CHAPTER-4: Rapid and cheaper protocol for DNA extraction for molecular approaches

Introduction

The discovery of the DNA structure in early 1950’s has led to remarkable developments in the field of modern biology called Biotechnology. With present knowledge, desired genes can be isolated and inserted in another DNA molecule at a desired position by the action of restriction enzymes. This product is known as recombinant DNA and the technique thus is called genetic engineering/recombinant DNA technology (Patel and Jasrai, 2007). Due to various secondary metabolites, a study involving biochemistry becomes difficult for cotton plant tissue (Bell, 1986). A great success in molecular manipulation of cotton through genetic engineering (Firrozabady et al, 1987; Umbeck et al, 1987) has led to a need for a detailed molecular map of cotton plant genome.

In plants, a breakthrough in DNA extraction came in 1980 with the development of the CTAB protocol (Murray & Thompson, 1980). CTAB is a cationic detergent that is compatible with the high salt concentrations often used to dissociate DNA from chromosomal proteins, and can also be used to selectively precipitate nucleic acids. CTAB based DNA extraction is now routinely used (Taylor and Powell, 1982; Rogers and Bendich, 1985; Watson and Thompson, 1986; Doyle and Doyle, 1987) for mapping (Shen et al, 2004; Yang et al, 2005; Jena et al, 2006) and cloning (Feng et al, 2004), which have led to much of what we know about how plant genes function. It is also widely used in the polymerase chain reaction (PCR), to detect small amounts of a specific sequence in complex samples. CTAB-extracted DNA is also
used to detect transgenes and to identify transgenic plants without the need for selection (Mannerlof and Tenning, 1997; Holm et al, 2000; Mishra et al, 2005), to screen plants for the presence of pathogen-DNA (Oliveira et al, 2002; Brandfass and Karlovsky, 2006) and to screen for plants lacking transgenes (Ritala et al, 2002; Foti et al, 2006).

There is a need for appropriate reproducible and inexpensive DNA extraction method that can easily be adapted to a 96-well format for high-throughput analysis (Flagel et al, 2005). The success of CTAB extraction protocols for plant tissues led to its use with many other organisms that are otherwise recalcitrant to DNA extraction. It gets also helpful in isolating the crude DNA from other forms including bacteria (Caccavo et al, 1994; Xu et al, 2003), fungi (Saleh and Leslie, 2004; Thuan et al, 2006) and animals (Shahjahan et al, 1995; Appelbee et al, 2003). DNA extracted with CTAB is now in use in an increasing number of diverse fields such as ecology (Chiang et al, 2006; Shimono et al, 2006), evolution (Nasrallah et al, 2004; Zhang et al, 2004; Alonso and Ecker, 2006; He et al, 2006) and forensic sciences (Ye et al, 2004)

Extraction of DNA from cotton seed is notoriously recalcitrant to many otherwise common extraction methods. Because of high levels of polyphenolic compounds (Permingeat et al, 1998). Cells get disrupted during sample grinding, phenolic compounds present interacts with protein and nucleic acid which leads to their oxidation and degradation (Dabo et al, 1993). Several methods for DNA isolation
have been developed (Baker et al, 1990; Callahen and Mehta 1991; Paterson et al, 1993) which requires fresh tissue or tissue stored at low temperature (-50 to -70°C), or they are time consuming, requires costly column inputs, etc.

The method demonstrated here for isolation of DNA from cotton seed is highly efficient, require less time and performs at lower cost. The principle of this isolation technique is to release the DNA present in the matrix into the aqueous solution and further concurrently/subsequently purifying the DNA from PCR inhibitors which are generally present in the crude extract techniques. The CTAB buffer contains sufficient amount of NaCl in order to release the DNA from the sample material in the solution and helps in removal of polysaccharides (Fang et al, 1992) by binding with polysaccharides, cell wall debris and denatured proteins. Mercaptoethanol is used as an antioxidant which helps in preventing oxidation caused by phenolic compounds (Permingeat et al, 1998). Use of phenol: chloroform: isoamyl alcohol in the ratio of 24:24:1 helps in removal of lipophillic molecules, proteins and CTAB-polysaccharide complex. Iso-propyl alcohol (IPA) is used for precipitating the crude DNA extract. Proteinase-K and RNase-A were also used to get rid of the interfering RNA and protein compounds. DNA can be isolated from very small quantity of cotton seed material (20-30mg) approx. This method provides us with pure form of DNA which is free from RNA and protein impurities. Moreover, it involves fewer steps, less time and lower cost as compared to other procedures (Saha et al, 1997; Paterson et al, 1993).
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Review of literature

Cotton (Gossypium spp) is known to contain high concentrations of polyphenolic substances and other contaminants such as polysaccharides, RNA, cell debris, polyphenols, and other secondary compounds. Also, seed tissues have very high lipid content, in some cases accounting for 50% of the seed dry mass (Yarosh and Megorskaya, 1975). Cotton contains potential PCR-inhibitory polysaccharides as well as other inhibitory compounds that pose problems (Scott and Playford, 1996) during DNA extraction. Polysaccharides can cause anomalous reassociation kinetics (Merlo and Kemp, 1976). The addition of certain ingredients to the extraction buffer improves the quality of DNA for example polyvinylpyrrolidone (PVP) binds to the phenolic compounds (Couch, 1990; Guillemaut, 1992; Paterson, 1993). Di-ethyl-di-thio-carbamic acid inactivates oxidation of phenol (Couch, 1990; Peterson, 1993), ascorbic acid and 2-mercaptoethanol are used as antioxidants (Webs, 1990; Gawel, 1991; Paterson, 1993, Permingeat et al, 1998) in the isolation technique.

The cleaning of crude DNA is essential for its utilization in PCR for amplification and restriction analysis. Before cleaning, the DNA appears brownish and contains contaminants which interfere in the molecular techniques (Zhang et al, 2000). The DNA dissolved in Tris-EDTA buffer undergoes a single cleanup procedure to remove polysaccharides, polyphenols, and other coloured materials (Permingeat et al, 1998). In the cleaning procedure, they get removed by CTAB, Rnase A, NaCl, PVP and 1,10-phenanthroline (Fang et al, 1992; Jena et al, 2006). Co-precipitation of such
contaminants results in genomic DNA that is of poor quality and quantity. According to some reports, this happens because phenolic compound gets oxidized and covalently bind to proteins and nucleic acids during the homogenization step of DNA extraction, thus rendering the DNA unsuitable for most research applications (Porebski et al, 1997; Chaudhry et al, 1999). 1,10-phenanthroline is a chaotrophic and metal chelating agent that helps reduce intermolecular binding in the extraction and cleaning buffer. Studies showed that this chemical inhibits the degradation of DNA and protects DNA from damaging effects in the presence of Fe ions (Wajahatallah et al, 1997).

According to Elsalam et al (2007) the use of an extraction buffer with 250 mM NaCl considerably reduced the amount of polysaccharides in the isolated DNA samples. Generally, RNase treatment is the accepted method to remove RNA; otherwise the contaminated RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikbart and Villeponteau, 1993).

Paterson et al (1993), Dabo et al (1993), Permingleat et al (1998) and Zhang et al (2000) developed procedures for cotton DNA extraction, but the proposed procedures resulted in low yields of DNA. Moreover, most of these protocols require large amounts of cotton tissue for efficient DNA isolation.

Zhang and James (2000) extracted DNA by a macro-prep CTAB method (Altaf et al, 1997) and used successfully for amplified fragment length polymorphism (AFLP)
analysis. The extractions can be carried out in microcentrifuge tubes for minimizing the chances of contamination and loss of DNA (Cenis, 1992; Stewart and Via, 1993).

Elsalam et al (2007) claimed that his method is much more efficient than the fast-prep methods previously reported for cotton DNA extraction proposed by Permingeat et al (1998).

**Materials and Method**

Plant material:

Seeds of field grown cotton (Mahyco-1612) were collected for DNA isolation. The seeds were crushed with mortar and pestle into fine powder. The powder (average 20-30mg) was transferred to the 1.5 ml microcentrifuge tube.

Chemicals required:

a) NaCl (AR, Ranbaxy Fine Chemicals Ltd, India)

b) Hexadecyltrimethyl-Ammonium Bromide (CTAB) (AR, Ranbaxy Fine Chemicals Ltd, India)

c) Tris HCl (AR, Ranbaxy Fine Chemicals Ltd, India)

d) Na$_2$-EDTA (AR, Ranbaxy Fine Chemicals Ltd, India)

e) Proteinase K (Banglore Genie, Banglore, India)

f) RNase A (Banglore Genie, Banglore, India)

g) β-mercaptoethanol (AR, Ranbaxy Fine Chemicals Ltd, India)

h) Phenol (Banglore Genie, Banglore, India)
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i) Chloroform (AR, Ranbaxy Fine Chemicals Ltd, India)

j) Iso-amyl alcohol (AR, Ranbaxy Fine Chemicals Ltd, India)

k) Iso-propyl alcohol (AR, Ranbaxy Fine Chemicals Ltd, India)

l) Sodium acetate (AR, Ranbaxy Fine Chemicals Ltd, India)

Instruments utilized:

a) Temperature controlled Micro-centrifuge Machine (Eltek, Bombay, India)

b) Water bath (Lab Tech, Ahmedabad, India)

c) PCR (Biorad, USA)

d) Micropipette (volume: 0.5 to 10µl, 10 to 100 µl, 100 to 1000 µl) (Biohit, Finland)

e) Electrophoresis Unit with power pack (Hoefer Inc, USA)

f) UV-transilluminator (Banglore Genie, Banglore, India)

Chemical Preparation:

- Extraction Buffer: (1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris-Base, 0.015 M EDTA)

  For 1 liter extraction buffer, weigh out 81.8 gm NaCl, 20 gm CTAB, 12.1 gm Tris-Base and 5.84 gm EDTA in an appropriate beaker. Add about 800 ml de-ionized water. Stir until all reagents are dissolved and adjust pH with HCl (pH 8.0). Adjust volume to 1 liter with de-ionized H₂O. Autoclave and store at room temperature.

- Proteinase-K: (100 mg/ml)
For 10 ml Proteinase-K solution, dissolve 1gm Proteinase-K in 10 ml de-ionized water. Store at -20°C.

RNase-A: (91 mg/ml)
Dissolve 0.5 gm RNase-A in 5 ml 0.01 M Sodium acetate (pH 5.2), make aliquots in 1 ml portions, boil for 15 min to inactivate DNases, cool slowly to room temperature and add 100 μl 1M Tris-HCl (pH 7.4) to each aliquot. Store at -20°C.

1x TE buffer: (10 mM Tris, pH 8.3, 1 mM EDTA)
For 100 ml 1x TE buffer combine 1 ml 1 M Tris (pH 8.3) and 200 μl 0.5 M EDTA (pH 8.0) and adjust the volume to 100 ml with de-ionized water. Autoclave and store at room temperature.

For 100 ml phenol:chloroform:iso-amyl alcohol (24:24:1) combine 49 ml phenol, 49 ml chloroform and 2ml iso-amyl alcohol. Mix properly and store at room temperature under the fume hood.

Method:
1. DNA was extracted from 20-30 mg of seed material. 500 μl extraction buffer is added to 1.5 ml micro-centrifuge tube containing the seed powder. To that add 2 μl of proteinase-K to dissolve the protein constituent and 2 μl RNase-A was added for getting rid of RNA from the DNA extract. 25 μl of β-mercaptoethanol was also added to the above mixture. This was kept in the water bath for 1 hr at 60°C.
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2. The micro-centrifuge tubes were removed after 1 hr, and to that 500 μl of phenol:chloroform:iso-amyl alcohol (24:24:1) was added, so as to precipitate the cell debris and protein present in the crude extract. The micro-centrifuge tubes were mixed gently by inverting for 5 min and kept for centrifugation at 10,000 rpm for 12 min at low temperature (4°C).

3. After centrifugation, 250 μl of the upper phase was pipetted out, and transferred to new micro-centrifuge tube. To this, 25 μl of 3M sodium acetate and 250 μl of iso-propyl alcohol was added. The mixture was swirled gently and carefully. In this step, the DNA get precipitated and appears like a white coil in the solution, and left at -20°C for 1 hr.

4. These tubes were centrifuge at 10,000 rpm for 10 min (4°C). Discard the supernatant by inverting the tubes. A translucent pallet appears on the wall of the tube as the pure DNA. The tubes were kept upside down on tissue paper to dry the sample for 1 hr. Removing the residual ethanol is critical, especially if the DNA is to be used directly for PCR. The dried samples are stable at RT overnight. If air drying is used, a minimum of 1 hr drying time is advised, but an overnight drying at RT can be used.

5. Warm (50°C) TE buffer in the volume of 200 μl was added to the DNA pellet and incubated for another 10 min at same temperature. The DNA was solubilized by tapping gently and stored at -20°C until further use. TE contains a chelator that can affect reactions such as PCR, or restriction digests. DNA in TE should be
suitably diluted before use in such reactions. It can also be resuspended in H$_2$O depending on what the DNA will be used for. If the DNA will be used directly for PCR, H$_2$O is preferred. However, if long-term storage is desired, TE is more preferable. Samples in TE can be stored at 4 or -20°C. Dried pellets of high molecular weight DNA may require an extended time to dissolve completely. Aliquots suitable for PCR or restriction analysis can be taken after about 30 min. For applications where complete solution is critical, however, it is recommended that the DNA be allowed to dissolve overnight at 4°C.

**Results and Discussion**

The genomic DNA from cotton seeds was isolated by the anonymous (2006) method (Fig 43 a). The DNA pellet was dissolved in 100µl of TE buffer from which 10 µl of the solution was taken and mixed with 1 µl of 10X bromophenol blue dye. The mixture was carefully loaded on 0.8% agarose gel containing ethedium bromide and was subjected to electrophoresis for about 30 min so as the DNA travels towards the anode in the gel. Disconnect the power supply and remove the gel from the electrophoresis buffer reservoir. The gel was placed on UV-transilluminator for observation. Total 14 samples were loaded from which only 8 samples contained DNA and only 3 samples contained high quantity of impurities (Fig. 43 a).

Similarly the sample isolated by the second method (Saha *et al*, 1997) was loaded from which 12 samples showed the result. 5 samples were rich in polysaccharide and protein impurities (Fig. 43 b)
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Fig. 43 c depicts the picture of high quality and quantity of DNA isolated with the technique described here. In the image, all the 14 samples showed a very good result. Pure form of DNA is visible in good quantity and without the presence of PCR-inhibitors like polysaccharides, polyphenolic compounds, proteins and RNA.

Isolation of DNA from seed sample is very tedious as seed is hard and dry material. There are few protocols available for DNA isolation from the seed (Anonymous, 2006). Generally for molecular studies, leaf and other soft tissues are being used for DNA extraction. Method available for cotton seed (Anonymous, 2006) includes lengthy procedure (more than 25 steps) and take 8-10 hr and requires costly columns. In some samples the DNA get lost during the extraction procedure. In this regard, the proposed method is simpler that requires less time, expenditure and that gives good results. It was found that addition of RNase-A and Proteinase-K enzymes which otherwise require 2 hr incubation can be combined with the initial step of CTAB buffer at 60°C.

The quantity of seed material plays an important role in DNA extraction technique as excess quantity of sample may have more impurities like protein, RNA, polysaccharides, polyphenolic compounds etc. Thus, sufficient quantity (20-30 mg) of cotyledon was used to reduce impurities from the beginning. With the proposed
Fig. 43: DNA profile of isolated DNA-extracted through different methods
a. Anonymous method (2006); b. Saha et al method (1997); c. the proposed method.
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procedure, extraction of DNA from half a cotyledon is possible. In the earlier protocols, 1 gm of seed is recommended for extraction. Sometime individual seed weighs less than 1 gm and would create confusion in deciphering the result from the individual seed. Since, transgenic cotton (BT cotton) has been cultivated in large tracts of the progressive state-Gujarat, it is essential to test absence or presence of introduced gene in the seed. Earlier method (Callahan and Mehta, 1991) has used commercial kit (Boehringer Mannheim biochemicals, product 1218620) which recommends extraction under liquid nitrogen which is an expensive proposition. In fact, use of liquid nitrogen is not at all essential for such extraction procedure (Saha et al, 1997).

The method proposed here is efficient enough and suitable for the extraction of DNA form 120 cotton seed samples per day by a person. The cost of this extraction is approximately 40-50 ₹ per sample (Table-10). A similar method described by Colosi and Schaal (1993) was capable of grinding one sample at a time using vortex mixture. The above described method can grind 60 samples per hr. The finely ground material can be used immediately for DNA isolation or can be kept at room temperature for further use.

The quality and quantity of DNA was checked with the help of UV Spectrophotometer (at wavelengths 260 and 280 nm). Quality of extracted DNA was also confirmed by the agarose gel electrophoresis (Fig. 43).
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**Table-10: Comparative study of DNA extraction method with the proposed method**

<table>
<thead>
<tr>
<th>Parameters Overviewed</th>
<th>Anonymous, 2006</th>
<th>Saha et al, 1997</th>
<th>Proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steps involved</td>
<td>28</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Cost/Sample (₹.)</td>
<td>558</td>
<td>100-120</td>
<td>40-50</td>
</tr>
<tr>
<td>Time consumed/48 Samples (Hr)</td>
<td>24-26</td>
<td>18-20</td>
<td>6-8</td>
</tr>
<tr>
<td>Type of sample</td>
<td>Seed</td>
<td>Leaf</td>
<td>Seed</td>
</tr>
<tr>
<td>Amount of material used (mg)</td>
<td>1000</td>
<td>1000</td>
<td>20-30</td>
</tr>
<tr>
<td>Quantity of DNA obtained (µg/1gm plant material)</td>
<td>265</td>
<td>400</td>
<td>800-1000</td>
</tr>
<tr>
<td>Presence of impurities</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Type of Impurities</td>
<td>Polysaccharides</td>
<td>Protein</td>
<td>--</td>
</tr>
<tr>
<td>Result obtained/14 samples</td>
<td>8</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>

The extracted DNA template was tested for molecular analysis using RAPD primer (OPB-01, 5’-GTTTCGCTCC-3’). Reaction mixture containing 50 ng of purified DNA, 10 pM of primer, 0.25 mM of each dNTP, 1.5 U Taq polymerase, 1X PCR buffer and 1.25 mM of MgCl₂. For DNA amplification, the reaction was performed in a thermal cycler (Biorad, USA). The PCR amplification for RAPD analysis was performed according to Williams et al (1990) with certain modification. Amplification conditions were maintained 4 min at 94°C, and then 40 cycles of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C. The last cycle was followed by 10 min incubation at 72°C. Amplified products were electrophoresed on 1.2% agarose gel in 1X TAE buffer at
60 V. 1 kb ladder was used as a molecular size standard. Agarose gel electrophoresis system (Hoefer Inc, USA) was used and after electrophoresis, finely resolved PCR products were visualized under UV light and photographed (Fig. 44).

![Image of gel electrophoresis](image)

**Fig. 44**: PCR amplification by OPB-1 primer (S); 1 kb ladder (M)

Compared with the other methods, this method improves the overall extraction rate by two folds. It allows a high-throughput of samples in a short time without freezing or lyophilizing the plant material. More than 120 samples could be handled by one person in a day and thus the method is useful for rapid DNA extraction. The DNA extracts can be maintained at ~20°C for more than one year without deleterious effects. The quantity and the quality of the DNA extracted by this method from 20-30 mg plant material is enough to perform 150 PCR reactions and is suitable for further studies like RFLP, AFLP, RAPD, SSR and other PCR techniques. The quantity and quality of DNA obtained is a major concern for the techniques like RFLP and AFLP marker assays. The routine extraction method leaves tannins, polysaccharides,
pigmens behind that interferes in the PCR amplification and are unsuitable for
RFLP and AFLP analysis as these techniques are highly sensitive of restriction
enzymes. Hence, the DNA isolation method described here is fast, cheaper, simple,
and useful for obtaining DNA of high quality and quantity for molecular analysis.