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Influence of Two Genomic DNA Extraction Methods on DNA Quantity and Quality Extracted from Barley in Jordan

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Abstract: Two methods were evaluated for the extraction of DNA from barley (*Hordeum vulgare* L.). A CTAB-based method and a nuclei isolation and extraction method. Results showed that barley DNA extracted by nuclei genomic DNA method was of a High Molecular Weight (HMW) with a very slight shearing. Whereas, DNA extracted by CTAB method gave a High Molecular Weight (HMW) with the heaviest DNA shearing. All nuclei DNA extracted samples did not show RNA contamination. However, all CTAB extracted samples, showed RNA contamination. Results obtained from the gel of Pulse Field Gel Electrophoresis (PFGE) and Agarose gel electrophoresis were almost similar except for High-Lo™ DNA marker (60-10000 bp) that did not appeared in PFGE due to the long period of the run. Furthermore, the two DNA extraction procedures seemed to be simple and efficient in extracting genomic DNA. But, the nuclei method is more effective in extracting intact and HMW of genomic DNA.

Key words: Barley (*Hordeum vulgare* L.), DNA extraction, CTAB, cetyltrimethylammonium bromide, nuclei isolation, PFGE

INTRODUCTION

Barley is one of the most important field crops in Jordan with annual average production during the last year of 34524.2 metric tons (FAOSTAT, 2005). Rapid progress in cultivar improvement through traditional breeding methods is restricted because the process is costly and labor intensive. Therefore, molecular breeding approaches provide additional tools in the improvement of these field crops. The cloning of important genes requires the extraction of large insert genomic DNA. The isolation of intact, high-molecular-mass genomic DNA is essential for many molecular biology applications including long PCR, endonuclease restriction digestion, Southern blot analysis and genomic library construction (Michiels *et al.*, 2003). Many protocols are available for the extraction of DNA from plant material. A cetyltrimethylammonium bromide (CTAB) protocol has been developed for isolation of genomic DNA. In a more commonly used procedure, polysaccharide co precipitation is avoided by adding a selective precipitant of nucleic acids, i.e., cetyltrimethylammonium bromide (CTAB) to keep polysaccharides in solution (Doyle and Doyle, 1987; 1990). Several successful DNA extractions methods for plant species containing polyphenolic compounds and polysaccharides have been developed (Tel-Zur *et al.*, 1999; Peterson *et al.*, 1997; Aljanabi *et al.*, 1999; Burr *et al.*, 2001) and its modifications (Huang *et al.*,

2000). Some publications demonstrated the use of hydrolytic enzymes (Rether *et al.*, 1993) or ion-exchange resins (Guillemaut, 1992; Marechal and Guillemaut, 1995) to remove polysaccharides from nucleic acid solutions. CTAB DNA purification methods extract high quantities of pure DNA from a variety of different plant tissues, e.g., cotton, blackcurrant, ferns, fruit trees and conifers (Woodhead *et al.*, 1998; Dempster *et al.*, 1999; Kim *et al.*, 1997).

A simple and rapid method based on CTAB extraction for isolating high-quality intact DNA was established by modifying existing methods. With this technique, the absorbance ratio (A₂₆₀/A₂₈₀) of DNAs obtained from fresh and/or dried roots of medicinal plant (ginseng) tissues enriching with polyphenols and polysaccharides was 1.8 approximately. The results demonstrated that the modified technique was efficient and reliable in isolating high-quality and high-molecular-weight DNAs (Luo *et al.*, 2001). Improved CTAB method, low pH medium with high salt method (LPHS) and urea method were used for DNA extraction. These methods produced high quantity and quality of DNA (Zhang *et al.*, 2004). A quick, simple and reliable CTAB DNA extraction method (method is a modification of Doyle and Doyle (1990). Modified by the use of NaCl to remove polysaccharides and PVP to eliminate polyphenols during DNA purification. Results showed that DNA yield from this procedure is high (up to 1 mg g⁻¹ of leaf tissue) (Muhammad *et al.*, 1994).

Rogers and Bendich (1988) obtained low yields of grapevine DNA with a CTAB-based extraction procedure. Collins and Symons (1992) developed a method that could successfully isolate nuclear DNA from fresh leaves of grapevine, but it was very arduous and the yield was also low. Four DNA extraction protocols (two protocols represented classical procedures and two were a combination of classical CTAB lysis followed by anion exchange chromatography) were evaluated for ability to extract DNA from several species: oak, elm, pine, fir, poplar and maize (fresh materials). Test results indicated superiority of the combination treatment which enabled DNA extraction from all seven species (Csaikli *et al.*, 1998).

Unlike conventional DNA preparation like the CTAB method, the mega base DNA must be protected from physical shearing during preparation. One of the popularly used approaches for doing so is to isolate protoplasts (plants), cells (animals), or nuclei (plants and animals) and then embed them in low-melting-point (LMP) agarose in the form of plugs or micro beads (Zhang, 2000). The agarose acts as a solid yet porous matrix, which allows the diffusion of various reagents for DNA purification and subsequent manipulations while preventing the DNA from being sheared (Schwartz and Cantor, 1984). Although the protoplast method yields mega base-size DNA of high quality, the process is costly and labor intensive (Ganal and Tanksley, 1989). However, recently developed nuclei method works well for several divergent plant taxa for its simplicity and cost-effectiveness to isolate high molecular weight DNA (Zhang *et al.*, 1995; Zhang, 2000). The predominant problems involved in trying to isolate plant nuclear DNA are ones that animal researchers do not typically encounter. For example, (a) plant cell walls must be physically broken or enzymatically digested without damaging nuclei, (b) chloroplasts must be separated from nuclei and/or destroyed, (c) volatile secondary compounds such as polyphenols must be prevented from interacting with the nuclear DNA and (d) carbohydrate matrices that often form after tissue homogenization must be prevented from trapping nuclei (Peterson *et al.*, 1997). Large amounts of polyphenolics in cotton leaves make it difficult to obtain high quality genomic DNA during extraction. A procedure to isolate nuclear DNA was developed. It consists of rapid isolation of stable nuclei, which hinders covalent interactions with phenolics, followed by DNA extraction. The yield and quality of the resulting DNA is satisfactory and the protocol can be scaled up or down according to sample size (Chaudhry *et al.*, 1999). Hein *et al.* (2005) developed a novel nuclei extraction method that allows for the

extraction of high molecular weight DNA from leaves of woody perennial soft-fruit species that contain high levels of carbohydrates and polyphenolics. It utilized a modified buffer system including 4% (w/v) (PVP)-10 and a combination of nylon filters and Percoll gradients to purify nuclei extracts prior to embedding in agarose plugs. Extracted DNA was of high quantity and quality suitable for bacterial (BAC) library construction. Wang *et al.* (1996) adapted the procedure of isolating nuclear DNA from the silica-gel dried leaves with the following improvements (as high as 6% PVP and 2% β -mercaptoethanol were employed to avoid oxidation of polyphenols and 2.5 M NaCl effectively removed polysaccharide rides). They were able to isolate nuclear DNA from the silica-gel dried leaves. Nuclei were separated by low-speed centrifugation (4000 g) from cytoplasm containing secondary compounds. Most genomic DNA extraction protocols require the use of liquid nitrogen and/or freeze-drying (lyophilization). A very simple, fast, universally applicable method to extract high quality mega base genomic DNA was applied to extract complex genomic DNA from different (fresh tissues) from wheat, barley, potato, beans, pear and almond leaves, fungi, insects and shrimps. The amount of tissue required by this method is 50-100 mg. The quantity and the quality of the DNA extracted by this method were very high (Salah and Iciar, 1997). The objectives of this study are:

- To evaluate the CTAB and the nuclei isolation and extraction methods in extracting high molecular weight (HMW) genomic DNA.
- To compare the two methods.
- To detect differences and/or similarities between the results obtained by Pulse Field Gel Electrophoresis (PFGE) and the Agarose Gel Electrophoresis.

MATERIALS AND METHODS

Plant materials: The certified barley (*Hordeum vulgare* L.) cultivar ACSAD 176 was used in this study. Seeds were germinated and grown in polystyrene tray under greenhouse conditions. One month old, fresh and healthy leaves were used in nuclei isolation and high molecular weight DNA preparation. The upper third of leaves blade was cut and used.

Reagents: SEB (Sucrose-based Extraction Buffer): 10% v/v TKE (0.1M Tris, 1.0 M KCl, 0.1 M EDTA, pH 9.1), 500 mM sucrose, 4 mM spermidine, 0.1% w/v ascorbic acid, 2.0% w/v PVP (polyvinylpyrrolidone. MW 40, 000) and 0.13% w/v sodium diethyldithiocarbamate.

SEB^{+BME}: 0.2% v/v β -mercaptoethanol (BME) in SEB. β -mercaptoethanol was added just before use.

Lysis Buffer: 1% w/v sodium lauryl sarcosine, 0.1 mg mL⁻¹ proteinase K, 0.1% w/v ascorbic acid, 6 mM EGTA (Ethylene glycol tetra acetic acid), 200 mM L-lysine and 0.13% w/v sodium diethyldithiocarbamate in 500 mM EDTA, pH 9.1. Proteinase K was added just before use.

Nuclei isolation: Nuclei were prepared from barley plants according to Zhang *et al.* (1995), with some modifications. About 20 g of fresh leaves was ground into powder in liquid nitrogen using a mortar and pestle. The powder was immediately transferred into 200 mL ice-cold SEB^{+BME} in 250 mL beaker and incubated on ice for 12 min with gently swirling for 20 sec every 2 min. The homogenate was filtered two times through 4 layers of cheesecloth into new ice-cold 250 mL flask. Ten milliliter Triton X-100 (final concentration 5% v/v) was added to the homogenate and incubated on ice for 10 min with gently swirling for 20 sec every 2 min. The mixture was transferred into three 85 mL tubes and centrifuged with fixed-angle rotor at 650 x g for 15 min at 4°C. The supernatant was gently decanted and 10 mL of SEB^{+BME} was added. The pellet was resuspended with assistance of small paint brush. The nuclei suspensions were consolidated into one 85 mL tube then, SEB^{+BME} was added to complete its volume. The nuclei were pelleted by centrifuge at 650x g for 15 min at 4°C. The supernatant was gently decanted and the pellet was resuspended in 20 mL of SEB. The nuclei were pelleted by centrifuge as described above. All but 1-2 mL of the supernatant was removed with a large pipette. The pellet was resuspended in the residual SEB.

Agarose-embedded nuclei preparation: The nuclei were prewarmed to 45°C in incubator for 10 min before being embedded in agarose. To embed nuclei in agarose plugs, the nuclei were mixed with an equal volume of 1.5% low melting point agarose (BioRad) in SEB using large-bore tip pipette. The nuclei/agarose mixture was aliquot into pre-chilled plug mold (BioRad) on ice with standard yellow tip with its tip cutoff. The agarose was allowed to completely solidify at 4°C for 30 min while still on ice. The solidified plugs were transferred to 50 mL of lysis buffer and incubated at 50°C for 48 h with the lysis buffer replaced after 24 h. Then, the plugs were incubated overnight in 50 ml of fresh Lysis buffer at 4°C. Two plugs from each species were retained in lysis buffer and the rest were equilibrate for four hours with 50 mL of 70% ethanol at room temperature before being stored at -20°C in 70% ethanol.

Preparation of (1% TBE-Agarose gel): Weight 1.5 g of Pulse field certified agarose- BioRad, put them in flask, add 150 mL of 0.5X TBE buffer. Dissolve the agarose using microwave for 1 min to melt the agarose until a clear transparent solution is obtained. Poured the melted solution into a gel casting apparatus (14×13 cm) with 15-tooth gel comb inserted. Allowed the gel to solidify. Stain the gel by ethidium bromide. In suitable container, add 5 μ L of ethidium bromide to 50 mL 0.5 X TBE buffer. Cover the container with lightproof cover. Allow to stain on a rocker shaker for 30 min. Distain by distilled water for 30 min (10-20 is enough) on rocker shaker. Reset the running setting: Block = 1, Volts = 6 v cm⁻¹, initial switch time = 5 sec, final switch time = 15 sec, running time = 20 h. Transfer to photo documentary unit.

DNA extraction by CTAB (Cetyl trimethyl ammonium bromide) method following (Doyle and Doyle, 1990):

- Reagents: CTAB 2 % v/v, Tris-HCl, pH 8.100 mM, EDTA, pH 8 25 mM, NaCl 1.5 M, PVP 1.0 % w/v, β -mercaptoethanol 0.2 % w/v.
- Preparation of CTAB extraction buffer (In 10 mL falcon tube put about 4 mL sterilized H₂O, add the above constituents, dissolve and mix by vortex. Bring the volume to 10 mL by sterilized H₂O).
- RNase preparation (Put the RNase tube in boiling water for 5 min. then on ice until using).

DNA extraction by CTAB (*Cetyl Trimethyl Ammonium Bromide*) method from barley plants is implemented according to Doyle and Doyle (1987), with some modifications. About 4 g of leaves was ground into powder in liquid nitrogen with a mortar and pestle. The powder was immediately transferred into 2 mL tube, add 1.6 mL CTAB extraction buffer. Incubate for 60 min. at 60°C in hybridization oven. It is supplied by rotors that continuously mix the sample, other wise, mix by hand every 2 min. Centrifuge at 4000 rpm for 5 min at 4°C. Take the supernatant (H₂O phase) from barley into 2 new 2 mL tubes. Add 0.8 mL of chloroform: octanol (24:1) under fume hood. Shake tubes by vortex for 10 sec. Centrifuge at 4000 rpm for 5 min at 4°C. Take the supernatant (H₂O phase) into new 2 mL tube. Add boiled RNase (from 10 mg mL⁻¹ stock) to a final concentration of 20 μ g mL. Incubate at RT for 30 min. Re-extract with 0.8 mL of chloroform: octanol (24:1) under fume hood. Shake tubes by vortex for 10 sec. Centrifuge at 4000 rpm for 5 min at 4°C. Take the supernatant (H₂O phase) into new 2 mL tube. It was light green in color. Add 0.6 volume ice cold isopropanole. Tubes were incubating at -20°C for 10

min. Centrifuge for 10 min at 4°C at 14000 rpm. Discard supernatant. Wash the pellet with 50 µL of 70% ethanol then centrifuge for 10 min at 4°C at 14000 rpm. Discard ethanol and air dry in the Laminar-Flow. Resuspend the pellet in 50 µL of sterile distilled water and Store at 20°C until needed. The tubes were labeled B1, B2.

Agarose mini-gel (0.7%) preparation in TAE buffer (Use 15 teeth comb): Weight 0.5 g of agarose, put them in flask and add 50 mL of 1 X Tris-acetate buffer (TAE buffer). Dissolve the agarose using microwave for 1 min to melt the agarose until a clear transparent solution is obtained. Add ethidium bromide 5 µL (it is added when the temperature become around 40°C). The melted is poured solution into a mould with 15 teeth comb inserted. When gel solidifies, remove the comb in the laminar hood. Take barley plugs out the lysis buffer. Insert the plugs in pre designated gel wells. Cover the plugs with milted LMP agarose. Prevent air bubbles. Put the gel in the electrophoresis apparatus filled (to the required height) with the previous buffer (TAE running buffer). Run at 120 v for 30 min. Transfer to photo documentary unit

RESULTS AND DISCUSSION

Agarose Gel Electrophoresis: DNA yield was determined by the tow methods described earlier (Fig. 1). Marker banding pattern was good. Barley plug (DNA extracted by nuclei method) showed large DNA quantity at the gel well and slightly extended smear. This indicates presence of high molecular weight DNA. Whereas, barley extracted by CTAB first sample (B1) gave heavy smear from the gel well to 10 kbp and less shiny smear from 10 to 1.4 kbp indicating DNA shearing, but still the yield of DNA is high this came in agreement with the results of (Woodhead *et al.*, 1998; Dempster *et al.*, 1999; Kim *et al.*, 1997; Luo *et al.*, 2001; Zhang *et al.*, 2004; Muhammad *et al.*, 1994; Csaikli *et al.*, 1998). But, barley extracted by CTAB the second sample (B2) gave almost one band slightly above 10 kbp. All CTAB extracted samples, showed RNA contamination. CTAB extracted barley sample (B1), showed higher DNA quantity due to less DNA shearing when compared to sample (B2), this disagreed with results obtained by (Rogers and Bendich, 1988; Collins and Symons, 1992) who indicated low yields of DNA with a CTAB-based extraction procedure. Nuclei extraction method for barley (plugs) seem to be more efficient in extracting high molecular weight DNA due to the very rare shearing effect of this method as compared to CTAB method. These results agreed with results

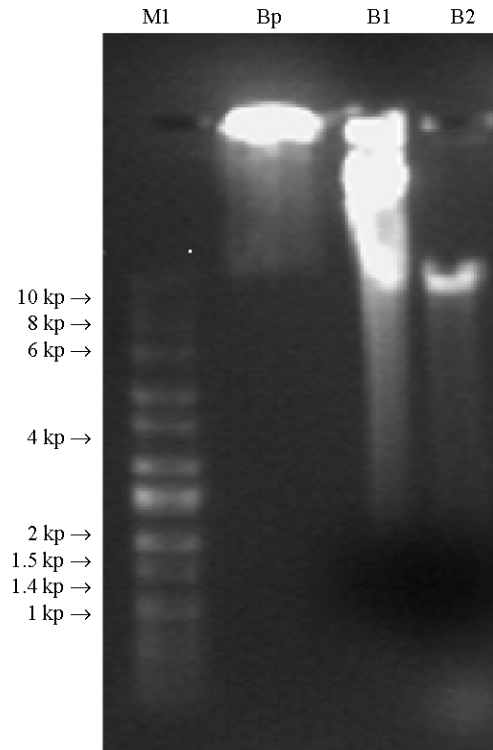


Fig. 1: Agarose gel electrophoresis of genomic DNA for: Nuclei isolation DNA (plugs) extraction and CTAB extraction methods from barley: M1: High-Lo™DNA marker (60-10000 bp), Bp: barley plug, B2 and B1: barley extracted by CTAB. Gel run at 120 voltages for 30 min

obtained by (Schwartz and Cantor, 1984; Zhang, 2000; Zhang *et al.*, 1995; 2000; 2004; Chaudhry *et al.*, 1999; Salah and Iciar, 1997; Hein *et al.*, 2005) who reported higher molecular weight DNA extracted by nuclei method with less shearing effects.

Pulse field gel electrophoresis: DNA yield was determined by the tow methods described earlier (Fig. 2). Low rang marker gave bands similar to the brochure. High-lo™ marker gave no bands because it escaped outside the gel due to the long period of the run in the PFGE apparatus. Wheat extracted by CTAB method showed no bands appearance indicating that the DNA is highly sheared. Barley extracted by nuclei method (plug) showed high DNA at the gel well and smear between the gels well and 200 kbp. This indicates a good DNA quantity or the DNA is not move freely. Barley extracted by CTAB the second sample (B2) showed smear from the gel well to the end but very shiny from ≈50 kbp to lower size. However, barley extracted by CTAB first sample

CONCLUSIONS

These DNA extraction procedures promises simplicity, speed and efficiency, both in terms of time and the amount of plant sample required. In addition, these methods do not require expensive facilities for plant genomic DNA extraction.

There are many advantages in using nuclei genomic DNA extraction method to obtain HMW DNA quantity and quality in barley over the CTAB method. From the present study the following are concluded:

- DNA run on both gels (Pulse Field Gel Electrophoresis (PFGE) and Agarose gel electrophoresis) were almost similar, except for High-Lo™ DNA marker (60-10000 bp) that did not appeared in PFGE due to the long period of the run.
- Barley DNA extracted by both nuclei genomic DNA and CTAB methods showed HMW DNA quantity with a very rare shearing, however, the DNA extracted by CTAB method exhibited a heavy shearing.
- All nuclei extracted genomic DNA did not show RNA contamination. Whereas, all CTAB extracted genomic DNA showed RNA contamination.

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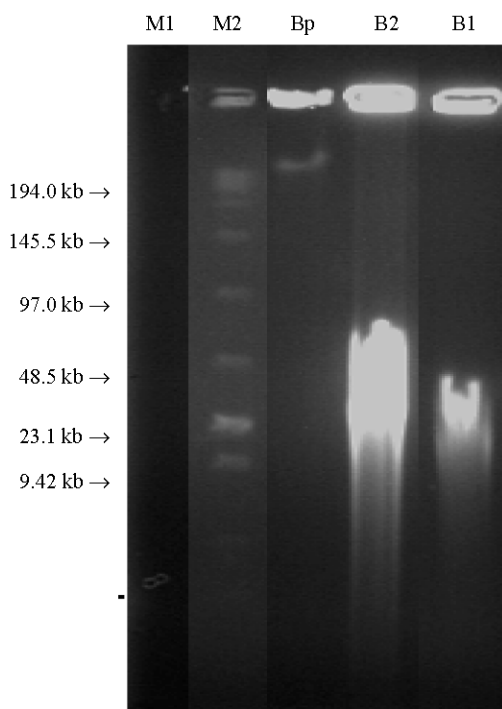


Fig. 2: Pulse Field Gel Electrophoresis of genomic DNA PFGE: Nuclei isolation DNA (plugs) extraction and CTAB extraction methods from: M1: High-Low DNA marker (60-10000 bp), M2: Low Range Marker plug Bp: barley plug, B1 and B2: barley extracted by CTAB. PFGE running settings: Block = 1, Volts = 6 v cm⁻¹, initial switch time = 5 sec, final switch time = 15 sec, running time = 13 h

(B1) exhibited a very shiny and thick long smear from around 50 kbp to lower weight size. In general, the DNA extraction by the nuclei method (plug) revealed a large quantity of DNA that did not leave the gel well as compared to the CTAB extraction method. These results agreed with results obtained by Schwartz and Cantor (1984), Zhang *et al.* (1995), Zhang (2000), Chaudhry *et al.* (1999), Salah Iciar (1997) and Hein *et al.* (2005) who reported higher molecular weight DNA extracted by nuclei method with less shearing effects and with for the CTAB method who indicated low yields of DNA with a CTAB-based extraction procedure (Rogers and Bendich, 1988; Collins and Symons, 1992). Generally speaking results obtained from the gel of Pulse Field Gel Electrophoresis (PFGE) and Agarose gel electrophoresis are almost similar except for High-Low DNA marker (60-10000 bp) that did not appeared in PFGE due to the long period of the run.

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