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RESEARCH PAPER

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DNA Extraction Protocol without Liquid Nitrogen of Cold Tolerant Rice

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ABSTRACT

Marker assisted selection (MUS) of cold tolerant rice requires rapid DNA extraction protocol for large number of samples in a low cost approach. Cetyltrimethyl ammonium bromide (CTAB) method is simple and fast compared to other methods and no liquid nitrogen is required. The extracted DNA is stable and applicable to marker assisted selection for evaluating genetic diversity, DNA fingerprinting to evaluate genetic variation, quantitative traits loci (QTL) analysis, RT-PCR, screening of transformants and enzymatic digestion. The objective of this study was to develop a simple and rapid method to isolate DNA under normal laboratory condition (room temperature) from small amount of tissue for large number of samples of cold tolerant rice. The present study has the potential applications in future breeding programme for the genetic improvement of cold tolerant rice.

Key words: DNA extraction, Cold and QTL.

INTRODUCTION

Rice (***Oryza sativa*** L.), one of the most world's most important staple crops, feeds more than 2.7 billion people worldwide and is extensively grown by more than half of the world's farmers .Due to its origin in tropical and subtropical regions, rice is more sensitive to cold stress than other cereal crops such as wheat (***Triticum aestivum*** L.) and barley (***Hordeum vulgare*** L.). Therefore, in temperate areas, the production of rice is severely limited by cold stress. Since January is the coldest month in Bangladesh have to tackle cold-related diseases affecting their crops. Cold injury of Boro (dry season irrigated crop) rice is a common physiological condition for northern part of Bangladesh. Large number of rice farmers suffers significant losses every year as none of the existing varieties can cope with extreme cold. As the development of molecular markers and linkage maps progresses, marker-assisted selection becomes an effective way to breed cold tolerant cultivars. Many cold-tolerance related QTL have been identified in the past 20 years. The *qCTP11* is related to cold tolerance at the germination stage; and *qCtss11* and *qCTS4a* are related to cold tolerance at the seedling stage. Because breeding for abiotic-stress resistance is urgently needed progress in the identification of cold tolerance-related QTL has been a significant development for facilitating molecular marker-assisted selection (MAS). Several authors (Muray and Thompson,1980;Dellaporta et al., 1983; Doyle and Doyle, 1990) described DNA extraction methods which are widely used in plant molecular biology, but most of the protocols are time consuming, comparatively expensive and requires liquid nitrogen for grinding (Allen et al, 2006). The cetyltrimethyl ammonium bromide (CTAB) method is one of the most popular protocols for cold tolerant rice. The objective of this study was to develop a simple and rapid method to isolate DNA under normal laboratory condition (room temperature) from small amount of tissue for large number of samples of cold tolerant rice
More importantly given the agronomic characteristics of the donor parent, the size of the donor chromosomal segment containing the target locus was reduced to ensure that there were minimal changes to the genetic composition of the recipient variety. This practical example of marker assisted selection clearly illustrates the superiority of using MAB compared to conventional backcrossing because obtaining such a small donor region within only a few backcross generations would be impossible using conventional methods (Ray et al., 2014)

MATERIALS AND METHODS

DNA was extracted from the leaves of each genotype using the CTAB mini-prep method at Biotechnology Lab., Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. The simplified CTAB mini-prep for DNA isolation in developed at IRRI (IRRI, 1997) was followed.

Collection of leaf sample

Young, vigorously growing fresh leaf samples were collected from 21 day old seedlings to extract genomic DNA. Initially healthy portion of the youngest leaves of the tiller were cut apart with sterilized scissors and washed in distilled water and ethanol and dried on fresh tissue paper to remove spore of microorganisms and any other source of foreign DNA. The collected leaf samples were then put into polythene bags and kept on ice in an ice box. After that stored at -80^{0}C freezer.

Reagents preparation for DNA extraction (Stock solution)

Extraction buffer (500 ml)

For the preparation of 500ml DNA extraction buffer, 50 ml 1M Tris (p^H 8.0) was mixed with 20 ml of 0.5M EDTA and added to 50 ml 5M NaCl in a 1000 ml measuring cylinder. Finally, sterilized ddH_2O was added to make the volume up to 500 ml, mixed well and autoclaved (Figures-1).

20% SDS (100 ml)

To prepare 100 ml 20% SDS, 20 gram SDS was mixed with 100 ml ddH_2O in a 250 ml beaker. SDS is hazardous, so the mixture was mixed well but not autoclaved.

10X CTAB (100 ml)

For the preparation of 100 ml 10X CTAB, 10 g CTAB was added in 100 ml 0.5M NaCl, heat and stirred and then autoclaved.

5M NaCl (100 ml)

For the preparation of 5 M NaCl, 29.22 g of NaCl was added in 100 ml ddH_2O in a 250 ml volumetric flask, mixed well and autoclaved.

Chloroform-Isoamyl alcohol (24:1) (25 ml)

To prepare 25 ml of Chloroform-Isoamyl alcohol (24:1) Chloroform-Isoamyl alcohol (24:1) 1 ml of Isoamyl alcohol was added in 24 ml Chloroform.

70% Ethanol (300 ml)

90 ml ddH_2O was added in 210 ml absolute ethanol.

1X TE (100 ml)

For the preparation of 100 ml 1X TE, 10 ml 1M Tris (p^H 8.0) was mixed with 0.2 ml EDTA (p^H 8.0) in a 250 ml beaker. Finally water (ddH_2O) was added to make volume up to 100 ml and autoclaved.

1M Tris P^H 8.0 (100 ml)

To prepare 100 ml 1M Tris(P^H 8.0), 12.112 g Trizmabase was added to 80ml ddH_2O in a 250 ml volumetric flask while p^H was adjusted to 8.0 by adding 6NHCl. Then sterilized water (ddH_2O) was added to make the volume up to 100 ml. Finally the solution was autoclaved.

0.5M EDTA p^H 8.0 (100 ml)

For the preparation of 100 ml 0.5M EDTA (p^H 8.0) ddH_2O was taken in a 250 ml beaker and 18.612 g $Na_2EDTA.\ 2H_2O$ was added to it and stirred vigorously. Then 2g NaOH pellet was added for p^H adjustment. Sterilized ddH_2O was added to make the volume up to 100 ml and then the solution was autoclaved.

Note: $Na_2EDTA.\ 2H_2O$ will not dissolve until p^H adjusts to 8.00 by NaOH pellet.

Ice Cold Isopropanol (Iso propyl alcohol)

It was done imply by keeping the isopropanol in -20^0C

Genomic DNA extraction

DNA was extracted from the leaves of each genotype using the Cetyl Trimethyl Ammonium Bromide (CTAB) mini-prep method at Biotechnology Lab., Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. The simplified mini scale procedure for DNA isolation in PCR analysis developed at IRRI was followed. The quality of the isolated DNA in the protocol was sufficient for PCR analysis.

The leaf samples were ground with pestle and mortar to collect DNA. Strict hygiene was maintained during the DNA extraction by autoclaving all glassware, micropipette tips, PCR tubes, distilled water, reagents and buffer solutions. The following steps were followed for DNA extraction.

The leaf samples were cut into 2-3 cm pieces and ground the sample with 670 µl extraction buffer and 50 µl 20% SDS were added. The mixture was vortexed for 20 second and incubated for 10 minutes at 65^0C in the hot water bath. 100 µl 5M NaCl was added and inverted gently shaking by hand to suspend the samples evenly. Then added 100 µl CTAB and mixed well with vortexed for 20 second and incubated for 10 minutes at 65^0C in the hot water bath. 900 µl chloroform (chloroform: isoamylalcohol = 24:1) was added and mixed well. The samples were spinned down at 12000 rpm for 15 minutes and then transferred the supernatant into a new eppendorf tube. 600 µl ice-cold isopropanol was added to the supernatant and shaken slowly. At this point, DNA became visible as white strands by flicking the tube several times with fingers. The mixture was again Spinned down at 12000 rpm for 15 minutes by centrifuge. The supernatant was discarded and washed the pellet with 200 µl 70% ethanol and then spinned down at 12000 rpm for 5 minutes. Then the ethanol was removed and the pellets were allowed for air-dried overnight. The pellet was then suspended in 30µl 1X TE buffer. Finally, the DNA samples were stored at - 20°C (Figures-1).

Precautions

All glassware, micropipette tips, centrifuge tubes, glass pipettes, distilled water and buffer solutions were properly autoclaved to keep away from DNAase contamination. Scissors and forceps were sterilized with absolute ethanol.

Confirmation of DNA samples

Isolated genomic DNA contains a large amount of RNA and pigments, which cause over estimation of DNA concentration during spectrophotometer reading. Therefore, the DNA samples were evaluated both quantitatively and qualitatively using spectrophotometer and agarose gel electrophoresis, respectively.

DNA confirmation using agarose gel electrophoresis

Reagents preparation for agarose gel electrophoresis

10X TBE (500ml)

- 54g Tris base was taken in 350ml ddH_2O
- Then the mixture was stirred for some time
- Then 27.5 g boric acid was added
- Then again stirring was done for several hrs
- After that 20 ml 0.5M EDTA (p^H 8.0) was added
- Finally sufficient water (ddH_2O) was added to make 500ml

1X TBE (2000ml)

1x TBE was made by adding 200 ml 10X TBE in 1800 ml ddH_2O

Preparation of 1.2% Agarose gel (40 ml)

40ml 1X TBE (electrophoresis buffer) was taken in a flask and 0.48 g of agarose was added to it. The mixture was then kept in micro oven and cooked for 5 minutes to dissolve it. The gel was kept in room temperature for 10-15 min to cool down at tolerable level. The gel was then poured into gel mold carefully. Meanwhile four combs were placed on the gel within 30 minutes the gel was solidified .The combs were removed from the gel .The gel was submerged into 1X TBE buffer in the gel tank. The gel was then ready for loading the DNA sample.

Preparation of 10 mg/ml ethidium bromide (10 ml)

To prepare 10 mg/ml ethidium bromide, 0.1g ethidium bromide was added in 10 ml ddH_2O and stirred vigorously for several hrs. The container was wrapped in aluminum foil and kept at room temperature and placed in dark place.

DNA sample preparation and electrophoresis

Reagents required

Loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA)

DNA marker (λ DNA ladder)

Procedure

Loading dye (2µl) was placed on a piece of parafilm paper using micropipette and 2 µl extracted DNA sample was added to it and mixed well and then the mixture was loaded in the well of the gel. The known DNA ladder (λ DNA) was loaded in the first lanes of the gel. The gel tank was covered and the electrophoresis apparatus were connected to the power supply unit. After switching on, the DNA migrated from negative to positive electrode (black to red). Electrophoresis was carried out at 80v for 80min to reach the dye ¾ of the gel length. When DNA migrated sufficiently the power supply was switched off (Figures-2).

Documentation of the DNA samples:

After electrophoresis the gel was stained with ethidium bromide for at least 15- 20 minutes. After staining, the gel was taken out from the staining tray with care and placed on gel doc system for visualizing the DNA bands under UV light. Then the observed DNA as band (smear) was saved in hard disk as records (Figures-2).

Precautions

As Ethidium Bromide (Et Br) is a powerful mutagen and carcinogenic, hand gloves were used when handling the gel.

PCR analysis for microsatellite markers

Polymorphism survey for primer selection

Polymorphism survey of BRRI dhan36 germplasms was carried out using 55 microsatellite markers RM556, RM562, RM594, RM7075, RM1287, RM3843, RM5806, RM27877, RM28102, RM28502, RM180, RM206, RM209, RM217, RM219, RM228, RM231, RM234, RM241, RM247, RM24330, RM25022, RM25181, RM25519, RM26063, RM26416, RM26652, RM27694, RM208, RM211, RM35, RM51, RM80, RM127, RM134, RM138, RM140, RM149, RM164, RM169, RM249, RM276, RM296, RM300, RM314, RM336, RM337, RM400, RM407, RM443,RM493, RM494, RM508, RM515, RM520.

Polymerase chain reaction (PCR)

The PCR cocktail including DNA had total volume of 10 µl/reaction based on rice protocol, was placed in the PCR tubes and run in the DNA thermal cycler.

Components of PCR cocktail (for each reaction):

The following components were used to prepare PCR cocktail (Table 1). The total volume of PCR cocktail for this study was 10 µl per sample.

Two µl genomic DNA was added with 8.0 µl PCR cocktail and finally, total volume was 10 µl.

Preparation of 1mM dNTPs (1ml)

Equal amounts (10 µl) of dATP, dTTP, dCTP and dGTP (each with concentration of 100 mM) were mixed in a 1.5 ml eppendrof tube and 960 µl ddH_2O was added. So, 1 ml dNTPs was prepared.

Procedure for Preparation of PCR cocktail (for each sample)

The following procedure were followed

i. At first 3.20 µl of sterilized ddH_2O, 1.0 µl of PCR Buffer (10X), 1.00 µl of dNTPs were taken in a 0.2 ml PCR tube.
ii. Then 1 µl of primer forward and 1 µl of primer reverse were added
iii. The mixture was then vortexed
iv. At last 0.2 µl of Taq DNA polymerase was mixed with it
v. Thus the PCR cocktail was ready to use
vi. 2.0 µl of each template DNA samples were pipetted into the wells of the PCR tubes for PCR amplification.

PCR amplification profile

The PCR tubes were set on the wells of the thermocycler plate. Then the machine was run according to the following setup

1) Initial denaturation at 94^oC for 5 min
2) Denaturation at 94^oC for 30 sec
3) Annealing at 55^oC for 30 sec
4) Polymerization at 72^oC for 2 min
5) Cycle to step 2 to 4 for 30 more time
6) Incubation at 72^oC for 5 min
7) Hold at 4^oC- forever

Agarose gel electrophoresis of PCR products

The PCR products (2.0µl) were mixed with 2.0 µl of loading dye. PCR products were run in 1.2% agarose to check for amplification (figures-2). After electrophoresis, the gel was stained with ethidium bromide for about 20-25 minutes. DNA bands were visualized under UV light using a Gel DOC. A molecular weight marker DNA (20bp or 1kb) was loaded on either side of the gel. Finally the photograph was saved in computer and subjected to data analysis.

RESULTS AND DISCUSSION

Cold tolerant rice was analyzed using a highly repeatable PCR based fingerprinting assay known as Simple Sequence Repeat (SSRs) or microsatellites markers. The microsatellite DNA markers produce a higher level of DNA polymorphism in rice. The microsatellite enriched libraries were constructed using the standard procedure with some modifications. The quality of extracted genomic DNA was also checked by 1.2% agarose gel electrophoresis (Figures-2). Furthermore, to check the suitability of the extracted DNA, DNA was amplified with SSRs markers such as RM556, RM562, RM594, RM7075, RM1287, RM3843, RM5806, RM27877, RM28102, RM28502, RM180, RM206, RM209, RM217, RM219, RM228, RM231, RM234, RM241, RM247, RM24330, RM25022, RM25181, RM25519, RM26063, RM26416, RM26652, RM27694, RM208, RM211, RM35, RM51, RM80, RM127, RM134, RM138, RM140, RM149, RM164, RM169, RM249, RM276, RM296, RM300, RM314, RM336, RM337, RM400, RM407, RM443, RM493, RM494, RM508, RM515, RM520(Figures-2). In the described protocol, no liquid nitrogen was required for the storage and grinding of the tissues. In addition, the expensive chemicals have not been used. To trim down the time, the extraction buffer was directly added to the rice flour, while rice leaf tissues were ground with extraction buffer. PCR product was also used to test the stability but it is not linked primer of cold tolerant rice. Amplified microsatellite loci were analyzed for polymorphism

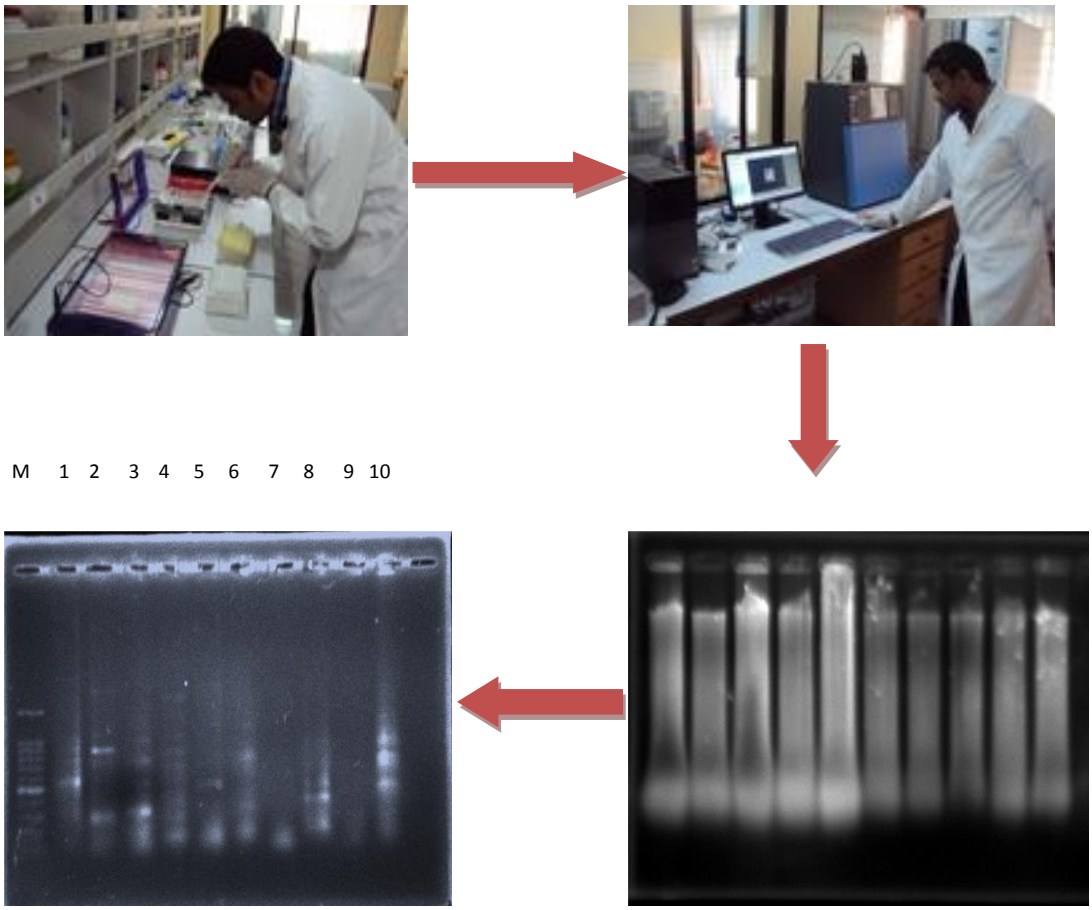
using agarose gels electrophoresis or PAGE and the result revealed that all the primers detected polymorphism such as this primers RM556, RM562, RM594, RM7075, RM3843, RM28502, RM219, RM228, RM231, RM241, RM247, RM25022, RM25181, RM26652, RM27694, RM134, RM138, RM140, RM249, RM276, RM296, RM300, RM314, RM336, RM337, RM400.

In this study, very common chemicals were used for DNA extraction instead of costly chemicals. Several authors used expensive chemicals such as Rnase (Ahmadikhah, 2009) and proteinase K (Kang et al., 1998) for rapid and simple DNA extraction.

Table 1. Components of PCR cocktail (for each reaction).

SL	Component	Quantity (For single reaction)
1	ddH_2O	3.20 µl
2	PCR Buffer (10X)	1.00 µl
3	dNTPs(1mM)	1.00 µl
4	Mgcl2 (25mM)	0.60 µl
5	Taq polymerase	0.20µl
6	Primer forward (5µM)	1.0 µl
7	Primer reverse (5µM)	1.0 µl
8	DNA sample (ng/µl)	2.0 µl
	Total	**10.00 µl**

Figures 1. Extraction Buffer, Leaf crushing in grinding plate and Centrifugation of microfuge tubes and three layers after centrifugation.



Figures 2. Microsatellite profiles of BRRI dhan36 at loci (M) Molecular weight marker (20 bp DNA ladder), Lane 1: RM556; Lane 2: RM562; Lane 3: RM594; Lane 4: RM7075; Lane 5: RM1287; Lane 6: RM3843; Lane 7: RM5806; Lane 8: RM27877; Lane 9: RM28102; Lane 10: RM28502.

And the quality of extracted DNA was high enough to PCR amplification for marker assisted selection and genetic diversity analysis without RNase and proteinase K. The amplified PCR products of cold tolerant rice DNA showed quality banding patterns and intensity like leaf (Figures-2). The amplification of expected bands with SSR were evident of good quality genomic DNA without RNase and proteinase K. The complete digestion of PCR product indicated that the extracted DNA is also useful for genetic manipulation. The extracted DNA samples and PCR products in the present study were stable but extracted DNA was unstable in a rapid DNA extraction protocol developed by Warner et al. (2001). Commercial DNA extraction kit is not economic for marker assisted selection or diversity analysis in which large number of samples is used. DNA was extracted by Miniscale DNA Extraction (Zheng et al., 1995) and it was observed that concentration was too low to amplify. Therefore, this protocol was found to be potential for DNA extraction using different tissues of cold tolerant rice.

Cetyltrimethyl ammonium bromide (CTAB) method is simple and fast compared to other methods and no liquid nitrogen is required. DNA fingerprinting to evaluate genetic variation, quantitative traits loci (QTL) analysis, RT-PCR, screening of transformants and enzymatic

digestion. The objective of this study was to develop a simple and rapid method to isolate DNA under normal laboratory condition (room temperature) from small amount of tissue for large number of samples of cold tolerant rice. The present study has the potential applications in future breeding programme for the genetic improvement of cold tolerant rice.

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