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A Modified and Efficient Method for Economical and Rapid Extraction of Genomic Dna for Marker-Assisted Selection in Wheat Hybridization

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Abstract

Marker-assisted selection (MAS) for cloning in crop breeding and genetic studies is an important tool to improve crop plants. For that, isolation of good quality DNA from a huge population of plants require in a short period of time. Metabolites interfere with DNA isolation procedure and yield poor quality DNA. Here we present a modified and efficient DNA extraction technique that is reliable free from protein, polysaccharide and polyphenols contaminants from wheat crop. The method involves using CTAB and high salt concentration to precipitate the polysaccharides. A_{260}/A_{280} and A_{260}/A_{230} ratios were calculated from the spectrophotometric readings to confirm the purity of genomic DNA. The approach is fast, re-producible, and can be practical for PCR base markers identification, such as AFLP marker.

Keywords: DNA extraction method, molecular markers, Triticumaestivum

Introduction

Among cereals wheat is the utmost significant, staple food crop for the majority of world's populations. Globally wheat disbursed nearly 55% of carbohydrates and 20% of food calories (Breiman and Graur, 1995).

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It is predicted that world's population in 2030 will be reached about 8.3 billion, the earth will nourish extra two billion peoples and large part of the population (90%) is living in unindustrialized countries (Bruinsma, 2003). It is therefore essential to produce ample food to nourish the escalating population, but also easily manageable to all. Hybrid cultivars have potential to meet the needs of future food demands (Sun *et al*., 1996).

Efficient breeding program require characterization and early progeny selection to speed the amalgamation of new genetic material into leadinggermplasm using molecular marker techniques. Favorable alleles can be detected in the primary stages of crop development through molecular markers as a result substantialdrop of breeding population (Liebhard*et al*. 2003). Marker assisted selection momentouslyupsurge the productivity of crop plant and animal breeding (Ribaut and Hoisington 1998;Dekkers and Hospital 2002).

To meet the needs of successful application of these crops upgrading schemes is the skill to isolated good amount of high value genomic DNA which can be used successfully for PCR reactions and downstream process (Clark,1997). Due to the presence of proteins, polysaccharides and DNA polymerase inhibitors such likepolyphenols, tannins and alkaloids in the genomic DNA and its isolation is generally quite difficult in cereal crops. These impurities often make DNA samples non-amplifiable and drastically reduce the quality and quantity of DNA (Jobes, D.V, *et al*., 1995). Quality DNA isolation from large number of population samples is the pre-request for the use of full potential of marker–based selection. The DNA obligationis high quality and steady during storage. Thus, the aim of this research was to ripen a low cost, high-throughput DNA isolationformula that fulfill the need for wheat breeding program and generous quality for use in repeatable PCR reactions.

Material and Method

Plant Material

In this investigation male sterile lines [ms (K)-77(2), ms(V)-77(2), ms(Ven)-77(2), ms(B)-77(2), ms(S)-77(2)] crossed withA-90-110 and A-8222 of wheat crop respectively.These lines are hetero-cytoplasm with same nucleus (Table1). These sterile lines were derived from recurrent backcross after 20 generations.

The wheat seed was saturated in water for 4-6 hours; budded wheat seed were grown in plastic pots on soil over a period of three weeks at room temperature understander agronomic culture practices.

Table 1: Wheat Accessions (Hybrid Wheat lines)

Sr.No.	Wheat Accessions		Type
	Name	Cross with Maintainer line	
1.	K-90-110	ms(K)-77(2)×A-90-110	*Ae.kotschyi*
2.	V-90-110	ms(V)-77(2)×A-90-110	*Ae.variabilis*
3.	Ven-90-110	ms(Ven)-77(2)×A-90-110	*Ae.ventricosa*
4.	B-90-110	ms(B)-77(2)×A-90-110	*Ae.bicornis*
5.	S-90-110	ms(S)-77(2)×A-90-110	*T.spelta*
6.	A-90-110	A-90-110 (Maintainer line)	*T.aestivum*
7.	K-8222	ms(K)-77(2)×A-8222	*Ae.kotschyi*
8.	V-8222	ms(V)-77(2)×A-8222	*Ae.variabilis*
9.	Ven-8222	ms(Ven)-77(2)×A-8222	*Ae.ventricosa*
10.	B-8222	ms(B)-77(2)×A-8222	*Ae.bicornis*
11.	S-8222	ms(S)-77(2)×A-8222	*T.spelta*
12.	A-8222	A-8222 (Maintainer line)	*T.aestivum*

Genomic DNA Isolation

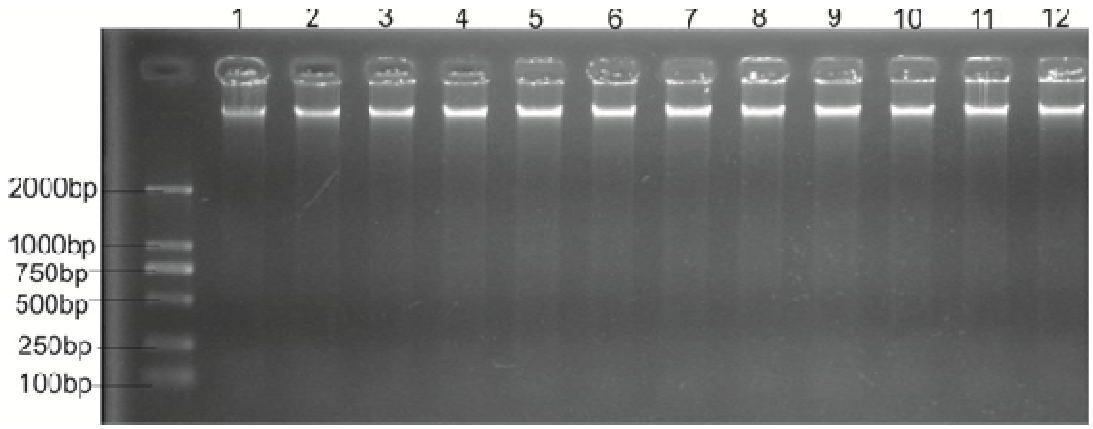
Genomic DNA was isolated from individual wheat plants following improved CTAB protocol of Doyle and Doyle (1987). 0.3gram of well-expended young leaves was taken, washed painstakingly with water and blot dried. The leaves were homogenized to a fine powder in liquid nitrogen. Ground powder reassigned to 800μL of pre warmed DNA extraction buffer (100mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.8 M NaCl, 2.3% w/v CTAB, 2% w/v Polyvinylpyrrolidone (PVP) and 2% β- mercaptoethanol). PVP and β-mercaptoethanol added before use. The homogenate was mixed meticulously by inverting the tube numerous times and incubated at 65°C for 60min. The DNA was then scrubbed with 2M/L NH_4-acetate and phenol/chloroform/isoamyl alcohol (25:24:1), pH 6.7 and centrifuged at 12000rpm for 10min on room temperature (Repeat the step). DNA was rinsed with Chloroform/Isoamyl alcohol (24:1) and centrifuged at 12000rpm for 10min at room temperature. RNA was removed from the samples with 10μg/μLDNase-free RNase enzyme (Tian Gen) and incubated at 37°C for 30min. Refine the DNA by added ice cold anhydrous ethanol, 1/10th of 3M CH_3COONa and incubated at -70°C for 60min.

Pelleted DNA by centrifuged at 12000rpm for 15min on 4°C, washed with ethanol (70% and 96%), dry at room temperature and re-dissolved in TE-buffer (10mMTris and 1mM EDTA pH 8.0) store at -20°C. Recovered DNA was quantified fluorometrically on 1.5 % agarose gel using ethidium bromide staining (Fig. 1).

Table.2: Spectrophotometer Value of Wheat Accessions

Sr.No.	Wheat Accessions	A_{260}/A_{280}	A_{260}/A_{230}	ng/µl
1.	K-90-110	1.99	2.09	949
2.	V-90-110	1.81	1.90	950
3.	Ven-90-110	1.92	1.99	895
4.	B-90-110	1.89	2.00	1050
5.	S-90-110	1.96	2.05	904
6.	A-90-110	1.98	2.10	950
7.	K-8222	1.94	2.05	1100
8.	V-8222	1.91	1.99	1203
9.	Ven-8222	1.86	1.97	1105
10.	B-8222	1.92	1.93	967
11.	S-8222	1.96	2.03	1133
12.	A-8222	1.81	194	989

Fig.1 Results of Genomic DNA from Wheat Accessions



M: Marker **1:**V-90-110, **2:** K-90-110, **3:** Ven-90-110, **4:** B-90-110, **5:** S-90-110, **6:** A-90-110, **7:** K-8222,**8:** V-8222, **9:** Ven-8222, **10:** B-8222, **11:** S-8222, **12:** A-8222,

Quality testing were measured the DNA at A_{260} nm and A_{280} nm. OD values were calculated and ratio of A_{260}/A_{280} and A_{260}/A_{230}, using UV spectrophotometer for purity analysis (Table 2).

Digestion and ligation of genomic DNA

Nearly250ng of genomic DNA of each sample was digested with *Mse*I, *EcoRI*1μL eachand buffer in 20μL volume PCR tube at 37°C for 180min after these 150min at 65°C, the reaction was stopped by incubated at 70°C for 10min. 10μLof restricted and digested mixture were used for ligation, added 1μLeach of MseI, EcoRI adapter (Table 3), 3.5 μL T4 DNA ligase buffer, 400 U T_4 DNA ligase and 3.5μL distilled water. The reaction was incubated for 720min at 16°C and stopped by heating at 65°C for 10min. The ligated DNA was stored at −20 °C until pre-amplification.

Pre-Amplification Reactions

Pre-amplification reactions were accomplished in 20μL reaction volume containing 5μLligation mixtures, 1μL each primer (*Mse*I00, *EcoRI*00), 10μL master mix and 3μL distilled water (Table 3). PCR- amplifications were performed using following program: opening denaturation step at 94°C for 3min, followed by 32 cycles of 94°C for 30s, 56°C for 1:45min and 72°C for 1:20min and a final elongation step at 72°C for 6min. Pre-amplification product was diluted (1:30) with 1 x TE buffer (pH 8.0) preceding to selective amplification.

Selective Amplification

Selective amplification were implemented in a total of 20μL reaction mixturecontaining 5μL of diluted pre-amplification template, 10μL master mix, 1μL each of selective primers (*Mse*I and *EcoRI*)and 3μL distilled water (Table 3). Selective amplification was introducedwith denaturation at 94°C for 2min, followed by cycle of 94°C for 30s,65°C for 45s and 72°C for 2min. The annealing temperature was lowered by0.7°C per cycle during the next fifteen cycles after which 23 cycles were performed at 94°C for30s, 56°C for 45s and 72°C for 1min, followed by one last elongationstep at 72°C for 6min.

Denaturing Polyacrylamide (PAGE) Gel Electrophoresis

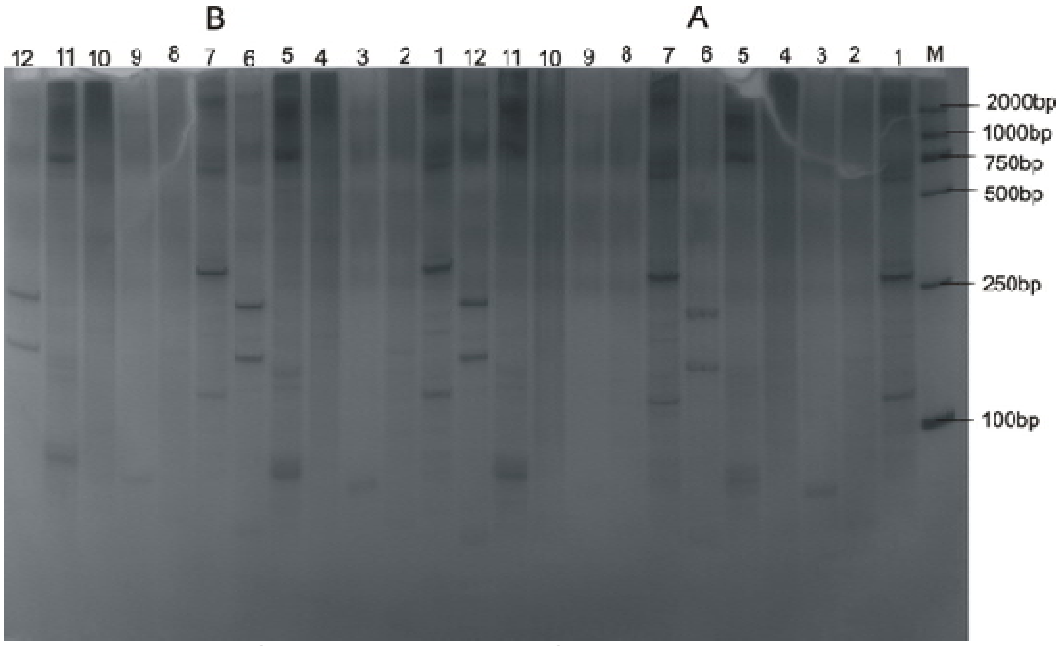
PCR products of selective amplification mixture 5μL were assorted with 3μL (10X loading buffer) buffer and separated through 6% denaturing polyacrylamide (PAGE) gel.

Electrophoresis was completed at a constant power of 20W for roughly three hours.Silver staining for DNA visualization of polyacrylamide gels were performed using stander protocol (Fig. 2). AFLP fragment extentswere resolute by judgmentwith 2000bpDNA ladder (Fermentas).

Table 3: Amplified Fragment Length Polymorphism (AFLP) Primer Sequence

Code	Primer sequence
EAF (Adopter)	5'-GTA GAC TGC GTA CC-3'
EAR (Adopter)	5'-AAT TGG TAC GCA GTC TAC-3'
MAF (Adopter)	5'-GAC GAT GAG TCC TGA G-3'
MAR (Adopter)	5'-TAC TCA GGA CTC AT-3'
E00 （Pre amplifier primer）	5'-GTA GAC TGC GTA CCA ATTC A-3'
M00 （Pre amplifier primer）	5'-GAC GAT GAG TCC TGA GTAA C-3'
E1	5'-GTA GAC TGC GTA CCA ATTC AAC -3'
M1	5'-GAC GAT GAG TCC TGA GTAA CAA -3'
M2	5'-GAC GAT GAG TCC TGA GTAA CAC -3'

Fig.2: Results of AFLP Marker from Wheat Accessions.Primer Combinations



A:E1/M1 **B:** E1/M2**.**

M: Marker **1:**V-90-110, **2:** K-90-110, **3:** Ven-90-110, **4:** B-90-110, **5:** S-90-110, **6:** A-90-110, **7:** K-8222, **8:** V-8222, **9:** Ven-8222, **10:** B-8222, **11:** S-8222, **12:** A-8222.

Results

Isolation of genomic DNA from outsized number of plant accessions is difficult because high levels of polysaccharides and secondary metabolites (Pandey et al., 1996). A simple, rapid, economical, and effective DNA extraction method is highly desirable. The DNA was extracted from fledgling leaves of wheat hybrid and their restorer line (Table 1), used the method of Doyle and Doyle (1987) with some amendments. RNA was removed from the samples using RNase I enzyme (Tian Gen), incubated at 37°C for 30 min (Fig. 1). The DNA was restricted with AFLP marker; two informative primers were used to check the DNA quality. Primer E1/M1 amplified about 20 clear bands and primer E1/M2produced about 22bands (Fig. 2).

The quality of DNA was tested with spectrophotometric values (A_{260}/A_{280} and A_{260}/A_{230} were in between 1.99 to 1.81, 1.90 to 2.10 respectively) shows the quality and quantity of DNA is good (Table 2). Meyer (2003) reported that higher value of A_{260}/A_{280} ratio is indicative of an RNA contamination, whereas a lower value is encountered when a contamination with proteins. The A_{260}/A_{280} ratio is usually used to determine the purity of isolated DNA. This ratio for pure double-stranded DNA is customarily taken to be between 1.8 and 1.9 (Sambrooket al., 1989).

Discussion

The quality of DNA is more important than its quantity in molecular work. But isolation of quality DNA is very difficult because presence of polysaccharides,RNA and other metabolites. The isolation process involvesdigestion of cell wall to discharge the cellular constituents. It is monitored by rupture of the cell membranes to extract the DNA into the extraction buffer. Cetyl-methyl ammonium bromide (CTAB) was used to disrupt the membrane. Puchooa,(2004) reported that the primaryisolated DNA often comprise a hefty amount of polysaccharides, RNA, proteins, tannins and pigments which may interfere andtough to dispersed. Chloroform and phenol are commonly used to detached most proteins by denaturation and precipitation from the extract. RNAs waseroded by heat-treated RNase I enzyme from the extract.

We increase the quantity of CTAB (2.3%) and NaCl (1.8M) to eradicate the polysaccharides.Scott and Playford(1996) reported that the polysaccharides weremore problematic to eliminatefrom the samples.

These can obstruct the action of assured DNA-modifying enzymes and may also restrict in the quantification of nucleic acids by spectrophotometric approaches (Wilkie et al., 1993). Do and Adams,(1991) described that acidic polysaccharides inhibit the digestion of DNA by certain endonucleases like HindIII and PCR amplification by inhibiting TaqDNA polymerase activity. Sodium chloride at concentration of more than 0.5 M together with CTAB is identified to eradicate polysaccharides (Murray and Thompson, 1980; Paterson *et al*., 1993; Demke, and Adams, 1992; Fang *et al*., 1992). The concentration arrayrevealed in fictionfluctuates between 0.7 M (Clark, 1997) and 6 M (Aljanabi*et al*., 1999),it dependent on the plant species under examination. The polyphenols are more abundant in green and over matured tissue than etiolated leaves (Sharma *et al*., 2000).DNA purification also contains high levels of various types of secondary metabolites affecting the quality. Phenolic compounds oxidized and irreversibly bind to nucleic acid during tissue homogenization (Aljanabi et al., 1999).

This irrevocable binding was rigid to separate from organelles and the DNA becomes incompatible for PCR amplification and restriction enzyme digestion analyses (Porebski*et al*., 1997). Phenolics compounds were removed by using β-mercaptoethanol, and PVP (Dawson and Magee, 1995;Clark, 1997).

The spectrophotometer mean values at A_{260}/A_{280} and A_{260}/A_{230} ratio of this method were in between 1.99 to 1.81, 1.90 to 2.10 respectively(Table 2). These values indicate good quality DNA. According to Clark, (1997) DNA purity was determined by calculating the absorbance ratio A_{260}/A_{280}. Pure DNA has a ratio of 1.8 ± 0.2.

Further we inspected the DNA quality by restricted with AFLP marker. Two informative primer pair (Table 3) wereused to investigate the quality of DNA. , Primer E1/M1 amplified about 20 clear bands and primer E1/M2 produced about 22bands it also confirmed the good excellence of genomic DNA (Fig. 2).

Several ways to achieve these objectives have been reported (Edwards *et al*., 1991; Tomas and Tanksley*et al*., 1989), but necessitate a great amount of plant tissues to be ground in liquid nitrogen.

High-quality DNAwith small amount of tissues in short time is essential in many studies of PCR-based markers from plant material and ensures the success of breeding program.

Conclusion

The approach we amended for isolationgenomic DNA from leaves of wheat crop within a short period of time using small amounts of plant tissues and yielding a high quality DNA suitable for restriction digestion and PCR-based analysis.With these problems in mind, we made substantial modifications to make the traditional cetyltrimethylammonium bromide (CTAB)based method more rapid and economical with small samples of plant tissue.

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