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Full Length Research Paper

Optimization of DNA isolation, ISSR-PCR system and primers screening of genuine species of rhubarb, an important herbal medicine in China

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To estimate genetic diversity and to authenticate the three endangered and official genuine species, *Rheum officinale*, *Rheum palmatum* and *Rheum tanguticum*, of herbal medicine of rhubarb, the optimization of DNA isolation methods including modified CTAB and isolation kit, PCR system of inter-simple sequence repeats (ISSRs) and primers screening were investigated in the present work. Modified CTAB was a preferable choice compared to isolation kit, ISSR protocol was optimized based on the use of the concentration of $MgCl_2$ (1.5 mM), lower concentrations of primer (0.4 µM), dNTPs (0.25 mM), *Taq* DNA polymerase (1.0 U) and 50 ng of template DNA, resulted optimal amplification. Reproducible amplifiable products were observed in all PCR reactions. According to this PCR system, sixteen out of one hundred primers were chosen for their high clarity and repetition. Thus, the results indicated that the optimized protocol for DNA isolation and PCR system was amenable to three genuine species of rhubarb which is suitable for further work on genetic diversity analysis. Furthermore, here the suitable DNA isolation protocol for ISSR-PCR analysis can be used to study the genetic variation in the future in *Rheum* grown in China.

Key words: Rhubarb, DNA isolation, ISSR-PCR condition, primer screening.

INTRODUCTION

Rhubarb is a widely used traditional Chinese medicinal herb and one of the important ingredients in Chinese traditional prescriptions. It has been used for more than one thousand years in China. As described in the Chinese Pharmacopoeia, rhubarb consists of the roots and rhizomes of *Rheum officinale* Baill., *R. palmatum* L. and *R. tanguticum* Maxim. ex Balf. (Polygonaceae) (Chinese Pharmacopoeia Committee, 2005), all of which are endemic to China (Bao and Grabovskaya-Borodina, 2003). *R. officinale* mainly distributes in Hubei, Henan, Shannxi, Sichuan, Chongqing, Guizhou and northwest part of Yunnan provinces. The main distribution of *R. palmatum* is in southeast part of Gansu, Ningxia, Qinghai, west part of Tibet, Sichuan and Shannxi provinces and *R. tanguticum* is in Sichuan, Gansu, Qinghai and east part of Tibet provinces. Rhubarb has many pharmacological actions, such as purgation, anti-inflammatory, antibacterial, antipyretic, hepatoprotective, antineoplastic and antipasmolytic effects and also as an agent to reduce blood-lipid, blood pressure, obesity and blood urea nitrogen (Chinese Pharmacopoeia Committee, 2005; Tseng et al., 2006; Huang et al., 2007). The usage of rhubarb has been recently extended into the functional food due to the advent of new functional and biologically active compounds. Therefore, the market request of rhubarb is increasing, the wild resource is decreasing significantly and the distribution of resources has continued to become narrow (Wang and Ren, 2009). To date, the previous studies mainly focused on the distribution, components analysis and pharmacological properties (Shen et al., 1996; Xu et al., 2002; Ye et al., 2007) and few efforts have been made to the genetic

diversity of one genuine species of rhubarb that is, *R. tanguticum*, using simple sequence repeats (SSRs) markers and inter-simple sequence repeats (ISSRs) (Wu et al., 2008; Chen et al., 2009). As the three genuine species of rhubarb are morphologically indistinguishable, therefore, it is necessary to authenticate these species using DNA-based technology. On the other hand, in order to establish effective conservation strategies of rhubarb, assessment of its genetic diversity and population structure of the genuine species is urgent. Yet, the first step of these studies is the isolation of high quality DNA. Total DNA was extracted from leaves of *R. tanguticum* using the isolation kit by Wu et al. (2008) while the modified 2-CTAB (cetyl trimethyl ammonium bromide) procedure (Doyle and Doyle, 1987) was adopted by Chen et al. (2009). Any commercial kit is of high per sample cost and CTAB is time-cost. Here we compared the isolation kit method and DNA isolation protocol derived from a method originally developed for other plants (Doyle and Doyle, 1987) in order to develop an effective but simple and inexpensive method to isolate high quality DNA from the three genuine species of rhubarb.

ISSRs by Zietkiewicz et al. (1994) are a modification of the SSR approach that use a single primer based on SSR (microsatellites) that are common in the genome. ISSRs polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats. Compared with the RAPD method (random amplified polymorphic DNA), the longer primer (16 - 20 bp) can precisely target the template DNA and improve reliability and reproducibility (Reddy et al., 2002). In addition, ISSRs are significantly lower cost than AFLP (amplified fragment length polymorphism). Thus, ISSRs are widely used to reveal the genetic variation of medicinal plants (Xiao et al., 2006; Qiu et al., 2008).

The aims of the present study are: (1) to estimate efficient, simple and inexpensive DNA isolation method from the genuine species of rhubarb; (2) to form an optimal protocol of ISSR-PCR reaction condition and (3) to screen one hundred primers and select those with high clarity and repetition for further downstream applications.

MATERIALS AND METHODS

Plant material

The materials of *R. officinale* (voucher: Nanchuan, Chongqing, alt. 1832m, 29°00.008′N, 107°11.769′E, Xu-mei Wang and Xiao-qi Hou 09072609, SANU), *R. palmatum* (voucher: Wuwei, Gansu, alt. 2575m, 38°10.000′N, 101°26.000′E, Xu-mei Wang 08071807, SANU) and *R. tanguticum* (voucher: Maqin, Qinghai, alt. 3580m, 34°38.821′N, 100°14.189′E, anonymous, LZU) were collected in 2008. These provinces are both main distribution regions and cultivated drug producing areas. Fresh leaf samples, five wild individuals for each species, were collected and preserved in zip-lock bags with silica gel until required for DNA isolation.

Solutions and reagents

Extraction buffer I [1 M Tris HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5.0 M NaCl], extraction buffer II [2% CTAB (w/v), 1M Tris HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5.0 M NaCl] were prepared. Polyvinylpyrrolidone (PVP, 0.1 g/1 g of leaf tissue, added while grinding), Ascorbic acid (0.1 g/1 g of leaf tissue, added while grinding), 2-mercaptoethanol, Chloroform: Isoamylalcohol (24:1), Isopropanol, and Ethanol (70%, 100%) were also prepared and stored.

DNA isolation

The dried leaf tissues of *R. officinale*, *R. palmatum* and *R. tanguticum* were used for DNA isolation and their total DNA were extracted to obtain optimal protocol by evaluating the two kinds of isolation methods, isolation kit and modified CTAB. The first method was according to manufacturer's protocol provided by isolation kit (QIAGEN, Germany). The modified CTAB method was used the following steps. Approximately 0.1 g the dried leaf tissues of rhubarb were ground in liquid nitrogen using a mortar and pestle along with small amounts of quartz sand, ascorbic acid and PVP powder. The powder was transferred into a 2 ml centrifuge tube and mixed with 2 ml cold Extraction buffer containing 10 µl 2-mercaptoethanol (added just before used). Then the mixture was incubated at 0°C for 30 min and centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the precipitation was suspended in 800 µl preheated extraction buffer II (10 µl 2-mercaptoethanol was added into buffer II just before used). The mixture was incubated at 65°C for 60 min with gently intermittent shaking.

After incubation the mixture was centrifuged at 11,000 rpm for 6 min at 4°C. The supernatant was carefully decanted and transferred to a new 2 ml centrifuge tube and an equal volume of chloroform: isoamylalcohol (24:1) and one tenth volume of preheated extraction buffer II was added and mixed gently by inversion for 10 min, followed by centrifugation at 12,000 rpm for 10 min at 4°C. The aqueous layer was carefully transferred to a fresh tube, and re-extracted with one tenth volume of preheated extraction buffer II and the chloroform: isoamylalcohol (24:1) by centrifuging at 12,000 rpm for 10 min (repeat the step by 1 - 2 times). The upper aqueous layer was taken to a new 1.5 ml centrifuge tube and two volumes of ice-cold isopropanol were added. It was then kept at -20°C for 60 min and centrifuged at 10000 rpm for l0 min at 4°C. The DNA pellet was washed with 70% ethanol twice and air dried. Finally, DNA pellet was re-suspended in 100 µl sterilized double distilled water.

Detection of DNA

DNA quantifications were performed by UV-spectrophotometer (ND-2000, NanoDrop, USA) at 260 and 280 nm and the purity was then determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (OD_{260}/OD_{280}). DNA concentration and purity was also determined by electrophoresis on 1.0% agarose based on the intensities of band when compared with 1 kb plus DNA ladder as marker. The re-suspended DNA was then diluted in sterilized double distilled water to the concentration of 50 ng/µl for use.

Optimization of ISSR-PCR system and primers screening

For the optimization of ISSR-PCR reaction using DNA extracted from three plant species of rhubarb, one hundred ISSR primers synthesized by Shanghai Sangon Biological Engineering Technology and Service (China), according to the primer set

Table 1. Optimization of the ISSR-PCR reaction parameters for three genuine species of rhubarb.

PCR parameter	Tested range	Optimum conditions
DNA template concentration (ng)	10, 20, 30, 40, 50, 75, 100, 150, 175, 200	50 ng
Magnesium chloride (mM)	1.0, 1.5, 2.0,2.5, 3.0	1.5 mM
Deoxynucleotide triphosphates (dNTPs) (mM)	0.05, 0.1, 0.15, 0.2, 0.25, 0.3	0.25 mM
Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5	0.4 µM
Taq DNA polymerase (units)	0.5, 1.0, 1.5, 1.75	1 U
Annealing temperature (°C)	50~58	51, 52 or 56°C for different primer

published by University of British Columbia, Canada (UBC set No. 9) were used for amplification to standardize the PCR conditions.

The reactions were carried out in a DNA Thermocycler PTC-200 (MJ Research, USA). Five factors including Taq DNA polymerase, dNTP, primer concentration, Mg^{2+} and annealing temperatures were investigated as described in Table 1. Reactions without DNA were used as negative controls. Each 25 µl reaction volume contained about 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.4µM primer, 1 unit Taq Plus DNA polymerase (Tiangen, Beijing, China) and approximately 50 ng template DNA (Table 1). The thermocycler was programmed for an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at different annealing temperature for each primer (Table 2), extension at 72°C for 1 min and final extension at 72°C for 10 min and a hold temperature of 4°C at the end. 8 µl of PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TBE Buffer at 110 V for 1.5 h and stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed in UV light by using Bio-Rad Gel Documentation System (Bio-Rad Laboratories, UK). DL2000 ladder (TaKaRa Biotechnology, China) was used as DNA molecular weight.

RESULTS

DNA isolation and detection

White deposit pellet could be obtained in most samples by using both modified CTAB and isolation kit, but the DNA quantity obtained by isolation kit was less than modified CTAB. Nucleic acid detection results showed that all ratios of OD_{260}/OD_{280} ranged from 1.6 - 1.9 when detecting purity of DNA extracted by two methods. Neither lagging and unbinding band nor impurity was found in each lane by electrophoresis. Figure 1 showed the electrophoresis result of genomic DNA extracted from five individuals of *R. officinale*. The other two species showed the similar results (no illustration).

ISSR-PCR system and primers screening

Six tested parameters for ISSR-PCR like the concentration of template DNA, primer, magnesium chloride, *Taq* DNA polymerase, dNTPs and annealing temperature were also optimized which also had an effect on amplification, banding patterns and reproducibility. The optimized conditions for ISSR-PCR protocol are given in Table 1.

From the preliminary screening, twenty-two primers out of one hundred that could amplify visible bands were selected for further examination. Consequently, sixteen ISSR primers that produced clear, reproducible and relatively high polymorphism bands were selected (Table 2). Fifteen DNA samples extracted by modified CTAB method from three species were used to detect the amplification of these primers (Table 2). A total of 150 bands were amplified, among which 107 were polymorphic (71.3%). Mean 9.4 bands were amplified by each primer. The electrophoresis result by primer UBC848 was displayed in Figure 2.

DISCUSSION

In order to obtain the efficient authentication, management and conservation of the endangered important herbal medicine of rhubarb, extensive research on DNA-based molecular markers such as the genetic diversity is necessary. Among all dominant markers, ISSRs have its advantages with high annealing temperature, repetition and lower cost and have been widely used for the genetic studies of medicinal plants. The present optimized protocol for DNA isolation and ISSR-PCR technique may serve as an efficient tool for further molecular studies.

CTAB (Doyle and Doyle, 1987) is a universal DNA isolation method, but it is time-cost with so many steps. In recent years isolation kits are welcomed for its concise steps and shortening time in which all necessary buffers or solutions were provided. The results from the present studies showed that two isolation methods could effectively extract DNA with higher quality and yield, but the DNA quantity obtained by modified CTAB was more than isolation kit, and the extracted DNA was of high quality as it showed a reading of between 1.6 - 1.9 after calculating the 260/280 nm absorbance. The steps in the modified CTAB method were less than that of the originally developed ones by Doyle and Doyle (1987). However, the isolation kits can be chosen if efficiency and speediness was foremost without regard to funding

Table 2. Sequences and amplification band numbers of 16 selected ISSR primers.

Primer code	Sequence (5'→3')	T_A (°C)	N_{PL}/N_L	*PPB* (%)
UBC807	AGAGAGAGAGAGAGAGT	51	7/12	58.3
UBC810	GAGAGAGAGAGAGAGAT	51	5/10	50.0
UBC816	CACACACACACACACAT	52	7/9	77.8
UBC834	AGAGAGAGAGAGAGAGYT	52	7/10	70.0
UBC835	AGAGAGAGAGAGAGAGYC	52	8/10	80.0
UBC841	GAGAGAGAGAGAGAGAYC	52	8/9	88.9
UBC842	GAGAGAGAGAGAGAGAYG	56	4/6	66.7
UBC847	CACACACACACACACARC	51	3/6	50.0
UBC848	CACACACACACACACARG	51	10/12	83.3
UBC873	GACAGACAGACAGACA	52	5/7	71.4
UBC880	GGAGAGGAGAGGAGA	52	5/7	71.4
UBC881	GGGGTGGGGTGGGGT	52	7/10	70.0
UBC888	BDBCACACACACACACA	52	2/7	28.6
UBC889	DBD ACACACACACACAC	52	10/12	83.3
UBC890	VHVGTGTGTGTGTGTGT	56	8/9	88.9
UBC891	HVHTGTGTGTGTGTGTG	52	11/14	78.6

R = (A, G); Y = (C, T) ; B = (C, G, T) ; D = (A, G, T) ; H = (A, G, T) ;V = (A, C, G); N_L: No. of loci scored; N_{PL}: No. of polymorphic loci scored; *PPB*: Percentage of polymorphism bands.

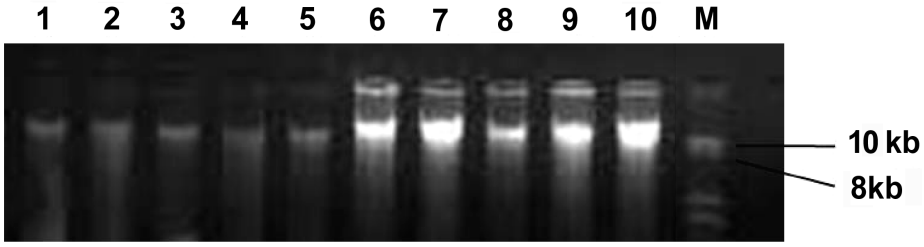

Figure 1. Electrophoresis result of genomic DNA extracted from five individuals of *Rheum officinale* by using isolation kit and modified CTAB method. (M: l kb plus DNA ladder size standard marker; Lane 1 - 5: DNA extracted by isolation kit; Lane 6 - 10: DNA extracted by modified CTAB.)

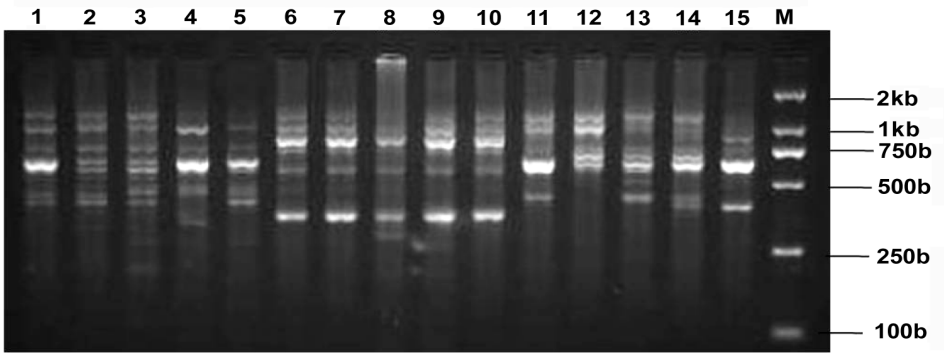

Figure 2. ISSR-PCR results of fifteen DNA samples from three genuine species of rhubarb amplified by primer UBC848. (M: DL 2000 ladder marker. Lanes 1-5: *Rheum palmatum*; lanes 6-10: *R. officinale*; Lanes 11-15: *R. tanguticum*.)

(ca. 4 US dollar per sample in China). On the other hand, the modified CTAB was the better choice especially for a large of DNA samples which was necessary for the study on genetic diversity, but it often need to be slightly modified even if it had been reported in other laboratory. This may be attributed to compounds difference of leaf tissue in different regions, chemicals and solutions manufactured by different company.

Phenolic compounds occur in many plants and are one of the major bioactive constituents in rhubarb (Ye et al., 2007). When the tissue was grinded and the cell was broken, phenolic compounds were released and oxidized by binding covalently with the total DNA, resulting in browning effect and an overall loss of DNA activity (Porebski et al., 1997). The way to remove the phenolic compounds is to prevent their oxidation in the initial extraction stage, and then separated them with the total DNA.

In the present study, enough PVP and ascorbic acid were added when the tissue was grinded. Addition of PVP along with CTAB may bind to the phenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent. Repeated chloroform: isoamylalcohol treatment ensured removal of chlorophyll, other colouring substances such as pigments and proteins etc. DNA degradation was avoided to some extent by carrying out all the steps at 4℃. Thus, we concluded that present protocol described a reliable, rapid, simple and consistent DNA isolation method for the genuine species of rhubarb.

After obtaining a total genomic DNA with high purity and yield, primers still need to be screened if their screening has not been reported because the same primer may exhibit different amplification results in different species. Therefore, primers screening and optimal PCR system based on each selected primer are necessary. In the present study, DNA isolated by modified CTAB method yielded strong and reliable amplification products showing its compatibility for the sixteen selected primers of ISSR-PCR. The present optimized protocol for ISSR technique may serve as an efficient tool for further molecular studies. On the other hand, *Rheum* L. contains about 60 species, 38 of which distribute in China (Bao and Grabovskaya-Borodina, 2003). Many species of *Rheum* have medicinal values, and some of them are eatable both in some east and the west countries or have other economic values. However, the species of the genus which has economic values is in a narrow distribution, and the amount of resources is limited.

Therefore, studies on the genetic diversity of these plants are very necessary, and the method used in the present study can be also applied in the genetic diversity studies of other traditional Chinese medicinal plants in the same genus.

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