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Optimization of DNA Extraction for ISSR Studies in Seven Important Rose Species of Iran

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ABSTRACT

Rose is one of the most commonly cultivated ornamental plants in the world. DNA isolation from rose species is particularly difficult because of their large amounts of polysaccharides and polyphenols and other compounds. These substances not only decrease the yield but the quality of DNA is almost unusable. To overcome this problem, a simple but efficient method for DNA isolation was designed. In this method, an extraction buffer is used to reduce the secondary metabolite levels. The use of high concentrations of EDTA, CTAB and β-mercaptoethanol in extraction buffer and high concentration of polyvinylpyrrolidone and NaCl in clean up buffer remove polyphenols and polysaccharides. Isopropanol and sodium acetate precipitation of polysaccharides and elimination of proteins by chloroform: isoamyl alcohol resulted a more pure DNA. DNA isolated using this method was checked biophotometrically: A_{260}/A_{230} (polyphenol and polysaccharide residuals) and A_{260}/A_{280} (protein contamination). Quantities of 750-1000µg DNA per gram of fresh leaf tissue were obtained which is enough for most of genetic assays. Considering extraction time, DNA yield and purity and PCR-ISSR amplifications, this procedure emerged to be the best for molecular diversity analysis of roses.

Key words: DNA isolation, ISSR, rose, polyphenols, polysaccharides.

Introduction

The genus *Rosa* comprises hundreds of species and thousands of cultivars. Rose is one of the most commonly cultivated ornamental plants in the world. Rose production encompassing cut flowers, garden and potted plants, is increasing worldwide (Gudin, 2000; Khosh-Khui and Teixeria, 2006 and Oyant *et al.*, 2008). The genetic basis of cultivated roses is narrow in comparison with the vast spectrum of genetic resources existing in the wild (Oyant *et al.*, 2008). Although there are more than 140 *Rosa* species, it is believed that only 11 of them have contributed to the origin of modern cultivars (Oyant *et al.*, 2008). Today, they are common approaches in both applied and fundamental science fields, such as in crop breeding, especially in studies on genetic diversity and gene mapping (Xu *et al.*, 2004). Molecular marker studies require large amount of high quality genomic DNA, emphasizing screening of inexpensive, rapid and simple DNA extraction methods (Narayanan *et al.*, 2006). Roses contain high levels of polysaccharides, polyphenols and other secondary metabolites (Xu *et al.*, 2004). Polyphenols and polysaccharides bind firmly to nucleic acids during DNA isolation and interfere with subsequent reaction (Aljanabi and Martinez, 1997; Dehestani and Kazemi Tabar, 2007; Peterson *et al.*, 1997 and Pirtillä *et al. 2001*). These compounds co-precipitate with DNA during purification and make the DNA preparation highly viscous and refractory to the enzymatic reactions used for molecular techniques (Dixit, 1998; Khan *et al.*, 2004 and Khanuja *et al.*, 1999).

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A number of methods for DNA isolation from plants containing high levels of polyphenols and polysaccharides have been developed (Aljanabi and Martinez, 1997; Dixit, 1998; Peterson *et al*., 1997 and Xu *et al*., 2004). Most of the above methods are time-consuming, expensive and result in a low yield of DNA. With these factors in mind, a simple, rapid and cost-effective method was developed for isolation of DNA from seven species of *Rosa*.

Materials and Methods

Plant material

Total DNA was isolated from the mature leaves of seven species of *Rosa* including: *Rosa banksiae* Ait., *Rosa canina* L., *Rosa chinensis* Jacq. Var. *minima*, *Rosa damascena* Mill., *Rosa foetida* Herm, *Rosa ×hybrida* L. and *Rosa moschata* Herm.

Solution and reagents

Extraction buffer: 120 mM Tris-HCl, pH 8.0; 80 mM EDTA pH 8.0; 4% β-mercaptoethanol; 2% CTAB
Clean up buffer: 120 mM Tris-HCl pH 8.0; 10 mM EDTA pH 8.0; 2% β-mercaptoethanol; 2% PVP;
1.5 M NaCl; 0.2% CTAB
5M Potassium acetate pH 4.8
3M Sodium acetate pH 5.2
70% (v/v) ethanol
Chloroform- isoamyl alcohol (24: 1 v/v)
10 mg/ml RNAase
TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0

DNA extraction procedure

Samples (fresh mature leaves 4 g) were rinsed in tap water following by sterilized distilled water. They were then air-dried and stored at -80°C if not used immediately. The leaves were ground in liquid nitrogen in a sterile prechilled mortar and pestle. Extraction buffer (at a ratio of 5 ml per gram of fresh sample) was added to each tube, incubated the tubes at 60°C water bath for 40 min and vortexed the tubes vigorously 5 min interval. The tubes were centrifuged at 8000g for 5 min and discarded the supernatant. 20 ml clean up buffer was added to each pellet and after resuspension, were incubated at 60°C water bath for 30 min with occasional inversion.

After the tubes were cooled to room temperature, 20 ml of chloroform-isoamyl alcohol (24:1 v/v) and 2 ml of 5M potassium acetate was added and the tubes were shaken vigorously to form and emulsion. Tubes were centrifuged at 8000 g at room temperature for 20 min. After centrifugation the aqueous phase transferred to a new tube and added 20 ml of chloroform-isoamyl alcohol (24:1 v/v), centrifuged and the aqueous phase transferred to a new tube and added 20 ml cold isopropanol with 2 ml of 3M sodium acetate, mixed and incubated at -20°C for 30 min. The DNA was precipitated by centrifugation at 8000 g at room temperature for 10 min.

Resulting pellets were washed 3 times with an equal volume of 70% ethanol and dried the pellet and resuspended in 2 ml of TE buffer with 10 µl of RNAase and incubated at 37°C for 4 h.

The quality and concentration of DNA were checked with biophotometry and electrophoresis.

PCR amplification

For each of isolated DNA samples, PCR was carried out in a 15 µl vol. of reaction mixture. A reaction tube contained 30 ng of template DNA, 1 U of *Taq* DNA polymerase enzyme (Fermentase Co.), 0.2 mM of each dNTP (Cinnagen Co.), 1 × *Taq* amplification buffer (Fermentase Co.), 2 mM $MgCl_2$ and 1µM of ISSR primer (UBC-834 [$(AG)_8$ YT](Cinnagen Co.). Amplification were carried out by using a DNA thermal cycler (Eppendorf, mastercycler gradient) programmed as: 94˚C for 5 min, 35 cycles at 94˚C for 30 s, 45˚C for 45 s and 72˚C for 2 min and a final extension at 72˚C for 10 min. PCR products were separated on 1% agarose gel and stained in ethidium bromide, observed under UV light and photographed using gel documentation unit.

Results and Discussion

DNA suitable for a broad range of molecular biology application was successfully extracted from seven different rose species containing high levels of secondary metabolites.

Following the protocol described in this paper, a yield of 750-1000µg DNA per gram of fresh leaf tissue was obtained. This is higher than the DNA yield obtained by other methods (Dixit, 1998, Dehestani and Kazemi Tabar, 2007 and Khan *et al*., 2004).

A DNA isolation procedure was judged by the quantity and quality of the DNA obtained. By using this method, we successfully isolated good quality DNA from seven rose species. Figure 1 shows that the extracted DNA was not degraded. The A_{260}/ A_{230} absorbance ratio indicates polysaccharide or polyphenolic contamination and the A_{260}/ A_{280} ratio indicates the protein contamination (Dehestani and Kazemi Tabar, 2007; Dixit, 1998 and Khan *et al*., 2004). We obtained that A_{260}/ A_{230} ratios ranged from 1.90 to 2.22 (Table 1), indicating little contamination of polyphenols and polysaccharides, whereas the A_{260}/ A_{280} ratios ranged from 1.78 to 1.96 (Table 1), indicating little contamination by proteins and macromolecules. This extraction procedure was found suitable for ISSR analysis of rose species (Figure 2).

Table 1: Biophotometric analysis of extracted DNA from seven rose species. Results are expressed as the mean of 3 samples (standard error).

Sample	A_{260}/ A_{230}	A_{260}/ A_{280}	Yield µg/g of fresh weight
Rosa banksiae	1.95 (0.022)	1.78 (0.087)	767 (22.4)
Rosa canina	2.05 (0.042)	1.86 (0.046)	912 (17.1)
Rosa chinensis	1.97 (0.060)	1.95 (0.024)	971 (25.0)
Rosa damascena	1.90 (0.055)	1.87 (0.068)	816 (28.0)
Rosa foetida	2.05 (0.078)	1.80 (0.080)	901 (18.7)
Rosa ×hybrida	1.98 (0.019)	1.96 (0.085)	759 (20.8)
Rosa moschata	2.15 (0.060)	1.89 (0.079)	927 (23.0)

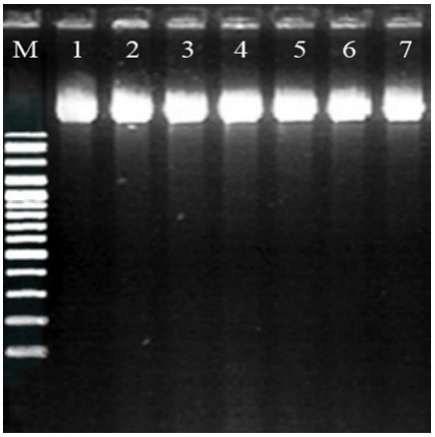

Fig. 1: Genomic DNA isolated from seven rose species. The lanes numbering from left to right, are: M: Weight marker, lane 1: *Rosa banksiae*, lane 2: *Rosa canina*., lane 3: *Rosa chinensis*, lane 4: *Rosa damascena*, lane 5: *Rosa foetida*, lane 6: *Rosa ×hybrida* and lane 7: *Rosa moschata*.

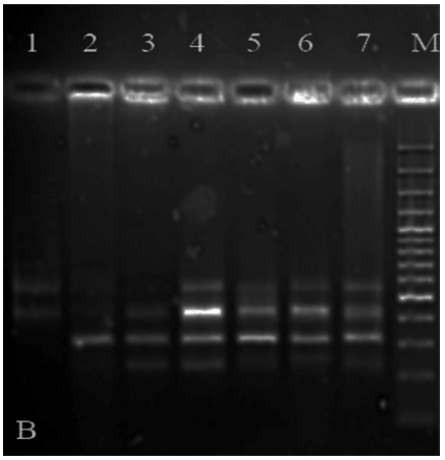

Fig. 2: PCR amplification of genomic DNA from seven rose species with primer UBC-834. Lane 1: *Rosa banksiae*, lane 2: *Rosa canina*., lane 3: *Rosa chinensis*, lane 4: *Rosa damascena*, lane 5: *Rosa foetida*, lane 6: *Rosa ×hybrida* and lane 7: *Rosa moschata* and M: Weight marker.

A large part of inhibitor compounds in the first step was eliminated. In present procedure, β-mercaptoethanol and high concentration of EDTA in the extraction buffer, made some temporary pores in the cytoplasm membrane, so a noticeable portion of soluble polysaccharides, polyphenols and pigments were removed. These conditions have also been found to improve DNA quality in other plants (Dehestani and Kazemi Tabar, 2007; Xu *et al.*, 2004 and Zhang and Stewart, 2000). The use of high levels of PVP and β-mercaptoethanol in the clean up buffer had a considerable effect on neutralization of polyphenols and prevent oxidization of the secondary metabolites. These chemicals have also been used to improve DNA quality in cotton (Zhang and Stewart, 2000) and *Rosa roxburghii* (Xu *et al.*, 2004). CTAB is used as a detergent in the extraction buffer to separate polysaccharides from DNA. This reagent has improved DNA quality in medicinal and aromatic plants (Pirtillä *et al.*, 2001). Contaminants such as polysaccharides and polyphenols are removed by a clean up buffer containing NaCl at high concentration.

In our procedure, chloroform-isoamyl alcohol extraction of DNA at room temperature is enough to eliminate co-precipitation of proteins and polysaccharides. The combined use of sodium acetate and isopropanol at room temperature during the precipitation of DNA was found to be efficient in removing most of the secondary metabolites and polysaccharides from DNA. These chemicals were efficient in high quality DNA extraction of other plants (Pirtillä *et al.*, 2001 and Xu *et al.*, 2004). The procedure also eliminates the necessity of phenol, which makes the method less hazardous. Polyphenols are easily oxidized in the presence of phenol and covalently bind to nucleic acid firmly, which would greatly reduce the DNA yield (Khanuja *et al*., 1999 and Xu *et al.*, 2004).

In conclusion, considering extraction time, DNA yield and purity and PCR-ISSR amplifications this procedure emerged to be the best for molecular diversity analysis of roses.

Because different plants can vary considerably in the number and types of produced secondary compounds, it is unlikely that any one technique for isolating contaminant-free nuclear DNA will ever be developed. It is likely that our rose DNA isolation protocol can be used to isolate nuclear DNA from a variety of other plant species.

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