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Rapid DNA Extraction Method for PCR Based Downstream Application of Genomic DNA from Mungbean Seeds (*Vigna radiata*)

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Abstract

The isolation of good quality of DNA is the pre-requisite for molecular research. Although many DNA extraction protocols have been developed for mungbean but none of them used 'seed' as plant material for DNA extraction. To avoid the problems related to preservation and use of liquid nitrogen, we examined grinding of dried seeds of mungbean into fine powder. Use of lithium chloride instead of RNase has been studied for the removal of RNAs in order to get pure DNA for PCR amplification. Five SSR primers for gene specific microsatellite regions of mungbean genome viz. SSR4849, SSR4243,SSR5253,SSR5657 and SSR6061 were used to conduct PCR reaction, which confirm the high quality amplification of DNA. This method of DNA isolation will help the molecular breeders and researchers for genetic purity evaluation and other PCR based downstream applications for mungbean genotypes at seed level using molecular markers without using liquid nitrogen and beta-mercaptoethanol. This method of DNA isolation is devoid of requirement of particular temperature for seed germination. It is very simple, easy and quick isolation protocol with modified CTAB extraction buffer.

Key words : *Mungbean seeds, DNA extraction, CTAB, lithium chloride, SSR primers.*

Introduction

Mungbean are tropical grain legumes widely grown in Southeast Asian countries. It belongs to genus *vigna* under *Phaseoleae* tribe and is a diploid crop (2n = 2x = 22) with a genome size of approximately 560 Mb (1). Mungbean seed is highly nutritious containing 24-28% protein, 1.0-1.5% ash and 59-65% carbohydrates on dry weight basis. Mungbean protein is considered to be easily digestible for children and persons with underlying health conditions (2).

Good quality of DNA is prerequisite for the most of the plant molecular biology experiments. Although several protocols have been developed for the extraction of DNA from different tissues which have high costs, complex procedures, consume excessive time or result in poor quality of DNA. Extraction of nucleic acids direct from seed are to be favored because they are easy to handle, applicable for high sample throughput, relatively inexpensive, and provide high DNA quantity, good quality, and purity which are pre-requisites for successful PCR amplification and long-term storage of nucleic acids in biorepositories.

DNA markers have been utilized for germplasm evaluation, genetic diversity and phylogenetic analysis, high-density linkage map construction, genome mapping, and marker-assisted selection to investigate structural genomics in crop plants (3). Simple sequence repeat markers are co-dominant, PCR-based markers that are easy to generate, highly polymorphic and effectively used to detect genetic variation based on repeat-lengths. In 1999, the first SSR markers for mungbean were reported by (4) based on a search of the Gene Bank database, the SSRs could potentially be used to construct linkage maps and for genetic improvement of mungbean (5, 6). Simple sequence repeat markers have been widely used to identify disease resistance QTLs in legume species, which could be used in marker-assisted selection in crops.

The present investigation was designed to develop a CTAB based protocol to extract DNA, directly from seeds of mungbean genotypes in cost effective manner. SSR primers were used to test the quality of extracted DNA for the PCR amplification.

Materials and Methods

The present investigation was conducted at molecular biology laboratory, department of agricultural biotechnology and molecular biology, DR. Rajendra Prasad central agricultural university, pusa, Bihar, India. A total of 18 genotypes of mungbean seeds were selected for the extraction of DNA with this method. Among 18 genotypes, 17 were procured from Tirhut College of Agriculture, Dholi, Dr.RPCAU, Pusa, Bihar and one genotype was procured from NBPGR, New Delhi (table-1).

Seeds of mungbean were washed with tap water and oven dried at 50^0C for 30 minutes and cooled at room temperature. The dried seeds were powdered with sterile motor pestle. 15 mg of fine powder was taken in 2 ml tube

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Table-1 : List of SSR primers used for PCR amplification.

Primers	Sequence F (5' to 3')	Sequence R (5'to 3')	Annealing temperature (Ta)
SSR4849	ACACATTTGCAGACAACCAATC	TGAGAGAGAGAGACGAAAAGGG	52°C
SSR4243	CTGTTTCTGCATGTGATGTTT	CAAAAGCAAGACCCATTCCTAC	50°C
SSR5253	AGAGAAGTGGGGAAAATGCTTA	ATTGATGGCACAGGATAACTGA	53°C
SSR5657	TAACCTTCTGCATTTCCTTGGT	AGACAGCTTCAACATCATCGAG	48°C
SSR6061	CACAGGGTGAGTTAGGCTTTTA	AATGAAACAGTACGAGTGCCAG	48°C

Table-2 : Composition of PCR reaction mixture (20 µl).

Constituents	Amount/quantity
DNA template (20ng/ µl)	1.5 µl
Taq DNA polymerase (1 unit)	0.2 µl
dNTPs mix (1 mM)	0.5 µl
$MgCl_2$ (10 mM)	0.1 µl
Primer F (10 µM)	0.5 µl
Primer R (10 µM)	0.5 µl
PCR Buffer (5X)	2.0 µl
Nuclease free water	Rest

and 650 µl of extraction buffer (2.5% CTAB, 3% PVP, TRIS-(PH-8)-4%, EDTA (PH-8.5)-25mM, 1.4mM NaCl) was added and homogenize properly. The tube was incubated at 65^0C for 1 hour in water bath and centrifuged at 14000 rpm for 10 minutes. Supernatant was transferred in another 2ml tube and then 750µl phenol/ chloroform/ isoamyl alcohol (25:24:1) was added. After gentle stirring, the tube was centrifuged at 14,000 rpm for 10 minutes. Now supernatant was transferred to 1.5ml tube and 100 µl of 8M LiCl was added and kept at 4^0C for 15 min and then centrifuged at 12000 rpm for 5 minutes. The resulting pellet was RNA while DNA present in supernatant. Supernatant was taken in another 1.5 ml tube and 700 µl of ice chilled isopropanol added, mixed well and centrifuged at 12000 rpm for 10 minutes. The pillet was washed with 200 µl 70% ethanol and air dried, then dissolved in 40 µl TE buffer and Stored at -20^0C for further use.

Determination of DNA purity and concentration : DNA concentration and purity was quantified by benchtop nanaodrop spectrophotometer. The DNA was diluted in TE buffer to make dilution factor of 10 (1 µl of DNA and 9 µl TE). The quantification of DNA was done in triplicates. The OD values at 260 and 280 nm by the instrument were taken for the purity measurement. Ratio of 260/280 and concentration in µg/ml was measured by the instrument. DNA samples were also subjected to electrophoresis (0.8% agarose gel) in 0.5X TBE buffer for 30 minutes at 90V. The visualization and documentation were done by gel documentation system (Alpha Innotech, USA)

SSR-PCR amplification of isolated DNA : PCR amplification reaction was performed using Agilent thermo cycler using five SSR primers (Table:3) following the programme viz. 1 cycle of 94^0C for 4 minutes followed by 35 cycles of 94^0C for 1 minute, 49^0C for 1 minute, 72^0C for 1 minute with final extension of 5 minutes and hold on 4^0C. The final volume of PCR reaction mixture was 20 µl. The amplified product was subjected to 1.8% agarose gel for 1 hour and visualize with gel doc system.

Results and Discussion

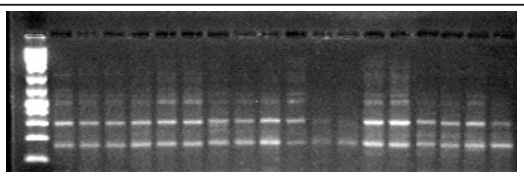
Under the present investigation mungbean seeds were used as plant material for DNA isolation from modified CTAB method. DNA extracted from mungbean seeds was found great quality and enough quantity. The ratio of 260/280 (1.8 is generally accepted as pure DNA) is commonly used to assess DNA purity with respect to protein contamination since aromatic amino acid in proteins tend to absorb at 280nm. We observed the ratio range from 1.76-1.85. Absorbance at 230nm shows salt contamination. The ratio of 260/230 nm should be 2.0-2.2. We found this ratio with range of 1.9-2.1. The amplification result with PCR using two SSR primers was found high quality. DNA from all genotypes isolated from this protocol using seed as plant material provides good amplification result (table-3).

Pvp forms complex hydrogen bond with polyphenolic compounds (Maliyakal,1992; kim1997). Sodium chloride helps to remove protein that are bound to DNA (7). 1.0-2.5 M NaCl is found very effective for the removal of polysachharides by increasing their solubility in ethanol so that they could not co- precipitate with DNA. Lithium chloride has been used to eliminate RNA. Lithium choride precipitate selectively RNA without binding with protein and DNA. In case of RNAase treatment, RNA is enzymatically degraded but not removed from extracts. Here the use of -mercaptoethanol (removes disulphide bond) was avoided as mungbean seeds are deficient of sulphur containing amino acids that is methionine and cysteine (8).

In mature seeds the major storage protein is 8S globulin in which no disulphide linkage exists due to lack of cysteine content (9). Presently the peroxidase activity and potassium hydroxide tests were performed for cultivars testing. The drawback of this method is that the enzymes activity is widely dependant on temperature and depends on cell environment so accurate analysis is quite difficult and also of high cost and time consuming. Outcrossing and inadvertent mixing of seed can compromise seed quality therefore genetic purity tests are critical tools for seed producers and plant breeders.

Table-3 : PCR amplification results of extracted DNA.

SSR4849



SSR4243

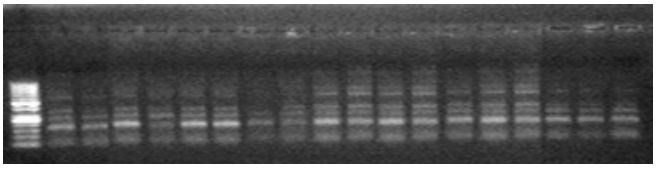
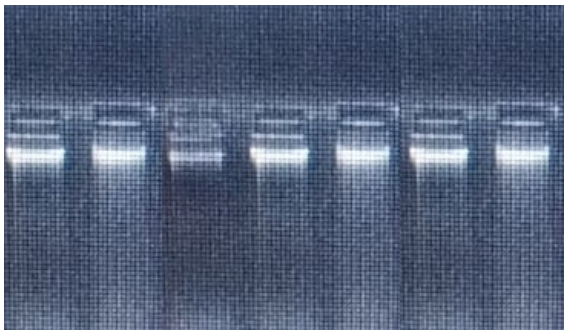


Table-4 : Determination of DNA purity and concentration.

S.No.	Genotypes	Concentration of DNA (ng/µl)	Agarose gel electrophoresis result of some DNA samples from selected genotypes
1.	PLM 380	100.12	
2.	SML 18-31	98.21	
3.	TRCRM-141	102.43	
4.	WBSM-48-5	112.23	
5.	IPM 99-125	99.05	
6.	IPM 610-2	100.04	
7.	SML-1825	108.55	
8.	PANT M-5	98.50	
9.	PUSAM-19-32	101.22	
10.	PUSA-BM-8	101.54	
11.	PM-14-19	103.43	
12.	IPM-2-3	102.54	
13.	IPM-101-102	102.76	
14.	PM-15-28	98.60	
15.	IPM-701-4	99.86	
16.	PM-15-21	99.65	
17.	IPM-604-1	100.23	
18.	SAMRAT	100.98	

Conclusions

Although many DNA extraction protocol has been developed for mungbean but none of them used seed for DNA extraction. This method is also quit suitable for screening large number of progenies directly from seeds of filial generations in a breeding population. It is also quit suitable for seed purity testing without undergo germination process for seeds. This method of DNA isolation is devoid of requirement of particular temperature for seed germination Germination process requires proper temperature and favorable environmental condition, so using this technique of DNA isolation researchers and plant breeders can get advantage to save time and effort. This method of DNA isolation will also help DNA based genetic purity evaluation the molecular breeders to characterize the mungbean varieties for verification of genetic purity at seed level using molecular markers. It is very simple, easy and quick isolation protocol with modified CTAB extraction buffer.

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