TITLE: **Pollen Tube Pathway Mediated Genetic Transformation Studies in Cotton (*Gossypium hirsutum* L.)**

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ABSTRACT

Although use of pollen tube pathway to deliver foreign DNA in to embryo sacs first reported by Zhou et al. (1983), in cotton was encouraging the overall quality of the data was very weak. Most results did not demonstrate integration of the transgene in to the plant genome. Therefore our attempts to modify this method yielded fruitful results. Out of 5619 flowers which were treated with *Agrobacterium*, where in the stigmatic surface was treated with 5 percent sucrose along with 20mg/l boric acid prior to application of *Agrobacterium* resulted in a bollset of 32.5 percent. Stringent *in solium* kanamycin screening of the treated plants in T_1 & T_2 generations yielded seven PCR positive transformants, which was also confirmed by insect bioassay studies. Transformation efficiency of Pollen Tube Pathway (PTP) mediated transformation was 0.3062 percent.

KEY WORDS:

Agrobacterium tumefaciens, pollen tube pathway, transformation

The choice of pollen tube pathway transformation stems from the underlying assumption that at fertilization the egg cell accepts the donation of entire genome from the sperm cell and it might thus be appropriate stage to deliver transgenes. This concept was demonstrated by the successful transformation method developed for *Arabidopsis*. Based on this principle, *Agrobacterium* harbouring the plasmids containing donor DNA are applied directly on to the previously treated stigma (with 5% sucrose and 40 mg/l boric acid) allowing for the formation of pollen tubes extending from the style into the ovule.

MATERIALS AND METHODS

The genotypes Sahana and BC-68-2 belonging to *Gossypium hirsutum* were planted at ARS, Dharwad farm on 3rd August 2003, in the transgenic greenhouse.

Preparation of *Agrobacterium* culture used for induction. Forty-eight hrs old *Agrobacterium* cultured on yeast extract mannitol agar (YEMA) medium containing kanamycin was used for the purpose. The bacteria were scraped from the culture plate and a slurry was made using 200 μM acetosyringone. In other set of experiment wherein the liquid *Agrobacterium* culture was used, 48 hrs old agroculture grown on YEMA media containing kanamycin was directly used for inoculation.

Flower buds, which would open the next day, were chosen as pollen recipients and the entire corolla along with anthers were removed carefully without damaging the gynoecium, and the stigma of the flower. The stigmatic surface was treated with 5 percent sucrose, boric acid at different concentrations ranging from 1-30 mg/l and combination of 5 percent sucrose and 20 mg/l, of boric acid in independent experiments during evening hrs after the sunset. Pollen was dusted onto treated stigmatic surface on the following morning. Inoculated plants were tagged, labelled and grown in pest and disease free conditions under containment. Seeds were harvested and sown in the next season for screening the transformants.

Different pollen tube pathway mediated transformation methods attempted to effect successful fertilization and boll setting. Under *in planta* transformation, four methods were attempted as seen in the table above. Method No. 4 involving liquid culture, pollinated during morning hours has yielded maximum boll set percentage. Therefore method 4 was employed for large scale *in planta* transformation studies. There are different methods available for *in planta* transformation. These are advantages over *in vitro* transformation methods as they are bereft of inherent problems associated with tissue cultural procedures. With a view to harness these benefits, *in planta* cotton transformation was attempted in this study.

RESULTS AND DISCUSSION

All the experiments were performed in the late evening on flowers that were emasculated during the previous evening and agroculture was applied to the exposed stigma, which was previously treated with 5 per cent sucrose and 20mg/l boric acid. Time of pollination and type of agroculture was varied in four sets of independent experiments. In methods 1 and 2 when pollination was carried out during evening hours, irrespective of the nature of agroculture, whether solid or liquid, there was very poor boll set (3-5%). Since the pollen was not treated with pollen germination medium and also, as the stigma receptivity was poor during evening hours in cotton, leading to abortive pollination, nevertheless pollination during morning hours resulted in successful fertilization leading to boll set of 21 to 28.5 percent (Table 1).

Another reason for poor boll set could be due to contamination by *Agrobacterium*. Like any other infectious agent, *Agrobacterium* is alien to plant system, infecting the ovules, leading to boll shedding. Boll set was significantly higher (28.5%) when the plants were infected with liquid agroculture when compared to the boll set resulting from the use of solid agroculture. This difference can perhaps be attributed to the fact that the bacterial load is more in solid culture

because of higher inoculum density resulting in decreased cell viability. These observations appear to give credence to the hypothesis that each plant cell binds to a finite number of bacteria (Gutlitz et al., 1987). Beyond this threshold, it appears that cell viability may be compromised resulting in lower proportion of boll set. Moreover, from the broth bacteria can move with the pollen tube path more easily than from agar medium and reach the embryosac to effect fertilization. This indicates that the precise guidance of the pollen tube to the embryosac is critical to the successful sexual reproduction in flowering plants.

Research needs to be focused to investigate the exact cause for the reduced bollset. Using different inoculum densities of *Agrobacterium* to treat the stigmatic surface so as to avoid heavy bacterial load could be a promising proposition, which may aid in increasing the recovery of transformants.

Factors influencing boll set during *In Planta* transformation in cotton. Effects of various factors on the growth of pollen tubes in the preliminary investigation were examined. With the treatment of stigmatic surface by 5 percent sucrose solution, pollination success dramatically improved up to 23.5 percent. Tetsuya et al. (1998) have also reported that sucrose concentration of 5 per cent appeared to be optimum for pollen tube growth. Reddy et al. (2004) have also observed that sucrose level was more crucial for the pollen penetration. Further, there was marginal increase in rate of boll set by addition of boron; Tetsuya et al. (1998) have also achieved improvement in the growth of pollen tubes by increasing the amount of boric acid from 0.5 to 10 mg/l. Encouraged by the results of sucrose and boric acid inclusion in the treatment, combined effect of both were tried in subsequent experiments and improvement in the boll set increased to 32.5% (Table 2). Further in the large scale PTP mediated transformation studies, these strategies were adopted.

Large scale PTP mediated transformation studies. A total of 5619 flowers were treated with Agrobacterial culture harbouring PCAMBIA AC, *cry1 Ia* $_5$ *, cry1 Aa*$_3$ *, and cry1 F* gene constructs. The boll set ranged from 23.1 to 29.9 percent. The bollset ranged from 23.1 to 29.9 percent. The low rate of boll set was due to combined effect of abortive pollinations and insect damage.

Out of 22861 seeds harvested only 6954 seeds germinated, which accounts for 30.4 percent (Table 3).

In solium selection of these cotton transformants resistant to kanamycin resulted in 521 kanamycin resistant plants in T_1 generation. From these plants 5692 healthy seeds were harvested and forwarded to T_2 generation. Out of these only 1199 seeds germinated (Table 4). All the seedlings emerged were further subjected to stringent *in solium* kanamycin screening in the transgenic green house. From the gene integration studies, it was clear that only seven plants were PCR positive (Figs. 1, 2 and 3). The transformation efficiency was 0.3062 percent.

Insect bioassay studies. Insect bioassay studies revealed that the transgenic plants showed notable resistance to second instar larvae of *Helicoverpa armigera* after seventy two hours of infestation compared to the non-transformed plants. Larval mortality in positive control was 76 percent and that of the transgenics ranged from 68 to 76 percent (Table 5).

The larval mortality was zero in negative control, the larvae survived and grew well in the negative control. It is not only the larval mortality and growth inhibition that is important to be considered, but also the nature and extent of damage, resulting from larval feeding has a serious bearing on the conclusion drawn. There was voracious feeding in the negative control and the damage increased with the progress in duration of feeding. Only the veins and vein lets were left unfed by the larva within 72 hrs. Nevertheless the leaf bits of Bt positive plants showed only the pinhole size shot holes (Fig. 4). Deterrence to feeding was noticed with the

advance in duration of feeding and the damage was static. This feeding inhibition was accompanied by decrease in larval weight. These results are in accordance with the studies conducted by Chakraborthy et al. (1987), where in the larvae showed different feeding patterns and growth in control and transgenic leaf discs of cabbage.

Southern blots and ELISA. Southern hybridization of PCR positive T_3 Plants was carried out in order to confirm *cry* gene inheritance and integration. All the transgenic plants showed the presence of a single copy of the introduced transgene. When transgenic plants were tested for *cry*1 Aa_3 protein using ELISA assay. The amount of protein ranged from 1.56 to 2.23 microgram per gram of the total soluble proteins. The over all transformation frequency was 0.3062 percent. From the gene integration study it was clear that out of seven transformants obtained, four transformants carried *cry*1 Ia_5 genes. One tranformant had *cry*1 Aa_3 and two plants were transformed with *cry*1 F gene. It can thus be concluded that PTP mediated transformation methods involving simpler procedural steps and in expensiveness have inspired renewal of efforts to adopt these methods to the development of cotton transformation.

REFERENCES

Chakraborthy, S., and S.C.M. Roy. 1987. Quick *in vitro* production of multiple plants lets in cabbage (*Brassica oleracea* L.) for commercial purpose. Ind. J. Exp. Biol. 25: 61-65.

Gutlitz R.G.H., P.W. Lamb, and A.G. Matthsse. 1987. Involvement of carrot cell surface proteins in attachment of *Agrobacterium tumefaciens*. Plant Physiol. 83: 564.

Potrykus I. 1990. Gene transfer to cereals: An assessment. Biotechnology 8: 535-542.

Reddy, N., S. Zhu, J.Y. Rong, and T.X. Hong. 2004. Pollen tube pathway mediated genetic transformation of cotton. p. 356-360. *In* Strategies for sustainable cotton production – a global vision". Proc. Intern. Symp., 23-25 Nov. 2004, UAS, DWR, KRK.

Tetsuya, H., H. Kuroiwa, and K. Shigeyuki. 1998. Plant Cell 10: 2019-2032.

Zhou, G.Y., Y.S. Weng, J.G. Huang, S.Y. Qian, and G.L. Liu. 1983. Introduction of exogenous DNA into cotton embryos. p. 433-481. *In* R. Wu et al. (eds.) Methods in enzymology. Academic Press, New York, NY.

Table 1. Effect of type of Agrobacterium culture and time of pollination on pollen tube pathway transformation.

Method	Type of Agrobacterium culture used	Time of emasculation and application of Agrobacterium	Time of pollination	No. of bolls set	% boll Sat
1	Solid culture	Evening	On the same evening	13.0[z]	3.25
2	Liquid culture	Evening	On the same evening	22.5[y]	5.00
3	Solid culture	Evening	Next day morning	84.0[x]	21.0
4	Liquid culture	Evening	Next day morning	114[w]	28.5
		Mean			
		S.Em.		1.38	
		CD (5%)		2.89	
		CD (1%)		4.53	

z
y
x
w

Note: 400 pollinations were carried out in two replications.

Table 2. Combined effect of application of boron and sucrose to stigmatic surface on boll set

Sl.No.	Treatment	No. of pollinations carried out	No. of bolls set	% Success of pollination
1	5% Sucrose + 20mg/l Boric acid	200	63	32.5

Table 3. Large scale pollen tube pathway transformation studies

Sl.No.	Gene construct	No. of pollns.	No. of abortive pollns.	No. of bolls dropped due to insect damage	No. of bolls formed	Percent boll formn.	Total No. of seeds harvested (T_1)	No. of plants established	% germination
1	P C_{AMBIA} Ac	1858	880	421	557	29.9	6684	2531	37
2	Cry1 I_{a5}	2068	1063	526	479	23.1	8622	2174	26.2
3	Cry1 A_{a3}	804	458	118	228	28.3	4104	1265	30
4	Cry1F	889	492	194	203	22.8	3451	984	28
	Total	5619	2893	1259	1467	26.10	22861	6954	30.4

Table 4. Screening of T_1 generation plants with Kanamycin resistance

Sl.No.	Gene construct	No. of T_1 plants treated with Kanamycin	No. of T_1 plants found to be Kanamycin resistant	No. of healthy seeds harvested	No. of plants established	% germination
1	PCAMBIA Ac	2531	189	2042	411	20.1
2	Cry1 I_{a5}	2174	162	1971	385	19.5
3	Cry1 A_{a3}	1265	94	733	186	25.3
4	Cry1F	984	76	946	217	22.9
	Total	6954	521	5692	1199	21.06

Table 5. Larval mortality of *Helicoverpa armigera* in leaf bit bioassay

Sl.No.	Sample	Genotype / gene construct	No. of replications	Larval mortality within 24 hrs	Larval mortality b/n 24-48 hrs	Larval mortality after 72 hrs	Corrected larval mortality %	Nature of damage
1	Positive control	RCH-2Bt	25	3	0	22	76	Shot hole
2	Negative control	DCH-32	25	2	0	1	0	Voraciously fed
3	Plant-1 BT_1	Cry1 I_{a5}	25	3	0	21	72	Shot holes
4	Plant-2 BT_2	Cry1 A_{a3}	25	1	0	22	76	Shot holes
5	Plant-3 BT_3	Cry1 I_{a5}	25	0	0	21	72	Shot holes
6	Plant-4 BT_4	Cry1 F	25	3	0	22	76	Shot holes
7	Plant-5 BT_5	Cry1 I_{a5}	25	2	0	21	72	Shot holes
8	Plant-6 BT_6	Cry1 F	25	2	0	22	76	Shot holes
9	Plant-7 BT_7	Cry1 I_{a5}	25	3	0	21	72	Shot holes
10	Plant-8 BT_8	Cry1 I_{a5}	25	2	0	20	68	Shot holes

$$\text{Corrected Larval Mortality} = \frac{\text{Larval mortality after 72 hrs.} - \text{Larvae died in negative control}}{\text{Total No. of Larvae}} \times 100$$

FIGURE CAPTIONS

Figure 1. *nptII* amplifications of the transformed plants M; 1Kb marker lane, 1, 3 12 and 14 lanes show positive amplification; + plasmid DNA, - Untransformed plant.

Figure 2. *nptII* amplifications of the *cry1A*a_3 transformed plants M; 1Kb marker lane, lane 25 positive amplification; + plasmid DNA, - Untransformed plant.

Figure 3. *nptII* amplifications of the *cry1I*a_5 transformed plants M; 1Kb marker lane, lane 28, 30 show positive amplification; + plasmid DNA, - Untransformed plant.

Figure 4. Nature and extent of damage of transformed and untransformed leaf bits consequent to feeding by II instar larvae of *Helicoverpa arimigera*.

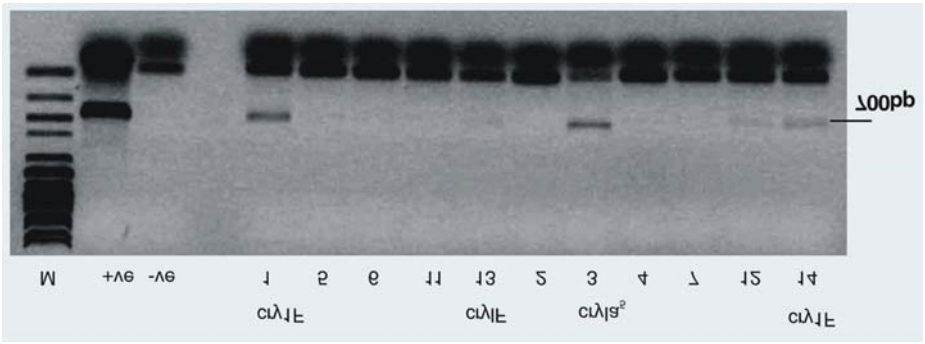

Figure 1.

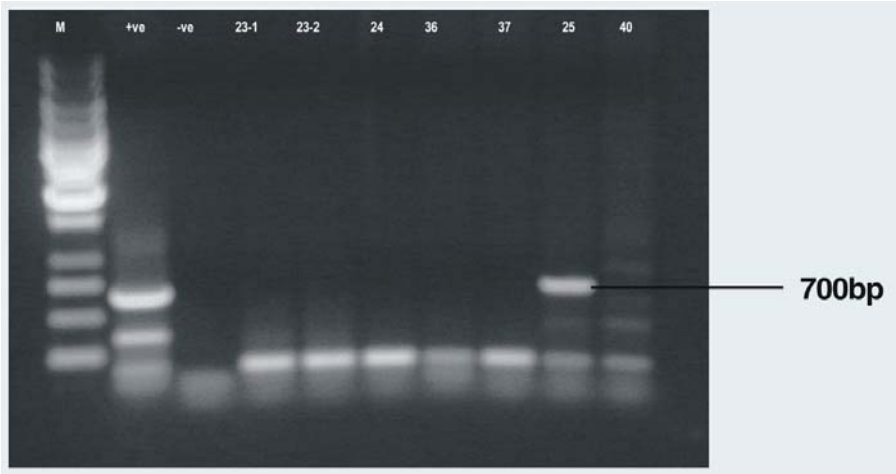

Figure 2.

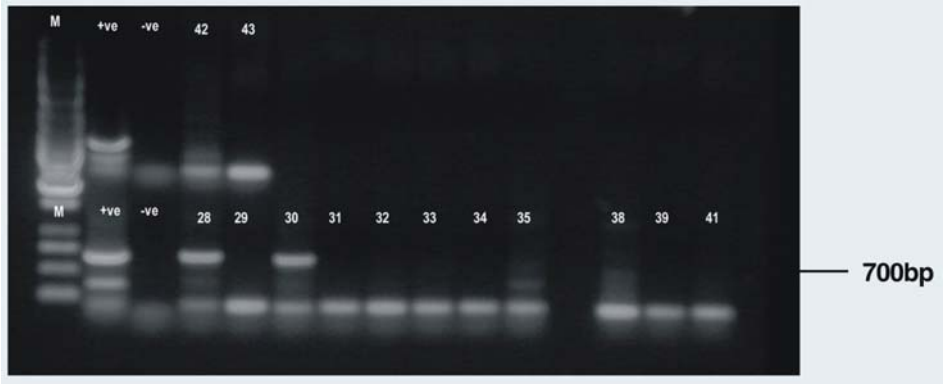

Figure 3.

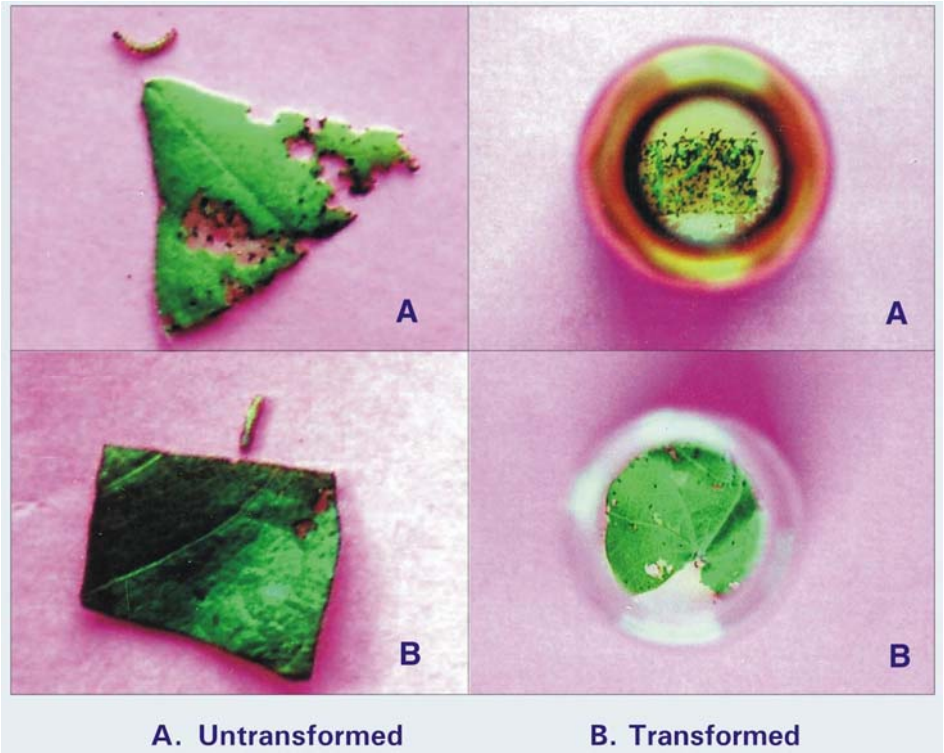

Figure 4.