Biotechnology

P1-96/1-ICR-F-30/0430:

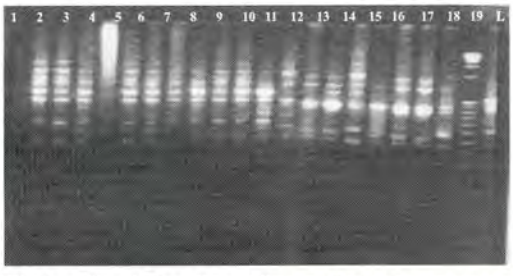
Evaluation of cotton germplasm through molecular techniques (A. B. Dongre, J Amudha, S. B. Nandeshwar and V. V. Singh).

One hundred germplasm lines of *G.hirsutum, G.arboreum* and wild species were subjected to molecular evaluation by using DNA based markers such as RAPD, ISSR and Microsatellite.

The study revealed that these accessions inspite of being morphologically similar were found to have considerable variation at the level of molecular loci. Similarities were found in the clustering of ISSR and RAPD analysis. However, all the three markers used showed separate clustering for diploid and tetraploid. No duplicates were observed in the stock analyzed.

Microsatellites and ISSR markers based molecular characterization of genetic diversity in *Gossypium spp.* (Diploid and Tetraploid)

The results of cluster analysis indicated not only the separation of genotypes of two major species (*G. arboreum* and *G. hirsutum*) into separate groups, but also the separation of the genotypes among each group. The similarity coefficient calculated by UPGMA method based on ISSR and microsatellite markers analysis, ranged from 0.59 to 0.90 and 0.59 to 0.93 respectively, suggesting a considerable genetic variation, between the cotton species studied.



ISSR profile of 19 cotton cultivars obtained with primer IS 7. Lane 1 19 corresponds to cultivars listed in Table 1. Lane 20, L = 1 kb, 100 bp ladder.

Microsatellite (SSR) profile of 19 cotton cultivars obtained with primer JESPR 25. Lane 1 19 corresponds to cultivars listed in Table 1. Lane 20, L = 1 kb, 100 bp ladder.

Molecular evaluation of grey mildew immune lines:

Grey mildew immune lines viz., G-135- 49, 03805, 30814, 30826, 30838, 30856, EC-174-092 and susceptible check AKA 8401 were subjected for RAPD analysis. OPERON kit primer OPC and OPD were used for PCR amplification. A total of 762 products were obtained out of which 28 % were polymorphic in nature. OPC 2 primer produced unique fragment of 1.5 Kb size which is present invariably in all immune lines and absent in the susceptible check. The PCR derived RAPD product $OPC02_{1500}$ can be used as diagnostic tool for identifying the grey mildew resistant line in the germplasm gene pool.

Molecular evaluation of lines with good fibre length:

Seven lines with good fibre were subjected for genomic DNA isolation and RAPD analysis. Out of 567 RAPD fragments obtained only 12 were polymorphic and mostly ranged from 1.0 Kb 2.5 Kb with different set of primers. All other fragments were found to be monomorphic. Diversity among them was obtained by analyzing the RAPD data and the dendrogram was constructed using UPGMA analysis method. The mean hallow length (MHL) ranges from 33-37mm.

P1-91/1-ICR-F30-30/0430:

Development of tissue culture protocol for use in breeding and genetic transformation (S. B. Nandeshwar and A. B. Dongre).

Regeneration protocol was standardized in *G. arboreum* viz. PA 255, PA 183 and

DLSA 24. Shoot tip and embryonic axis explants excised from three day and seven day old germinated seedlings were manipulated and induction of multiple shoots were obtained.

Both the procedures of regeneration viz. embryogenic axis and the shoot tip showed differential response in different varieties as well as for different procedures employed for explant preparation for co-cultivation.

Out of three varieties used viz. LRK 516, LRA 5166 and MCU 10, LRK 516 responded well for both explants (shoot tip and embryogenic axes).

However, it was observed that embryogenic axes respond better in comparison to shoot tip in all the three cultivars.

Amongst different procedures adopted for preparation of explants for co-cultivation, side cut method of explant preparation appears to be better. Embryogenic axes were found to respond well for root induction as compared to shoot tip.

P1-2003/1-ICR-F-30 /0430:

Molecular mapping of leaf curl virus resistance gene in cotton genome (J. Amudha, D. Monga, G. Balasubramani).

Young healthy leaves of CNH 123, CNH 1012 (resistant lines) and CNH 1020, CNH 120 (susceptible lines) were collected from the plants grower in green house and genomic DNA was isolated by Paterson method. The DNA isolated was amplified by Operon random primers. The resistant and susceptible lines were amplified and identification of molecular polymorphism among the parents (CLCuV resistant and susceptible lines) was carried out. The F_2 mapping population was developed and advanced to F_3. Ten resistant and susceptible F_2 DNA were pooled for bulk segregant analysis. The F_2 segregating RAPD loci were mapped using Mapmaker programme into ten groups. F_2 plants were tested for CLCuV incidence. Whiteflies were released after primary leaves appeared (7th day) and on 21st day the incidence was recorded. That the CLCuV resistant gene is governed by Mendelian segregation (3:1) pattern as indicated by the observed and expected frequencies.

(i) CNH 123(resistant line) X CNH 1020(susceptible line)

↓

F_2: F_3

(ii) CNH 1012 (resistant line) X CNH 120 (susceptible lines).

↓

F_2 : F_3

F_2 derived F_3 (F_2: F_3) population is suitable for mapping recessive genes as the cotton leaf curl virus resistant gene is governed by recessive gene. Out of 692 fragments produced 35% of fragments were polymorphic and SCAR marker was developed.

Young healthy leaves of F_2 segregating population were collected from Sirsa. Mapping populations are as LRA 5166 X Laxmi, PIL 8 X F 84 b, Laxmi X RS 921, LRK 516 X RST 9 , LRK 516 X RS 921

Genomic DNA was isolated and resistant and susceptible lines were amplified with the designed SCAR forward and reverse primer among the parents (CLCuV resistant and susceptible lines) for the validation of the SCAR marker developed in all the populations.

National Agricultural Technology Project

MM 4 : Development of Bt transgenic cotton for insect resistance (A. B. Dongre, S. B. Nandeshwar, G. Balasubramani and K. R. Kranthi).

G. hirsutum varieties viz. LRA 5166 and

LRK 516 were taken for co-cultivation with indigenously synthesized gene Cry I Aa 3, Cry I A 5 and Cry I F. In LRA 5166, 543, 286 and 789 explants were used for transformation with gene construct Cry I Aa 3, Cry I A 5, Cry I F respectively. With Cry I Aa 3, 11 explants are in rooting medium and six hardened plants in pot-house. With Cry I A 5 construct, 4 explants are in rooting medium and nine are hardened plants in pot-house. With Cry I F, 8 plants are on rooting medium.

In LRK 516 variety, total 100 explants with Cry I A 5 and 912 explants with Cry I F were co-cultivated for transformation. Out of that only 9 putative transformants of Cry I F are in rooting medium.

Anjali (LRK 516) explants (embryonic axes and meristematic tissues) were transformed with Bt *Cry* I A (c) gene by *Agrobacterium* mediation. Of the 31 putative transformants produced by direct shoot organogenesis. 17 plants were found positive with ELISA test (table 8). The Cry protein expression was found to be 0. 4 2. 4 μg /g of fresh leaf protein. Seeds from T_0-generation plants were collected and raised in the restricted field condition. T_1 - generation plants showed around 10 ng of Cry protein expression by ELISA test. Activation of *vir* gene present in the helper plasmid was confirmed by the acetosyringone treatment and excision of LB and RB sequence from the binary plasmid was observed.

RCPS 10: Development of Bt transgenic diploid cotton against bollworm (S. B. Nandeshwar and A. B. Dongre).

G.arboreum cultivars PA 255, PA 183 and DLSA 24 and one *G.hirsutum* cv PKV 081 were evaluated for regeneration and their embryo axes were co-cultivated with *Agrobacterium* containing Cry I Ac , Cry I F and Cry I Aa3 genes. The Cry 1F was used in PA 183 ,Cry I Aa3 in DLSA 24 and Cry I Ac in PKV 081.

In cv. PA 183 , 838 embryo axes were co-cultivated and 271 were isolated on antibiotics medium. Out of 271 explants survived on kanamycin medium, 231 responded to regeneration and 8 rooted plants were obtained.

In cv. DLSA 24, 769 embryo axis explants were co-cultivated with *Agrobacterium* containing Cry I A a3 gene and 242 explants survived on selection medium. The transformed embryo axes on transfer to regeneration medium produced 18 rooted shoots. While in case of PA 255, 793 explants were co-cultivated, 232 were selected on kanamycin medium, 64 plants were regenerated . Out of which 15 rooted plants were obtained and 8 were established in the soil.

Testing of leaf samples for gene expression in RG 8

The leaf sample of transformed RG-8 were

Table : 8. Summary of transformation and regeneration of cv Anjali in MS media with BAP and kinetin for direct shoot organogenesis

Cultivar inoculated	No. of explants transformants	No. of putative frequency	Transformation	ELISA test
Anjali				
Embryonic axes	3150	28	0.8	16
Meristomatic tissues	720	3	0.4	1
Albinism	-	6 (EA)	-	-
	0 (MT)			
Total	3870	31	0.8	17

tested for the Bt- protein by ELISA. In all 98 samples were tested and 36 were found positive.

NIC CGP 1 / 41 : Induction of para-nodules in cotton with nitrogen fixing bacterium *Azorhizobium caulinodans* (G. Balasubramani and J. Amudha).

Rhizobium fredii isolated from soybean (*G. max)* showed virulence on nodule induction in cotton. Six individual plants of Anjali and two of LRA 5166 produced nodules in their root system. Nodules were characterized but nitrogenase activity estimated by AR-method, showed no positive results.

Technology Mission on Cotton

MMA 6 : Overcoming incompatibility barrier in interspecific hybridization (S. B. Nandeshwar).

The long staple *arboreum* PA 255 and PA 183 were crossed with *G.thurberii*, *G.trilobum*, *G.anomalum*, *G.aridum*, *G.raimondii*, *G.capitis-viridis* and *G.harknesii* as pollen parents. The following results were obtained.

1. The cross PA 183 X *G.trilobum* , PA 183 X *G.harknesii* and PA 183 X *G.capitis-viridis* did not show setting of seeds and bolls.
2. In cross PA 255 X *G.trilobum*, four bolls were set containing two seeds.
3. In PA 255 X Coker 312, three bolls were set without any seeds. Similar situation was seen in crosses made with *G.anomalum*, *G capitis-viridis* and *G.harknesii* respectively.
4. In crosses PA 183 X *G.anomalum* , PA 255 X *G.aridum*, PA 255 X *G.raimondii* and PA 183 X *G. raimondii*, the seed set range was 8-56.

Embryo culture in interspecific hybridization

Four crosses were used in embryo culture studies. They are PA 183 X *G.aridum*, *G. thurberii* and PA 255 X *G.anomalum*, *G.aridum*.

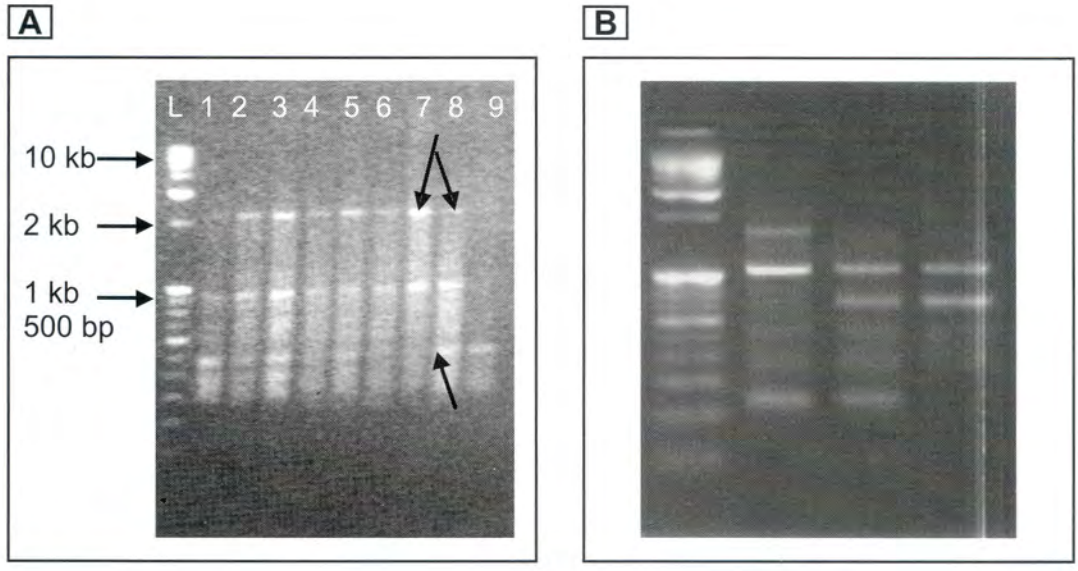
In cross PA 183 X *G.thurberii* 10,12,15 days after pollination (DAP) ovules were isolated and cultured in four media combinations. The responses of *in-vitro* culture of ovules were observed mostly in MS+ 2,4-D + Kin (0.1: 0.1 mg/L) + GA3 1mg/L .This was followed by MS+ 2,4-D + Kin (0.5:0.5 mg/L) + GA3 1 mg/L.

In second cross (PA 255 X *G.anomalum*) 12,8,10 DAP ovules were isolated and cultured for regeneration. When 52 ovules were cultured in MS+2,4 -D + Kin (1.0 :0.5 mg/L) there was response, while in 2,4 - D + Kin (0.5:0.5 mg/L) +GA3 1 mg/L 37 per cent of the ovules responded. In 2,4 -D + Kin (0.2 :0.2 mg/L) + GA3 1 mg/L medium there was 29 per cent response to ovule growth while in 2,4 -D + Kin (0.1:0.1 mg/L) +GA3 1mg/L 41 per cent response was recorded.

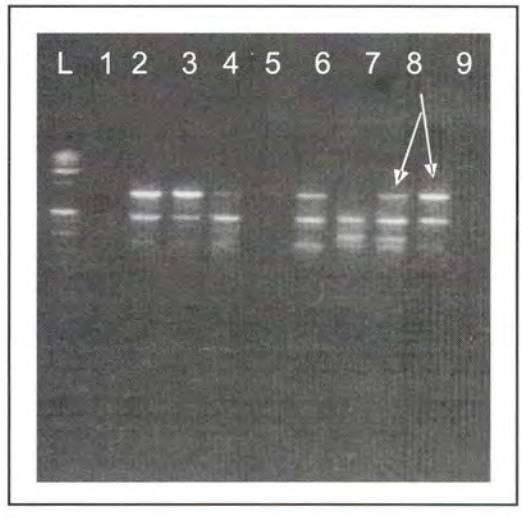
In cross PA 183 X *G.aridum* 6,10,12 DAP ovules when cultured showed 44 per cent response in 2,4-D + Kin (0.1 : 0.1 mg/L) + GA3 1 mg/L.

MM 1.5 : Maintenance breeding, seed production, enhancement of seed viability and studies on marker based purity evaluation (A. B. Dongre).

40 RAPD primers and 12 ISSR were used for the identification of the F_1 hybrids Surya, Savitha and Shruthi and their parents. The RAPD polymorphic primer OPA 13 and OPB 14 and ISSR polymorphic primer IS 2 led to the confirmation of hybridity of Shruthi and hence can be used as a discriminating marker for testing of genetic purity of hybrid. The ISSR polymorphic primer IS 10 confirms the hybridity of Surya.



RAPD profile of Shruthi by A) OPA 13 and B) OPA 14. Male, hybrid and females of Surya (Lane - 1 2 3), Savitha (4 5 6) and Shruthi (7 8 9) L- DNA ladder (1kb + 100 bp)



ISSR profile of Shruthi by IS 2. Male, hybrid and female of Surya (Lane - 1 2 3), Savitha (4 5 6) and Shruthi (7 8 9). L- DNA ladder (1kb + 100 bp), showing the hybridity of Shruthi.