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Research Article

A RAPID METHOD FOR RICE GENOMIC DNA EXTRACTION WITHOUT LIQUID NITROGEN AND PHENOL

SHAMIM M.Z.*, MANZAR H. AND SHARMA V.K.

Department of Agricultural Biotechnology & Molecular Biology, Rajendra Agricultural University, Pusa, 848125, Bihar, India
*Corresponding Author: Email-mzshamim85@gmail.com

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Abstract- A rapid, safe and inexpensive method for good quality DNA extraction from rice leaves is the basic requirement for molecular breeding in rice. Here we described a faster, safe and inexpensive method for genomic DNA extraction from rice leaves after modification of CTAB method. In the described method liquid nitrogen and Phenol is fully avoided for DNA extraction. This method yields approximately 110 ng/µl-420 ng/µl of DNA from 300 mg of fresh leaves tissue, depending on rice varieties and grinding of leaf samples. It can be completed within 4-5 hrs. The extracted DNA is stable and it can be used for DNA fingerprinting, quantitative traits loci analysis, marker-assisted selection, and genetic diversity analysis.

Keywords- CTAB method, DNA extraction, PCR, Rice.

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Introduction

Isolation of DNA from plants through efficient, rapid and inexpensive method is necessary for marker assisted selection, diversity assessment, quantitative trait loci analysis, germplasm and transgenic plants identification as well as screening. Several researchers [1-3] described DNA isolation methods, which are extensively used in plant molecular biology, but most of the methods are time consuming, laborious, expensive due to multiple steps, requires liquid nitrogen for sample grinding and Phenol for DNA extraction [4-6]. The Cetyl-trimethylammonium bromide (CTAB) method is one of the most popular methods for rice genomic DNA isolation, including other plants [7], bacteria [8], fungi [9] and animals [10]. Most of the purposed modified methods for DNA extraction method are the modifications of CTAB method described by prior research workers [1,2]. The main cause of diversity and modification in CTAB method is the composition of cell walls and intra-cellular as well as extra-cellular components. Lignification of cereal cell wall makes degradation difficult, which results in restricted DNA extraction [6]. To overcome this problem in the case of rice leaves some researchers has developed methods for DNA extraction [11]. Some other methods have been also reported to minimize the DNA extraction steps but these methods need liquid nitrogen [12] and Phenol. Continuous liquid nitrogen supply is a problem in many developing countries because purchasing time is unpredictable from overseas [7,13]. Phenol is highly corrosive (can cause severe burns) to skin, toxic (absorbed phenol act as a systemic toxin) and mutagen. We have optimized a simple and rapid method for isolating high-quality plant DNA without liquid nitrogen and Phenol at room temperature from the small amount of rice leaves tissues.

Materials and Methods

Plant material

Eighteen rice varieties [Table-1] were grown under the green house in different pots for DNA extraction. All leaves samples were collected in polyethylene bag on ice and stored at -20°C.

Reagents and Chemicals

- Extraction buffer: 100mM Tris-HCl (pH=8.0), 20mM EDTA (pH=8.0), 2.0 M NaCl, 3% (w/v) CTAB, 2% (w/v) PVP, 1% (v/v) β-mercaptoethanol. The β-mercaptoethanol should be added immediately in extraction buffer before use to avoid possible oxidation.
- Chloroform: Isoamyl alcohol (24:1)
- Isopropanol (Ice cold)
- 70% Ethanol
- TE buffer [10mM Tris-HCl (pH=8.0), 1mM EDTA (pH=8.0)]
- RNase (250 µg/ml)
- 5X TBE buffer: 54 gm of Tris base, 27.5 gm of Boric acid and 20 ml of 0.5 M EDTA (pH=8.0) was dissolved in distilled water and volume was maintained one liter.

DNA extraction procedure

(i) 300 mg of 15 days old fresh leaves were grinded in 1ml extraction buffer by mortar and pestle and transferred 2 ml centrifuge tube.
(ii) The homogenate was incubated at 65°C for 30 minutes in water bath and centrifuged at 13500 rpm for 10 minutes.
(iii) The supernatant was transferred to a new 1.5 ml tube and 700 µl of Chloroform:Isoamyl alcohol mixture (24:1) was added.
(iv) The content was then mixed by gently turning the tubes upside-down and subsequently, the mixture was centrifuged at 13500 rpm for 10 minutes.
(v) After centrifugation the supernatant was transferred into new 1.5 ml tube.
(vi) Chloroform: Isoamyl alcohol (600 µl) was again added and then centrifuged at 13500 rpm for 10 minutes and supernatant was transferred to a new tube.
(vii) DNA was precipitated by mixing 0.6 volume of ice-cold isopropanol and then pelleted by centrifugation at 13500 rpm for 10 minutes.
(viii) The supernatant was removed, the pellet was washed with 300 µl 70%

ethanol and centrifuged at 10000 rpm for 5 minutes to remove the salts and then pellets was dried in air. Pellet was then dissolved in 50 µl of T.E buffer.

(ix) Finally, 1.0 µl (250 µg/ml) of RNase was added and incubated at 37°C in water bath for 30 minutes.

(x) The DNA was stored at -20°C for further use.

Agarose gel electrophoresis

(i) 0.8% agarose gel was prepared in 0.5X TBE buffer. Agarose was dissolved by heating the solution and then allowing it to cool around 60°C.

(ii) Ethidium bromide was added and solution was poured into sealed gel casting unit on gel casting plate. The comb was placed at the top of the gel and allowed to solidify at room temperature for 30 minutes.

(iii) After solidification the comb was removed carefully and gel plate (along with gel) was placed in migration chamber containing 0.5X TBE buffer. In each well, DNA sample along with the gel loading dye (0.25% Bromophenol blue, 0.25% Xylene cyanol and 30% glycerol in H_2O) was loaded carefully.

(iv) Electrophoresis was carried out with the help of Genei-200V power pack at 70 V for 40 minutes so that the dye moved to approximately five cm from the well.

(v) The gel was viewed under U.V (332nm) light and the quality of DNA was detected.

Quantification and quality determination of extracted DNA

The absorbance of purified DNA sample was recorded with the help of Varian Cary 50 U.V-visible Spectrophotometer at 260 nm and 280 nm in order to determine the DNA concentration and also to check its purity. The following steps were followed for DNA quantification:

(i) 1ml of TE buffer was taken in quartz cuvette used as blank and the spectrophotometer was calibrated at 260 nm as well as 280 nm wavelengths.

(ii) 5 µl of DNA sample was added in a quartz cuvette and mixed with 995 µl TE buffer, mixed properly and the optical density (OD) was recorded at both 260 nm and 280 nm.

(iii) DNA concentration was estimated employing the following formula:

$$\text{Concentration (µg/µl)} = \frac{A_{260} \times 50 \times Dilution\ factor}{1000}$$

Where,

$$\text{Dilution factor} = \frac{\text{Volume of diluted samle used for DNA quantification}}{\text{Volume of samle used for dilution}}$$

A_{260} = Absorbance at 260 nm.

The concentration of double-stranded DNA at 1 unit O.D. at 260 nm = 50 µg/ml.\

(iv) The purity of DNA sample was determined on the basis of A260/A280.

(v) If this ratio (A_{260}/A_{280}) was

1.8-2.0 = Pure DNA

More than 2.0 = RNA contamination

Less than 1.8 = Protein and phenol contamination

Amplification of extracted genomic DNA

Three SSR markers i.e., RM 171, RM 317 and RM 332 were used to check the amplification property of extracted genomic DNA. Polymerase Chain Reaction (PCR) was conducted in 15 µl reaction volume containing 1X PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTPs), 2mM of $MgCl_2$, 0.4 µM of each forward and reverse primer, 1 unit Taq DNA polymerase (Fermentas) and 40ng of template DNA. The amplification of DNA was carried out using a Thermocycler (Biometra). Thermocycler was programmed to 1 cycle of 5 minutes at 94°C as an initial hot start and strand separation step. This was followed by 30 cycles of 1 minute at 94°C for denaturation, 1 minute for annealing temperature depending on the marker used (48°C-60°C) and 2 minute at 72°C for primer elongation. Finally, 10 min at 72°C was used for final extension then PCR was hold at 4°C. Amplified products were stored at -20°C for further use. The reproducibility of amplification products was checked twice for each primer. The amplified product was electrophoretically resolved on a 2.0% agarose gel in 0.5X TBE buffer at 100V for 1.5 hrs. A 50 bp DNA ladder was used as size marker to compare the molecular weight of amplified products. DNA fragments produced were visualized under UV light using a Gel documentation system.

Results

The amount of DNA in the genomic DNA samples extracted from eighteen rice varieties was determined by measuring the optical density of DNA samples at 260 nm. The data recorded on optical density of DNA samples of different rice varieties is presented in [Table-1]. The absorbance was recorded to be the minimum (0.011) in the case of rice variety Rajshree, whereas the maximum absorbance was registered in the case of rice variety Richharia (0.042). The data indicated the presence of the minimum amount of DNA in sample obtained from Rajshree (110 ng/ µl), whereas the maximum amount of DNA was present in the case of the sample extracted from Richharia (420 ng/ µl). In most of the cases, the absorbance ratio (ratio of absorbance at 260 and 280 nm) was found to fall within the range of 1.85 to 1.95, reflecting the superior quality of DNA in the samples extracted from the leaves at seedling stage of different varieties used in the present study [Fig-1]. DNA isolated from eighteen rice varieties following this method was amplified using SSR markers RM 171, RM 317 and RM 332. The amplification was observed in all samples tested with little variation in intensity of the amplified bands. After amplification RM 171 produced 321 bp to 353 bp [Fig-2], RM 317 produced 161 bp to 173 bp [Fig-3] and RM 332 produced 189 bp to 213 bp [Fig-4] polymorphic products.

Table-1 *Absorbance of DNA samples and amount of DNA in samples of eighteen rice varieties used in the study*

Sl.No.	Variety	O.D.$_{(260)}$	O.D. $_{(280)}$	A_{260}/A_{280}	Amount of DNA (ng/ µl)
1	Prabhat	0.031	0.017	1.824	310±20.00
2	Rajendra Bhagwati	0.028	0.015	1.866	280±12.50
3	Rajshree	0.011	0.006	1.833	110±10.00
4	Rajendra Shweta	0.026	0.014	1.857	260±6.03
5	Dhanlakshmi	0.022	0.012	1.833	220±9.02
6	Richharia	0.042	0.022	1.909	420±17.50
7	Gautam	0.018	0.010	1.800	180±10.00
8	Rajendra Mahsuri	0.039	0.020	1.950	390±15.00
9	Satyam	0.028	0.015	1.866	280±16.62
10	Rajendra Suwasni	0.022	0.012	1.833	220±12.58
11	Rajendra Kasturi	0.028	0.015	1.866	280±12.06
12	Vaidehi	0.022	0.012	1.833	220±9.02
13	Katarni	0.020	0.011	1.818	200±11.06
14	Super Katarni	0.022	0.012	1.833	220±22.03
15	Super Sugandha	0.210	0.011	1.909	210±15.50
16	Sudha	0.019	0.010	1.900	190±10.00
17	Sambha Mahsuri	0.015	0.008	1.875	150±15.01
18	Nata Mahsuri	0.021	0.011	1.909	210±10.50

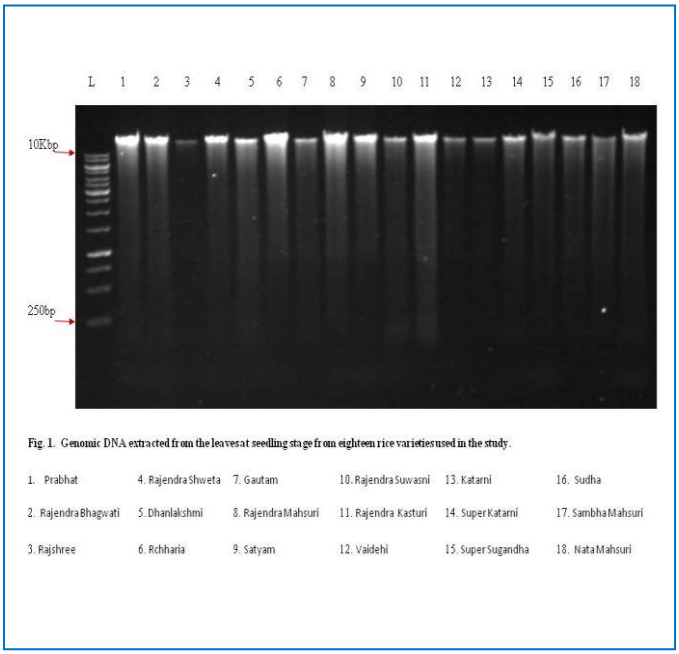

Fig. 1. Genomic DNA extracted from the leaves at seedling stage from eighteen rice varieties used in the study.

1. Prabhat	4. Rajendra Shweta	7. Gautam	10. Rajendra Suwasni	13. Katarni	16. Sudha
2. Rajendra Bhagwati	5. Dhanlakshmi	8. Rajendra Mahsuri	11. Rajendra Kasturi	14. Super Katarni	17. Sambha Mahsuri
3. Rajshree	6. Rchharia	9. Satyam	12. Vaidehi	15. Super Sugandha	18. Nata Mahsuri

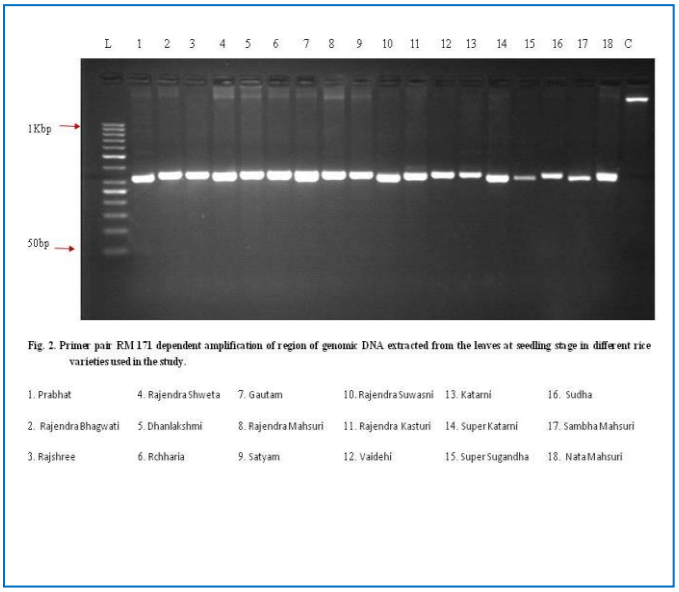

Fig. 2. Primer pair RM 171 dependent amplification of region of genomic DNA extracted from the leaves at seedling stage in different rice varieties used in the study.

1. Prabhat	4. Rajendra Shweta	7. Gautam	10. Rajendra Suwasni	13. Katarni	16. Sudha
2. Rajendra Bhagwati	5. Dhanlakshmi	8. Rajendra Mahsuri	11. Rajendra Kasturi	14. Super Katarni	17. Sambha Mahsuri
3. Rajshree	6. Rchharia	9. Satyam	12. Vaidehi	15. Super Sugandha	18. Nata Mahsuri

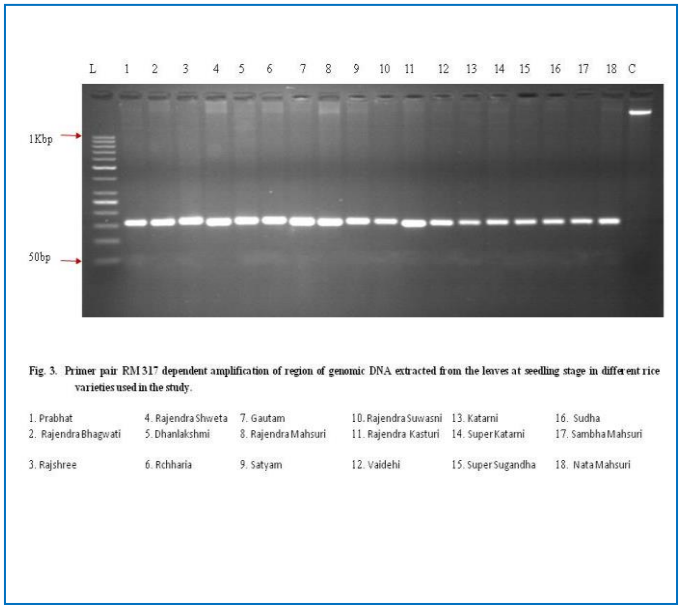

Fig. 3. Primer pair RM 317 dependent amplification of region of genomic DNA extracted from the leaves at seedling stage in different rice varieties used in the study.

1. Prabhat	4. Rajendra Shweta	7. Gautam	10. Rajendra Suwasni	13. Katarni	16. Sudha
2. Rajendra Bhagwati	5. Dhanlakshmi	8. Rajendra Mahsuri	11. Rajendra Kasturi	14. Super Katarni	17. Sambha Mahsuri
3. Rajshree	6. Rchharia	9. Satyam	12. Vaidehi	15. Super Sugandha	18. Nata Mahsuri

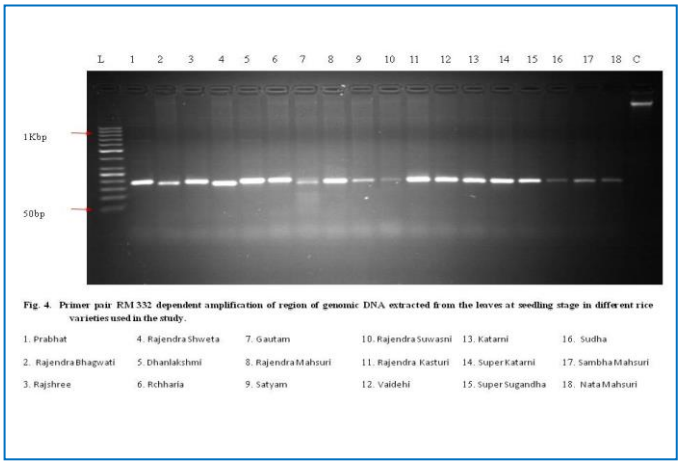

Fig. 4. Primer pair RM 332 dependent amplification of region of genomic DNA extracted from the leaves at seedling stage in different rice varieties used in the study.

1. Prabhat	4. Rajendra Shweta	7. Gautam	10. Rajendra Suwasni	13. Katarni	16. Sudha
2. Rajendra Bhagwati	5. Dhanlakshmi	8. Rajendra Mahsuri	11. Rajendra Kasturi	14. Super Katarni	17. Sambha Mahsuri
3. Rajshree	6. Rchharia	9. Satyam	12. Vaidehi	15. Super Sugandha	18. Nata Mahsuri

Discussion

No liquid nitrogen was required in the described method for grinding of the leaf tissues. In addition the hazardous chemical like Phenol was also not been used during DNA extraction. In this method, most of the common chemicals were used instead of costly and harmful chemicals like Proteinase K. The quantity of extracted DNA was high to PCR amplification for genetic diversity analysis and molecular breeding. The variation in the quantity of extracted DNA was obtained due to measuring error and differential grinding of leaf samples taken from different varieties. The extracted DNA samples by this method were stable more than two years. All three SSR markers show very good amplification and polymorphism with the DNA extracted through this method. Therefore, this method should be recognizing as a good rapid and inexpensive method for DNA extraction from rice leaves.

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Conflict of Interest: None declared

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