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MOLECULAR CHARACTERIZATION OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS*) MUTANT LINES

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ABSTRACT

In the present investigation, eight morphologically distinct mutants of winged bean and control were evaluated for the assessment of genetic alterations induced through mutagenesis.
Out of the nine ISSR primers used, 3 primers were able to amplify and out of 45 fragments, 37 were polymorphic in nature (82.22 %). Hence it is inferred that the mutants of winged bean are not only morphologically /biochemically different but genetically also they are variable. The actual quantum of genetic variation achieved through induced mutation has been studied by using ISSR molecular marker. The *xantha* mutant can be used in breeding programme of ornamental garden plants for developing attractive leaf colour. The long pod and early maturing mutants can be used beneficially in further breeding programme of winged bean for developing its novel recombinant plant types having commercial utility value.

Introduction

"DNA markers are a class of molecular markers, which utilize the DNA polymorphism between organisms." The DNA polymorphism can result from the induced mutation, recombination, inversion, deletion and duplication in genomic DNA. Marker assisted selection can be used as a powerful tool for the differentiation and identification of mutant plant types from the mutant populations at seedling stage (George, 2006).

Genetic engineering and biotechnology hold great potential for plant breeding as they promise to expedite the time taken to produce crop varieties. With the use of molecular techniques it is now possible to hasten the transfer of desirable genes from related species. In addition the polygenic characters which were previously very difficult to analyze by using traditional plant breeding methods, can now be easily tagged using molecular markers.

There are various types of DNA markers presently available to evaluate DNA polymorphism in sample genomes. Selection of a correct marker system depends upon the type of study to be undertaken and the marker system used. These markers are generally classified as (a) hybridization based markers in which the DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to a labelled probe, which is a DNA fragment of known or unknown sequence and (b) the PCR based markers.

In case of PCR based markers, the primers of known sequence and length are used to amplify genomic DNA sequences which are visualized by gel electrophoresis technique. The PCR which is a versatile and extremely sensitive technique, (Saiki et al., 1995) that uses a thermostable DNA polymerase (Saiki et al, 1988) has changed the total scenario of molecular biology with new possibilities in molecular marker research. Some of the hybridization as well as PCR based marker systems have been detailed below:

RFLP – Restriction Fragment Length Polymorphism

In RFLP analysis restriction endonuclease digested genomic DNA is resolved by gel electrophoresis and then blotted on to a nitrocellulose membrane (Southern, 1975). Specific banding patterns are then visualized by hybridization with a labelled probe.
STS – Sequence Tagged Sites

In this, RFLP probes specifically linked to a desirable trait can be converted into PCR based STS oligonucleotide primers, based on nucleotide sequence of the probe giving a polymorphic band pattern. This is extremely useful for studying the relationship among several species at specific locus (Bustos et al., 1999)
EST – Expressed Sequence Tag Markers

These are introduced by Adams et al., (1991) and are obtained by partial sequencing of random CDNA clones.

1. Microsatellites and Mini satellites

It is known that about 30 to 90 percent of the genome of virtually all eukaryotic species is made up of repetitive DNA, which is highly polymorphic in nature. One major form of repetitive DNA, has been microsatellites and minisatellites. Minisatellites are tandem repeats with a monomer length of about 11-6 bp while microsatellites are short tandem or simple sequence repeats of 1-6 bp length, repeated several times.

RAPD – Randomly Amplified Polymorphic DNA

Welsh and McClelland (1990) introduced RAPD technique for the first time. Here, a single primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. A discrete DNA product is obtained after PCR amplification, if these priming sites are within the amplification range of each others.
AFLP – Amplified Fragment Length Polymorphism

This technique was developed by Vos et al., (1995). It is based on selective amplification of restriction enzyme digested DNA fragments with specific primers. Multiple bands are generated in each amplification reaction that contain DNA markers of random origin.

ISSR – Inter Simple Sequence Repeats

Ziethkiewicz et al., (1994) reported this technique for the first time, where microsatellites anchored at the 3` end are used for amplifying genomic DNA. The anchor also allows only a subset of the targetted inter-repeat regions to be amplified, thereby reducing the high number of PCR products expected from the primers of inter-repeat region.

ISSR marker was found simple and capable of producing reproducible and greater polymorphism than other marker system. (Wolfe et al.,1998). The efficiency of ISSR markers than RAPD markers is higher, which is well visualized from higher frequency bands and polymorphic information content values (Chattopadhyay et al., 2005).

Review of literature

The winged bean is classified under family *Leguminosae*. Botanically it is known as *Psophocarpus tetragonolobus* (L.) DC. The genus *Psophocarpus* was first proposed by Neckner in 1790 (Verdcourt and Halliday, 1978). It is commonly known as Goa bean, Asparagus bean and four angled bean.

The genus *Psophocarpus* comprises nine species. Verdcourt and Halliday (1978) have suggested a classification for this genus. The different species like the *Psophocarpus* scandens (Endl) Verdc, *Psophocarpus grandiflorus* Wilczek and Psophocarpus palustris

Desv are closely related to the winged bean. Eight species of Psophocarpus are believed to be native to tropical Africa and the cultivated winged bean (*Psophocarpus tetragonolobus*) is derived from these eight species in south east Asia.

Molecular marker study

Genetic variation of a species or any mutated population can be assessed phenotypically in field or by studying molecular markers in the laboratory. A combination of these two approaches is required for reliable and confirmed results. Molecular markers are the identifiable DNA sequences, found at specific locations of the genome and they are associated with the inheritance of a trait or linked genes. The polymorphic DNA bands help to confirm the additional phenotypic characters of mutant lines.

Analysis of genetic diversity and its use in crop improvement

Genetically modified crops like wheat, rice, cotton, maize, soybean and chickpea are under cultivation for improved traits. For example, transgenic tomatoes with delayed ripening, potato for higher starch and the golden rice for higher B-carotene content.

It has also become possible to clone the mutated genes that are responsible for a certain phenotype; this part of genetics is better known as forward genetics (Barr and Emmanuel, 1990).

George (2006) used the RAPD and ISSR molecular markers for the genetic diversity analysis of soybean mutant and breeding lines, with reference to lectin content of seeds. She detected 69% DNA polymorphism through ISSR and 53.5% through RAPD protocols

Molecular markers have several advantages over the traditional phenotypic markers which were earlier available to plant breeders. They offer great scope for improving efficiency of conventional plant breeding by carrying out selection not directly on the trait of interest but on molecular markers linked to that trait. (Souframanien and Gopalkrishna, 2004).

RAPD markers have been used for assessing the genetic diversity among cultivars of several crops like cowpea (Mighouna et al., 1998), pea (Hoey et al., 1996), mungbean (Lakhanpaul, 2000; Chattopadhyay, 2005), and blackgram (Ali et al., 2007).

RAPD markers were also used for studying genetic variability in induced mutants of groundnut (Bhagwat et al; 1997), chick pea (Banerjee et al., 1999), moth bean (Khadke, 2005) and for mapping mutations in *Arabidopsis thaliana* (Williams et al; 1993).

Boukar et al; (2004) have developed AFLP – derived SCAR markers associated with Striga gesnerioides resistance in cowpea. Kulkarni (2007) succeeded in developing SCAR marker for genetic male sterility in cotton.

Ray et al; (2006) evaluated genetic stability of three economically important micropropagated banana (Musa spp.) cultivars as assessed by RAPD and ISSR markers. Among the two marker systems used, the ISSR fingerprinting revealed more polymorphism than the RAPD.

The genetic relationships among Turkish cultivars and breeding lines of Lens culinaris have been analyzed by Yuzbasiogly et al., (2006). They noted highest genetic distance of 58.3% in these lines.

The future possibilities of crop improvement would include development of new and more efficient plant ideotypes with improved quality traits (including nutritional traits), which earlier did not receive the adequate attention of plant breeders. The objectives of the present day plant breeding can be successfully achieved, if the conventional plant breeding is supplemented with molecular breeding approaches including both, the transgenic crops and marker –assisted selection (MAS) methodology (Gupta and Roy, 2002).

Keeping the foregoing in mind the need for genetic analysis of winged bean mutant lines through marker studies was felt necessary. It was visualized that this study will reveal the actual quantum of genetic diversity prevailing in winged bean mutant lines generated through earlier mutation breeding program. It was further noted that the work on molecular marker studies in winged bean as such has been quite scanty and hence it has become necessary to undertake the marker–assisted selection in winged bean so as to facilitate the future breeding program of the winged bean crop.

Materials and methods

Fourteen true breeding M_6, M_7 and M_8 mutant lines of variety EC 38955-A of winged bean obtained from the earlier mutation breeding programme (Kulthe, 2003) were taken for the morphological studies, besides quantitative /qualitative biochemical aspects and molecular marker studies.

The list of mutants of winged bean used in the present study is as follows:

Mutants used for ISSR marker studies.
1.Control (EC-38955-A)
2.Long pod
3.Early maturing
4.La.L./high yielding
5.FP/La.L.
6.LP/black seed
7.FP/LP
8.Dwarf
9.*Xantha*

Molecular studies

1. The genomic DNA isolation

Genomic DNA was extracted from selected mutants of winged bean using liquid nitrogen according to standard protocol with necessary modifications. Isolation of genomic DNA from plant leaf samples was carried out by using CTAB method (Doyle and Doyle, 1987).

Preparation of buffers and chemicals 2X CTAB (1liter)

- 20 g of CTAB
- 81.82g NaCl
- 100 ml 1M Tris pH8.0
- 40 ml 0.5M EDTA pH 8.1

The final volume was made up to 1000ml, autoclaved and stored at room temperature. 40µl β-mercaptoethanol per 20 ml solution was added just before use.

3M Sodium Acetate (pH 5.2)

- 40.83 g of Sodium Acetate
- 80 ml of Distilled water
- P^H was adjusted to 5.2 and final volume was made to 100 ml

TAE (Tris-Acetate-EDTA)

- Tris base 48.4 g
- Acetic Acid11.42 ml
- 0.5 M EDTA 20 ml
- P^H was adjusted to 8.5 using KOH and final volume was made to 1000ml.

TBE (Tris-Borate-EDTA) (10X)

- Tris base 108 gm
- Boric Acid 55 gm
- Na_4EDTA 9.3 gm

P^H was adjusted to 8.3 using KOH and final volume was made up to 1000ml.

10 TE (10X)

- 100 ml 1 M TRIS (P^H 8.0).
- 20 ml 0.5 M EDTA (P^H 8.0).

Final volume was made up to 1 liter. The solution was autoclaved before use.

Protocol used

1. Young leaf tissues were ground in liquid nitrogen. About 100 mg ground tissue was taken into 1.5 ml microcentrifuge (MCF) tubes for DNA isolation.
2. 500 µl of 2x CTAB buffer was added to all MCF tubes and warmed to 65°C.
3. 5 µl of ß-mercaptoethanol was added into each MCF tube, mixed thoroughly and incubated at 65°C for 1 hour.
4. After cooling 500 µl chloroform-isoamyl alcohol (24:1) was added, mixed the content and kept at room temperature for 20 minutes. Centrifuged at 7000 rpm for 10 minutes.
5. The aqueous phase (top phase) was decanted into a new 1.5 ml MCF tube, 500 µl of isopropanol was added and incubated at -20°C for 30 minutes
6. All the MCF tubes were centrifuged at 5000 rpm for 10 minutes, isopropanol was removed, and pellets were washed with 500µl 70% ethanol.
7. Pellets were dissolved in (100 µl) TE buffer, 1 µl RNase (10mg/ml) was added and incubated at 37 °C for 30 minutes.
8. The 1/10 volume of 3M sodium acetate and 2 volumes of absolute ethanol were mixed. It was incubated at -20 °C for 1 hour.
9. Centrifuged at 5000 rpm for 15 minutes, drained and washed the pellets with 70% ethanol. The pellets were dried and dissolved in 100 µl TE.
10. The stock solution was stored at – 20°C and the working solution at 4°C.
11. A small aliquot of isolated DNA was run on a 1% (w/v) TAE gel to check the quality of DNA sample

2. Quality check of isolated DNA

For checking the quality of isolated DNA, 2 µl DNA was diluted with 7 µl of TE buffer. After adding 1 µl 6x loading dye, 10 µl sample was loaded in a slot of 1% agarose gel containing 0.05 µg/ml of ethidium bromide. The agarose gel electrophoresis was carried out for nearly one hour at 50Ma. The gel was visualized and photographed by gel documentation system. The intact double stranded DNA forming a thick single band of high molecular weight confirmed the good quality of genomic DNA extracted.

3. A. ISSR marker

Inter Simple Sequence Repeats (ISSR), a molecular technique, was used for the assessment of genetic variability of selected mutants. Total nine mutants were genetically analysed by this technique.

ISSR Primers -

ISSR primer sets were ordered from University of British Columbia (UBC). Total nine ISSR primers were used for initial screening. Out of nine primers, 3 primers gave the amplification (in terms of repeatability, scorability) and were selected for identification.

The selected 3 primers were with various di and tri nucleotide SSR repeats.

Following primers gave the amplification. The primer sequences were as follows.
Primer 816- 5' CAC ACA CAC ACA CAC AT 3'
Primer 818 - 5' CAC ACA CAC ACA CAC AG 3'
Primer 821 - 5' GTG TGT GTG TGT GTG TT 3'

3. B. PCR amplification of DNA with ISSR primers

PCR technique has promoted the development of a range of molecular assay systems which detects polymorphism at molecular level. In this study the most widely adopted PCR based ISSR marker technology has been used. PCR reactions were carried out in a Finnzyme make thermal cycler.

Preparation of master mix for PCR

Taq buffer (10X)	- 2.0µl
DNTP's (10mM)	- 2.0µl
Taq polymerase (3U/ul)	- 0.4µl
Tween 20	- 0.2µl
MQ water	-3.4µl
Primer (3pmol/ ul)	- 2.0µl
Template DNA	-3.0µl

PCR program used

- 94 °C for 3 min - Hot start Denaturation
- 94 °C 15 sec – Denaturation (38 cycles)
- Annealing for 30 sec – temperature vary according to primer
- The reaction for 30 to 38 cycles.
- 72 °C for 1 minute – Extension (38 cycles)
- Last 72 °C for 5 minutes – final extension.
- Total 30 reactions
- After completing 38 cycles, samples were loaded on 1% Agarose gel in TBE.

3. C. Agarose gel electrophoresis

The PCR products were separated by using 1% agarose gel in 1X TBE buffer.

Protocol used

1. The 0.8g agarose was dissolved in 100ml of 1X TBE by gentle heating on magnetic stirrer with hot plate.
2. Gel was prepared by pouring hot agarose in agarose gel tray situated in agarose gel apparatus supplied by Bangalore Genei Pvt. Ltd.
3. The comb was inserted in such a way that 1mm gap between the teeth and surface of tray could be made easily and this was hold for about 30-40 minutes.
4. After solidifying the gel, the comb was removed gently. The 1x TBE buffer was poured into the buffer tank so as the gel should be immersed about 5mm in this buffer.
5. The 3 µl amplified DNA samples and 3 µl of 6X gel loading buffer(0.25% bromophenol blue, 0.25% xyline cyanol FF, 20% glycerol in deionised water) were added and spun for 2-5 seconds.
6. Samples were gently loaded into the wells using microsyringe and run at 60 V until samples migrated to end of the gel.
7. Gel was carefully removed and kept for staining.

3. D. Gel staining and documentation

1mg/ml ethidium bromide staining solution was used and the gel was photographed using Alpha Imager gel documentation system.

Band Scoring and Data Analysis

For each sample, each fragment / band that was amplified using ISSR primers was treated as a unit rearrangement in genome. The primers which gave scorable and consistently reproducible amplicons were considered. The gel pictures were taken and documented to computer by using Alpha Imager gel documentation system and size of each amplicon was measured by using Alpha Imager Software with respect to standard molecular weight DNA ladder and the molecular weight of each of the potential specific band was calculated using the software program Alpha Imager.

Phylogeny Analysis

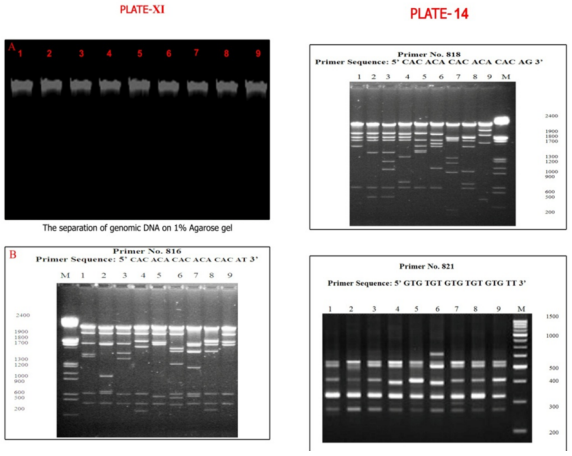
The Dendrogram was plotted by using bioinformatics phylogeny Free Tree and Tree View of DNA fingerprint analysis tool. Distance Similarity Matrix was calculated by using Neighbor-joining tree construction method of Nei and Li (1979) Dice index.

RESULTS

Molecular studies

In present studies the eight morphological mutants and control plant types were used for DNA isolation. The DNA of all mutants

was amplified on 1% agarose gel.

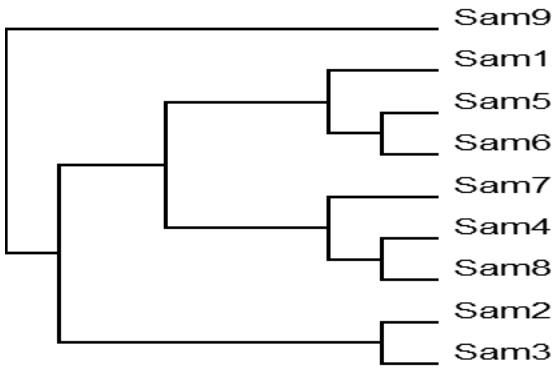


1. ISSR-marker studies (Plates XI A & B, XII A & B)

The genetic status of winged bean mutants was evaluated by ISSR molecular marker technology. The dendrogram and phylogeny of mutants have been studied.

Dendrogram

The Dendrogram analysis was carried out by using bioinformatics phylogeny tool Free Tree and Tree View of DNA fingerprint analysis. The dendrogram tree was as follows.



2. Phylogenetic Analysis

Distance Similarity Matrix:

Distance Similarity Matrix was calculated by using Neighbor-joining tree construction method of Nei and Li (1979) Dice index.

Table 01: Distance Similarity Matrix of winged bean mutants.

	Sam1	Sam2	Sam3	Sam4	Sam5	Sam6	Sam7	Sam8	Sam9
Sam1		0.18919	0.18919	0.24324	0.222	0.28205	0.38462	0.24324	0.31429
Sam2	0.18919		0.10526	0.26316	0.243	0.35	0.35	0.31579	0.27778
Sam3	0.18919	0.10526		0.26316	0.243	0.35	0.35	0.26316	0.27778
Sam4	0.24324	0.26316	0.26316		0.351	0.35	0.4	0.21053	0.33333
Sam5	0.22222	0.24324	0.24324	0.35135		0.28205	0.48718	0.35135	0.37143
Sam6	0.28205	0.35	0.35	0.35	0.282		0.47619	0.35	0.36842
Sam7	0.38462	0.35	0.35	0.4	0.487	0.47619		0.35	0.47368
Sam8	0.24324	0.31579	0.26316	0.21053	0.351	0.35	0.35		0.38889
Sam9	0.31429	0.27778	0.27778	0.33333	0.371	0.36842	0.47368	0.38889	

Out of 9 ISSR primers used, 3 were able to amplify and the number of amplified fragments ranged from 8 (UBC 821) to 19 (UBC 816) which varied in size from 285bp to 2260bp. Amplification of genomic DNA of nine genotypes, using ISSR markers yielded 45 fragments, of which 37 were polymorphic while the remaining were monomorphic in nature. Of the 45 amplified bands, 37 bands (82.22%) were polymorphic with an average of 12.33 polymorphic fragments per primer. ISSR based dendrogram indicated that the 9 mutants used in the present study could form 3 clusters (I, II & III) having 2, 6 & 1 mutants, respectively.

Cluster I comprised of 2 mutants. The cluster II carried 2 sub clusters, namely, IIa and IIb, with 6 mutants getting grouped altogether in this cluster. The cluster III comprised of only one mutant, namely, *xantha* (sam9) which was significantly different in the entire group of mutants used in present study.

Conclusion

The ISSR molecular marker study of winged bean mutants has shown genetic alteration achieved through induced mutation in winged bean. The *xantha* mutant can be used in breeding programme of ornamental garden plants for developing attractive leaf colour. The long pod and early maturing mutants can be used beneficially in further breeding programme of winged bean for developing its novel recombinant plant types having commercial utility value.

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