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Extraction of total DNA and optimization of the RAPD reaction system in *Dioscorea opposita* Thunb.

G.Q. Wen[1], J. Li[1], X.H. Liu[1], Y.S. Zhang[1] and S.S. Wen[2]

[1]College of Life Science, China West Normal University, Nanchong, China
[2]Rice and Sorghum Research Institute, Sichuan Academy of Agricultural Sciences, Deyang, China

Corresponding author: X.H. Liu
E-mail: liuxiaohong0817@aliyun.com

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ABSTRACT. *Dioscorea opposita* Thunb. has been used as health food and herbal medicinal ingredients in traditional Chinese medicine. In this study, the total DNA of *D. opposita* Thunb. was extracted using an improved cetyltrimethylammonium bromide (CTAB) method, and the extracted DNA was further used for random amplified polymorphic DNA (RAPD) reaction system by design of the L_{16} (4^4) orthogonal diagram. The results showed that the improved CTAB method can be used to isolate high-quality and high-concentration DNA, and the optimized protocol can overcome the instability of RAPD reaction system. The knowledge stated here can be used to study the genetic diversity of *D. opposita* Thunb.

Key words: Improved cetyltrimethylammonium bromide method; *Dioscorea opposita* Thunb.; Random amplified polymorphic DNA; Orthogonal design

INTRODUCTION

Dioscorea opposita Thunb. (Chinese name shanyao or yam), included in the family Dioscoreaceae, has been an important invigorant in traditional Chinese medicine for many years. It has been applied as an important ingredient for invigorating spleen and stomach, promoting the production of body fluids, benefiting the lung, and invigorating the kidney (Yuan, 2008; Wang et al., 2010; Kim et al., 2011). It is widely distributed in the world and includes at least 600 species. There are many *D. opposita* Thunb. cultivars that are grown in China (Wei et al., 2008; Hua et al., 2009; Li et al., 2009). Lichuan *D. opposita* Thunb. is a particular local variety that is mainly distributed in Lichuan City, Hubei Province, China. In 2007, it had been officially listed as a national product of geographical indication and was classified as a key protection species in China (Sun et al., 2010).

According to the literature, the species classification of *Dioscoreae* mainly relied on their tuber shapes, which had great limitations and resulted in many different species being regarded as the same species, and Lichuan *D. opposita* Thunb. is no exception (Li et al., 2009). Molecular markers of DNA can be used to reveal the differences, correlation, and genetic relationships among different varieties of species (Hou et al., 2006). For example, Terauchi and Konuma (1994) analyzed a wild yam species with microsatellite polymorphisms, Mignouna et al. (2002) constructed a genetic linkage map of guinea yam and water yam with amplified fragment length polymorphism markers, and Zhou et al. (2005) analyzed the genetic diversity of 28 cultivar varieties of *D. opposita* Thunb. using inter-simple sequence repeat markers. Bousalem et al. (2006) analyzed chromosomal segregation patterns of the American *D. opposita* Thunb. using 8 simple-sequence repeat markers. Lichuan *D. opposita* Thunb. acts as a featured product in Lichuan City, Hubei Province. However, to date, very little knowledge is known about the variety's origin, phylogeny, diversity, and genetics.

Random amplified polymorphic DNA (RAPD) is an important molecular tool, which was widely applied to study the origin, evolution, relationship analysis, and genetic diversity of species, as well as the construction of genetic linkage maps and gene localization (Gichuki et al., 2003; Liu, 2010; Xing, 2010). Although many reports noted the deficiency known as instability, it can be overcome by strict experimental conditions such as the optimization of the RAPD reaction system. Some experiments demonstrated that the RAPD reaction system is mainly influenced by the concentration of Mg^{2+}, dNTP, *Taq* DNA polymerase, and primer (Yao et al., 2009; Wang et al., 2011; Yang et al., 2011). Orthogonal design is a commendable and effective tool to analyze multifactor and multilevel experiments; it can be used to explore the interactions between different factors (Ming, 2005). Therefore, a standardized orthogonal table was used to arrange the experiment to optimize the RAPD reaction system in this study. The knowledge stated here is beneficial for studying the genetic diversity of *D. opposita* Thunb.

MATERIAL AND METHODS

Solutions and reagents

Taq DNA polymerase was obtained from Takara Biotech, Dalian, Co. Ltd., China. RAPD primers were synthesized by SBS Genetech, Co. Ltd., China. Extraction buffer I consisted of 100 mM Tris-Cl, pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA), and 500 mM NaCl, pH 8.0. Just prior to use, the solution was adjusted by the addition of 2%

(v/v) β-mercaptoethanol (β-ME). Extraction buffer II contained 3% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-Cl, pH 8.0, and 0.5 M EDTA, pH 8.0. Similarly, 100 µL 2% (v/v) β-ME was added before use. Additional solutions included 2.5 M potassium acetate (KAc), isopropanol, 70% ethanol, chloroform:isoamyl alcohol (24:1), and sterile water.

Plant materials

The seeds of Lichuan *D. opposita* Thunb. were collected in Lichuan City, Hubei Province. Afterward, the seeds were sowed in our experimental field (China West Normal University, Nanchong City, China). Before the experiment, the young leaves of *D. opposita* Thunb. were collected for DNA extraction.

Modified protocol for genomic DNA extraction

An improved CTAB method was used to isolate DNA from *D. opposita* Thunb. and the basal procedures consisted of the following steps.

1) Approximately 1.0 g leaves and small amounts of polyvinylpyrrolidone powder (0.1 g per 1.0 g leaves) were ground into fine powder in a liquid nitrogen-chilled mortar.

2) The powder was then immediately transferred to a 2-mL centrifuge tube. Subsequently, 1 mL cold extraction buffer I was added to the tube, and the contents were homogenized quickly by vortexing and allowed to stand at room temperature for about 10 min. Afterwards, the tube was centrifuged at 4°C and 8000 rpm for 10 min.

3) The supernatant was discarded, and the precipitate was suspended in 1 mL preheated extraction buffer II. The mixture was incubated at 65°C for at least 1 h with occasional, gentle shaking. After incubation, the mixture was centrifuged at 4°C and 8000 rpm for 10 min.

4) The supernatant was carefully transferred to a new 2-mL centrifuge tube, and then an equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube. The tube was mixed gently by inversion for 1 min, kept at room temperature for 10 min, and centrifuged at 4°C and 10,000 rpm for 10 min.

5) The aqueous phase was carefully transferred to a fresh 2-mL centrifuge tube. About one-tenth volume of 2.5 M KAc solution, pH 5.2, was added to the tube, and the chloroform:isoamyl alcohol (24:1) treatment was repeated as above.

6) The upper aqueous phase was transferred to another new 2-mL tube, and two-thirds volume of ice-cold isopropanol was added to the tube. The tube was maintained at -20°C for more than 30 min and then centrifuged at 4°C and 12,000 rpm for 10 min.

7) The supernatant was discarded, and the pellet containing DNA was washed once or twice with 70% ethanol and dried under the natural environment.

8) The final DNA precipitate was dissolved in 50 µL sterilized double-distilled water.

Determination of the quantity and purity of DNA

The quality and quantity of the total DNA were determined by monitoring the A_{260}/A_{280} absorbance ratio using the NanoDrop 2000. The integrality of the sample was tested by 1.0% agarose gel electrophoresis with staining by ethidium bromide (EB). Finally, the DNA sample was diluted to a concentration of 50 ng/µL.

Establishment of the RAPD reaction system

The initial RAPD reaction system was designed as follows: 25 µL for one reaction including 2.5 µL 10X PCR buffer (Mg^{2+}-free), 2 µL dNTP (2.5 mM each), 2 µL $MgCl_2$ (25 mM), 1 µL DNA (50 ng/µL), 1 µL primer (20 µM), 0.25 µL *Taq* DNA polymerase (5 U/µL), and 16.25 µL ddH_2O. The RAPD reaction was performed using the following thermal cycle procedures: 94°C for 4 min; 40 cycles of 45 s at 94°C, 45 s at 36°C, and 60 s at 72°C; and a final extension step at 72°C for 7 min. Amplified PCR products were electrophoresed on a 1.0% agarose gel with EB in Tris/borate/EDTA (TBE) buffer and photographed on ultraviolet (UV) light. The primers that produced distinct bands were used for the RAPD analysis of the total DNA in *D. opposita* Thunb.

Optimization of the RAPD reaction system

Because a large number of studies that revealed RAPD results were influenced significantly by the concentration of Mg^{2+}, dNTP, DNA polymerase, and primer (Yao at al., 2009; Wang et al., 2011; Yang at al., 2011), we optimized the RAPD reaction system using the orthogonal design. In the 20-µL reaction system, genomic DNA (50 ng/µL) of *D. opposita* Thunb. acted as template and SBS A06 (GGTCCCTGAC) acted as primer. To simplify the experiments, the interactions between Mg^{2+}, dNTP, DNA polymerase, and primer concentrations are not taken into account. The experiments of the orthogonal design were designed with 4 factors and 4 levels using the L_{16} (4^4) array. Orthogonal test factors and level treatment combinations were presented in Tables 1 and 2, respectively. The amplification was performed as above. In this way, the experiment was repeated for the third time. Finally, the optimization of the RAPD reaction system was further confirmed with the SBS B07 (GGTGACGCAG) and the A19 (CAAACGTCGG) primers. Amplified PCR products were electrophoresed on a 1.0% agarose gel with EB in TBE buffer and photographed on UV light.

Table 1. Factors and levels designed for RAPD system.

Levels	Factors			
	Primer (µM)	dNTP (mM)	*Taq* polymerase (U/µL)	Mg^{2+} (mM)
1	0.2	0.10	0.1	1.0
2	0.4	0.15	0.15	1.5
3	0.6	0.2	0.2	2.0
4	0.8	0.25	0.25	2.5

RESULTS

Purity and integrity of genomic DNA from D. opposita Thunb.

During the DNA extraction, the physical characteristics of the final DNA pellet were white with no visible discoloration. The quality and purity of the extracted nucleic acid sample was assessed by spectrophotometry with 2 µL sample. Using this protocol, the A_{260}/A_{280} absorbance ratio of all DNA samples ranged from 2.0 to 2.2 (Table 3), indicating that the nucleic acid that was isolated by this approach was largely free of contaminating proteins. The A_{260}/

A_{230} ratio was higher than 1.6, indicating that the high-purity DNA samples did not have polyphenol and polysaccharide contamination. In addition, we also found that the DNA quality could be further improved when the DNA samples were precipitated with isopropanol again after washing with 75% ethanol and being dissolved.

Table 2. Orthogonal design for RAPD system [L_{16} (4^4)].

Test number	Factors			
	Primer (μL)	dNTP (μL)	*Taq* DNA polymerase (μL)	Mg^{2+} (μL)
1	1 (0.2)	1 (0.8)	1 (0.4)	1 (0.8)
2	1	2 (1.2)	2 (0.6)	2 (1.2)
3	1	3 (1.6)	3 (0.8)	3 (1.6)
4	1	4 (2.0)	4 (1.0)	4 (2.0)
5	2 (0.4)	1	3	2
6	2	2	4	1
7	2	3	1	4
8	2	4	2	3
9	3 (0.6)	1	4	3
10	3	2	3	4
11	3	3	2	1
12	3	4	1	2
13	4 (0.8)	1	2	4
14	4	2	1	3
15	4	3	4	2
16	4	4	3	1

Table 3. Purity and yield of the genomic DNA extracted from *Dioscorea opposita* Thunb. by the modified CTAB method.

Samples	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/μL)
1	10.04	5.08	1.98	1.78	501.9
2	8.74	4.11	2.13	1.59	437.2
3	7.67	3.72	2.03	1.89	383.4
4	14.94	7.04	2.12	2.21	747.0

To determine the integrity of the DNA, 6 μL of each sample was electrophoresed on a 1.0% agarose gel in TBE buffer. As displayed in Figure 1, the band of the genomic DNA by this protocol is very homogeneous, and no other contamination band appeared, which further confirmed the high purity of the DNA that was extracted by this method and that the DNA was free from polysaccharide and polyphenol contamination. The result of Figure 1 agrees with the results of Table 3.

We found that the RAPD system had poor repeatability when the selected primers were used again. Consequently, the optimization of the RAPD system for *D. opposita* Thunb. is very necessary.

Optimization of the RAPD reaction system

The L_{16} (4^4) array was used to arrange the tests, and the total test number of a set of the orthogonal design was only 16. The result was analyzed according to Xie's method (Xie et al., 2005). As displayed in Figure 2, there are significant differences among the 16 different combinations of the orthogonal design. Moreover, some combinations did not even result in

bands, including the first, second, third, eighth, twelfth combinations; additionally, some combinations displayed bad bands such as the tenth and thirteenth combinations. Of these combinations, only the seventh and the ninth combinations displayed good bands, but the band from the ninth combination is more excessive and darker than that from the seventh combination. Therefore, the seventh combination was chosen and used for additional study. That is, the best treatment system consisted of 2.0 mM Mg^{2+}, 0.25 U/μL *Taq* DNA polymerase, 0.1 mM dNTP, and 0.6 μM primer.

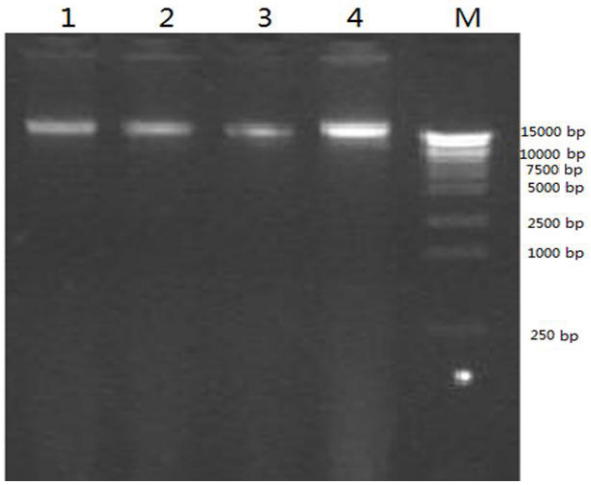

Figure 1. Electrophoresis analysis of the extracted genomic DNA from *Dioscorea opposita* Thunb. by the modified CTAB method. *Lanes 1-4* = DNA samples; *lane M* = DNA marker.

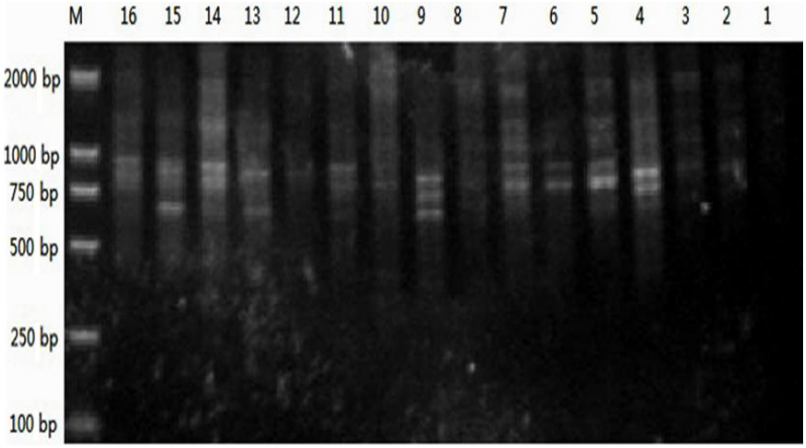

Figure 2. Electrophoresis analysis of the RAPD products based on the orthogonal design diagram. *Lane M* = DNA marker; *lanes 1-16* = 16 combinations by orthogonal design.

Stability of the optimal reaction system

The optimized RAPD reaction system was further confirmed using primers SBS B07

(GGTGACGCAG) and A19 (CAAACGTCGG). The result showed the abundant and legible bands that were displayed in Figure 3, and 3 experimental results were nearly identical. These results showed that the optimized reaction system presented better repeatability, which is suitable for the RAPD analysis of *D. opposita* Thunb.

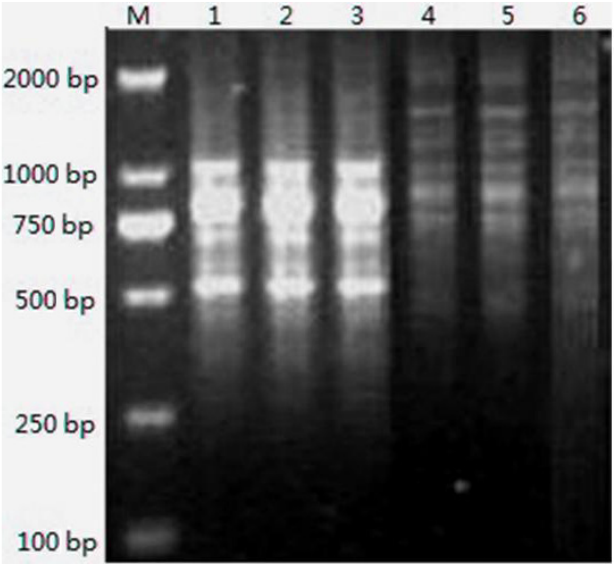

Figure 3. Electrophoresis analysis of RAPD products obtained from the selected best treatment system. *Lanes 1-3* = RAPD products using primer SBS B07; *lanes 4-6* = RAPD products using primer SBS A19; *lane M* = DNA marker.

DISCUSSION

It is well-known that high-quality DNA extraction is a critical determinant of molecular biological procedures, such as PCR and gene library construction. However, the extraction methods are probably unsuitable for specific plants because of the physiological and structural differences of the plant itself and the interference of secondary metabolites (Li et al., 2007a; Hu et al., 2009). A great number of studies revealed that *D. opposita* Thunb. contained abundant mucus that belongs to a complex of mannan-protein and other secondary metabolites (Yuan, 2008; Li et al., 2009). In this study, extraction buffer I was used to partly eliminate polysaccharides. In the extraction and precipitation process, 2.5 M KAc was used to further remove polysaccharides. The results showed that high-quality DNA was obtained successfully. The isolated DNA was used for many molecular experiments.

The optimization of the RAPD reaction system had been reported for many species (Yao et al., 2009; Wang et al., 2011; Yang et al., 2011). However, previous studies mainly focused on a single factor, which ignored the interactions among multiple factors (Li et al., 2007b). More importantly, the RAPD reaction system is different among the different species and different family members of the same species (Ballard et al., 1996). For instance, Wang et al. (2011) and Yang et al. (2011) optimized the RAPD reaction system of *Fritillaria*

unibracteata and *Sophora alopecuroides* L., respectively. These optimized reaction systems optimized never agreed. Here, the orthogonal design form L_{16} (4^4) was utilized to arrange the tests to optimize the RAPD reaction system, and the total test number of a set of the orthogonal design was up to 16. It is obvious that the orthogonal design can significantly reduce the experimental workload and improve the accuracy and efficiency. Finally, a good reaction system for *D. opposita* Thunb. was established. The optimized system is stable and reliable, and it has good repeatability.

In conclusion, the method that we described is an efficient and reliable method to isolate total DNA from *D. opposita* Thunb. At the same time, a suitable RAPD reaction system for *D. opposita* Thunb. was developed. An optimal 20-µL reaction system consisted of 2.0 mM Mg^{2+}, 0.25 U/µL *Taq* DNA polymerase, 0.1 mM dNTP, and 0.6 µM primers. The results described here provided some useful information for other related studies on effective DNA extraction and RAPD reaction.

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