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Molecular characterization of elite cotton cultivars using ISSR markers

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Genetic variability and relationship between varieties are of great importance for cotton breeding. ISSR marker systems were used for identification and genetic diversity analysis of elite *G. hirsutum, G. arboreum* and introgressed lines. 12 cotton genotypes were subjected to ISSR analysis using 55 ISSR primers. PCR products were subjected to agarose gel electrophoresis and the banding patterns were compared among 12 elite cotton varieties of diploid, tetraploid and introgressed cotton. Out of 55 ISSR primers tested, 15 were scorable, producing 101 marker bands with 83 being polymorphic. The primer IS-08 generated the greatest number of polymorphic markers. The ISSR markers were found to be reproducible and polymorphic. A dendrogram constructed from ISSR data classified 12 cotton genotypes into two major clusters, one containing six genotypes belonging to *G. hirsutum* cultivars and the other contained 4 genotypes belonging to *G. arboreum* cultivars. Two introgressed cultivars PAIG-8/1 and PAIG-27 showed highest level of genetic similarity with G. arboretum varieties. ISSR technique was thus found to be efficient method for detecting DNA polymorphism useful for DNA fingerprinting and genetic diversity analysis in cotton.

Key words : Genetic diversity, Molecular markers, ISSR, Cotto

INTRODUCTION

Cotton 'the white gold' is the world's leading natural fiber crop and it is the corner stone of textile industries world wide. The cultivated cotton include *Gossypium arboreum* (L) and *Gossypium herbacium* (L), Old World species, both diploid species with an AA genome native to southern Asia, Africa and two allotetraploid species *Gossypium barbadense* (L) and *Gossypium hirsutum* (L),New World species with AD genome from Central, North and South America. Although small gains in yield and fiber quality continue to be made by conventional breeding programs, genetic improvement of agronomic traits is beginning to plateau as a result of an increasing narrow germplasm base for selection. Genetic diversity is desirable for long term crop improvement and reduction of vulnerability to important crop pests. Genetic diversity resulting from interspecific introgression can be evaluated with morphological characteristics, seed proteins, isozymes and DNA markers. To have reliable estimates of genetic relationship, a large number of polymorphic markers are required. This limits the use of morphological characteristics and isozymes, which are few, or lack adequate levels of polymorphism in *Gossypium* spp. Therefore, there is a need to study polymorphism at the DNA level which can be indicative of genetic diversity in cotton. DNA markers have proven to be valuable in crop breeding especially in studies of genetic diversity and in cultivar identification.

Polymerase chain reaction (PCR) based molecular markers, e.g. ISSR, RAPD, SSR, STS, AFLP etc. are useful for various applications in the plant breeding. Inter Simple Sequence Repeats (ISSR) are arbitrary markers in which only one primer is used. The ISSR technique involves amplification of a DNA segment present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite strands. This technique uses microsatellites, usually 16 to 25 bp long, as primers in the single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter SSR sequences of different size (Reddy *et al.*, 2002). The primers used can be repeated of di, tri, tetra or penta nucleotides anchored with one or two base sequences at 3'or 5' end (Zietkiewich *et al.*, 1994). Unanchored primers also can be used (Gupta *et al.*, 1994). ISSR are reproducible markers with 92-95 per cent efficiency (Reddy *et al.*, 2002). The present molecular analysis was carried out to analyze genetic relationship and genetic diversity of the cultivars.

MATERIALS AND METHODS

Plant material and DNA extraction:

The list of elite cotton cultivars used in the present study is as below.

Elite G. hirsutum cultivars.

1. PH-93 2. PH-325 3. PH-348 4. NH-452 5. NH-

545

Elite G. arboreum cultivars:

1. PA-402 2. PA-255 3. PA-405

Elite introgressed cultivars:

1. PAIG- 8/1 2. PAIG-27

Parents of introgressed cultivars:

1. PA-140 *(G. arboreum) 2. Poornima (G. hirsutum)*

The seeds of the above 12 cotton cultivars were obtained from the Cotton Research Station, Nanded; Cotton Research Station, Mahboob Baugh Farm; and the Cotton Research Scheme, Marathwada Agricultural University, Parbhani. Total genomic DNA was extracted from 4g of bulked leaf sample by a modified procedure of Edwards *et al.* (1991) in which 2% PVP (polyvinyl pyrrolidone) was added to the DNA extraction buffer to avoid co-isolation of phenolics and polysaccharides in the DNA. A total of 55 ISSR primers from Banglore Genei, India were used. Among them only 15 primers were scorable on 1.6 per cent agarose gel.

Molecular analysis:

ISSR amplification:

ISSR amplification was performed in 25µl reaction volume containing 40ng genomic DNA, 2.5µl of reaction buffer (10X Taq polymerase buffer with 15mM $MgCl_2$), 200ìM of each dNTPs (Q -Biogene), 15ng of ISSR primers (synthesized by Bangalore Genei, India), 1.5 U Taq polymerase (3U/ìl, Bangalore Genei, India). The reaction profile was preceded by a single 94°C soak for 5 minute, 45 cycles consisting each of a denaturing step of 1 minute at 94°C, a primer annealing step of 45 seconds at 49°C and a primer extension step of 2 minutes at 72°C. At the end of 45 cycles, a single 72°C extension was applied for 5 minutes for polishing the ends of PCR products. PCR amplified products were separated on a 1.6 % agarose gel containing 0.1ìg/ìl of ethidium bromide for about 5 hours at 60 Volts. Gels were photographed under UV light with a Tracktel GDS-2 gel documentation system.

Data analysis:

DNA fragment size was estimated by comparing the DNA bands against a Lambda/ Hind III, pUC 18 / Sau 3A-pUC 18/ Taq I base pair ladder (Bangalore Genei, India). The amplified DNA bands were scored on gel under a UV transilluminator as 1 for the presence and 0 for the absence of bands and assembled in the data matrix table. The pair wise comparisons were calculated using Nei and Li's coefficient (Nei and Li, 1979).The similarity values found were utilized to group individuals via the unweighted pair group method with arithmetic average (UPGMA). NTSYS-PC (Rolf, 1993) was used to perform all the analysis.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below:

Polymorphism as detected by ISSR analysis:

Initially 55 ISSR primers were screened, of these 15 primers were scorable on 1.6 per cent agarose gel. A total of 101 ISSR markers were amplified, out of which 83 were polymorphic (82.1%). Average number of polymorphic loci amplified per primer was 5.5. The size of ISSR amplicons was between 100bp in IS 09 to 3000 bp in IS 07. The number of polymorphic markers generated by each ISSR primer are given in Table 1. The ISSR primer IS 16 generated the least number of markers (3). The maximum number of polymorphic markers (12) was generated by IS 8 (Fig. 1a). Highly polymorphic profiles were obtained with 9 of the primers used *viz*., IS 4, IS 5, IS 6, IS 7, IS 8, IS 9, IS 11, IS 12 and IS 14. None of the primers individually was so informative as to differentiate all the cultivars. Primers IS 7 and IS 8 were polymorphic in all the cultivars. IS 7 generated a specific band of 800bp for the cultivar PH 93 and another of 585bp for cultivar PH 348 (Fig 1b). IS 9 produced a specific band of 1 kb for cultivar PA 140.

Introgression study:

ISSR analysis:

The ISSR analysis revealed that both introgressed cultivars had more specific band homology with parent PA-140 (9). Only one specific band for Poornima was generated by the primer IS-14 (Table 2). The primer IS-14 generated 800bp band in both introgressed cultivars which is specific to parent Poornima .

Cluster analysis:

Nei and Li (1979) similarity coefficient between 12 cotton cultivars using ISSR markers ranged from 0.49 to 0.93. *G. hirsutum* cultivars PH 325, NH 452 and Poornima were highly similar. Moreover, high similarity was found between the two introgressed cultivars PAIG –8/1 and PAIG-27. Maximum ISSR diversity was evident between PA-402 (*G. arboreum*) and the *G. hirsutum* varieties PH 325, NH 425 and Poornima.

A dendrogram based upon cluster analysis of similarity coefficients from the ISSR analysis revealed two major groups (I and II) (Fig. 2). In group I, 5 *G. hirsutum* cultivars: NH 545, PH 93, PH 325, NH 425 and Poornima clustered separately from the *G. hirsutum* cultivar PH 348. In group II, the 2 introgressed cultivars PAIG 8 /1, PAIG 27 and 3 *G. arboreum* cultivars: PA 405, PA 140 and PA 402 clustered separately from the *G. arboreum* variety PA 255.

In order to apply molecular techniques for genetic diversity analysis one needs to know the level of polymorphism revealed by the different techniques in the species under study. Chowdhary *et al.* (2002) were able to identify only 6 chickpea cultivar specific markers out of 19 cultivars studied due to low level of intra-specific polymorphism in chickpea.

The level of polymorphism was high using ISSR primers (Table 1). A higher level of polymorphism for ISSR compared to RAPD primers was reported in chickpea (Chowdhari *et al.*, 2002) and in citrus (Fung and Roose, 1997). Reproducibility of the ISSR markers was found to be good. ISSR markers are superior to RAPD markers in cotton in term of cost and speed. Yang

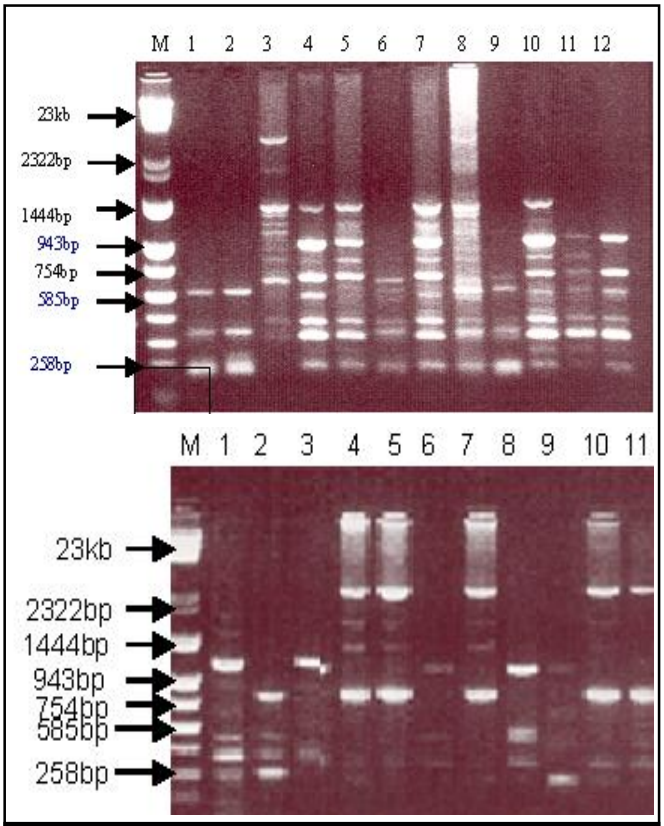

Fig.1. (1a & 1b) : RAPD Polymorphism among 12 Cotton Varieties using primer a) IS-08 b) IS-07. Details of lanes : 1. NH-545 (G.h.) 2. PH-93 (G.h.) 3.PH-325 (G.h.) 4. PA-255 (G.a.) 5. PA-405 (G.a.) 6. NH-452 (G.h.) 7. PA402 (G.a) 8. PH-348 (G.h.) 9.Poornima (G.h.) 10. PAIG-8/1 (I) 11. PAIG-27 (I) 12.PA-140 (G.a) and M for marker 1 Kb ladder and Lambda/hindIII, pUC18/Sau 3AI-pUC18/Taq I Digest (Note-G.h - G.hirsutum and G.a - G. arboreum. and I - Introgressed cotton varieties)

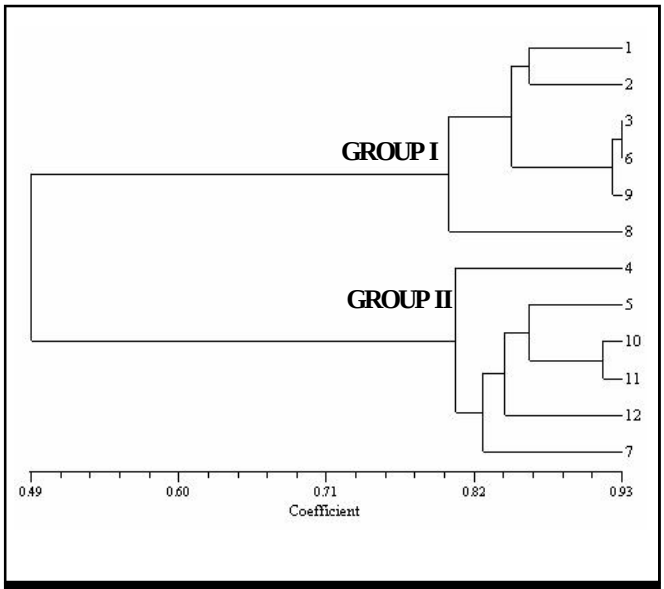

Fig. 2 : Dendrogram generated by ISSR analysis of Diploid, Tetraploid and Introgressed Cotton varieties : Details of lanes : 1. NH-545 (G.h.) 2. PH-93 (G.h.) 3. PH-325 (G.h.) 4. PA-255 (G.a.) 5. PA-405 (G.a.) 6. NH-452 (G.h.) 7. PA-402 (G.a.) 8. PH-348 (G.h.) 9. Poornima (G.h.) 10. PAIG - 8/1 (I) 11. PAIG-27 (I) 12. PA-140

Table 1 : List of ISSR primers used, level of polymorphism detected among 12 cotton genotypes

Primer code generated	No. of markers generated	No. of polymorphic markers
IS-1	4	3
IS-2	6	4
IS-3	4	3
IS-4	7	6
IS-5	10	9
IS-6	7	6
IS-7	8	8
IS-8	12	12
IS-9	9	7
IS-11	7	5
IS-12	6	4
IS-13	8	6
IS-14	6	5
IS-15	4	3
IS-16	3	2
Total	101	83 (82.1%)

Table 2 : Parent specific bands generated by introgressed lines by ISSR analysis

Primer	Introgressed variety PAIG-8/1	Parent – 1 (Poornima) Specific Band	Parent – 2 (PA140) specific band	Introgressed variety PAIG-27	Parent – 1 (Poornima) Specific band	Parent – 2(PA-140) Specific band
IS-01	2	0	1	2	0	1
IS-14	3	1	0	3	1	0
IS-07	2	0	2	2	0	2
IS-09	7	0	5	6	0	4
IS-12	3	0	0	2	0	0
IS-15	1	0	0	1	0	0
IS-03	1	0	0	1	0	0
IS-13	2	0	1	3	0	2
Total	21	1	9	20	1	9

et al. (1996) in their comparison of DNA marker systems report a lower relative cost and time for ISSR's than RAPD's. ISSR markers clustered the G. hirusutum and *G. arboreum* cultivars into separate groups. Iqbal *et al.* (1997) also found distinct cluster formation of accessions belonging to *G. hirsutum* and *G.arboreum* groups except for one variety based on RAPD analysis. The cultivar PH-348, a *G. hirsutum* genotype is most diverse from the other *G. hirsutum* cultivars based on ISSR markers. Two introgressed cultivars PAIG-8/1 and PAIG-27 also clustered in the *G.arboreum* group. These two introgressed cultivars were developed from interspecific crosses between *G. arboretum* (PA-140) and *G. hirsutum* (Poornima). Polyploidy was induced in the diploid cotton species *G. arboretum* (2n=26) by colchicine treatment prior to their being crossed with *G. hirsutum* (2n=52). To recover the maximum genes from *G. arboreum*, the resultant F1 was then back crossed with 4n *G. arboreum* in the C4 generation. PAIG 8/1 and PAIG-27 are the selection lines from back crossed F1 populations (Deshpande and Baig, 2002). Since the maximum number of genes of *G. arboreum* have been recovered in the introgressed cultivars, these showed the highest level of genetic similarity with *G. arboretum*. Although a few genes like those for fibre quality, boll size and ginning outturn have been introgressed from *G. hirsutum*.

The results of the present investigation confirmed the efficiency of ISSR markers in estimation of genetic relatedness among cotton genotypes and these cultivars can be identified using profile of several markers in future.

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