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Full Length Research Paper

Small and large scale genomic DNA isolation protocol for chickpea (*Cicer arietinum* L.), suitable for molecular marker and transgenic analyses

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Chickpea is an important food legume crop with high nutritional value. Lack of appropriate DNA isolation protocol is a limiting factor for any molecular studies of this crop. The present report describes a rapid and efficient protocol for small and large scale preparation of superior quality and quantity of DNA from four cultivars (JG62, WR315, C235 and ICCV89314) compared to that of earlier reports. The yield of DNA through both the methods was estimated to be approximately 80 g per g of plant tissue. Both small and large scale preparations were essentially suitable for PCR and Southern blot hybridization analyses, which are the key steps in crop improvement programme through marker development and genetic engineering techniques.

Key words: *Cicer arietinum* L., phenolics, restriction enzyme digestion, PCR amplification, Southern hybridization.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important food legume, cultivated in over 40 countries. The emergence of plant transformation and molecular marker analyses in genome studies has greatly enhanced the speed and efficacy of crop improvement and breeding programme. A prerequisite for taking advantage of these methods is the ability to isolate genomic DNA of superior quality and quantity for analyzing through PCR, restriction enzyme digestion and subsequent Southern blot hybridization. To fulfill this criterion a rapid, simple and reliable DNA isolation method is highly solicited. Since size, content and organization of genome and contents of metabolites of different plant systems vary from each other to a great extent, a single DNA isolation protocol is not likely to be applicable for all plant systems (Loomis, 1974).

Chemotypic heterogeneity among species may not allow optimal DNA yield with a single protocol, thus even closely related species may require different isolation protocols (Weishing et al., 1995).

The isolation of good quality DNA from chickpea is complicated due to the presence of phenolic compounds, highly viscous polysaccharides and DNA degrading endonucleases. During tissue homogenization, phenolics become oxidized and irreversibly bind to the protein and nucleic acids (Loomis, 1974). This irreversible binding produces a gelatinous material, which is hard to separate from organelles and the DNA becomes unsuitable for PCR and restriction enzyme digestion analyses (Porebski et al., 1997). Polysaccharides are also problematic (Scott and Playford, 1996), as acidic polysaccharides inhibit digestion of lambda DNA by certain endonucleases like *Hin*dIII (Do and Adams, 1991) and classical 2-primer PCR amplification (Demeke and Adams, 1992; Pandey et al., 1996) by inhibiting *Taq* DNA polymerase activity (Fang et al., 1992), whereas, neutral polysaccharides are non-inhibitory (Do and Adams, 1991 and Pandey et al., 1996). In addition, polysaccharides can cause anomalous reassociation kinetics of DNA sample (Merlo and Kemp, 1976). They co-precipitate with DNA during alcohol precipitation to form a highly viscous solution (Do and Adams, 1991) making the DNA unsuitable for restriction enzyme digestion and Southern blot hybridization. The DNA tends to stick to the wells of the gel during

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Abbreviations: CTAB, Hexadecyltrimethylammonium bromide; ISSR, inter-simple sequence repeat; PCR, polymerase chain reaction; Tris, tris (hydroxymethyl)-aminomethane.

electrophoretic analysis. The contamination of polysaccharide can be overcome by increasing the volume of extraction buffer, thereby diluting the inhibitory polysaccharides (Pandey et al., 1996). However excessive dilution makes it inconvenient for further analysis. The problem of polyphenols and polysaccharides is exacerbated if green, over matured tissue is taken rather than etiolated leaves (Sharma et al., 2000).

In spite of several attempts we were unsuccessful to isolate DNA of consistent amount of good quality and quantity suitable for amplification through PCR and restriction analyses from both field and glass-house grown chickpea plants following the available methods (Dellaporta et al., 1983; Rogers and Bendich, 1985; Porebski et al., 1997; Sharma et al., 2002). Mature leaves have high level of polyphenols, polysaccharides and other secondary metabolites giving worse results, in case, young, expanding leaves and shoots are not available (Porebski et al., 1997). Thus it was necessary to devise an appropriate protocol for DNA extraction from chickpea. Here we describe simple, rapid, inexpensive and efficient large scale and small scale protocols that can be routinely used in PCR based molecular marker studies and analysis of transgenics through PCR, restriction enzyme digestion and subsequent Southern hybridization analysis.

MATERIAL AND METHODS

Plant samples for DNA isolation

Seeds of cultivars JG62, WR315, C235 and ICCV89314 were obtained from the ICRISAT Patancheru, India. Seeds were germinated in 25-30 cm diameter pots containing soil, sand and organic manure (6:3:1) in glass house and field. Plant tissue samples were collected from both field and glass house, frozen in liquid nitrogen and stored at -80ºC for future use. DNA was extracted from both fresh and stored tissue samples.

DNA isolation protocol

The plant material was ground in liquid nitrogen (2 g and 400 mg fresh tissue for large scale and small scale respectively). For large and small scale preparation the materials were transferred to 40 ml polypropylene tube and 2 ml polypropylene tube, respectively, and 5 ml and 1 ml of freshly prepared extraction buffer [100 mM Tris-Cl, pH 8.0, 25 mM EDTA, 1.5 M NaCl, 2.5% CTAB, 0.2% -mercaptoethanol (v/v) and 1% Polyvinylpyrrolidone, MW 40,000 (PVP) (w/v)] were added, respectively, and mixed by inversion to slurry. The mixture was incubated at 60ºC in a shaking water bath (100 rpm) for 30 min. Equal volume of phenol : chloroform (1:1) was added and mixed by gentle inversion for about 10 min. The mixture was spun at 7200 g for 5 min at 25–30ºC. Upper clear aqueous layer was carefully transferred to another tube. Equal volume of chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversion for about 5 min and spun at 7200 g for 5 min at 25–30ºC. The upper clear aqueous layer was transferred to another tube. One-tenth volume of 3 M Sodium acetate, pH 5.2 and double volumes of 100% chilled ethanol was added and allowed the mixture to stand at room temperature for 30 min. Fibrous nucleic acid was scooped and transferred to a 1.5 ml microfuge tube. Alternatively, after mixing with sodium acetate and ethanol, the samples can be centrifuged at 11200 g for 10 min. The supernatant was discarded and the pellet washed with 70% ethanol. The resultant pellet was dried in a vacuum for 15 min or by keeping the tube for 1-2 h inside the laminar airflow and dissolved in 400 µl (for large scale) and 40 µl (for small scale) of TE (10:1) buffer. 8 µl and 2 µl of RNase A (10 mg/ml) for large scale and small scale, respectively, were added and incubated at 37ºC for 30 min. The mixture was extracted with equal volume of phenol: chloroform (1:1). The aqueous layer was transferred to a fresh 1.5 ml microfuge tube and double volumes of 100% chilled ethanol was added, mixed and kept at -20º C for 20 min and spun at 11200 g for 10 min at 25–30ºC. The pellet was washed with 70% ethanol. The pellet was vacuum dried for 15 min or the tube may be kept for 1-2 h inside the laminar airflow for drying the pellet and dissolved in 200 µl and 30 µl of TE buffer for large scale and small scale preparation, respectively.

Monitoring of the quantity and quality of DNA

The yield of DNA per gram of tissue extracted was measured by running aliquots (2 µl) of DNA on an 0.8% agarose and comparing the band intensities with known standards by gel analysis software (Quantity one- 4.2.2, Bio-Rad) or monitoring the OD in Beckman Coulter UV-Vis spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. 2 µg of DNA samples were digested with *Eco*RI and *Hin*dIII at 37ºC for 10 h and electrophoresed on a 0.8% agarose gel in 1X TAE buffer containing 0.5 µg ml^{-1} of ethidium bromide and photographed by Bio-Rad gel doc system.

PCR amplification

Polymerase chain reactions for amplification of inter-simple sequence repeat (ISSR), using UBC primers no 807 and 818 (5'-AGA GAG AGA GAG AGA GT - 3' and 5' - CAC ACA CAC ACA CAC AG - 3'; the Nucleic Acid-Protein Service Unit, Biotechnology Laboratory University of British Columbia, Vancouver, BC, Canada) were carried out in a 25 µl volume for all DNA preparations. A reaction tube contained 1 X PCR reaction buffer (Roche Diagnostics Corporation) 50 ng DNA, 1 unit Taq DNA polymerase (Roche Diagnostics Corporation), 100 µM each of dNTPs, 1.5 mM $MgCl_2$ and 200 nM primer. PCR reaction conditions were 1 cycle of 5 min at 94ºC followed by 35 cycles of 1 min at 94ºC, 45 s at 40ºC and 1 min at 72ºC, then a final extension step of 10 min at 72ºC in My Cycler (Bio Rad). The amplified products were loaded in a 1.4% agarose gel and photographed by Bio-Rad gel doc system.

Southern blot analysis

Southern blot analysis was carried out according to Sambrook et al. (1989) with little modifications. 20 µg of chickpea genomic DNA digested individually with *Eco*RI, *Hin*dIII, and *Kpn*I and *Nicotiana tabacum* genomic DNA digested with *Hin*dIII were separated on a 0.8% agarose gel and blotted onto positively charged nylon membrane (Hybond N+) (Amersham Biosciences) following depurination, alkali denaturation and neutralization; hybridized overnight at 68ºC using [^{32}P] dCTP (Perkin Elmer) labeled PCR purified actin sequence of chickpea. For probe preparation ~600 bp actin sequence (NCBI Accession no. AJ012685) was PCR amplified using the above PCR conditions and chickpea specific primers (forward: 5' - CAT TAG GAA GGA TCT GTA TGG - 3', reverse: 5'-CTA GCC TTC ATG CTC TTA TCC-3') and eluted. After hybridization, the membrane was washed thoroughly using 2X SSC

(3 M Sodium Chloride and 0.3 M Tri sodium citrate, dihydrate) and 0.1% Sodium dodecyl sulphate (SDS) at room temperature for 1 h and at 68ºC for another hour using 0.1X SSC, 0.1% SDS and then exposed to Kodak X-ray film, stored at -80ºC for 7 days and subsequently developed.

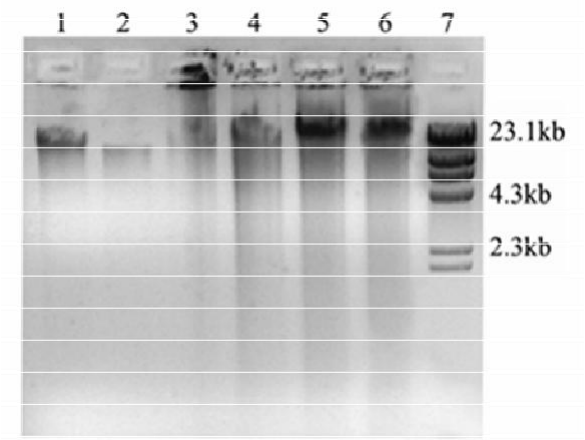

Figure 1. Electrophoretic analysis of total DNA isolated from chickpea cultivar ICCV89314 by six different methods. Lane 1, Rogers and Bendich (1985); lane 2, Dellaporta et al. (1983); lane 3, Sharma et al. (2002); lane 4, Porebski et al. (1997); lane 5, large scale isolation (this work); lane 6, small scale isolation (this work); lane 7, HindIII digested lambda DNA marker.

RESULTS AND DISCUSSION

Comparative analysis of earlier reported and presently described protocols

The small scale and large scale methods described here can be used consistently to obtain good quality of genomic DNA from chickpea. We used a modified CTAB method, the key steps of which are (1) extraction with high salt CTAB buffer to remove polysaccharides, (2) use of -mercaptoethanol (0.2%) and PVP (1%) to remove polyphenolic compounds, (3) phenol:chloroform extraction to remove proteins (4) chloroform:isoamyl alcohol extraction to remove remaining phenols. Genomic DNA was isolated from tissues of glass house and field grown plants of 4 cultivars using the presently described small scale and large scale methods as well as several published protocols and the efficiency were compared.

The total genomic DNA isolated by methods described by Rogers and Bendich (1985) and Dellaporta et al. (1983) provided intact DNA but yield was low while Sharma et al. (2002) and Porebski et al. (1997) methods produced large amount of sheared DNA (Figure 1). On the contrary, DNA isolated by presently described method produced good quality and high quantity of intact DNA. The DNA yield of both the methods from 4 cultivars of chickpea was approximately 80 g per g of tissue. The $A_{260/280}$ ratio was 1.6-1.8 indicating the absence of contaminants (Pich and Schubert, 1993). The purity of the DNA was confirmed by means of complete *Eco*RI and *Hin*dIII digestion and monitoring the banding profile of the digested DNA after incubating the reaction mixture at 37ºC for 10 h (Figure 2). This indicated that isolated DNA was amenable for further downstream applications.

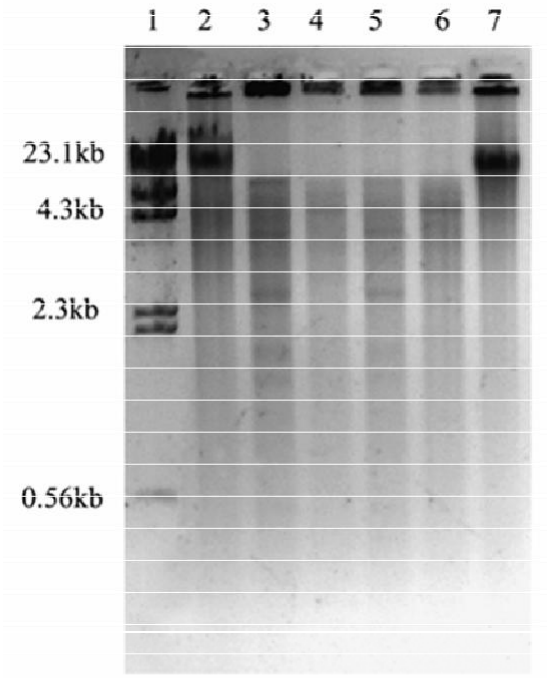

Figure 2. Restriction digestion analysis of chickpea cultivar ICCV89314 DNA. Lane 1, *Hin*dIII digested lambda DNA marker; lane 2, Uncut DNA isolated by large scale method; lanes 3 and 4, large scale DNA digested with *Eco*RI and *Hin*dIII respectively; lanes 5 and 6, small scale DNA digested with *Eco*RI and *Hin*dIII respectively; lane 7 Uncut DNA isolated by small scale method.

Qualitative analyses of DNA through PCR and Southern blot analyses

Using DNA of present protocols in PCR amplification with the ISSR primers further proved about the authenticity of the DNA quality. The results showed the distinct amplification of genomic DNA at the molecular weight range of 500 bp to 3 kb (Figure 3). Reproducible amplification was observed in PCR reaction in several independent extractions and replicates.

In Southern hybridization using ~600 bp actin sequence as probe, positive signals were obtained for all three digestions while no signal was visible in case of digested *Nicotiana tabacum* genomic DNA which was used as

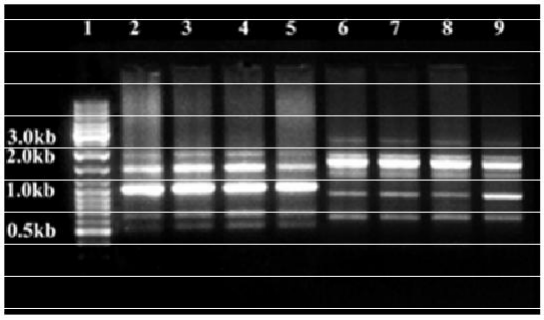

Figure 3. Electrophoretic analysis using 1.4% agarose gel of PCR amplified ISSR products using UBC primers and template of DNA of cultivars JG62, WR315, C235 and ICCV89314.Lane 1 gene ruler marker; lanes 2-5, amplification by UBC primer 818; lanes 6-9, amplifiication by UBC primer 807.

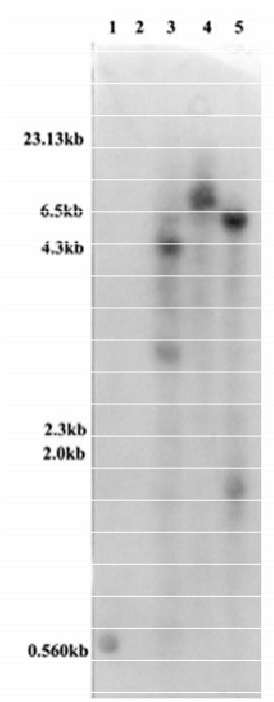

Figure 4. Southern hybridization developed on film after probing the digested total DNA with ~600 bp chickpea specific actin coding sequence. Lane 1, eluted PCR amplified ~600 bp actin coding sequence as positive control; lane 2, *Hin*dIII digested total DNA from *Nicotiana tabacum* as negative control; lanes 3,4 and 5, *Eco*RI, *Hin*dIII and *Kpn*I digested total DNA from chickpea cultivar ICCV89314.

negative control (Figure 4). Both *Eco*RI and *Kpn*I digested DNA generated two bands due to the presence of their cutting sites within the actin sequence. Southern hybridization requires high quality, intact genomic DNA free from any polysaccharides, phenolics or other inhibitors as contaminants, which might lead to the shea ring of DNA, unsuitable for restriction digestion and Southern hybridization.

CONCLUSION

Thus, we conclude that present protocol describes a reliable, rapid (can be completed within 3 h), simple and consistent DNA isolation method for chickpea that yields large amount of pure, intact DNA amenable for restriction digestion, PCR and Southern hybridization analyses as compared to the previous reports (Sharma et al., 2002; Mace et al., 2003) without any ultra centrifugation or column purification steps. The small scale method can be used for PCR based marker studies and screening of transgenics whereas the large scale method is ideal for Southern hybridization analysis.

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