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A Modified CTAB Method for Quick Extraction of Genomic DNA from Rice Seed/Grain/Leaves for PCR Analysis

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ABSTRACT

Each 5 degree increase in temperature, the life span of seed is halved and every 2% increase in seed moisture content the life of seed is halved. This variation in temperature and humidity lowers initial seed viability and a faster drop in seed germinability. These problems related to seed persist due to poor storage of seed. In such condition DNA isolation from rice leaves is difficult due to poor germination of seeds. Here we optimized a modified CTAB protocol for the isolation of good quality of DNA from rice seed/grains for PCR analysis with Rice Microsatellite (SSRs) primers. However, this protocol is also tested for DNA isolation from rice leaves at seedling stage and vegetative stage. This protocol is also cost effective, rapid, simple, high throughput and PCR compatible.

INTRODUCTION

The most severe problem with rice is that the mean minimum temperature of 12^0C for 30 days after seeding is critical and temperature below 12^0C resulted in high risk of crop failure due to poor germination, poor seedling growth or insufficient seedlings (Sipaseuth *et.al*.2007). In case of germination failure, DNA extracted directly from endosperm tissues can be effectively used in genotyping, genetic purity testing and seed quality evaluation (Mutou *et. al.* 2014). Pure and rapid DNA extraction is pre-requisite step for the most advanced techniques such as genetic mapping, DNA fingerprinting, marker assisted selection etc. DNA isolation from rice grains can be very time consuming and difficult procedure. However, PCR-based techniques for molecular analysis requires efficient recovery of good quality and quantity of DNA. The CTAB based method and its modifications have been used to obtain good quality of DNA for PCR-based downstream applications (Yari *et.al*.2013, Chuan 2010, Ahmadikhak 2010).

DNA-based molecular markers have been used extensively to assess the genetic diversity in most crop species. Condit and Hubbell (1991) were among the first to report that SSRs occur abundantly in plant species. SSR or microsatellite markers consist of di-, tri-, or oligonucleotide tandem repeats and are distributed throughout the eukaryotic genome (Struss and Plieske, 1998). Due to high efficiency, reproducibility, easy-to-use, co-dominance nature and high degree of polymorphism, or simple sequence repeats (SSRs) are widely used as molecular markers for fingerprinting germplasm to assess genetic diversity, pedigree analysis, evolutionary studies and genome mapping. (Yang *et.al*., 1994; Garland *et.al.* 1999). Hence, the aim of this work was to standardize a DNA isolation protocol for rice seeds/grains which would be simple, cost-effective, a high throughput, be suitable for PCR, and needs a few plant tissues without using liquid nitrogen. The protocol was also tested for several tissues of rice (seeds, grains, leaves of rice seedlings and leaves from vegetative stage) for their DNA yield.

MATERIALS AND METHODS

Plant material: Good healthy seeds of three selected genotypes i.e. NDRK 11-6, CR2218-64-1-327-4-1 and CR 2814-2-4-3-1-1-1 were used in this study. The genotypes were collected from CSSRI, Karnal, India.

Genomic DNA extraction:

25 rice seeds/grains or 3.7 mg of green leaves are washed with distilled water and soaked in 3ml of extraction buffer (8% CTAB, 8% PVP, 1.4 M NaCl, 0.1M Tris (pH 8), 25Mm EDTA) with 15 µl β-mercaptoethanol for 45 minutes in sterilized mortar pestle (soaking is not required for leaves). Mortar pestle with soaked seeds is now transfer in -20 for 5 minutes to harden the seed tissue for crushing. Soaked seeds are homogenized in mortar pestle till tissue disintegrates (small pieces of leaves were homogenized with plastic pestle in 2ml test tubes). Then homogenate is transferred in 2ml centrifuged tubes (600 microlitre homogenate in each tube). 600 microlitre phenol:chloroform (1:1) is added, mix well by inversion and centrifuge at 13000 rpm for 6 minutes. The supernatant is transferred into another 2 ml centrifuge tubes and 600 microliters of chloroform:isoamyl alcohol (24:1) is added and centrifuged at 13000 rpm for 6 minutes. Supernatant is transferred into 1.5 ml tubes and 700 microlitre ice chilled isopropanol is added and mix it by inversion and leave for 10 minutes and centrifuge at 12000 rpm for 10 minutes. Supernatant are now washed with 70% ethanol. Air dry the pellet for 1 hour and dissolve it in TE buffer (pH 8). RNase treatment is given for 30 minutes at 37^0C. Now the DNA is used for PCR analysis.

DNA Quantification and visualization:

The concentration of DNA obtained (ng/µl) by this protocol was determined on spectrophotometer. The purity level for all the genotypes under investigation were assessed by measuring optical density at A_{260} and A_{280} by UV spectrophotometer. Samples were subjected to electrophoresis in 0.5X TBE buffer for 45 minutes at 80V. 6 µl of isolated genomic DNA was loaded on 0.8% agarose gel stained with ethidium bromide (7µl/100ml of gel) to check DNA quality. The gels were photographed under gel documentation system (*BIO-RAD*). (table2)

PCR and product analysis:

Amplification reaction was carried out in a thermal cycler (Eppendorf) with 15µl of PCR reaction mixture containing 2µl 10X PCR buffer, 0.15µl 5 u/µl taq, 0.15µl 50mM $Mgcl_2$,0.5 µl 2mM dNTPs, 2µl DNA, 1µl of each forward and reverse rice microsatellite primers at a concentration of 10 pmol/µl. The thermal cycling conditions for the first cycle were 94^0C for 4

min and T_a for 1 min. For the next 30 cycles, the temperature regime was 94^0C for 1 min, T_a for 1 min and 72^0C for 2 min. The final extension was at 72^0C for 5 min. The amplified products were resolved in 1.8% agarose gel and documented in gel documentation system. A total of 6 SSR (RM 493, RM 1108, RM 25519, RM 10764, RM10864, RM 10748) primers have been used for assessment of PCR amplification of isolated genomic DNA of rice seed/grain by discussed protocol.

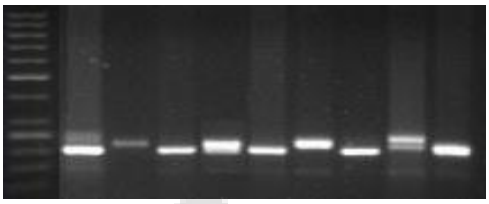
	L 1 2 3 4 5 6 7 8 9 		
Primers	**Genotypes**		
	NDRK 11-6	CR2218-64-1-327-4-1	CR 2814-2-4-3-1-1-1
RM 493	**Lane 1**	**Lane 2**	**Lane 3**
RM 11008	**Lane 4**	**Lane 5**	**Lane 6**
RM 25519	**Lane 7**	**Lane 8**	**Lane 9**

Fig1: PCR amplified product with SSR primers RM493, RM11008, RM25519

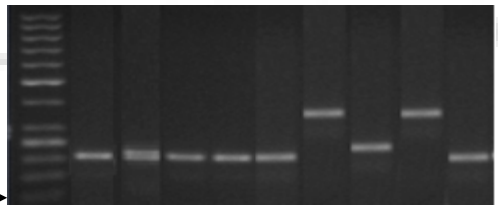
	L 1 2 3 4 5 6 7 8 9 		
Primers	**Genotypes**		
	NDRK 11-6	CR2218-64-1-327-4-1	CR 2814-2-4-3-1-1-1
RM 10864	**Lane 1**	**Lane 2**	**Lane 3**
RM 10764	**Lane 4**	**Lane 5**	**Lane 6**
RM 10748	**Lane 7**	**Lane 8**	**Lane 9**

Fig 2: PCR amplified products with SSR primers RM10864, RM10764, RM10748

Table 1: List of 6 SSR primers used for validation of amplification by PCR of isolated genomic DNA.

Sr. No.	Primers	Repeat motifs	Sequence	Annealing temperature	Ch. No.
1.	RM493	(CTT)9	TAGCTCCAACAGGATCGACC(F) GTACGTAAACGCGGAAGGTG(R)	58	1
2.	RM11008	(TTC)12	TTTGGATGGTCATTAGCCTCTGG(F) ATCAACCTTGCATGCTGTCTTCC(R)	58	1
3.	RM25519	(TA)42	GGTGATTAATTACTGGTCGGAAGG(F) GCTGGTTTGATCGGAATTACAGG(R)	58	10
4.	RM10864	(GT)27	GAGGTGAGTGAGACTTGACAGTGC(F) GCTCATCATCCAACCACAGTCC(R)	60	1
5.	RM10764	(AT)28	AGATGTCGCCTGATCTTGCATCG(F) GATCGACCAGGTTGCATTAACAGC(R)	60	1
6.	RM10748	(AG)14	CATCGGTGACCACCTTCTCC(F) CCTGTCATCTATCTCCCTCAAGC(R)	60	1

Table 2: DNA yield from different tissues of rice

Sr. NO.	GENOTYPES	TISSUE USED	DNA YIELD OBTAINED IN MICROGRAM/ml	Remarks
1.	NDRK 11-6	Kernel	1.2	The yield of nuclear DNA from all the tissues is great. Most importantly the quantity of DNA from the seed/grain is appropriate for PCR amplification as shown in figs.
		Dehusked seed	1.45	
		green fresh leaves of seedling stage	1.8	
		Green leaves of vegetative stage	1.6	
2.	CR2218-64-1-327-4-1	Kernel	1.2	
		Dehusked seed	1.42	
		Green fresh leaves of seedling stage	1.8	
		Green leaves of vegetative stage	1.6	
3.	CR 2814-2-4-3-1-1-1	Kernel	1.2	
		Dehusked seed	1.45	
		Green fresh leaves of seedling stage	1.8	
		Green leaves of vegetative stage	1.6	

RESULTS AND DISCUSSION

The presented modified CTAB method can isolate DNA from various tissues of rice seeds in a short duration without using water bath and liquid nitrogen. This protocol isolates DNA of good quality and PCR compatible from rice grains/seeds. Isolated DNA from seed and other tissues gave excellent amplification product by using SSR primers. However this protocol gives highest amount of DNA from green fresh leaves of seedling stage and green leaves of vegetative stage i.e. 1.8 µl and 1.6 µl respectively (1.8 µg / µl: table), other tissues like kernels and dehusked seeds also gave good quantity of DNA which is required for the PCR amplification reaction.

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