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High efficiency transformation protocol for two Indian cotton (*Gossypium hirsutum*) varieties via pollentube pathway

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ABSTRACT

Genetically engineered resistance has been actively investigated in recent years as an alternative for genes from wild relatives. The development of a method to obtain transformants, which is independent of problems inherent to tissue culture procedures, has been the ultimate goal of plant breeders. The alternative method, which involves *Agrobacterium*, but does not require tissue culture steps, is *in planta* tansformation. Although use of pollen tube pathway to deliver foreign DNA in to embryo sacs first reported in cotton was encouraging the overall quality of the data was very weak. Therefore, our attempts to modify this method yielded fruitful results. Out of 5 619 flowers, which were treated with *Agrobacterium*, where in the stigmatic surface was treated with 5% sucrose along with 20 ppm boric acid prior to application of *Agrobacterium* resulted in a boll set of 32.5%. Stringent *in solium* kanamycin screening of the treated plants in T_1 and T_2 generations yielded 7 PCR positive transformants, which was also confirmed by insect bioassay studies and Southern blots. Transformation efficiency of Pollen Tube Pathway (PTP) mediated transformation was 0.30%.

Key words: *Agrobacterium tumefaciens*, PCR and *in solium* kanamycin screening, Pollen tube pathway, Transformation

Plant transformation using currently established protocols is a tedious, time consuming and expensive effort and sometimes may result in undesirable alterations such as somonoclonal variation. Therefore the aim of this study was to develop a transformation protocol that avoids the use of tissue culture**.** *In planta* transformation procedures have been used successfully for various plant species, e.g. *Arabidopsis* (Bent 2000). Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens* is reported by Zale *et al.* (2009) and the protocol has the potential to reduce the time and expense required for transformation. Further the optimal growth stage for transformation by floral dip is when plants contained numerous unopened floral buds (Clough and Bent 2008)

In nature, when pollen grain falls on stigma it germinates and pollen tube grows through stylar tissues and ultimately arrives at the ovary. It is also known that the pollen tube enters the ovule at the micropyle and then the nucellus cells form a column to facilitate passage of pollen tube. Exogenous DNA can pass through this pathway of pollen tube and enter the embryosac. This is indeed an intelligent method of introducing exogenous DNA directly into a germ line, which is sure to be inherited. The choice of the pollen tube pathway transformation stems form the underlying assumption that at fertilization, the egg cell accepts the donation of entire genome from the sperm cell and it might be thus an appropriate stage to deliver transgene. Therefore with the hypothesis that there is a possibility of placing *Agrobacterium* with TDNA into egg cell through germinating pollen tube, the present study has been carried out.

MATERIALS AND METHODS

The genotypes Sahana and BC 68-2 belonging to *Gossypium hirsutum* were planted at Agriculture Research Station, Dharwad farm during *kharif* 2008 in the transgenic greenhouse.

Agrobacterium harbouring PCAMBIA AC, *cry1 Ia*$_5$, *cry1 Aa*$_3$, *and cry1 F* gene constructs [s old] cultured for forty eight hours on yeast extract mannitol agar (YEMA) medium containing kanamycin was used for *in planta* transformation. The bacteria were scraped from the culture plate and a slurry was made using 200 M acetosyringone. In other set of experiment wherein the liquid *Agrobacterium* culture was used where 48 hr old agroculture grown on Yeast Extract Manitol(without agar) media containing kanamycin was

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directly used for inoculation. 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% sucrose, boric acid at different concentrations ranging from 1-30 ppm in independent experiments during evening hours after the sunset. Pollen was dusted onto treated stigmatic surface in 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.

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% sucrose and 20 mg/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% (Table 1).

The 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 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 need 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% sucrose solution, pollination success dramatically improved up to 23.5%. 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. Encouraged by the results of sucrose and boric acid inclusion in the treatment, combined effect of both were tried in subsequent experiments, the boll set increased to 32.5 % (Table 2). Further in the large scale PTP mediated transformation studies, these strategies were adopted.

A total of 5 619 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 low rate of boll set was due to combined effect of abortive pollinations and insect damage.

In solium selection of these cotton transformants resistant to kanamycin resulted in 521 kanamycin resistant plants in T_1 generation. From these plants 5 692 healthy seeds were harvested and forwarded to T_2 generation. Out of these only 1 199 seeds germinated (Table 3).The seedlings emerged were further subjected to stringent *in solium* kanamycin

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 set
1	Solid culture	Evening	On the same evening	13.0[d]	3.25
2	Liquid culture	Evening	On the same evening	22.5[c]	5.00
3	Solid culture	Evening	Next day morning	84.0[b]	21.0
4	Liquid culture	Evening	Next day morning	114[a]	28.5
		Mean		58.34	14.43
		SEm		1.38	
		CD (P=0.05)		2.89	
		CD (P=0.01)		4.53	

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

Treatment	No. of pollinations carried out	No. of bolls set	% success of pollination
5% sucrose + 20mg/l boric acid	200	63	32.5

screening in the transgenic green house. From the gene integration studies, it was clear that only seven plants were PCR positive (Fig 1 and 2). The transformation efficiency was 0.30%.

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 84% and that of the transgenics ranged from 72 to 80% (Table 4). Katageri *et al.* (2007) have also reported high degree of larval mortality in transgenic of *Bikaneri nerma.*

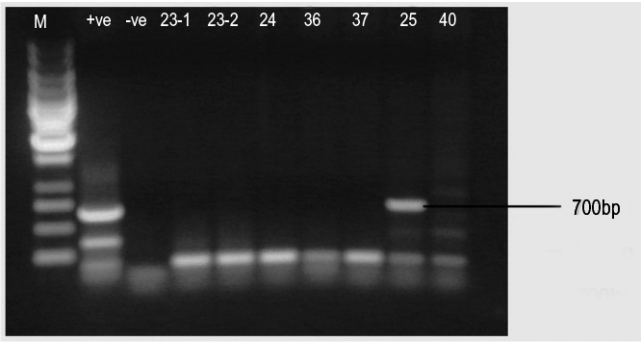

Fig 1 *nptII* amplifications of the *cry1A*a_3 transformed plants M; 1Kb marker lane, lane 25 positive amplification; + plasmid DNA, - untransformed plant

Table 3 Screening of T_1 generation plants with kanamycin resistance

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
PCAMBIA Ac	2 531	189	2 042	411	20.1
Cry1 I_{a5}	2 174	162	1 971	385	19.5
Cry1 A_{a3}	1 265	94	733	186	25.3
Cry1F	984	76	946	217	22.9
Total	6 954	521	5 692	1 199	21.06

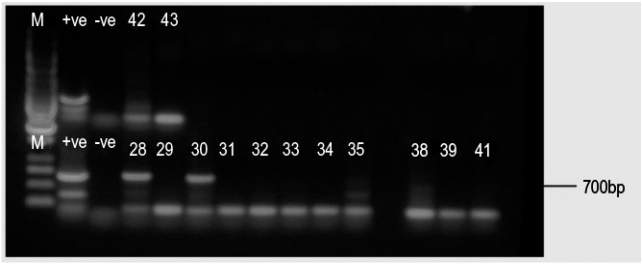

Fig 2 *nptII* amplifications of the *cry1I*a_5 transformed plants M; 1Kb marker lane, lane 28, 30 show positive amplification; + plasmid DNA, - untransformed plant

The larval mortality was nil 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 hr. Nevertheless the leaf bits of Bt positive plants showed only the pin hole size shot holes (Fig 3). 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.

Southern blots and ELISA

Southern hybridization of PCR positive T3 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 cry1Aa3 protein using ELISA assay, the amount of protein ranged from 1.56 to 2.23 µg/g of the

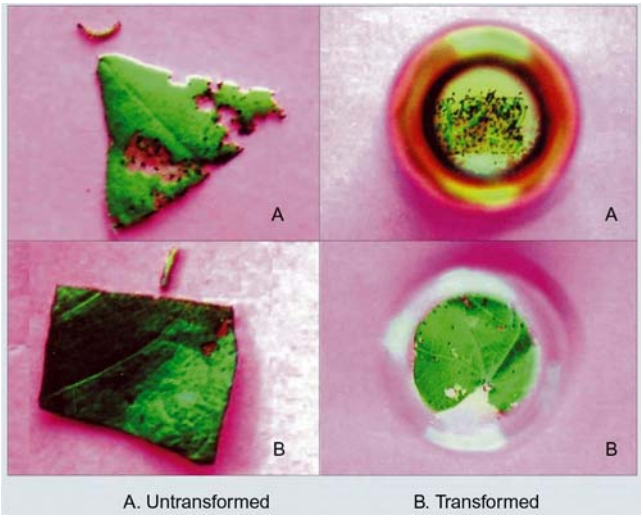

Fig 3 Nature and extent of damage of transformed and untransformed leaf bits consequent to feeding by II instar larvae of *Helicoverpa arimigera*

Table 4 Larval mortality of *Helicoverpa armigera* in leaf bit bioassay

Sample	Genotype/ gene construct	No. of replications	Larval mortality within 24 hr	Larval mortality b/n 24-48 hr	Mortality after 72 hr	Mortality %	Mortality damage
Positive control	RCH-2Bt	25	3	0	24	84	Shot hole
Negative control	DCH-32	25	2	0	3	0	Voraciously fed
Plant-1 BT_1	Cry1 I_{a5}	25	3	0	22	76	Shot holes
Plant-2 BT_2	Cry1 A_{a3}	25	1	0	23	80	Shot holes
Plant-3 BT_3	Cry1 I_{a5}	25	0	0	21	72	Shot holes
Plant-4 BT_4	Cry1 F	25	3	0	23	80	Shot holes
Plant-5 BT_5	Cry1 I_{a5}	25	2	0	22	76	Shot holes
Plant-6 BT_6	Cry1 F	25	2	0	23	80	Shot holes
Plant-7 BT_7	Cry1 I_{a5}	25	3	0	22	76	Shot holes

$$\text{Corrected larval mortality} = \frac{\text{Larval mortality after 72 hr} - \text{Larvae died in negative control}}{\text{Total no. of larvae}} \times 100$$

total soluble proteins. The overall transformation frequency was 0.30%. From the gene integration study it was clear that out of the seven transformants obtained, four transformants carried cry 1Ia5 genes. One transformant had 1Aa3 and two plants were transformed with cry 1F gene. It can thus be concluded that PTP mediated transformation methods involving simpler procedural steps and inexpensiveness have inspired renewal of efforts to adopt these methods to the development of cotton transformation.

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