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A Rapid (100 min) Method for Isolating High Yield and Quality DNA from Leaves, Roots and Coleoptile of Wheat (*Triticum aestivum* L.) Suitable for Apoptotic and Other Molecular Studies

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ABSTRACT

A rapid (less than 100 min) wheat DNA isolation protocol specifically modified for studying degradation of nuclear DNA during apoptosis in leaf, roots and coleoptile of wheat has been reported in this paper. DNA extracted was also exceptionally good for PCR amplification, RAPD and Micro-satellite analysis. The method involves no expensive equipment and chemicals, require fewer steps, tubes and allows DNA extraction from many samples per day per person, making this method economical than other published protocols. DNA yield from this protocol was incredibly high ranging up to 540μg g^{-1} fresh sample. Purity of extracted DNA was excellent as A_{260}/A_{280} ratio was 1.79-1.94 and A_{260}/A_{230} ratio was >2, suggesting that the preparations were sufficiently free of proteins and polyphenolics/polysaccharide compounds, respectively. Therefore no further purification was needed for molecular analysis of DNA samples.

Key Words: DNA; *Triticum aestivum*; Apoptosis

INTRODUCTION

Many plant molecular biology techniques are toil and time consuming. This is particularly true for DNA extraction techniques. Extraction of usable nucleic acids from plants has been difficult in some instances notoriously so. In general success in DNA extraction is measured by DNA yield and utility (Rogres & Bendich, 1994). For apoptotic studies in plants, extraction of intact and unsheared DNA is of utmost importance along with other qualities.

Characteristic biochemical and molecular changes (Gorczyca *et al*., 1993; Hiramine, 1997) especially inter-nucleosomal DNA cleavage (Loo & Rillema, 1998) is used as a marker for apoptotic studies. Therefore DNA for studding apoptotic DNA fragmentation should be extracted as such as in cells with out any shearing during extraction. As seedlings or separated organs are used for apoptotic studies (Bakeeva *et al*., 2001; Zamyatnina *et al*., 2002), an extraction procedure capable of isolating DNA with good yield along with integrity from small amount of plant material is required. The traditional macro-preparation of DNA usually requires from 0.5 to several grams of plant tissue, making it impractical to analyze individual plants during early seedling stage. Science many steps are involved, these methods are not only time consuming and laborious but also consumes a large amounts of hazardous chemical solvents.

Several procedures that allow small-scale DNA extraction and give DNA compatible with polymerase chain reaction (PCR) based technology have been reported (Berthomieu & Meyer, 1991; Oard & Dronavalli, 1992; Cheung *et al*., 1993; Chunwongse *et al*., 1993; Wang *et al*., 1993; Guidet, 1994; Thomson & Henry, 1995; Haymes, 1996; Lange *et al*., 1998). Most of these small-scale extraction procedures have been modified for specific purpose other than apoptotic studies while compromising the need to isolate high molecular weight unsheared DNA (Burr *et al*., 2001), therefore not much suitable for apoptotic studies.

Most important consideration in successful extraction of plant DNA for apoptotic studies is to avoid the shearing of DNA during extraction procedure. Many factors can cause shearing of DNA during extraction. Degradation of DNA due to endonucleases is one such problem encountered in the isolation and purification of high molecular weight DNA from plant, which directly or indirectly interfere with the enzymatic reactions (Weishing *et al*., 1995) during different molecular studies i.e. PCR, RFLP, and RAPD etc. DNA in solution during extraction can also be broken down by exposure to turbulence (Rogres & Bendich, 1994). For example, DNA molecule may under go shearing even if solution is quickly drawn through a small orifice. Furthermore the time between thawing of frozen, pulverized tissue and its exposure to the extraction buffer can cause nucleolytic degradation of the DNA and should be minimized (Rogres & Bendich, 1994).

Any DNA extraction protocol specifically modified for apoptotic studies in plants has not yet been reported. For apoptotic studies a reliable DNA extraction method should meet the following criteria: (i) require only a small amount

of tissue; (ii) use minimal number of pipetting in and out; (iii) eliminate all risk of DNA shearing during and post extraction; (iv) and yield large amounts of high-quality DNA even from small amount of plant material.

Here we describe a rapid DNA isolation protocol from leaves, roots and coleoptile of wheat using few milligrams of fresh plant tissues as the starting material. The protocol permitted isolation of DNA suitable for apoptotic studies along with high quality and quantity. The isolated DNA proved amenable to PCR amplification for RAPD and microsatellite analysis. The method involves no expensive equipment and chemicals, fewer steps and tubes and allows DNA extraction from many samples per day per person, so making method economic than other published protocols.

MATERIALS AND METHODS

Plant material. Similarly sized seeds (44.05± 3.07mg) of wheat strain 1076 (*Triticum aestivum* L.) were germinated for few days (3 to 8 days) at 25±1°C on wet filter paper in plastic petridishes. Seedlings of defined age (seedling age was estimated in days starting from the beginning of the seed soaking time) were thoroughly washed with water. Roots, initial leaf and coleoptile were separated and used for DNA isolation.

Solutions required

- Extraction Buffer: 100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 20 mM EDTA, 1% (w/v) Sodium Dodecyl Sulphate (SDS), 0.2% (v/v) 2-Mercaptoethanol (added immediately before use).
- Chloroform : Isoamyl Alcohol (24:1)
- 96% Ethanol
- TE buffer: **(**pH 8.0**)** 0.5mM EDTA, 10 mM Tris-HCl.
- Ribonuclease A (100 µg/mL)

Protocol

DNA isolation from wheat leaves, roots and coleoptile

- Quickly grind 0.1 to 0.3 g (fresh weight) plant material in liquid nitrogen to a fine powder using a mortar and pestle. (If liquid nitrogen is not available, overnight freezing of sample in mortar along with pestle at -80°C can do the same purpose).
- Transfer grounded material immediately and directly into pre warmed extraction buffer (600 µl) in labeled 1.5 mL microfuge tubes placed in a water bath at 65°C. Incubate the samples at 65°C for 10 min with gentle mixing after every 3 min.
- Take out microfuge tubes from water bath and leave them for 1-2 min at room temperature. Add 600 µl of chloroform-isoamyl alcohol to each tube and gently mix the solutions.
- Centrifuge at 4,000 rmp for 5 min. (equally weighed tube opposite to each other minimize shearing of DNA during centrifugation).
- Remove the upper aqueous phase with a wide-bore pipette to a new 1.5 mL microfuge tube (Cutting the tip of 1mL pipette tip with a sharp blade to make a wide-bore pipette tip; this will also reduce the chance of DNA shearing during extraction)
- Add two volumes of ice-cold 96% ethanol. Mix gently for 5 to 10 times to precipitate the DNA. Centrifuge at 10,000 rmp for 10 min to pellet the DNA.
- Carefully remove all ethanol and the vacuum dries the samples (approximately 5 to10 min). Dissolve the pellet in 20 µl of TE buffer.
- Treat the samples with DNase-free ribonuclease A (50 µg/mL) for 25 min at 37°C.
- Precipitated DNA with addition of three volumes of 96% ethanol. Centrifuge at 10,000 rmp for 10 min to pellet the DNA. Carefully remove all ethanol and vacuum dries the samples. Dissolve the pellet in 40 µl of TE buffer.
- Store DNA at -20°C till further use. (To avoid fragmentation of DNA by residual endonucleases during storage either DNA should be used immediately or for latter on use especially for apoptotic studies, it must be stored at -20°C.

Whole protocol takes less than 100 min. Method can be scaled up for large-scale extractions of plant DNA using large tubes or bottles.

Quantity and purity of DNA. Purity of isolated DNA was checked by obtaining the mean of the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} for proteins and polyphenolics/polysaccharide compounds, respectively. For quantification of DNA, absorbance was measured at 260nm. A solution with an OD_{260} of 1 contains 50 µg of DNA per milliliter (Sambrook *et al*., 1989).

PCR amplification. Approximately 100 ng of extracted DNA was used as a template for PCR amplification. A reaction mixture containing 1x reaction buffer, 1.5 mM $MgCl_2$, 100 ng DNA, 0.20 mM dNTPs (each one), 200 nM of RAPD and microsatellite primers, and 2.5 units *Taq* DNA Polymerase (Perkin Elmer™ was prepared in a total volume of 50 µL. PCR was performed for 30 cycles of 1 min denaturation at 95°C, 1 min annealing at specific tempratre for different primer and 1 min primer extension at 72°C in a Perkin Elmer™ thermocycler.

Purified DNA and amplification products were subjected to electrophoresis for 3-4 h in 1.5% agarose gels at constant Volts in 1X Tris-borate buffer, pH 8.3. After staining with ethidium bromide (0.5 µg/mL) the gels were visualized under UV illuminator. PCR amplification products were subjected to electrophoresis for 2-3 hours in 6% denaturing Polyacrylamide gels at 50mM current. After electrophoresis the gels were silver stained (Sambrook *et al*., 1989) and then dried in Speed Gel ™ SG210G.

RESULTS AND DISCUSSION

The method reported here is much faster than other published rapid DNA extraction protocols (Hugo *et al*.,

1998; Suman *et al*., 1999) and the whole DNA extraction procedure takes less than 100 minutes.

This protocol involves less steps, mirocentrifuge tubes and time therefore is more economic (as cost per sample is reduced) when compared to traditional techniques (Nickrent *et al*., 1994; Triboush *et al*., 1998). Moreover previously reported rapid DNA extraction techniques also involve the use of beads and shakers along with requiring several steps (Dilworth & Frey, 2000). No such beads or shakers were used therefore our method is rapid as well as more simple and easier to handle.

Isolating nuclei prior to extraction produced gives a higher quality DNA (Couch & Fritz, 1990; Collins & Symons, 1992; Dabo *et al*., 1993; Paterson *et al*., 1993), but this step is much laborious. In our protocol we avoided the need for prior extraction of nuclei as included in many previously reported protocols (Couch & Fritz, 1990; Webb & Knapp, 1990; Gawel & Jarret, 1991; Collins & Symons, 1992; Guillemaut & Marchal, 1992; Paterson *et al*., 1993; Dabo *et al*., 1993) that made the extraction simpler and less time consuming. Quality of extracted DNA was increased by changing the concentration of NaCl and b-mercaptoethanol in extraction buffer. It has been reported that high level of b-mercaptoethanol successfully removes the polyphenols (Suman *et al*., 1999). Therefore, high concentration of b-mercaptoethanol was used which made the protocol good for extraction of high quality DNA from plant species high in polyphenols. Further NaCl at a higher concentration can be used to remove high levels of polysaccharides during DNA extraction (Suman *et al*., 1999). For this reason we used a higher level of NaCl concentration in the extraction buffer. After these modifications the purity of extracted DNA was excellent. The A_{260}/A_{280} and A_{260}/A_{230} ratios were 1.79-1.94 and >2, respectively, suggesting that the preparations were free of proteins and polyphenolics/polysaccharide compounds, respectively. Therefore no further purification was required for molecular analysis of DNA samples.

DNA yield is of paramount importance when target tissue for obtaining DNA is limited because of plant uniqueness or when few days old wheat seedlings are used for studying apoptotic induction of DNA fragmentation (Loo & Rillema, 1998) in different organs i.e. roots leaves and coleoptile. The procedure described in materials and methods is capable of extracting DNA from milligrams of plant material from few days old seedlings. Fig. 1 shows DNA extracted even from 3-day-old wheat coleoptile. In this procedure we used a very small amount (0.05 to 0.2 g) of different wheat tissues. High quality DNA was obtained (Fig. 1) and the yields were up to 540µg/g of fresh tissue for all organ types. These yields are higher than those reported in the literature (Webb & Knapp, 1990; Couch & Fritz, 1990; Callahan & Mehta, 1991; Collins & Symons, 1992; Guillemaut & Marchal, 1992; Dabo *et al*., 1993; Paterson *et al*., 1993; Hugo *et al*., 1998).

Fig. 1. DNA extracted using our method and elecrtophorised after ribonuclease A treatment in 1.5% agarose gel at 30 volts for 3-4 hours and stained with ethidium bromide. DNA extracted from 5th (1) and 6th day (6) roots, from 5th (2) 6th, (5) and 8th day (4) leaf, from 6th (3), 3rd (7) and 4th day (8) coleoptile

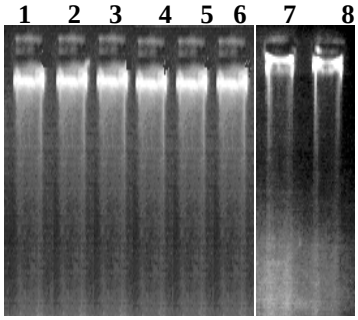
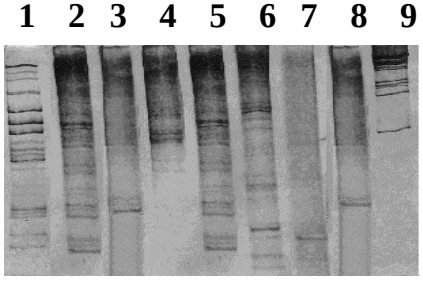


Fig. 2. PCR assay using isolated DNA as a template for amplification. Amplification products of decamer primers and 3D, 1A and 2A chromosome specific primer from wheat stain 1076 were subjected to electrophoresis for 2-3 hours in 6% Polyacrylamide gels at 50mM and silver stained. 100 ng of wheat genomic DNA extracted form roots leaves and coleoptile were added to each tube for reaction. Amplicons form S16 decamer primer (5'-AGGGGGTTCC-3) using DNA extracted from roots (2) and leaves (5), Amplicons form S08 decamer primer (5'-TTCAGGGTGG-3') using DNA extracted from roots (4), Amplicons form S03 decamer primer (5'-CAGAGGTCCC-3') using DNA extracted from coleoptile (6), Amplicon (84bp) from wheat chromosome 1A specific microsatellite primers using DNA extracted from roots (7), Amplicon (218bp) from wheat chromosome 3D specific microsatellite primers using DNA extracted from leaves (8), Amplicon (211bp) from wheat chromosome 2A specific microsatellite primers using DNA extracted from coleoptile (3). ϕX174 DNA digested with Hae III restriction enzymes (1) and Lambda DNA mixed digest (Sigma D-2916) (9) were used as a molecular weight markers



Extraction protocol mentioned in material and methods can be efficiently used for intact DNA isolation from roots, leaf and coleoptile of wheat seedlings to check

DNA fragmentation due to apoptosis. No DNA fragmentation due to shearing of DNA during extraction procedure was seen in any of samples and results were reproducible (Fig. 1). DNA was extracted from hundreds of samples and similar results were obtained every time.

DNA was suitable for amplification by Taq DNA polymerase. DNA extracted using this method (100 ng) was also used for PCR amplification for RAPD and microsatellite analysis for gene tagging (Fig. 2). No inhibition of Taq DNA polymerase activity was observed. It has been reported previously that shearing of DNA during extraction can directly or indirectly interfere with the enzymatic reactions (Weishing *et al*., 1995) during different molecular studies i.e. PCR, RFLP, and RAPD etc. As intact DNA isolation was main aim of modification of DNA extraction protocol, DNA extracted proved to be more compatible with polymerase chain reaction (PCR)-based technologies and other molecular studies.

In our protocol SDS was used instead of CTAB to overcome problems associated with CTAB based methods. CTAB based procedure described earlier (Doyle & Doyle, 1987; Porebski *et al*., 1997) however with (Suman *et al*., 1999) advantages like removal of high polysaccharide and polyphenol components, suffered from lower yield and the precipitation of DNA as CTAB left in the supernatant during incubation of the mixture in cold temperatures (Suman *et al*., 1999). Therefore we use SDS instead of CTAB in this protocol, as we could not compromise on yield while extracting DNA for later on electrophoresis for apoptotic studies while using a small amount of available plant material.

Degradation of DNA due to endonucleases is among one of problems encountered in the isolation and purification of high molecular weight DNA from plant (Suman *et al*., 1999), which directly or indirectly interfere with the enzymatic reactions (Weishing *et al*., 1995) during different molecular studies i.e. PCR, RFLP, and RAPD etc. So DNA must be protected from endogenous nucleases. The detergents are used for this purpose, as is EDTA. EDTA is used as a chelating agent that binds magnesium ions, generally considered a necessary cofactor for most nucleases (Rogers & Bendich, 1994). But in some grasses like, wheat and maize special nuclease is present that is stimulated by EDTA (Jones & Boffey, 1984). This may be a reason why out of the more than 100 tissues types from hundreds of species, only leaves but not embryos and seed of grasses for examples wheat and maize, yielded some times highly degraded DNA (Rogers & Bendich, 1994). Therefore, EDTA concentration in extraction buffer cannot be much increased or decreased and we used standard concentration as given in most of reported protocols. Quick and direct transfer of plant material child at -80^{o}C to an extraction buffer warmed at 65^{o}C minimized the chance of activation of all type of endogenous nucleases. Great care should be taken to avoid thawing of the shattered tissues; this minimizes the chance of nuclease activation and will yield undegraded DNA.

Wheat seedlings are supposed to be ideal system for studying apoptosis in plants and are widely used for such studies (Bakeeva *et al*., 2001; Zamyatnina *et al*., 2002) but suffered from problem of DNA degradation during extraction by endogenous nucleases (Rogers & Bendich, 1994). This protocol successfully yields unsheard DNA from all wheat organs, previously, which was problematic. Furthermore DNA extracted from some leaves i.e. wheat, maize and broad bean, that appeared to be undegraded when examined by agarose gel electrophoresis immediately after preparation or after storage at -20^{o}C sometimes appeared highly degraded after storage at 4^{o}C for several days. Residual nucleases are the apparent cause of this (Rogers & Bendich, 1994). It is therefore recommended that especially for apoptotic studies either DNA should be subjected to electrophoresis immediately after extraction or stored frozen at -80^{o}C for letter use. In DNA dissolving (TE) buffer we decrease the EDTA concentration to 0.5mM for the sake of DNA digestion during storage by residual EDTA activated nucleases (Jones & Boffey, 1984).

Taking together, the DNA isolation method described here is fast, economic and simple and produces DNA of high quality along with high yield when compared with other published protocols. Method allows small-scale extraction of DNA suitable for apoptotic and other molecular studies from many samples per day, and per person.

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