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Full Length Research Paper

An effective and low-cost method for DNA extraction from herbal drugs of *Rheum tanguticum* (polygonaceae)

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Rhubarb is an important traditional Chinese herbal drug with high secondary metabolites that interfere with DNA extraction procedures and downstream applications, such as DNA restriction and amplification. An effective and low-cost protocol for isolating genomic DNA from root of *Rheum tanguticum* is described in this paper. It involved a modified CTAB method with distilled water pretreating samples. The A_{260}/A_{280} absorbance ratio of extracted DNA, free from polysaccharide, polyphenols and tannins contaminants, ranged from 1.85 to 2.0 within the high level of purity. The DNA preparations were found suitable for analyses with restriction enzyme digestion and inter-simple sequence repeats (ISSR). This method is helpful for further studies on rhubarb at molecular level and is more cost-effective than commercial kit.

Key words: Rhubarb, *Rheum tanguticum*, DNA extraction, ISSR, secondary metabolites.

INTRODUCTION

Rheum tanguticum Maxim. ex Balf. (Polygonaceae), the rhizome and root of which is an important traditional herbal drug called rhubarb (Dahuang in Chinese), is endemic to China (Li, 1998; Wu, 2004). It is characterised of bitter flavor and cold property and is widely used as a purgative and anti-inflammatory agent (Chinese Pharmacopoeia Commission, 2005).

Confused with the adulterant species in medicinal use of rhubarb, it is important to effectively distinguish the official plants from the adulterants. However, based on traditional, morphological and histological traits, it is difficult to identify official rhubarb. Recently, the application of molecular technology has progressed rapidly over the last two decades, especially in the area of medicinal plants authentication (Zhang et al., 2001; Ren and Timko, 2001; Liu and Zhang, 2008; Vural and Dageri, 2009). Yet the first key step of the technology is the isolation of high quality DNA.

With the modified Doyle and Doyle (1987) CTAB method, it is feasible to extract qualified DNA from leaves of *R. tanguticum* (Zhang et al., 2007), but difficult from rhubarb crude drugs because of its high amounts of secondary metabolic substances such as polyphenols, polysaccharides, quinone and tannins, which would interfere with DNA extraction procedures and the activity of DNA enzymes and restriction endonucleases (Angeles et al., 2005; Padmalatha and Prasad, 2006; Mishra et al., 2008; Niu et al., 2008). Recently, commercial manufactures have developed a variety of kits such as DNeasy™ plant mini kit (Yang et al., 2004; Komatsu et al., 2006) that allow rapid and efficient isolation of high-quality DNA from rhubarb. However, any commercial kit is of high per-sample cost. The objective of this study was to develop an effective but low cost method to isolate high quality DNA from rhubarb.

MATERIALS AND METHODS

Plant material

Samples of rhubarb (*R. tanguticum* Maxim. ex Balf.) roots were collected from Dawu county, Qinghai province, China, and air-dried at room temperature.

Solutions and reagents

Extraction buffer I [200 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 250 mM NaCl] and extraction buffer 2 [100 mM Tris-HCl (pH

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Abbreviations: CTAB, hexadecyltrimetylammonium bromide; **ISSR**, inter-simple sequence repeats.

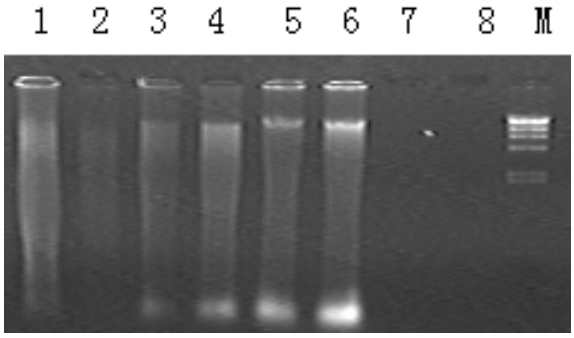

Figure 1. Comparison of genomic DNA extracted from rhubarb herbal drugs of *R. tanguticum* with different pretreat-ments. Lanes 1 and 2: samples pretreated with extraction buffer I at 0℃; lanes 3 and 4: samples pretreated with 95% ethanol at 0℃; lanes 5 and 6: samples pretreated with 0℃ distilled water; lanes 7 and 8 untreated. M represents the molecular weight marker λDNA HindIII digest.

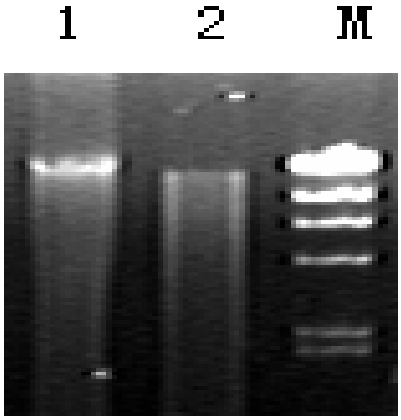

Figure 2. Restriction endonuclease digestion of DNA with *Eco*RI. Lane 1 is the unrestricted DNA, lane 2 represents DNA restricted by *Eco*RI. M represents the molecular weight marker λDNA HindIII digest.

8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB] were prepared. Polyvinylpyrrolidone (PVP), 2-mercaptoethanol, CTAB/NaCl solution (10% CTAB and 0.7 M NaCl) 3 M sodium acetate solution (pH 5.2), Chloroform: isoamylalcohol (24:1), Isopropanol, ethanol (75 and 95%) were also prepared and stored.

DNA extraction protocol

Roots samples were immersed in different solutions, distilled water, 95% ethanol and extraction buffer I at 0 for 24 h respectively and each solution was changed every 8 h and sample was done nothing as control. Then samples were cut into pieces of approximately 1 mm size and were sterilized with 75% ethanol.

Approximately 1.0 g of root tissues were ground using a mortar and pestle along with small amounts of quartz sand and PVP powder. The powder was transferred into a 2 ml centrifuge tube and mixed with 1 ml cold extraction buffer I containing 10 µl, 2-mercaptoethanol (added just before use). Then the mixture was incubated at 0℃ for 10 min and centrifuged at 7 000 rpm for 10 min at 4℃.

The supernatant was decanted and the precipitation was suspended in 1 ml preheated extraction buffer II (10 µl 2-mercaptoethanol was added into buffer 2 before usage). The mixture was incubated at 65℃ for 60 min with gently shaking occasionally. After incubation the mixture was centrifuged at 7 000 rpm for 10 min at 4℃. The supernatant was carefully transferred to a new 2 ml centrifuge tube and an equal volume of chloroform: isoamylalcohol (24:1) was added and mixed gently by inversion for 10 min, followed by centrifugation at 7 000 rpm for 10 min at 4℃. The aqueous phase was carefully transferred to a fresh tube, one tenth volume of CTAB/NaCl was added and the chloroform: isoamylalcohol (24:1) treatment was repeated. The upper aqueous phase was taken and two third volumes of ice-cold isopropanol and one tenth volume of sodium acetate solution were added. It was then kept at 20℃ for more than 30 min and centrifuged at 10 000 rpm for 10 min at 4℃. The DNA pellet was washed with 75% ethanol twice and air dried. Finally, DNA pellet was dissolved in 200 µl sterilized double distilled water.

DNA analysis

The quality of extracted DNA was analyzed by means of agarose gel electrophoresis stained with ethidium bromide. The purity was first estimated by using a UV-spectrophotometer at 260 and 280 nm, and further confirmed by restriction digestion with *Eco*RI (BioLabs) restriction endonuclease.

In order to check the suitability of extracted DNA for downstream analysis, ISSR-PCR analysis was done with UBC809 (5`-AGAGAGAGAGAGAGAGG-3`) and UBC888 (5`-BDBCACACACACACACA-3`) primers. 20 µl PCR reaction mixture consisted of 2.5 µl 10 × buffer (Tris-HCl (pH 8.3) 100 mM; KCl 500 mM), 3 mM $MgCl_2$, 0.1 mM dNTP, 10 pmol primer, 0.75U Taq DNA polymerase (TaKaRa) and 30 ng template DNA. PCR reaction was performed in a DyadDisciple™ peltier thermal cycler using the following profile with an initial step of 94℃ for 5 min followed by 38 cycles of 20 s at 94℃, 60 s at 50 - 58℃ and 80 s at 72℃ with a final extension for 6 min at 72℃. Amplified PCR products were electrophoresed in a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide in TAE buffer and photographed on UV light.

RESULTS

Pretreatments with different solutions had different effects on DNA extraction from rhubarb samples. Although DNA was partially degraded with 95% ethanol and buffer I and even not observed without pretreatment, yet integrated DNA could be perfectly extracted with distilled water, which was of high molecular weight compared with λDNA digested by HindIII (Figure 1).

The extracted DNA quality was assessed by spectrophotometry, restriction endonuclease digestion and PCR amplification respectively. The A_{260}/A_{280} absorbance ratio ranged from 1.85 to 2.0 for DNA extracted with the pretreatment of distilled water, indicating the isolated DNA was free from protein contamination (Weising et al., 2005). The suitability of extracted DNA for downstream molecular processes was further verified by restriction endonuclease digestion and ISSR-PCR amplification. As shown in Figure 2, the extracted DNA with the pretreatment of distilled water was amenable to restriction

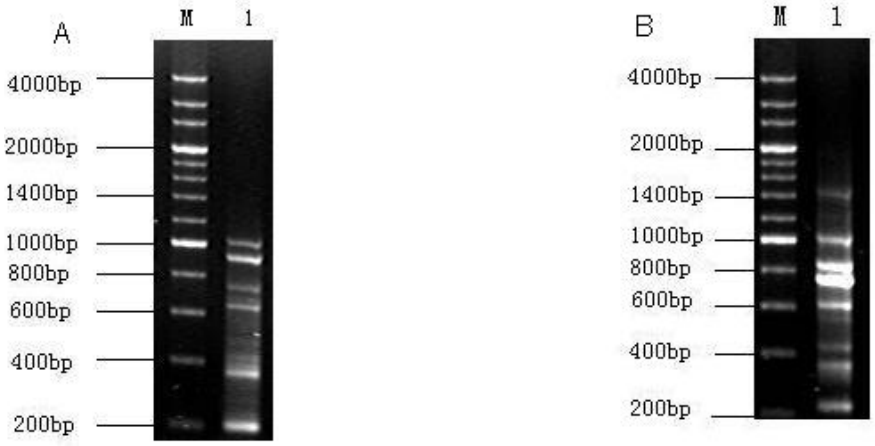

Figure 3. Gel electrophoresis (1.5% agarose) showing PCR profiles of amplified DNA. **A.** Primer UBC809: 5`-AGAGAGAGAGAGAGAGG-3` (lane 1). **B.** primer UBC888: 5`-BDBCACACACACACACA-3`(lane 1). M represents the molecular weight marker (200bp DNA Ladder Marker, *TaKaRa*)

digestion using EcoRI. Furthermore, it was highly amplified by PCR as indicated by the amplification pro-ducts resolved on 1.5% agarose gel (Figure 3). This further confirmed the purity of DNA extracted with this method, free from polysaccharide and polyphenol conta-mination. The results indicated that isolated DNA was amenable to further downstream applications.

DISCUSSION

One problem of medicinal plants DNA preparations is the presence of polysaccharides and certain secondary metabolites, which have been observed to interfere with DNA isolation procedure and inhibit the activity of DNA-modifying enzymes (Angeles et al., 2005; Padmalatha and Prasad, 2006; Mishra et al., 2008; Niu et al., 2008). DNA extraction was improved by modifying some steps in the original Doyle's (Doyle and Doyle, 1987) CTAB method to remove these secondary metabolites. Samples were pre-treated with distilled water at 0°C to remove water-soluble contaminations. Buffer I was used to get nucleolus and mitochondrion DNA by removing cell wall debris, proteins, lipid and many secondary compounds at low speed centrifugation. Besides, DNA was further purified by removing polysaccharides with CTAB/NaCl buffer in which DNA was soluble but polysaccharides were insoluble (Murray and Thompson, 1980). Despite no RNAse treatment, the extracted DNA could be used for restriction and ISSR-PCR analyses, so it is unnecessary to purify DNA with RNAse (Murray and Thompson, 1980).

The DNA extracted with our protocol from rhubarb samples was as reliable as that extracted by standard methods from other tissues like leaves (data not shown). To our knowledge, this is the first report of DNA extraction from rhubarb herbal drugs of *R. tanguticum* without commercial kit (Fushimi et al., 1997; Yang et al., 2004).

In conclusion, we have described an effective and low-cost DNA isolation protocol from rhubarb drugs. The protocol used in this paper involved a crucial step of immersing samples in distilled water at 0℃ at the beginning. The extracted DNA could be used successfully for downstream applications such as restriction digestion and PCR amplification. In addition, this procedure is affordable and does not require sophisticated equipment, making it a preferable choice relative to expensive commercial kit for DNA extraction. Consequently, this method would be helpful for further studies on rhubarb herbal drugs at molecular level.

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