, indicating that the template had an obvious impact on the ISSR-PCR reaction system. On the whole, when the template DNA was 15 ng, the bands amplified by ISSR-PCR were clearer and the polymorphism was relatively better. Therefore, the optimum dosage of template was 15 ng.

1.2.6 Optimization of annealing temperature

According to the Tm value of the synthesized primer UBC818, the temperature gradient (50.0 ℃~56 ℃) was set to amplify. The results of gradient PCR showed that the annealing temperature had little effect on the PCR of *Pinellia ternata*, and each temperature could amplify clear bands with good polymorphism. Relatively speaking, the annealing temperature of primer UBC818 set at 56.0 ℃ had better results (Figure 2F).

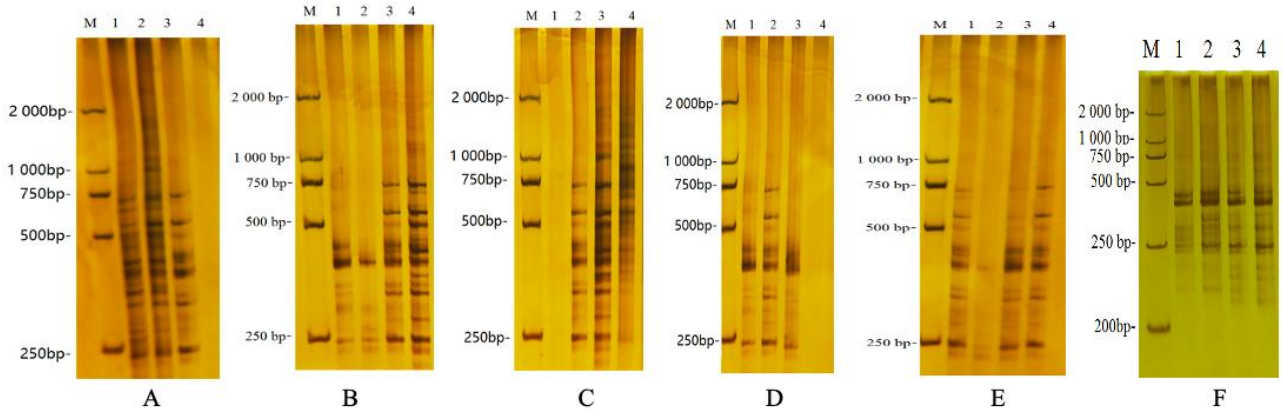

Figure 2 ISSR-PCR single factor experiment of *P. ternata*

Note: M: DL2000 DNA Marker; A: ISSR-PCR amplification results at different concentrations of dNTPs; B: ISSR-PCR amplification results of different primer concentrations; C: ISSR-PCR amplification results of different Mg^{2+} concentrations; D: ISSR-PCR amplification results of different *Taq* enzyme concentrations; E: ISSR-PCR amplification results of different DNA concentrations; F: ISSR-PCR amplification results of different annealing temperatures

1.3 Stability detection of the optimum system in tissue culture seedlings

The primer UBC818 and the optimized reaction system were selected for ISSR-PCR amplification of 8 randomly selected tissue culture seedlings (Figure 3). The results showed that the bands were clear and the polymorphism was good, indicating that the optimized system was stable in tissue culture seedlings and could be used for the correlation analysis of ISSR-PCR amplification reaction and genetic stability of tissue culture seedlings of *Pinellia ternata*.

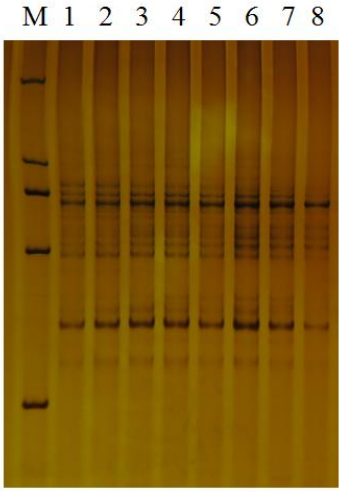

Figure 3 Detection of tissue culture seedlings by optimal system
Note: M: DL2000 DNA Marker; 1~8: Tissue culture seedlings of *P. ternata* randomly selected

2 Discussion

Because ISSR molecular marker technology has the advantages of rapidity, simplicity, stability and good polymorphism, it is widely used in various research fields, such as ISSR fingerprint of species, genetic diversity and genetic evolution. Because the PCR reaction system varies greatly due to different biological varieties, only when the conditions of various parameters in the reaction system are optimum, ISSR-PCR can maintain high stability (Li et al., 2008). The factors affecting the PCR reaction system mainly include primers, DNA templates, dNTPs, Mg^{2+}, *Taq* DNA polymerase, annealing temperature and so on. This study also confirmed that these factors had an impact on the effect of PCR. Therefore, optimizing the above influencing factors is the key to ensure the reliability of ISSR-PCR conclusion. Through $L_{16}(4^5)$ orthogonal test, the impact of various factors on the experimental results from strong to weak was as follows: dNTPs, primers, Mg^{2+}, *Taq* DNA polymerase, DNA template, and the optimum 20 μL reaction system was as follows: 0.3 mmol/L dNTPs, 0.6 μmol/L primer, 1.0 mmol/L Mg^{2+}, 0.075 U/μL *Taq* DNA polymerase, 10 ng DNA template, and annealing temperature 53.3℃. The results of orthogonal test provided a reference range for single factor test.

At present, there are few reports on ISSR analysis of *Pinellia ternata* at home and abroad. Only in the research of Zhang et al. (2007, Jiangsu Agricultural Sciences, (2): 154-157), the establishment and condition optimization of ISSR-PCR reaction system of *Pinellia ternata* using primer ISSR-76 were reported. The ISSR-PCR system of *Pinellia ternata* was preliminarily established through $L_9(3^4)$ orthogonal test and single factor test, However, no reference parameters of PCR reaction system were provided in the conclusion. In this experiment, the primer UBC818 was used for amplification test. Based on $L_{16}(4^5)$ orthogonal test, the single factor test was carried out to further optimize the PCR system. The optimum reaction system of ISSR-PCR of *Pinellia ternata* was as follows: the total system was 20 μL, 0.2 mmol/L DNTPs, 0.8 μmol/L primers, 2.0 mmol/L Mg^{2+}, 0.075 U/μL *Taq* DNA polymerase, 15 ng DNA template and 56℃ annealing temperature. The system was used to detect 8 randomly selected tissue culture seedlings. The results showed that the bands were clear and the polymorphism was good. The system was stable in tissue culture seedlings. Therefore, the optimized system was suitable for ISSR related studies such as genetic stability detection and genetic diversity of tissue cultured seedlings of *Pinellia ternata*.

3 Materials and Methods

3.1 Plant materials

On May 19, 2019, the young wild *Pinellia ternata* plants with petiole length of 5~8 cm and white bead buds just sprouted at the base were selected from the Sericulture Base in Wanmin Village, Mengshan Township, Wusheng County, Guang'an City, Sichuan Province (30°20′57.66″N, 106°17′45.49″E), and directly transplanted to the flower pot and placed in the laboratory for later use.

3.2 Extraction and detection of genomic DNA

Hangzhou BIOER (BioFastSpin plant genomic DNA extraction kit) was used to extract the genomic DNA of the whole wild *Pinellia ternata*. The analysis of electrophoresis results showed that the extracted genomic DNA of *Pinellia ternata* was of good quality, and the bands after electrophoresis results were neat, bright and clear, without tailing and protein pollution. This sample was suitable for ISSR-PCR experimental template (Figure 4).

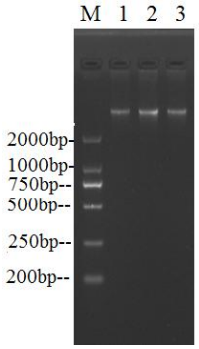

Figure 4 Electrophoresis detection of total DNA extracted from *P. ternata*
Note: M: DL2000 DNA Marker; 1,2,3: Total DNA of wild *P. ternata*

3.3 Amplification system and amplification procedure of PCR

Biospin plant genomic DNA extraction kit was purchased from Hangzhou BIOER and other common chemical reagents were purchased from Nanning GoldTech, with DL2000 Marker as the molecular weight standard. The primers were screened from 100 UBC-ISSR primers published by Columbia University in Canada. The screened primers were synthesized by Shanghai Sangon Biotech. The PCR reaction was carried out on the (Easy Cycler) gradient PCR instrument (AnalytikJena, Germany) (the annealing temperature was 54℃, and the amplification procedure was referred to relevant literature) (Zhou et al., 2019). By PAGE electrophoresis (the specific methods were referred to relevant literature) (Gao et al., 2019), the amplification results were recorded in 5200Multi (Shanghai Tanon) automatic chemiluminescence/fluorescence image analysis system.

3.4 Orthogonal test of ISSR-PCR reaction system

Firstly, the pre-experiment was carried out by orthogonal test. According to the instructions of TaKaRa *Taq* enzyme, the primary screening primer UBC818 (5′-CACACACACACACACAG-3′) was used for amplification. The total system of ISSR-PCR reaction was 20.0 μL. The orthogonal test $L_{16}(4^5)$ with five factors and four levels of genomic DNA template, primers, *Taq* DNA polymerase, Mg_2Cl and dNTPs was designed (Table 3). Each group was repeated three times, and the scores were scored according to the number and the quality of bands amplified by each reaction system. The average value of three scores was taken for each group, and the optimum reaction system of orthogonal test was analyzed by orthogonal teat assistant software (IatinII v3.1). Based on this system, a single factor test was designed to optimize the ISSR-PCR reaction system of *Pinellia ternata*.

3.5 Optimization of ISSR-PCR single factor test system

Using the primary screening primer UBC818 (5′-CACACACACAC ACACAG-3′) and single factor test method, four gradients (Table 4) were set for six factors, DNA template, primers, *Tap* enzyme concentration, concentration of Mg^{2+}, concentration of dNTPs and annealing temperature for ISSR-PCR amplification. The effect of amplified bands was observed in 5200Multi automatic chemiluminescence/fluorescence image analysis system by PAGE electrophoresis, and the diversity and quality of bands were analyzed to find out the optimum system.

3.6 Verification of the optimum system

The UBC818 was selected as the reaction primer, the reaction system optimized by single factor teat was selected to perform ISSR-PCR amplification of 8 randomly selected tissue culture seedlings. Through PAGE electrophoresis, the effect of amplification bands was observed in 5200Multi automatic chemiluminescence/ fluorescence image analysis system, and the diversity and the quality of bands were analyzed to verify the application of the optimized system in tissue culture seedlings.

Table 3 The orthogonal experimental design of ISSR-PCR for *P. ternata*

Level	Factors				
	DNA template (ng)	Primer (μmol/L)	*Taq* DNA polymerase concentration (U/μL)	Concentration of $MgCl_2$ (mmol/L)	Concentration of dNTPs (mmol/L)
1	5	0.2	0.050	1.0	0.1
2	5	0.4	0.075	1.5	0.2
3	5	0.6	0.100	2.0	0.3
4	5	0.8	0.125	2.5	0.4
5	10	0.2	0.075	2.0	0.4
6	10	0.4	0.050	2.5	0.3
7	10	0.6	0.125	1.0	0.2
8	10	0.8	0.100	1.5	0.1
9	15	0.2	0.100	2.5	0.2
10	15	0.4	0.125	2.0	0.1
11	15	0.6	0.050	1.5	0.4
12	15	0.8	0.075	1.0	0.3
13	20	0.2	0.125	1.5	0.3
14	20	0.4	0.100	1.0	0.4
15	20	0.6	0.075	2.5	0.1
16	20	0.8	0.050	2.0	0.2

Table 4 Different concentration of each factor in ISSR-PCR system of *P. ternata*

Level	Factors					
	DNA template ng)	Primer(μmol/L)	*Taq* DNA polymerase concentration (U/μL)	Concentration of $MgCl_2$ (mmol/L)	Concentration of dNTPs (mmol/L)	Annealing temperature (℃)
1	5	0.2	0.050	1.0	0.1	50
2	10	0.4	0.075	1.5	0.2	52
3	15	0.6	0.100	2.0	0.3	54
4	20	0.8	0.125	2.5	0.4	56

3.7 Data analysis

DPS 7.0 was used for orthogonal experimental design, and the data were analyzed by orthogonal test assistant software (IatinII v3.1) and Excel 2007.

Authors' contributions

YN and TYC were the experimental designers and executors of this study; LY completed data analysis and wrote the first draft of the manuscript; DW participated in the experimental design and analysis; YN was the conceiver and person in charge of the project, guiding experimental design, data analysis, manuscript writing and revision. All authors read and approved the final manuscript.

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