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An optimized mini-preparation method to obtain high-quality genomic DNA from mature leaves of sunflower

J.T. Li[1], J. Yang[1,2], D.C. Chen[1], X.L. Zhang[1] and Z.S. Tang[1]

[1]School of Life Sciences, China West Normal University, Nanchong, Sichuan, P.R. China
[2]Institute of Rare Animals and Plants, China West Normal University, Nanchong, Sichuan, P.R. China
Corresponding author: J. Yang
E-mail: yangjunlz@tom.com

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ABSTRACT. In order to investigate the mutation characteristics and to further examine the genetic variation of mutant sunflower (*Helianthus annuus*) obtained in plants grown from seeds exposed to space conditions, only the mature tissues such as leaf and flower could be used for DNA extraction after mutation characteristics were confirmed. The rich contents of polysaccharides, tannins, secondary metabolites, and polyphenolics made it difficult to isolate high-quality DNA from mature leaves of sunflower according to previous reports. Based on the comparison of the differences in previously reported protocols, a modified method for the extraction of high-quality DNA was developed. Using this protocol, the DNA isolated from sunflower was high in quality and suitable for restriction digestion *(Eco*RI, *Hin*dII, *Bam*HI), random amplified polymorphic DNA study and further molecular research. Therefore, the modified protocol was suitable for investigating the genetic variation of sunflower using mature leaf DNA.

Key words: CTAB, DNA extraction protocol, PVP, RAPD, Sunflower

INTRODUCTION

DNA-based assays such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism are the most widely used tools for the assessment of genetic variation (Singh et al., 1999; Belaj et al., 2002; Shan et al., 2005; Sarwat et al., 2006). All these techniques require a fair amount of DNA of good quality.

Rapid and efficient sunflower (*Helianthus annuus* L.) DNA extraction protocols have been previously developed (Brahm et al., 2000; Quagliaro et al., 2001; Yu et al., 2003; Horne et al., 2004). However, the materials used for DNA extraction were tender tissues in sunflower. Some mutant sunflower plants were obtained in plants grown from seeds exposed to space conditions. In order to investigate the mutation characteristics, especially the floral mutation characteristics, and to further analyze the genetic variation of these mutant plants, only the mature tissues such as leaf and flower could be used for DNA extraction after mutation characteristics were confirmed.

It is known that sunflower extract contains high concentrations of polyphenolics and other contaminants such as polysaccharides and tannins (Horne et al., 2004; Khan el al., 2004). If not eliminated, such contaminants could result in a genomic DNA sample of poor quality and quantity and of poor solubility, making it difficult to support polymerase chain reaction (PCR), genomic blot analysis, fingerprinting and other molecular analysis. Polyphenolics become oxidized and covalently bind to proteins and nucleic acids during the homogenization step of DNA extraction, making the DNA brown and unsuitable for most research application (Loomis, 1974; Guillemaut and Maréchal-Drouard, 1992; Porebski et al., 1997; Horne et al., 2004).

We tried to extract DNA from mature leaves of mutant sunflower according to previous reports, or even the latest modified protocols (Peterson et al., 1997; Aljanabi et al., 1999; Yu et al., 2003; Khan et al., 2004; Sarwat et al., 2006), but failed to obtain high-quality DNA. Thus, we compared various protocols of the common methods using cetyltrimethylammonium bromide (CTAB) and sodium dodecylsulfate (SDS) for DNA extraction from sunflower, and determined the differences among these methods to develop a method for extracting DNA from mature leaf. Therefore, an effective procedure was established for DNA extraction from sunflower; the protocol was efficient and gave high yields of clean DNA which was extremely pure and very suitable for molecular biological studies.

MATERIAL AND METHODS

Material

Mature leaves of mutant sunflower (*Helianthus annuus* L.).

Reagents and solutions

• Washing buffer: 100 mM Tris-HCl, pH 8.0; 50 mM ethylenediaminetetraacetic acid (EDTA); 1 M NaCl; 1% 2-mercaptoethanol; 1% polyvinylpyrrolidone (PVP) (k-30, S_{ABC})

• Extraction buffer: 2% CTAB; 1.42 M NaCl; 200 mM EDTA; 100 mM Tris-HCl, pH 8.0; 1% 2-mercaptoethanol; 1% PVP

- Chloroform-isoamylalcohol (24:1)
- Phenol-chloroform-isoamylalcohol (25:24:1)
- Ethanol (70% and absolute)
- TE buffer I: 0.1X Tris-EDTA (10 µL/mL RNase A (Calbiochem))
- TE buffer II: 0.1X Tris-EDTA

Modified protocol for genomic DNA extraction

The method consisted of the following steps:

1. Mature leaf samples of mutant and natural sunflower (600-700 mg) were harvested and placed in a mortar. The mortar including the pestle were wrapped in tinfoil, and then frozen rapidly at -80°C. The sample must be kept frozen for at least 2 h.

2. The frozen sample was taken out of the freezer, the leaves were crushed gently into pieces, and then ground to a fine powder.

3. The powder (200-300 mg) was transferred to a 1.5-mL tube. A volume of 1 mL washing buffer was added, and the tube was kept on ice with mixing for 5 min.

4. The tubes were centrifuged at 11,000 *g* for 10 min at 4°C.

5. The top aqueous layer was removed, and 700 µL preheated high-salt CTAB buffer (60-65°C) was added with uniform mixing to avoid clumping at the bottom.

6. The sample was incubated for 60 min at 65°C in a water bath. The samples were mixed by inversion 4-6 times during incubation.

7. The tubes were allowed to cool for 4-5 min, and then 500 µL chloroform/iso-amyl alcohol (24:1) was added. The tubes were then mixed well by inversion for 1 min and allowed to stand for 5 min at room temperature.

8. The tubes were centrifuged for 10 min at 11,000 *g*.

9. The top layer of about 750 µL was transferred to a new tube. Steps 7-9 were repeated once.

10. The aqueous phase was carefully transferred to a fresh tube, and 2 volumes of absolute alcohol were added. The tubes were mixed by gentle inversion to precipitate the DNA.

11. DNA was spooled out with a yellow plastic tip and put into a new 1.5-mL tube with 1 mL 70% ethanol to wash the DNA.

12. Step 11 was repeated once, DNA was placed in a fresh tube, centrifuged for an additional 1 min at 5000 *g*, and the remaining liquid was removed as much as possible with a pipette. The tubes were allowed to drain inverted to dry the pellet for 10 min at room temperature.

13. The DNA pellet was dissolved in 450 µL TE I by incubating for 1 h at 37°C.

14. An equal volume of phenol-chloroform-isoamyl alcohol (v/v, 25:24:1) was added to the tube. DNA precipitation was repeated once.

15. DNA was resuspended in 2.5 volumes of 100% absolute alcohol, with gentle mixing; there were some translucent floccules with some air bubbles present.

16. DNA was centrifuged for 30 s at 5000 *g*.

17. The top aqueous layer was poured off, and the DNA pellet washed twice with 70% ethanol.

18. The 70% ethanol was discarded, and 500 µL absolute alcohol was added, followed by mixing with gentle inversion for 1 min.

19. The tubes were centrifuged for 1 min at 5000 *g*, and the absolute alcohol was discarded.

20. The tubes were allowed to drain inverted, and the pellet was dried at 37°C for 15 min.

21. A volume of 150 µL TE II (RNase free) was added to dissolve the pellet and the DNA solution was stored at -20°C.

22. DNA quality was assessed by electrophoresis and spectrophotometry. The extracted DNA was subjected to three restriction enzyme digestions (*Eco*RI, *Bam*HI, *Hin*dIII) and to RAPD analysis.

Technical notes

1. The mortar and the pestle should be heated at 120°C for 40 min after clean out.

2. 2-Mercaptoethanol and PVP should be added to the washing buffer and CTAB buffer just prior to use. 2-Mercaptoethanol inhibits the oxidation of polyphenols, and PVP adsorbs polyphenols, thereby preventing their interaction with DNA (Loomis, 1974).

3. The leaf powder transferred to the tubes for DNA extraction should be less than 300 mg. If not, the larger amounts of polysaccharides, secondary metabolites and polyphenolics will be difficult to eliminate.

4. This protocol is partially based on the method described by Doyle and Doyle (1990).

5. DNA must be spooled out with a yellow tip and placed into a new 1.5-mL tube for washing after precipitation of the DNA with 2 volumes of absolute alcohol. Centrifugation should be avoided, lest the precipitated contaminants are recovered with the DNA, making them difficult to eliminate cleanly.

Quantity and purity of DNA

Purity of DNA was checked by means of absorbance ratios A260/A280 for protein contamination and A260/A230 for the presence of polyphenolic/polysaccharide compounds. Electrophoresis was used to determine the quantity of DNA extracted.

Analysis by random amplified polymorphic DNA

PCR analysis of RAPD was used for testing the fidelity of genomic DNA. PCR was performed using the Bio-Rad MyCycler™ PCR system (Bio-Rad, USA). PCR products were separated on 1.5% agarose gels, and then analyzed by Gel doc 2000 T_2A (Bio-Rad, USA).

Approximately 100 ng extracted DNA was used as a template for PCR amplification. A reaction mixture containing 1X reaction buffer, 0.75 mM $MgCl_2$, 100 ng DNA, 0.20 mM dNTPs, 100 nM RAPD primers, and 1.25 units *Taq* DNA polymerase (TaKaRa Ex Taq™ Hot Start Version) was prepared in a total volume of 25 µL.

RESULTS AND DISCUSSION

It is known that sunflower (*H. annuus*) contains high concentrations of polysaccharides, polyphenolics, tannins, and secondary metabolites (Horne et al., 2004). These secondary compounds

could hamper DNA isolation and further molecular analysis. For example, the presence of unusual substances could inhibit endorestriction enzymes (Khanuja et al., 1999). Therefore, these compounds must be eliminated during DNA isolation. When we extracted DNA from mature leaves of mutant sunflower according to previous reports or even the latest modified protocols (Peterson et al., 1997; Aljanabi et al., 1999; Yu et al., 2003; Khan et al., 2004; Sarwat et al., 2006), these compounds were not eliminated efficiently. Thus, the DNA extraction protocol for mature leaf of sunflower had to be modified. Based on the comparison of the CTAB method and SDS method (Figure 1), an efficient CTAB method for DNA extraction from mature leaf was obtained in part according to Doyle and Doyle (1990).

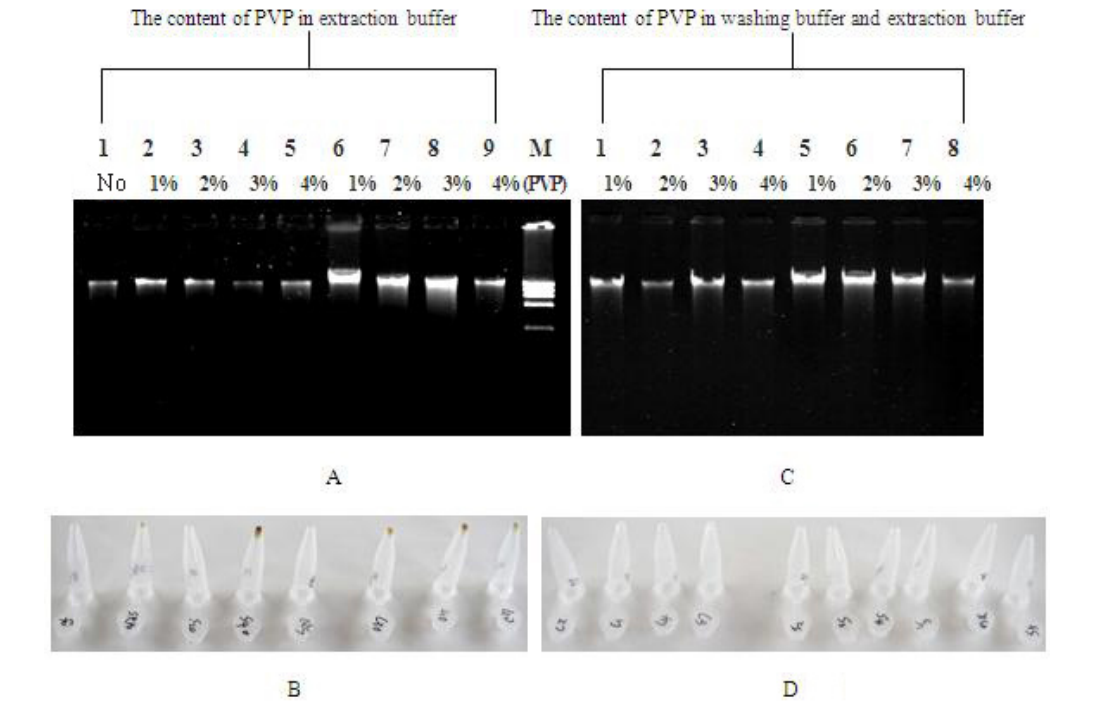

Figure 1. DNA was extracted using different protocols, and then 1 µL DNA was run at 80 V on a 0.7% agarose gel for 1 h to separation. **A,B.** Sunflower DNA extracted by means of a modified protocol without washing buffer. **C,D.** Sunflower DNA extracted by means of a modified protocol including the step of using washing buffer to wash powder. **A.** *Lanes 1-5*, SDS-based method; *lanes 6-9*, CTAB-based method; M = 1500-DNA marker. **C.** *Lanes 1-4*, SDS-based method; *lanes 5-8*, CTAB-based method. **B,D.** Open the lid and inverse it to dry pellet in tube. PVP = polyvinylpyrrolidone.

According to previous reports (Aljanabi et al., 1999; Hameed et al., 2004), PVP and 2-mercaptoethanol are absolutely necessary to eliminate the effect of polyphenol. In this protocol, 2-mercaptoethanol and different concentration of PVP were added to washing buffer and CTAB buffer, to test their capability of eliminating polysaccharides and polyphenols from tissues. Most polysaccharides, polyphenols and secondary metabolites could be removed by rinsing the tissue powder with washing buffer (Figure 1C,D), and 1% PVP was adequate to eliminate the compounds (Figure 1A,C). After this treatment, the extracted DNA was free of coloration and was lighter than that without washing in color (Figure 1C,D). To be mentioned, perfect results could be obtained with one washing. The problem arising from the presence of high levels of

polysaccharides could be overcome using NaCl at a higher concentration (1.42 M) and repeating the DNA extraction with phenol-chloroform-isoamyl alcohol (v/v, 25:24:1) after dissolving the pellet. Therefore, in this modified protocol, the following reagents were involved: washing buffer (1% PVP; 2% 2-mercaptoethanol), extraction buffer (2% CTAB; 1.42 M NaCl; 1% PVP; 2% 2-mercaptoethanol) and phenol-chloroform-isoamyl alcohol (v/v, 25:24:1).

Using the modified protocol (Figure 2A,B), 300-400 µg DNA was obtained from only a small amount tissue (200-300 mg) of mature leaf, 1.5-mL plastic tubes and tips were enough for the extraction process. Therefore, it is very convenient and highly efficient. The ratios of A260/A280 and A230/A260 were recorded as 1.80-1.89 and >2, respectively, which suggests that the preparations were free of proteins and polyphenolic/polysaccharide compounds.

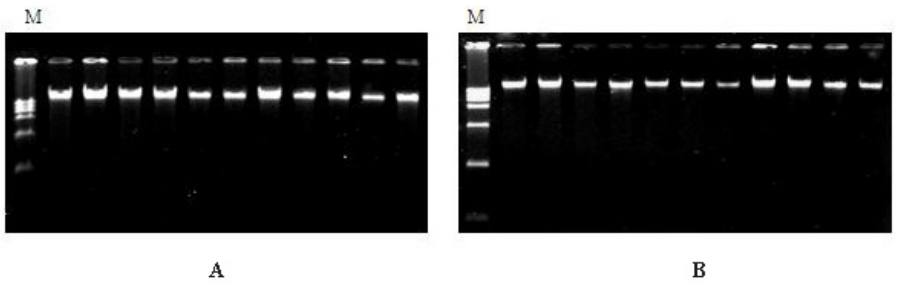

Figure 2. A,B. DNA were run at 80 V on a 0.7% agarose gel for 1 h for separation. DNA were extracted from varieties mature leaf of mutant sunflower with modified protocol. M: DNA marker DL15,000 (TaKaRa).

As the leaf sample, mortar and pestle were frozen prior to grinding, it was easy to grind the tissues into a very fine powder. The powder did not thaw for 5 min and did not stick to the porcelain mortar, so there was enough time to grind the samples. Using liquid nitrogen can produce the same result, but a liquid nitrogen source is needed during the grinding progress, which is sometimes not conveniently available in some situations. A porcelain mortar has several advantages over a glass one in hardness and non-stick surface. In this protocol, tissues and the porcelain mortar were frozen together prior to grinding.

It should be emphasized that the amount of mature leaf tissue used for DNA extraction should not exceed 300 mg per sample. Otherwise, it is difficult to effectively remove contaminants even when washing buffer, PVP and 2-mercaptoethanol are used in later steps (Figure 3).

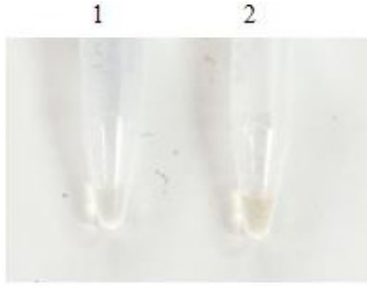

Figure 3. The extration DNA was dissolved with TE buffer II. **1.** Mature leaf tissue used for DNA extraction was under 300 mg. **2.** Mature leaf tissue used for DNA extraction was exceeding 300 mg. The color of the tube 1 is lighter than tube 2.

In this protocol, the extracted DNA was dissolved in 450 µL TE buffer I (containing 10 µg/mL RNase A), and then incubated for 1 h at 37°C for RNA digestion. The RNA was readily eliminated. It is necessary to extract the DNA once again after RNA digestion, using phenol-chloroform-isoamyl alcohol (v/v, 25:24:1). If not, the protein is excessive in the DNA solution.

After washing with 70% ethanol, DNA was washed again with absolute alcohol. This was helpful in removing water quickly, since the remaing ethanol was not easy to drain off after washing with 70% ethanol, and could affect the activity of restriction enzymes and PCR-based analysis. However, if DNA is washed again with absolute alcohol, it is easy to dry when placing in a 37°C incubator for a while, making the DNA easy to dissolve.

The purity and quality of isolated DNA were validated by restriction digestion and PCR amplification. It was found that the entire DNA could be digested completely by three different restriction enzymes (*Eco*RI, *Hin*dIII, *Bam*HI) (Figure 4). Furthermore, the DNA could be used as a sample for PCR, and the results of RAPD were very reliable (Figure 4). These results suggest that the DNA isolated was very pure and of high quality, and was suitable for further molecular research.

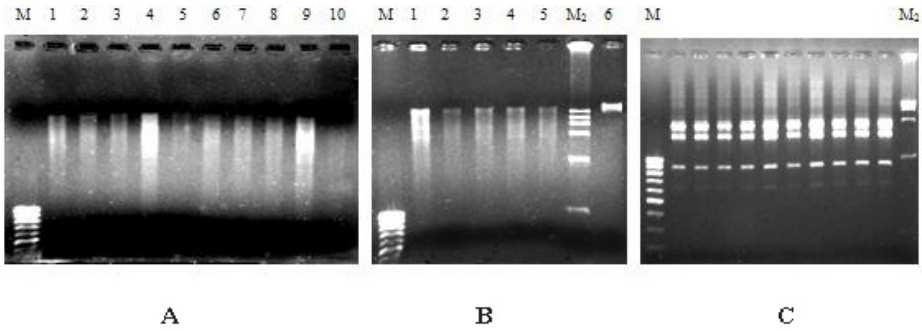

Figure 4. A. DNA was digested by two different restriction enzymes. *Lanes 1-5*. The DNA of different mutant sunflower were digested by *Eco*RI (TaKaRa). *Lanes 6-10*. The DNA of different mutant sunflower were digested by *Hin*dIII (TaKaRa). **B.** *Lanes 1-5*. The DNA of different mutant sunflower were digested by *Bam*HI (TaKaRa). *Lane 6*. Extraction genome DNA without digestion. **C.** Results of RAPD using the extracted DNA as sample. M: 100-bp DNA ladder (TaKaRa); M_2: DNA marker DL15,000 (TaKaRa).

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