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Molecular Analysis of Macroinjected Cotton Plants Through RAPD Markers

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Abstract: Among the OPM primers, OPM-17 did not give amplification, whereas the primers 01, 02, 03, 05, 08, 13, 14, and 15 did not show any polymorphism among the samples. Amongst the polymorphic OPM primers, the OPM-4, OPM11 and OPM-19 confirmed the introgression of DNA fragments of sizes, 750 bp, 1 kb and 750 by bands respectively of *G. arboreum* into *G. hirsutum* on molecular level. Whereas none of the 20 OPM primers showed polymorphism even between *G. hirsutum* and *G. barbadense* parents. Out of 12 OPB primers two primers OPB-3 and OPB-4 were found to be polymorphic between *G. hirsutum* and *G. barbadense* parents while 6 OPB primers (OPB-05, 06, 07, 08, 11 and 12) showed polymorphism between *G. hirsutum* and *G.arboreum* parents. However these polymorphic OPB primers could not detect polymorphism between parents and their transformed progenies. Therefore these studies have confirmed the introgression of *G. arboreum* DNA fragments into *G.hirsutum* through DNA macroinjection via DNA mediated embryo transformation technique.

Key words: Macroinjected cotton plants, molecular analysis, RAPD markers

Introduction

Cotton belonging to genus *Gossypium* is the world's leading fibre crop and the most valuable oilseed crop. Four *Gossypium* species (*G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L.) are cultivated. *Gossypium barbadense* L. is the most popular species for its superior quality fibre while *Gossypium arboreum L.* (A genome cotton) is best reported for high fibre strength, resistance to insects and diseases like cotton leaf curl virus etc. (Stanton *et al*., 1994; Ali, 1997). Cotton is very important cash crop of Pakistan and is being extensively used for research. Breeders are making a number of crosses/back crosses to evolve cotton varieties with desirable traits. Wide hybridization is also being carried out to transfer desirable gene(s)/characters but interspecific crosses are not easy and very successful and the chances of viable crosses are very low in number. This problem becomes more difficult when the species with different ploidy level are crossed (Nishiyama and Yabuno, 1978). Various plant transformation approaches like, *Agrobacterium* mediated transformation (Horsch *et al*., 1985), electroporation method of gene transfer (Fromm *et al*., 1985; Shillito *et al*., 1985), DNA microinjection (Crossway *et al*., 1986; De la Pena *et al*., 1987), particle bombardment method (Finer and McMullen, 1990) etc. can be used to overcome this problem. Moreover the additive type of gene action (Gawel and Robacker, 1990) controls the genotype- specificity. Whereas the DNA-mediated embryo transformation via DNA macroinjection is a straight forward *in vivo* approach, which involves the injection of exogenous DNA into the plant reproductive structure during the fertilization process, leading to the transformation of the developing embryo during zygotic cell divisions. Various scientists have already done such type of transformation work (Aslam *et al*., 1995, 1998b), but no molecular analysis has been done by anyone. Therefore it is a dare need to perform its molecular analysis.

Materials and Methods

A cultivated diploid species of cotton, *G. arboreum*, var. Ravi, (2n = 2x = 26) and a tetraploid long staple species, *G. barbadense* (2n = 4x =52) were used as donors. While a locally cultivated species of cotton *G. hirsutum,* var. N1AB-78, (2n = 4x = 56) was used as recipient. The irradiated (2.5 Gy. of gamma rays) donors DNA solutions were injected into the self fertilized flower/ovaries of the recipient at 24 hrs. post self-pollination through axial placenta with a macrosyringe. The D_o seed was collected from successful injections. The D_1 generation was raised from D_o seed. The D_1 population obtained from *G. hirsutum* × *G. arboreum* DNA treatments was studied for phenotypic and economic trait changes. The D2 population was grown from D_1 generation seed as plant progeny rows for evaluation and confirmation. Moreover D_1 and D2 populations originated from *G. hirsutum* × *G. arboreum* DNA treatments were exposed to CLCuV disease conditions for evaluation. The details of which have been published (Aslam *et al*., 1998a).

Plant material: Different macroinjected transformed plant progenies obtained through DNA injection treatments alongwith their parents (donor/recipient) were grown from 02 generation selfed seed. Young leaves from the following 02 derived 03 pooled genotypes were collected in liquid nitrogen and were used for RAPD analysis (Table 1).

DNA isolation: DNA was isolated from three to four young sprouting leaves. The leaves were ground to a very fine powder in liquid nitrogen and transferred into a 50 ml centrifuge tube. Then 15 ml of hot (65°C) 2xCTAB (2% cetyldimethyl triethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris-HCl (pH 8.0), 1% polyvinyl pyrolidone (PVP), 1% 2-mercapto-ethanol was added and incubated in the water bath for VI an hour at 65°C with occasional shaking. The mixture was emulsified with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged for 10 minutes at 9000 rpm. The upper phase was again treated with chloroform:isoamyl alcohol and spun for 10 minutes at 9000 rpm. The aqueous phase was removed

Table 1: List of macroinjected cotton plant progenies and parents used for RAPD analysis

Sample Code	Genotypes	
M-41	*G. hirsutum* L.	(recipient)
M-54	*G. barbadense* L.	(donor)
M-27	*G. hirsutum* L.	(FMC)
M-39	*G. bar.* DNA injected into	*G. hirsutum*
M-33		"
M-44		"
M-46		"
M-47		"
M 35		"
M-23		"
M-24		"
M-51	*G. arboreum* L.	(donor)
M-29	*G. arb.* DNA injected into	*G. hir.*
M-34		"
M-38		"
M-31	*G. arboreum* DNA injected into	D@
M-52		"
M-49	*G. arb.* DNA injected into	*G. hir.*
M-53	G. *arboreum* DNA injected into	D@

D@ = *G. hirsutum*, previously injected with *G. barbadense* DNA

and added 0.6 volume 2-propanol to precipitate the DNA. Then DNA pellet was transferred with the help of a sterilized loop into another 50-m1 tube containing 70% ethanol. The pellet was washed thrice with 70% ethanol. Then the ethanol was discarded from the tube. The DNA pellet was air dried and resuspended in 0.5 ml of 0.1x TE buffer. After RNase treatment, the DNA concentration was measured by DyNA Quant 200 Fluorometer. The DNA was diluted in sterilized distilled water to a concentration of 12.5 ng/µl to use in PCR reaction for RAPD analysis.

Random decamer primers (Operon Technologies Inc., Alameda, Calif., USA) were dissolved in sterilized distilled water at a concentration of 15 ng/µl. Thirty two primers belonging to Operon kits; OPM (20 primers) and OPB (12 primers) were used for PCR amplifications. Amplifications were carried out in a 25 µl reaction volume containing 10 mM Tris-HC1 (pH 8.3 at 25°C), 50 mM KCI, 3 mM $MgCl_2$, 0.1 mM each of dATP, dGTP, dTTP and dCTP, one unit of Taq DNA polymerase (Perkin Elmer, Norwalk, Conn.), 0.001% gelatin (Sigma, St-Louis, Mo.), 25 ng of template DNA and 30 ng of primer. The reaction mixture was overlaid with two drops of mineral oil in order to avoid evaporation. The amplifications were carried out in a Perkin Elmer Thermal Cycler 480, programmed for a first denaturation step of 5 minutes at 94°C followed by 40 cycles of 94°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes. After the completion of 40 cycles, the reactions were kept at 72°C for 7 minutes and then held at 4°C until the tubes were removed. PCR products were separated on a 1.2 per cent agarose gel with ethidium bromide in the gel, using 0.5x Iris Borate EDTA (TBE) buffer.

Results and Discussion

The results of D_1 and D2 generations originating from *G. hirsutum* × *G. arboreum* and *G. hirsutum* × *G. barbadense* DNA treatments indicated the enhanced introgression of donors gene(s) into the recipient where the DNA was irradiated at low doses (2.5 Gy) of gamma rays before injections. The transformed genotypes had higher yield, better fibre quality and superior other economic traits as compared to recipient. Some of the transformants originated from *G. hirsutum* × *G. arboreum* DNA treatments were found resistant to CLCV disease. Moreover introgression of some of the qualitative marker traits i.e. red petal spot, yellow pollen colour etc. from the donors i.e. *G. barbadense* and *G. arboreum* into *G. hirsutum* was noticed (Aslam *et al*., 1998a). The results of RAPD analysis of transformed genotypes are:

Thirty-two primers were used to amplify the genomic DNA of different cotton genotypes; consisting of 16 D2 derived D3 transformed progenies developed through DNA macroinjections and their respective parents for PCR analysis. Amongst twenty OPM primers, the primer OPM-17 did not give any amplification and the eight primers i.e. OPM-01, 02, 03, 05, 08, 13, 14 and 15 did not show any polymorphism among all the samples. The sample M-51 (*G. arboreum* var. Ravi) in lane 12 was found to be polymorphic as compared to all the other samples by using OPM-06, 07, 10, 12, 16, 18 and 20 primers. The results indicated that the primer OPM-09 gave a polymorphic band of size 750 by which was absent in sample M-51 (lane 12) belonging to *G. arboreum* var. Ravi and present in the other samples. Moreover a 100 by band was present in samples M-39, M-31 and M-52 and was absent in the recipient/donor and all other samples. Therefore this could not confirm the introgression of donor i.e. *G. arboreum* genes into *G. hirsutum* on molecular basis. The primer OPM-04 was found to be polymorphic among the samples (Fig. 1). A band of 750 bp, present in sample M-51 i.e. *G. arboreum* (lane 12) and also in M-49 (lane 18), a transformed progeny, while this band was absent in all other samples. Therefore this gave a clear evidence of introgression of *G. arboreum* DNA fragment into *G. hirsutum.*

An other primer, OPM-11 showed polymorphism among the samples (Fig. 2). A band of 1 kb present in sample M-51, *G.arboreum* (lane 12) was detected in sample M-49 (lane 18), a transformed progeny but this fragment was not detected in all the other samples which again confirmed the phenotypically observed introgression of *G. arboreum* DNA fragment into *G. hirsutum* on molecular basis. A third primer, OPM-19 revealed a 750 by band, present in sample M-51, *G. arboreum* (lane 12) and in sample M-34 (lane 14), a transformed progeny while this fragment was absent in all other samples which is also a clear confirmation of the phenotypically observed introgression of *G. arboreum* DNA fragment into *G. hirsutum* on molecular basis (Fig. 3). Moreover a 300 by band was also detected in all other samples, except sample M-51 i.e. donor *G. arboreum* (lane 12), therefore no conclusive results could be drawn from it. However the results of RAPID analysis indicated that none of the twenty OPM primers detected polymorphism between donor, *G. barbadense* and recipient, *G. hirsutum* and their phenotypically observed transformed progenies.

But among 12 OPB primers two primers i.e., OPB-03 and 04 were found to be polymorphic between *G. hirsutum* and *G. barbadense* and their transformed progenies. While six primers i.e., OPB-05, 06, 07, 08, 11 and 12 were found to be polymorphic between *G. hirsutum* and *G. arboreurn.* However all the individuals/progenies when treated with these primers were failed to produce polymorphism and all the progenies were like *G. hirsutum,* whereas within the transformed progenies, which were segregating for marker gene i.e. red petal spot and otherwise showed polymorphism but no

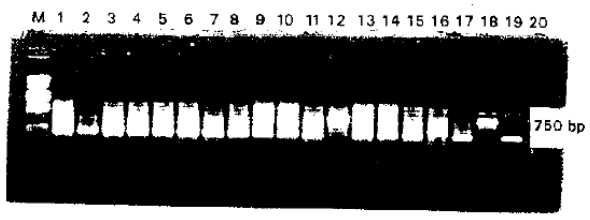

Fig. 1: (OPM-04)

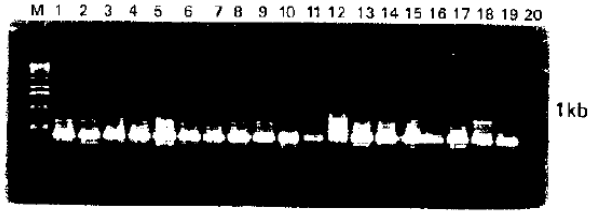

Fig. 2: (OPM-11)

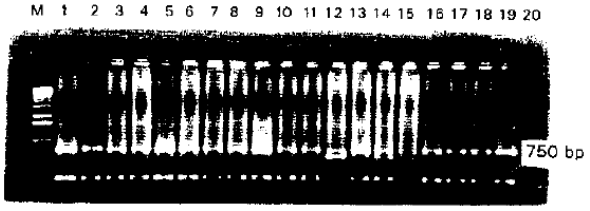

Fig. 3: (OPM-19)

conclusive results could be drawn from it. It may be concluded from the results of RAPD analysis that the incorporation of *G. arboreum* gene(s) into *G. hirsutum* following DNA macroinjections has been confirmed on molecular basis. Similar results for gene(s) incorporation in barley in tobacco using different biotechnological approaches have been reported. However these studies could not confirm the phyenotypically observed introgression of *G. barbadense* genes into *G. hirsutum* on molecular basis. Therefore further studies with some more polymorphic primers are required to confirm the introgression of *G. barbadense* gene(s) into *G. hirsutum* on molecular basis.

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