A Rapid Protocol for Isolating Genomic DNA from Tropical Grass Species Suitable for RAPD, ISSR and STS

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Summary Largely in apomictic tropical grasses molecular markers analyses including identifying genes through association genetics approach requires DNA from large numbers of samples in short span of time. A simple protocol for DNA isolation from 5 apomictic tropical grass species namely *Dichanthium annulatum*, *Heteropogon concortus*, *Sehima nervosum*, *Chrysopogon fulvus* and *Cenchrus glaucus* is described. The method allows DNA extraction from as little as 0.2 to 0.3 g of leaves ground in liquid nitrogen, followed by DNA isolation in 1.5 ml Eppendorf tubes involving modified cetyltrimethylammonium bromide (CTAB) procedure using 1% polyvinylpyrrolidone (PVP) to remove polysaccharides. By using the method, a DNA yield up to 345 µg/g leaf tissues was obtained. The quality of the DNA was quite suitable not only for PCR-based markers analyses but also for restriction enzyme digestion. No or insignificant DNA was obtained in plant samples extracted from the fixed solution (alcohol, alcohol and chloroform, alcohol and ethylene diamine tetra acetic acid (EDTA)), thus the use of liquid nitrogen was inevitable.

Key words Apomictic grass, CTAB, DNA isolation, Genomic DNA, ISSR, RAPD, STS

Facultative and obligate nature of apomixis in tropical grasses hinders the development of genetic stocks viz., F_2 , RIL and NIL. These are important starting materials required in molecular plant breeding for the development of genetic maps, identification of markers and QTLs for different agronomically important traits. Tackling such situations the association genetics are preferred which requires DNA from large numbers of individual plants. Good quality of genomic DNA is also required to construct the genomic DNA library to isolate abiotic stress related genes especially from those crops growing under extreme harsh and dry conditions.

Contamination with polysaccharides, phenolics and secondary metabolites is a common problem when isolating DNA from plant parts (Pandey *et al.* 1996), specifically from the plants species growing abettedly under harsh and extreme dry conditions. High contents of polysaccharides and lignin are some of the prominent specific problems observed in tropical grasses. The method involves a modified cetyltrimethylammonium bromide (CTAB) procedure based on that of Murray and Thompson (1980) and additional extraction with chloroform and correct proportion of 2-mercaptoethanol and polyvinylpyrrolidone (PVP). Surprisingly in case of *Cenchrus glaucus* this procedure did not yield good quality DNA. However, using SDS in place of CTAB, this species yielded good quality DNA. The DNA obtained through these 2 protocols is a good substrate for restriction endonucleases and polymerase chain reaction.

In light of IPR regime, DNA fingerprinting and cataloguing of the important and unique accessions, the preparation of good quality and quantity DNA has become a major concern. DNA extraction from tissues needs to be simple, rapid, inexpensive, and effective when many samples are used as in case of apomictic grasses where association genetics study requires genotyping several thousands of individual plants. To economize further to avoid the use of liquid nitrogen, method of iso-

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lating DNA after fixing them in alcohol, alcohol and chloroform, alcohol and ethylene diamine tetra acetic acid (EDTA) (Sharma *et al.* 2003) was attempted with tropical grasses. However, this method failed because DNA was unstable and degraded in course of isolation.

Materials and methods

Solutions

CTAB extraction buffer: 2% (w/v) CTAB, 1.4% (w/v) NaCl, 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 3–6% 2-mercaptoethanol (v/v), % PVP (w/v). Extraction buffer 'S': 2% (w/v) SDS, 100 mM Tris-HCl (pH 8), 50 mM EDTA (pH 8), 100 mM NaCl. Chloroform–isoamylalcohol 24 : 1 (v/v). TE buffer: 10 mM Tris-HCl (pH 8), 1 mM EDTA. Isopropanol. 70% ethanol.

DNA isolation

The leaves from the plants growing under natural conditions of *Dichanthium annulatum*, *Heteropogon concordus*, *Sehima nervosum*, *Chrysopogon fulvus* and *Cenchrus glaucus* were used in several treatment combinations to isolate genomic DNA. Leaves were homogenized in liquid nitrogen and fine powders were processed using extraction buffer. Extraction buffer 'S' was exclusively used with *Cenchrus glaucus* to isolate the DNA. The fixing solutions (5 ml/g tissue) namely (A) absolute alcohol, (B) alcohol–chloroform (70 : 30), and (C) alcohol–0.5 M EDTA, [pH 8] (70 : 30) (Sharma *et al.* 2003) were also used to isolate the DNA without grinding the leaf samples in liquid nitrogen. Treated tissue was removed from solution and homogenized with a mortar and pestle.

Using a pre-chilled mortar and pestle 250 mg of the sample was ground in the presence of liquid nitrogen. The frozen powder was transferred to extraction buffer (CTAB and buffer 'S') containing 2-mercaptoethanol (3 to 6%) and 1% PVP and then mixed well and incubated at 65°C for 1 h in a water bath with occasional mixing by gentle swirling. After removing from water bath was cooled the mixture to room temperature. Then equal volume of chloroform–isoamylalcohol (24 : 1) was added. The mixture was mixed by inversion for 10 min. After centrifugation at 10,000 rpm (8000g) for 10 min, the resulting aqueous phase was transferred to another tube. The extraction with chloroform–isoamylalcohol (24 : 1) was repeated. Then 0.6 volume of isopropanol was added to aqueous phase to precipitate the DNA. DNA was recovered by centrifugation 10,000 rpm for 10 min at 4°C. After decanting the supernatant, the sample was washed with 70% ethanol. The dried DNA was dissolved in 500 µl of 1×TE (pH 8) and then treated with RNase (10 µg/ml stock) for 1 h at 37°C. To this was added 250 µl of phenol and 250 µl of chloroform–isoamylalcohol (24 : 1) and mixed thoroughly for 10 min. Centrifugation was carried out for 15 min at 8000g. This procedure was repeated. After removing the organic phase, the DNA was precipitated by adding double the quantity of absolute alcohol. The DNA was washed with 70% ethanol and the DNA was resolved in 500 µl of 1×TE (pH 8). The DNA was diluted 1000 times in TE buffer and quantified by taking the optical density (OD) at λ_{260} with a spectrophotometer. Readings at λ_{280} was taken to obtain the $\lambda_{260}/\lambda_{280}$ ratio as an indicator of DNA purity (Sambrook *et al.* 1989). Purity of DNA was observed on 0.8% agarose gel after staining with ethidium bromide to ascertain its integrity.

RAPD analysis

Random amplification of polymorphic DNA (RAPD) analysis was performed with 5 primers using 25, 40, and 50 ng of genomic DNA. After standardization (without smear and stutter bands), RAPD analysis was performed using DNA isolated from different methods of each grass species (Williams *et al.* 1990). Each PCR amplification was performed in a final volume of 20 µl of reaction mixture containing 67 mM Tris-HCl (pH 8), 16.6 mM (NH₄)₂SO₄, 0.45% (v/v) Triton X-100, 4 µg BSA, 3.5 mM MgCl₂, 150 µM of each of dATP, dCTP, dGTP and dTTP, 7.5 pmol (15 ng) primer, 25, 40 and 50 ng genomic DNA templates and 0.5 unit *Taq* polymerase (Bangalore Genei,

India), and finally it was overlaid by 5–10 μ l light mineral oil. Amplifications were performed on a DNA thermal cycler PTC-200 (MJ Research, USA) with the cycling program consisting of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min for 40 cycles followed by 41st cycle at 37°C for 1 min and finally at 72°C for 10 min extension. The amplified products were kept at 4°C until loaded on to the gel. Amplification products were separated by electrophoresis on 1.6% agarose gel in 0.5×TBE buffer (pH 8) to which ethidium bromide (0.5 μ g/ml) was added for visualization with UV light, and photographed using AlphaImager (Alpha Innotech Corporation, USA). Along with the unknown samples 100 base pair DNA ladder was also loaded to know the size of the amplified products.

ISSR analysis

Inter-simple sequence repeat-PCR (ISSR-PCR) is a simple, cost efficient, robust, multilocus marker method, which is extensively useful in determining genetic variability. Therefore, isolated DNA was subjected to ISSR analysis. Single primer was used in each PCR reaction/experiment, which had a final volume of 25 μ l. The reaction mixtures contained 2.5 μ l of 10×buffer (67 mM Tris-HCl (pH 8), 16.6 mM (NH₄)₂SO₄, 0.45% (v/v) Triton X-100, 4 μ g BSA), 2.0 μ l of 25 mM MgCl₂, 1.5 μ l of 2.5 mM dNTP, 2.0 μ l primer (0.5 μ M), 0.1% gelatin, 1 unit *Taq* polymerase (Bangalore Genei, India) and 25, 40 and 50 ng genomic DNA. The PCR reactions were performed on thermal cycler PTC-200 (MJ Research, USA) with cycling program consisting 94°C for 4 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing for 1 min at 50°C, extension at 72°C for 90 s, and final extension at 72°C for 7 min. The amplification products were first mixed with loading buffer (40% sucrose and 0.25% bromophenol blue) and size separated by a standard horizontal electrophoresis on 1.6% agarose gel in 0.5×TBE buffer under room temperature at a constant voltage of 70 V and detected by ethidium bromide staining, and photographed using AlphaImager (Alpha Innotech Corporation, USA).

STS analysis

Sequence-tagged-sites (STS) primers are specifically used in studying the species relationships developed on the basis of gene sequences or single copy genes. The isolated DNA was also subjected to visualize the STS patterns, as it would generate DNA profile useful to explore the progenitors or genome analysis. The polymerase chain reaction (PCR) amplification for STS loci followed the procedure described by Liu *et al.* (1996) with modification in genomic DNA concentration and inclusion of primer extension step for 5 min as a last step of amplification. The total reaction volume was 25 μ l, which contained 78.2 mM Tris-HCL (pH 8.8), 19.4 mM (NH₄)₂SO₄, 0.53% (v/v) Triton X-100, 233 μ g/ml BSA, 5.8 mM MgCl₂, 130 mM dNTPs, 0.3 μ M of primers, 1.2 unit of *Taq* DNA polymerase (Bangalore Genei, India) and 25, 40 and 50 ng of genomic DNA templates. The reactions were overlaid with mineral oil. The PCR protocol was as follows: 32 cycles of 94°C/60 s, 55°C/60 s, 72°C/90 s; one cycle at 72°C/5 min; and 4°C soak in an MJ Research PTC-200. Amplification products were separated on 1.8% agarose gels in 0.5×TBE buffer and visualized by staining with ethidium bromide.

Restriction analysis of DNA

DNA was restricted by Hind III (Bangalore Genei, India) using 5 U/ μ g of DNA. The reaction mixture was incubated at 37°C overnight. Digested DNA was separated on 0.8% agarose gel, stained with ethidium bromide and observed under UV light.

Results and discussion

The major problem associated with tropical grasses are the presence of high level of polysac-

Fig. 1. Genomic DNA isolated from 5 species of tropical grasses applying the protocols mentioned in Table 1. Lanes 1–6: *Dichanthium annulatum*; lanes 7–12: *Heteropogon concordus*; lanes 13–18: *Chrysopogon fulvus*; lanes 19–24: *Sehima nervosum* and lanes 25–27: *Cenchrus glaucus*. DNA in lane numbers 6, 12, 18, 24 were isolated after fixing the tissues in alcohol.

charides including lignin which have been observed to interfere with DNA isolation procedure and inhibit the activity of a wide range of DNA-modifying enzymes, such as restriction enzymes, polymerase, and ligases. This has also hinders in grinding the sample when fixing solution was used to isolate the genomic DNA. This makes inevitable to use liquid nitrogen. The grinding of sample in absence of liquid nitrogen was very difficult and also leads to complete degradation of DNA.

The procedure reported yielded high molecular weight DNA (>40 Kb, Fig. 1) after grinding the leaves in liquid nitrogen and using 2×CTAB buffer with 3 to 6% 2-mercaptoethanol along with 1% PVP (Table 1). The $\lambda_{260}/\lambda_{280}$ ratio was greater than 1.7 (ranging from 1.68 to 2.4) indicating DNA purity (Sambrook *et al.* 1989, Henry 1997) (Table 2). DNA yield ranged from 110–345 $\mu\text{g/g}$ among 5 species of tropical grasses when 2×CTAB with 3% 2-mercaptoethanol and, 1×CTAB with 6% 2-mercaptoethanol was used (Table 1).

Tissues fixed in 3 fixing solutions A, B and C for a period of 30 to 60 min and ground in absence of liquid nitrogen surprisingly either did not yield or very insignificant amount of intact DNA. All DNA got degraded and thus not useful for any molecular biology work. However, exception was observed with *S. nervosum* which produced 115 $\mu\text{g/g}$ DNA with $\lambda_{260}/\lambda_{280}$ ratio of 1.76 (Table 1). Sharma *et al.* (2003) have reported the isolation of intact DNA after fixing the tissue is above fixing solution and argued that treatment was sufficient to denature enzyme and desiccate the leaves. However, in case of tropical grasses the protocol has not worked may be due to presence of high amount of polysaccharides including lignin hindering the complete desiccation of leaf samples. There is also likelihood that the presence of high polysaccharides slows down the penetration of fixing solution and thus slows tissue death. DNA from *C. glaucus*, specifically, was obtained only when buffer 'S' was applied. The yield of the DNA was 265 $\mu\text{g/g}$ and $\lambda_{260}/\lambda_{280}$ ratio was 2.03. As buffer 'S' has SDS instead of CTAB and therefore, possibly did not generate any complex substances which was otherwise formed when CTAB was used and finally hinders the release of DNA. Less than 1.8 $\lambda_{260}/\lambda_{280}$ ratio indicated impaired DNA quality and this may be due to difference in levels of polysaccharides, fibers and associated anti-quality factors in plant species.

Samples ground in liquid nitrogen produced good quality DNA. The concentration of 2-mercaptoethanol (3 to 6%) along with 1% PVP yielded good quality genomic DNA. The yield of the DNA was better when 3% 2-mercaptoethanol was used with 2×CTAB buffer (145 to 345 $\mu\text{g/g}$) over 1×CTAB with 3% 2-mercaptoethanol (120 to 315 $\mu\text{g/g}$). In case of *Dichanthium* and *Sehima* yield was even more when concentration of 2-mercaptoethanol was increased from 3 to 6%, however, the yield of DNA decreased in case of *Chrysopogon* and *Heteropogon* (Table 1). Overall less yield obtained with *Dichanthium* (120 to 180 $\mu\text{g/g}$) was because of higher level of polysaccharides present in the plant. We obtained DNA yield and quality similar to results reported with other protocols (Murray and Thompson 1980, Callahan and Mehta 1991, Henry 1997, Khan *et al.* 2004, Kumar *et al.* 2003, Sharma *et al.* 2003).

Table 1. Comparison among 3 protocols and over the standard

Item	Protocols					
	1	2	3	4	5	6
Amount of liquid nitrogen ground powder	200 mg	200 mg	200 mg	200 mg	200 mg	200 mg leaves fixed in alcohol
CTAB buffer conc., +1% PVP, condition and volume (in case of <i>C. glaucus</i> buffer 'S')	2×, cold, 400 µl	1×, pre-warm, 400 µl	1×, cold, 400 µl	1×, cold, 400 µl	2×, cold, 400 µl	1×, cold, 400 µl
2-mercaptoethanol	3%	3%	3%	4.5%	6%	3%
Incubation temperature and period	65°C, 45 min	65°C, 45 min	65°C, 45 min	65°C, 45 min	65°C, 45 min	65°C, 45 min
Chloroform–isoamylalcohol (24:1)	400 µl	400 µl	400 µl	400 µl	400 µl	400 µl
Precipitating solvent and volume	Isopropanol (0.6 volume)	Isopropanol (0.6 volume)	Ethanol (0.6 volume)	Isopropanol (1:1 volume)	Isopropanol (0.6 volume)	Isopropanol (0.6 volume)
DNA yield and quality	DNA yield is better than the standard procedure, quality is comparable					Low and degraded
<i>Dichanthium annulatum</i>	140 µg/g	140 µg/g	120 µg/g	210 µg/g	180 µg/g	Nil
<i>Chrysopogon fulvus</i>	190 µg/g	135 µg/g	160 µg/g	135 µg/g	160 µg/g	45 µg/g
<i>Heteropogon concortus</i>	345 µg/g	175 µg/g	315 µg/g	180 µg/g	110 µg/g	40 µg/g
<i>Sehima nervosum</i>	155 µg/g	240 µg/g	230 µg/g	175 µg/g	245 µg/g	115 µg/g
<i>Cenchrus glaucus</i>	265 µg/g	—	—	—	—	—

Table 2. DNA yield and purity obtained with the present protocol from different species of tropical grasses

Species/protocol no.	OD, λ_{260}	OD, λ_{280}	OD, $\lambda_{260}/\lambda_{280}$	Concentration, µg/l	DNA yield, µg/g
<i>D. annulatum</i> (1)	0.028	0.012	2.33	0.140	140
<i>D. annulatum</i> (2)	0.028	0.015	1.86	0.140	140
<i>D. annulatum</i> (3)	0.024	0.012	2.00	0.120	120
<i>D. annulatum</i> (4)	0.042	0.020	2.10	0.210	210
<i>D. annulatum</i> (5)	0.036	0.019	1.89	0.180	180
<i>D. annulatum</i> (6)	−0.020	−0.001	—	—	—
<i>C. fulvus</i> (1)	0.038	0.019	2.0	0.190	190
<i>C. fulvus</i> (2)	0.027	0.016	1.68	0.135	135
<i>C. fulvus</i> (3)	0.032	0.018	1.77	0.160	160
<i>C. fulvus</i> (4)	0.027	0.015	1.86	0.135	135
<i>C. fulvus</i> (5)	0.032	0.019	1.68	0.160	160
<i>C. fulvus</i> (6)	0.009	0.010	—	0.045	45
<i>H. concortus</i> (1)	0.069	0.037	1.86	0.345	345
<i>H. concortus</i> (2)	0.035	0.018	1.94	0.175	175
<i>H. concortus</i> (3)	0.063	0.035	1.86	0.315	315
<i>H. concortus</i> (4)	0.036	0.020	1.80	0.180	180
<i>H. concortus</i> (5)	0.022	0.012	1.80	0.110	110
<i>H. concortus</i> (6)	0.008	0.007	—	0.040	40
<i>S. nervosum</i> (1)	0.031	0.018	1.72	0.155	155
<i>S. nervosum</i> (2)	0.048	0.020	2.40	0.240	240
<i>S. nervosum</i> (3)	0.046	0.025	1.84	0.230	230
<i>S. nervosum</i> (4)	0.035	0.020	1.75	0.175	175
<i>S. nervosum</i> (5)	0.049	0.027	1.84	0.245	245
<i>S. nervosum</i> (6)	0.023	0.013	1.76	0.115	115
<i>C. glaucus</i>	0.053	0.026	2.03	0.265	265

Fig. 2. ISSR (A), RAPD (B) and STS (C) profiles generated for *Dichanthium annulatum* (lanes 1–6); *Heteropogon concordus* (lanes 7–12); *Chrysopogon fulvus* (lanes 13–18); *Sehima nervosum* (lanes 19–24). M. 100 bp DNA ladder as a molecular weight marker.

PCR analysis

DNA isolated from 5 tropical grass species was amplified using 10-mer random primers from Operon Biotechnologies, Inc. (USA), ISSR primer ([GACA]₄ GT) and 2 pairs of STS primers (Liu *et al.* 1996). DNA isolated through 6 treatments combination (Table 1) was used for PCR amplifica-

Fig. 3. *Hind*III cut genomic DNA isolated as per protocol mentioned in Table 1 from *Dichanthium annulatum* (lanes 1–6); *Heteropogon concortus* (lanes 7–12); *Chrysopogon fulvus* (lanes 13–18); *Sehima nervosum* (lanes 19–24). U=uncut DNA.

tions. DNA was diluted to 5 ng/ μ l in TE buffer, and 5 μ l, 8 μ l, and 10 μ l of diluted DNA was used for amplification with 4 primers in total. When 25 ng of DNA was used, bands without background smearing and upper DNA streaks were produced which were easily scorable (Fig. 2). A uniform DNA patterns was expected because DNA was from the same plants. Bands were uniform with all reactions, indicating good quality DNA. Genomic DNA isolated from *C. glaucus* also produced uniform patterns, regardless of different buffer used (buffer 'S').

Restriction analysis of genomic DNA

Genomic DNA from all 5 grass species was restricted by *Hind*III (5 unit of enzyme/ μ g of DNA, kept overnight at 37°C). The restricted DNA produced a good patterns on 0.8% agarose gel, indicating complete digestion of DNA samples from 2 buffers (Fig. 3).

In conclusion, genomic DNA isolated from 4 tropical grass species using 2 $\times$ CTAB buffer along with 3 to 6% 2-mercaptoethanol and 1% PVP, and buffer 'S' for *C. glaucus* DNA observed useful for routine molecular biological work. Grinding of leave tissues in liquid nitrogen found inevitable as alcohol fixed plant samples only yielded degraded and non-usable DNA for molecular biology work.

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References

- Callahan, F. E. and Mehta, A. M. 1991. A rapid alkaline extraction procedure for screening recombinant plasmid DNA: a method for preparing plasmid DNA. *Nucleic Acids Res.* **7**: 1513–1523.
- Henry, R. J. 1997. *Practical Applications of Plant Molecular Biology*. 1st ed. Chapman and Hall, London.
- Khan, L. A., Ahmad, A. A. and Khan, A. A. 2004. A modified mini-prep method for economical and rapid extraction of genomic DNA in plants. *Plant. Mol. Biol. Repr.* **22**: 89.
- Kumar, A., Pushpangadan, P. and Mehrotra, S. 2003. Extraction of high molecular weight DNA from dry root tissue of *Berberis lycium* suitable for RAPD. *Plant. Mol. Biol. Repr.* **21**: 309.
- Liu, C. J., Musial, J. M. and Smith, F. W. 1996. Evidence for a low level of genomic specificity of sequence tagged sites in *Stylosanthes*. *Theor. Appl. Genet.* **93**: 864–868.
- Murray, M. G. and Thompson, W. F. 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acids Res.* **8**: 4321–4325.
- Pandey, R. N., Adams, R. P. and Flournoy, L. E. 1996. Inhibition of random amplified polymorphic DNAs (RAPDs) by plant

- polysaccharides. *Plant. Mol. Biol. Repr.* **14**: 17–22.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular cloning: A Laboratory Manual*, 2nd ed. Nolan, C. (ed.). Cold Spring Harbor Laboratory Press Cold Spring Harbor, New York.
- Sharma, R., Mahla, H. R., Mohapatra, T., Bhargva and Sharma, M. M. 2003. Isolating plant genomic DNA without liquid nitrogen. *Plant. Mol. Biol. Repr.* **21**: 43–50.
- Williams, J. G. K., Kubelik, A. R., Livak, K. T., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531.
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