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Plant DNA Extraction and Amplification Procedure

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Genomic DNA was isolated from 39 wheat genotypes following CTAB (Cetyl Tri-methyl Ammonium Bromide) extraction method with some modifications as described by Helguera *et al.* (2005).

DNA Isolation

Reagents

1. CTAB DNA extraction buffer: 100 mM Tris-HCl (pH-8) containing 20 mM EDTA (pH 8), 1.4 M NaCl, 20 % CTAB (w/v) and 2 µl/ml β-mercaptoethanol.
2. Ice cold Isopropanol.
3. Chloroform: Isoamylalcohol (24: 1) (v/v)
4. Ethanol: (70 % and 100 %) (v/v)
5. RNase: 10 mg/ml
6. TE (10:1) buffer: 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA

Procedure

1. One gram young leaves of wheat genotypes were taken and powdered in liquid nitrogen (N_2) with pestle and mortar.
2. Powder was transferred to 2 ml polypropylene centrifuge tube containing pre-warmed 650µl CTAB (Cetyl Tri-methyl Ammonium Bromide) DNA extraction buffer and mixed well by inversion for 15 min.
3. Tubes were incubated for 60 min at 65^0C in a thermostatic water bath.
4. Contents in the tubes were mixed after every 15 min by inversion during incubation.
5. After incubation, the tubes were allowed to cool at room temperature.
6. An equal volume (650 µl) of Chloroform: Isoamyl alcohol (24:1) were added and mixed gently by inversion.
7. Tubes were then centrifuged at 10000 rpm for 10 min at 4^0C in high speed refrigerated centrifuge.
8. Aqueous phase was carefully removed and transferred in to a new 1.5 ml tubes.
9. A 0.6 volume of ice cold isopropanol (300µl) was added into the contents of fresh tube.
10. Tubes were incubated at -20^0C for 20 min for precipitation of DNA.
11. Tubes were centrifuged at 10000 rpm for 10 min for precipitation of DNA and the aqueous layer was discarded.
12. Ice cold 70 % ethanol (100 µl) was added into the tubes and centrifuged at 5000 rpm for 5 min, the pelleted DNA was once more rinsed with ice-cold 70 % ethanol. The pelleted DNA was air dried till the last traces of ethanol were evaporated.
13. Pellet was re-suspended in 100 µl of TE (10/1) buffer.

RNase Treatment

1. Isolated DNA of wheat genotypes were equally distributed in 1.5 ml centrifuge tubes and 5 µl of RNase (10 mg/ml) were added in each tube. The tubes were incubated in water bath at 37^0C for 1 hrs.
2. After one hour, purity of DNA sample were checked on 0.8 % agarose gel by electrophoresis.

DNA Quantification

Reagents

- 10 X TBE Buffer (Tris Base, Boric acid, EDTA) Exactly 108 g Tris (1M), 55 g of boric acid and 40 ml EDTA (0.5 M) were measured and dissolved separately. These were mixed separately and final volume was made to 1000 ml with sterile distilled water.
- Ethidium bromide: 10 mg/ml
- Tracking dye: 1 % (w/v) bromophenol blue + 20 % (w/v) Ficoll + 10 mM EDTA.
- 1X TBE Running Buffer (500 ml): added 50 ml 10X TBE buffer in 450 ml distilled water.

Procedure

2 µl of all DNA extracts were electrophoresed in 0.8 % (w/v) on agarose gel containing 0.5 µg/ml ethidium bromide. After electrophoresis the band intensity of genomic DNA was visualized on gel documentation unit (Flour Chem.^{TM} Alpha Innotech, USA) and compared to that of standard Lambda phage DNA. These gels also provided a visual measure of purity of DNA.

DNA Amplification

PCR amplification was carried out using Thermal Cycler PCR (Eppendorf, Master Cycler Gradient, Germany). Amplification reaction mixture was prepared in 0.2 ml thin walled flat capped PCR tubes, containing the following components. The total volume of each reaction mixture was 20 µl (Table 3).

Composition of PCR reaction mixture

PCR reaction component	Stock concentration	Final Concentration	Volume for one tube
Taq buffer B (Genei)	10 X	1 X	2 µl
$MgCl_2$	25 mM	1 mM	1.2 µl
dNTP mix	10 mM	3.2 mM	1.6 µl
Primer (F)	0.2 picomole /µl	0.32 picomole	1.6 µl
Primer (R)	0.2 picomole /µl	0.32 picomole	1.6 µl
Genei *Taq* DNA polymerase	3 U	1 U	0.33 µl
Sterilized distilled water	-	-	9.67 µl
Template DNA	25 ng/ µl	50 µl	2 µl
Total volume			**20 µl**

Procedure

The 20 µl reaction mixture was gently vortexed and spun down. The DNA amplification was carried out in a Thermal Cycler (Eppendorf, Master Cycler Gradient, and Germany). The temperature profiles set for PCR amplification of different primers are mentioned in Tables 4 to 8.

Temperature profile used for primer

Steps	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	45 sec	40
Annealing	55°C	45 sec.	
Extension/Elongation	72°C	1 min.	
Final extension	72°C	10 min	1
Final hold	4°C	-	-

Agarose gel electrophoresis of amplified PCR products

1. Electrophoresis unit (gel casting tray, gel combs, power pack).
2. UV trans-illuminator/ Gel documentation unit
3. Solutions required:
    - Ethidium bromide: 10 mg/ml
    - Bromophenol blue (loading dye)
    - Agarose
    - 10X TBE Buffer (Tris base, boric acid, EDTA; 1000 ml): Exactly 108 g Tris (1 M), 55 g boric acid glacial and 40 ml EDTA (0.5M) were weighed and dissolved separately. The final volume made 1000 ml with sterile distilled water.
    - 1X TBE Running Buffer (500 ml): Add 50 ml 10 X TBE buffer in 450 ml distilled water.

Procedure

1. Agarose powder (2g) was added to 100 ml of 1X TBE buffer and was melted by heating the solution in micro-oven.
2. Solution was cooled to about 55-60^{0}C and 5µl of ethidium bromide (0.5µl/ml) was added in it.
3. The melted agarose solution was poured into the gel casting unit after keeping the gel comb in the proper place.
4. The gel was allowed to solidify at room temperature.
5. The apparatus was filled with 1X TBE buffer in order to submerge the gel in the buffer to prevent the entry of air bubbles
6. Gel was placed in electrophoresis apparatus and gel combs were removed carefully.
7. The 10 µl sample which contain DNA 2 µl, tracking dye 4 µl, and sterile distil water 4 µl loaded in the wells of the agarose gel.
8. The unit was connected to power pack and electrophoresis was carried out at 80 volts.
9. The power supply was switched off when the dye is in front of about 2 cm from positive end.
10. The separated DNA samples were observed under UV transilluminator or gel documentation unit and image was captured.

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