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High Quality DNA Isolation Method for Chickpea Genotypes

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Abstract: In chickpea breeding genetic studies of individual plants need to be evaluated at the DNA level using molecular markers. A simple and reliable DNA extraction method is a prerequisite. This small-scale method is cetyltrimethylammonium bromide (CTAB)-based and extracts DNA from 1 to 3 folded young leaves processed in a 1.5 ml tube with 0.5 ml of extraction buffer and homogenized using an electric drill. Compared with the micro-prep method the improved mini-prep CTAB method is highly efficient and much cheaper in terms of time, chemical use and labor input. About 49 samples per day can easily be processed by one person. The DNA yield is greater (60 µg per 50-100 µg of fresh leaf tissue) than that obtained from the micro-prep method (50 mg from 5 g of fresh leaf tissue). High quality DNA was obtained and used successfully for restriction endonuclease digestion and polymerase chain reaction amplifications using the mini-prep CTAB method.

Key Words: Chickpea, DNA isolation, Molecular markers

Abbreviations: CTAB, Cetyltrimethylammonium bromide; ISSR, Inter simple sequence repeat; PCR, Polymerase chain reaction; RAPD, Random amplified polymorphic DNA; SSR, Simple sequence repeat.

Nohut Genotiplerinde Yüksek Kalitede Saf DNA İzolasyon Metodu

Özet: Moleküler markırlardan faydalanılarak ekonomik açıdan önemli birçok bitkinin ıslah edilmesi, günümüzde giderek önemi artan markır teknolojisinin ve markır yardımıyla seleksiyon uygulamalarının, artık rutin çalışmalar olabileceği konusunu gündeme getirmiştir. Bu nedenle, öncelikli olarak çalışmalarda kullanılacak ana materyal olan DNA'nın, kısa sürede ve saf olarak eldesi son derece önemlidir. Bunun için de güvenilir bir DNA izolasyon metodunun belirlenmesi gerekmektedir. Bu çalışmanın amacı, nohut ıslahında ve diğer genetik çalışmalarda kullanılacak olan DNA'nın, izolasyonunda mini-prep CTAB metodunun önemini kanıtlamaktır. Bu metod ile, tarla ve sera şartlarında yetiştirilen 49 nohut (Cicer spp.) genotipinin taze yaprak eksplantlarından alınan örnekler, öncelikli olarak içerisinde 0.5 ml ekstraksiyon buffer bulunan 1.5 ml'lik reaksiyon tüpü içinde elektrikli öğütücü ile homojenize edilerek suspansiyon sağlandı ve az sayıda olan materyalden fazla miktarda ve yüksek kalitede DNA izolasyonu yapıldı. Mini-prep metodu ile mikro-prep metodu karşılaştırıldığında mini-prep CTAB metodunun, hem zaman açısından hem de maliyet, iş gücü ve kimyasal malzeme kullanımı açısından son derece ekonomik olduğu da isbatlandı. Mini-prep CTAB metodu ile izole edilen DNA, UBC181 RAPD primeri kullanılarak PCR ile amplifiye edilerek izolasyon sonrası, uygun saf DNA'nın elde edildiği gözlenen bant profilleri ile de kanıtlanmış oldu.

Anahtar Sözcükler: Nohut, DNA izolasyonu, Moleküler marker

Introduction

The emergence of molecular marker analyses in genome studies has greatly enhanced the speed and efficiency of crop improvement and breeding. A prerequisite for taking advantage of these methods is the ability to isolate DNA of adequate quality and quantity. With the development of polymerase chain reaction (PCR) technology, molecular markers based on PCR soon found a wide application in plant genetics and breeding (1). To accommodate the need for PCR-based markers, a rapid, simple and reliable DNA preparation method is required to provide DNA for the analyses. Although numerous DNA extraction methods for plants have been reported in the literature, the CTAB extraction method, mini-prep, is used most often (2-4). The traditional DNA macro-preparation method usually requires a minimum of 0.5 g of plant tissue, making it impractical to analyze individual plants during the early seedling stage. In addition, the methods are time consuming and laborious due to their multi-step procedures. Furthermore, large amounts of hazardous chemical solvents are required. Modifications have been made for plant species such as chickpea, lentil, pea, soybean and barley, which are high in polysaccharides. These modified methods are usually employed to remove

polysaccharides (5). b-mercaptoethanol was found to improve extracted DNA quality (6). For PCR-based DNA markers used in marker-assisted selection a fast DNA extraction method is needed. In recent years, significant progress has been made in the use of molecular marker technology for plant breeding. In addition to their use in plant breeding, molecular markers offer specific advantages in genome mapping, DNA fingerprinting and the study of genetic diversity. PCR-based molecular markers (RAPDs, ISSRs, STMSs etc.) are preferred to hybridization markers like RFLPs for marker-assisted selection because they permit the breeder to use smaller amounts of more crudely prepared DNA from each plant being genotyped, thus reducing the time, labour and operational costs of DNA extraction (7). Molecular markers closely linked to numerous traits of economic importance have been developed in several crop plants (8) and will allow indirect selection for desirable traits in genotypes. Indirect selection is very effective because of the absence of a confounding effect of the environment, and also allows pyramiding of genes for characteristics, which is difficult to achieve through the use of conventional methods of plant breeding (9). Mini-prep methods require only a small amount of tissue, and use minimal numbers and amounts of chemicals. Furthermore, by this method is high-quality DNA in large quantities are extracted. Many mini-prep methods for obtaining DNA have been developed, including such modifications as no grinding and no centrifugation (10). To show the success of the presented CTAB mini-prep method, DNA samples were isolated from 49 chickpea genotypes by purification through a CsCl ethidium bromide gradient and the described mini-prep procedure. The DNA isolated by this CTAB mini-prep method compared favorably to the control CsCl cleaned DNA for use in RAPD reactions (11).

The present study reports a rapid, simple, reliable and inexpensive method for isolating DNA from chickpea genotypes, yielding DNA in a quantity and quality suitable for DNA marker analysis.

Materials and Methods

Plant materials

We successfully experimented with mature leaves of field and greenhouse grown plants (Table). However, for breeding applications, leaf tissue is preferred because its collection is the least destructive. Fresh leaf tissue was collected and placed on ice. Tissue can be used immediately or stored at -80 °C. Freeze-dried tissue can be used, but is not recommended because DNA yields are reduced. Fresh tissue (1 g) was used for the isolation. The tissue was frozen in liquid nitrogen and ground in a mortar and pestle. The ground tissue was subsequently suspended in 0.25 M sucrose, 0.03 M Tris and 0.05 M EDTA. The homogenate was then centrifuged at 500xg (2200 rpm) for 15 min.

DNA Extractions

Micro-prep method: Fresh tissue samples (growing buds) were collected into labeled 1.5 ml microfuge tubes and then mixed with 1.5 ml of extraction buffer per sample [0.1 M Tris-HCl, pH 8.0; 1.0 M NaCl; 0.02 M EDTA, pH 8.0; 2% (w/v) CTAB; 2% (w/v) polyvinlypyrrolidone-40; 0.4% β-mercaptoethanol] and incubated for 1 h at 65 °C. The solution was then twice extracted with equal volumes of chloroform:isoamyl alcohol (24:1) and centrifuged at 12, 000 x g at 4 °C for 10 min. The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with an equal volume of cold ethanol and kept at -20 °C overnight. The DNA was then spooled out and washed with 75% ethanol for 20 min with gentle shaking. The DNA pellet was air dried and dissolved in 5 ml of sterile TE buffer (Tris-EDTA buffer) by incubation at 60 °C for 20 min. The DNA was then subjected to an additional cleaning procedure. The cleaned DNA pellet was resuspended in Tris-EDTA buffer containing 1 µl of RNase (10 mg/ml)/100 µl of TE.

Mini-prep method: A g tissue sample was removed from the -70 °C ultracold freezer and placed on dry ice. The sample was immersed in liquid nitrogen and ground to a fine powder with a mortar and pestle, and the ground powder was transferred to a 50 ml corning tube containing 7.5 ml of ice cold extraction buffer. The tube was then capped and briefly shaken. To the tubes were added 7.5 ml of nuclei lysis buffer and 3 ml of 5% sarkosyl. After incubation at 65 °C for 20 min to 2 h, 18 ml of chloroform:isoamyl alcohol (24:1) was added and the tube was centrifuged at 500 x g and 4 °C for 15 min. The supernatant was transferred to a fresh tube and mixed with 15 ml of chloroform:isoamyl alcohol solution. After sequential washing with 75% and 95% ethanol, the DNA pellet was air dried and suspended in 5 ml of TE buffer, to which was added 5 µl of RNase. The DNA yield and quality were estimated by absorbance spectra at 260 nm. Genomic DNA was then electrophoresed on 1% and 1.4% agarose gels.

Table Chickpea genotypes used in DNA isolation reactions.

S. No	Species	Accession	Description	Origin
1	*C. arietinum*	AKN33	resistant to Ascochyta blight	CIFC
2	*C. arietinum*	AKN42	resistant to Ascochyta blight	CIFC
3	*C. arietinum*	AKN98	resistant to Ascochyta blight	CIFC
4	*C. arietinum*	AKN99	resistant to Ascochyta blight	CIFC
5	*C. arietinum*	AKN102	resistant to Ascochyta blight	CIFC
6	*C. arietinum*	AKN144	resistant to Ascochyta blight	CIFC
7	*C. arietinum*	AKN145	resistant to Ascochyta blight	CIFC
8	*C. arietinum*	AKN146	resistant to Ascochyta blight	CIFC
9	*C. arietinum*	AKN147	resistant to Ascochyta blight	CIFC
10	*C. arietinum*	AKN148	resistant to Ascochyta blight	CIFC
11	*C. arietinum*	AKN395	resistant to Ascochyta blight	CIFC
12	*C. arietinum*	AKN411	resistant to Ascochyta blight	CIFC
13	*C. arietinum*	AKN426	resistant to Ascochyta blight	CIFC
14	*C. arietinum*	AKN568	resistant to Ascochyta blight	CIFC
15	*C. arietinum*	87AK71114	resistant to Ascochyta blight	AARI
16	*C. arietinum*	ESER87	resistant to Ascochyta blight	AARI
17	*C. arietinum*	İZMİR92	resistant to Ascochyta blight	AARI
18	*C. arietinum*	MENEMEN	resistant to Ascochyta blight	AARI
19	*C. arietinum*	DAMLA89	resistant to Ascochyta blight	AARI
20	*C. arietinum*	GÖKÇE	resistant to Ascochyta blight	AARI
21	*C. arietinum*	KÜSMEN99	resistant to Ascochyta blight	AARI
22	*C. arietinum*	ER99	resistant to Ascochyta blight	AARI
23	*C. arietinum*	UZUNLU99	resistant to Ascochyta blight	AARI
24	*C. arietinum*	AKÇİN91	resistant to Ascochyta blight	AARI
25	*C. arietinum*	SARI98	resistant to Ascochyta blight	AARI
26	*C. arietinum*	AYDIN92	resistant to Ascochyta blight	AARI
27	*C. arietinum*	AZİZİYE94	resistant to Ascochyta blight	AARI
28	*C. arietinum*	AKN26	susceptible to Ascochyta blight	AARI
29	*C. arietinum*	AKN63	susceptible to Ascochyta blight	AARI
30	*C. arietinum*	AKN78	susceptible to Ascochyta blight	CIFC
31	*C. arietinum*	AKN79	susceptible to Ascochyta blight	CIFC
32	*C. arietinum*	AKN88	susceptible to Ascochyta blight	CIFC
33	*C. arietinum*	AKN89	susceptible to Ascochyta blight	CIFC
34	*C. arietinum*	AKN564	susceptible to Ascochyta blight	CIFC
35	*C. arietinum*	AKN566	susceptible to Ascochyta blight	CIFC
36	*C. arietinum*	AKN567	susceptible to Ascochyta blight	CIFC
37	*C. arietinum*	AKN570	susceptible to Ascochyta blight	CIFC
38	*C. arietinum*	AKN587	susceptible to Ascochyta blight	CIFC
39	*C. arietinum*	AKN589	susceptible to Ascochyta blight	CIFC
40	*C. arietinum*	AKN591	susceptible to Ascochyta blight	CIFC
41	*C. arietinum*	AKN592	susceptible to Ascochyta blight	CIFC
42	*C. arietinum*	AKN599	susceptible to Ascochyta blight	CIFC
43	*C. arietinum*	ESKVD17	susceptible to Ascochyta blight	AARI
44	*C. arietinum*	ESKVD18	susceptible to Ascochyta blight	AARI
45	*C. arietinum*	ESKVD45	susceptible to Ascochyta blight	AARI
46	*C. arietinum*	CANITEZ	susceptible to Ascochyta blight	AARI
47	*C. arietinum*	KIRMIZI NOHUT	susceptible to Ascochyta blight	AARI
48	*C. arietinum*	FLIP 84-92C(3)	resistant to Ascochyta blight	WSU
49	*C. reticulatum*	PI 599072	susceptible to Ascochyta blight	WSU

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PCR Analysis

PCR amplifications were performed in 10 mM Tris-HCl, pH 8.0; 50 mM KCl; 0.1% (v/v) Triton X-100; 1.5 mM $MgCl_2$; 100 µM dNTP; 0.24 µM primer; 20 ng of DNA and 1 unit of Taq DNA polymerase per 25 µl reaction using RAPD primer (UBC181-ATGACGACGG) (Invitrogen, England). RAPD reactions were performed with the following cycle repeated 40 times: denaturing at 94 °C for 20 s, annealing at 36 °C for 1 min with a ramp to 72 °C and elongation at 72 °C for 1 min. The final elongation segment was held for 8 min. PCRs were carried out in a PTC-100 thermocyler (MJ Research, USA). The amplification products were resolved by electrophoresis in 2% agarose gels. The bands were visualized using ethidium bromide staining.

Results

Genomic DNA from chickpea cultivars, breeding lines and parents was isolated using the mini-prep and micro-prep methods. In the mini-prep method, the yield averaged 150 µg of DNA/1 g of leaf tissue. DNA samples isolated using this method were amenable to PCR amplifications, and were used to initiate RAPD marker studies of chickpea genotypes (Figure). RAPD analysis employing 10 other base primers gave equivalent results for the methods of DNA preparation. A CTAB-based mini-prep method for DNA extraction process is undertaken in 1.5 ml tubes with 0.5 ml of the respective solutions. The DNA is separated by centrifugation. Furthermore, very high DNA yield and quality that can be used for PCR analysis are obtained. We describe a simple and efficient method for genomic DNA extraction from chickpea genotypes. Recently, the procedure was used to isolate DNA from samples of hundreds of accessions.

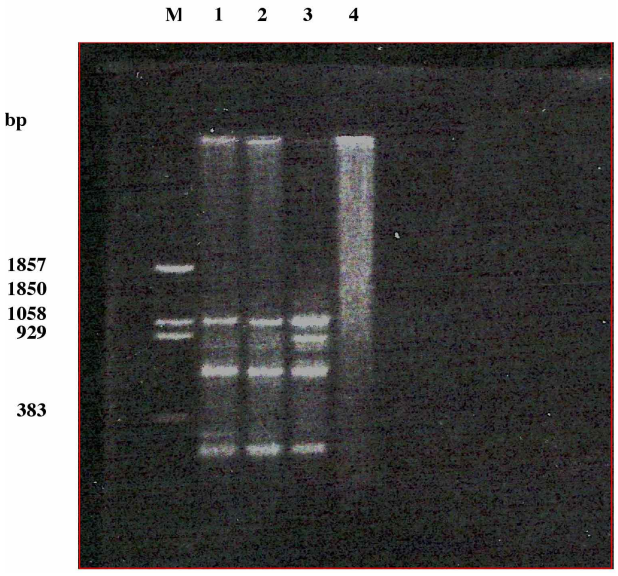

Figure. Single primer (UBC181) PCR amplification of mini-prep DNA from chickpea genotypes. Lane 1: M. Marker, pBR322BstN1 digest, Lane 2: FLIP84-92C(3), Lane 3: PI599072 (*Cicer reticulatum* L.), Lane 4: Akçin91, Lane 5: Landrace -Kırmızı Nohut (*Cicer arietinum* L.).

Discussion

The CTAB mini-prep method is rapid and yields DNA sufficiently pure for RAPD fingerprints. RAPD markers are generated by PCR amplification of random genomic DNA segments with single primers in an arbitrary sequence (12). They are usually dominant markers with polymorphisms between individuals defined as the presence or absence of a particular RAPD band (13). In performing population studies using RAPD, the time consuming step is often isolating DNA from numerous samples. For researchers using amplification techniques on hundreds of plant DNA samples, large yields are likely to be less important than speed and cost of sample preparation. This procedure, with minor modifications in equipment, may useful for DNA extractions in the field, and it can be directly applied to many different plants. Various approaches and protocols were developed to address the problem (for example, 3, 12, 14-16). The advantage of our mini-prep method is that leaf samples can be collected at any time during the growing season, as long as leaf buds are available, even in the late season when plants are infested with diseases. Originally, an initial mini-prep method was introduced and used to prepare soybean DNA directly from leaf disks for SSR analysis without DNA quantification. We successfully used DNA extracted by this method for RAPD analysis of isolated genes responsible for resistance. All the DNA templates produced clear, sharp and reproducible PCR banding patterns. Our method is comparable to conventional plant DNA isolation methods in terms of the speed of isolation, requiring 3-4 h from the fresh tissue up to the final DNA resuspension. We have found that this method generates DNA that yields more reproducible results in the RAPD system than does DNA generated by other isolation methods.

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