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RESEARCH PAPER

In planta genetic transformation in cotton (*Gossypium* Spp.)

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Abstract: The fidelity and reproducibility of a novel tissue culture-independent Agrobacterium tumefaciens-mediated transformation strategy was analyzed in cotton (*Gossypium* spp.) and the transmission study of the transgenes was done by doing analysis at progeny level. Each 90 seedlings of Sahana and 70 of BCS 23-18-7 were used to transfer *cry*1Ac gene respectively. Totally four and five plants of Sahana and BCS 23-18-7 were amplified with *cry*1Ac and *npt*-II specific primers. However, all progenies of the T_0 positive transformants failed to amplify in T_1 generation. This rationalization of the results indicates the instability or failure of this method towards integrating the transgene at nuclear genome.

Keywords: *Agrobacterium, Gossypium, In planta,* Transformation

Introduction

Cotton is considered as the foremost commercially important fiber crop and is deemed as the backbone of the textile industry grown throughout the world. It is grown as source of fiber, food, edible oil and feed. Since its release into commercial production, genetically-modified cotton altered for insect and herbicide resistance has ranked among the most successful genetically-modified organisms. Transgenic Bt cotton not only controls *Helicoverpa armigera* (cotton bollworm) but also may reduce its presence on other crops such as corn, peanuts, soybeans and vegetables and may decrease the need for insecticide sprays in general (Wu *et al.,* 2008). Although the tissue culture based genetic transformation in cotton is well studied, it mainly constrained because it is time consuming, expensive and requires specialized equipment and highly qualified personnel. Somatic mutation or somaclonal variation frequently occurs in plant cells during *in vitro* culture, and some plants are recalcitrant to regeneration, although transgenic cotton is regenerated readily. Coker remained choice for *in vitro* genetic transformation studies (Hu *et al.,* 2011). However, the low embryogenic potential, maturation and most of the SE regenerated transformed plants, show abnormalities remained as problem. Hence, this necessitates development of simple, tissue culture independent and reliable transformation protocols for cotton transformation for improvement, particularly in the Indian cultivars which are adapted to local conditions.

To tackle the problems pertaining to regeneration in cotton and certain other recalcitrant crops, alternate methods to eliminate the steps of regeneration is being standardized. These are called the *in planta t*ransformation protocols. The first *in planta* method was described by Feldmann and Marks in 1987 and consisted of the imbibition of seeds with *Agrobacterium.* This approach avoids the oided tissue culture related problems and takes short time to obtain entire transformed individuals. However, the mean frequency of transformants in the progeny of such inoculated plants is relatively low and variable. Several strategies had developed to simplify the foreign genes delivery directly into the plant genome itself. Further, they have used successfully for various plant species, *e.g.*, *Solanum lycopersicum* (Yasmeen *et al.,* 2009), *Gossypium hirsutum* (Liu *et al.*, 2013), apple, pear, peach, strawberry (Spolaore *et al.,* 2001), and citrus (Ahmad and Mirza 2005).

We tried a novel non-tissue culture based approach for transformation of cotton (*Gossypium s*pp.), which involved inoculation of *Agrobacterium* solution onto growing apical meristem by doing vertical cut damage.

Material and methods

Sahana (*Gossypium hirsutum* L.) and BCS 23-18-7 (*Gossypium barbadanse* L.), both of them with higher adaptability and potentially high seed cotton yielding, 18-22 q/ha, were chosen during 2014-15 study at Agriculture Research Station, Dharwad Farm (Hebballi), UAS, Dharwad. Delinted seeds were surface sterilized with 0.2% $HgCl_2$ for 20 minutes followed by the repeated washes with sterile distilled water under laminar airflow. Seeds were grown in 500gm capacity polythene bag. Shoot apex with apical meristems from the germinating seedlings were damaged using sterilized scalpel blades and transformation were followed. The disarmed Agrobacterium strain EHA-105 harbouring binary vector pBinAR, carrying *cry1*Ac gene linked to the CaMV35S promoter, OCS terminator II and *npt*-II gene under the control of nopaline synthase (nos) promoter and terminator was used in transformation studies (Fig. 1). *npt*-II is the selectable marker. This construct was obtained from National Research Centre on Plant Biotechnology, IARI, New Delhi.

Actively growing eight to ten days old seedlings were used as explants. In order to make minimum damage to growing apical meristem vertical cut with a sharp blade was given in such a way that only one cotyledon was remained. Immediately after cutting, 48 hours old *Agrobacterium* culture with 0.6 OD (600 nm) was applied on apical meristem for 4-5 minutes, followed by washing with sterile distill water to remove overload of *Agrobacterium* culture and seedlings were incubated in dark for additional 48 hours. Seedlings were kept in a plant growth chamber (65% RH) for two weeks, before shifting to natural conditions in a transgenic greenhouse.

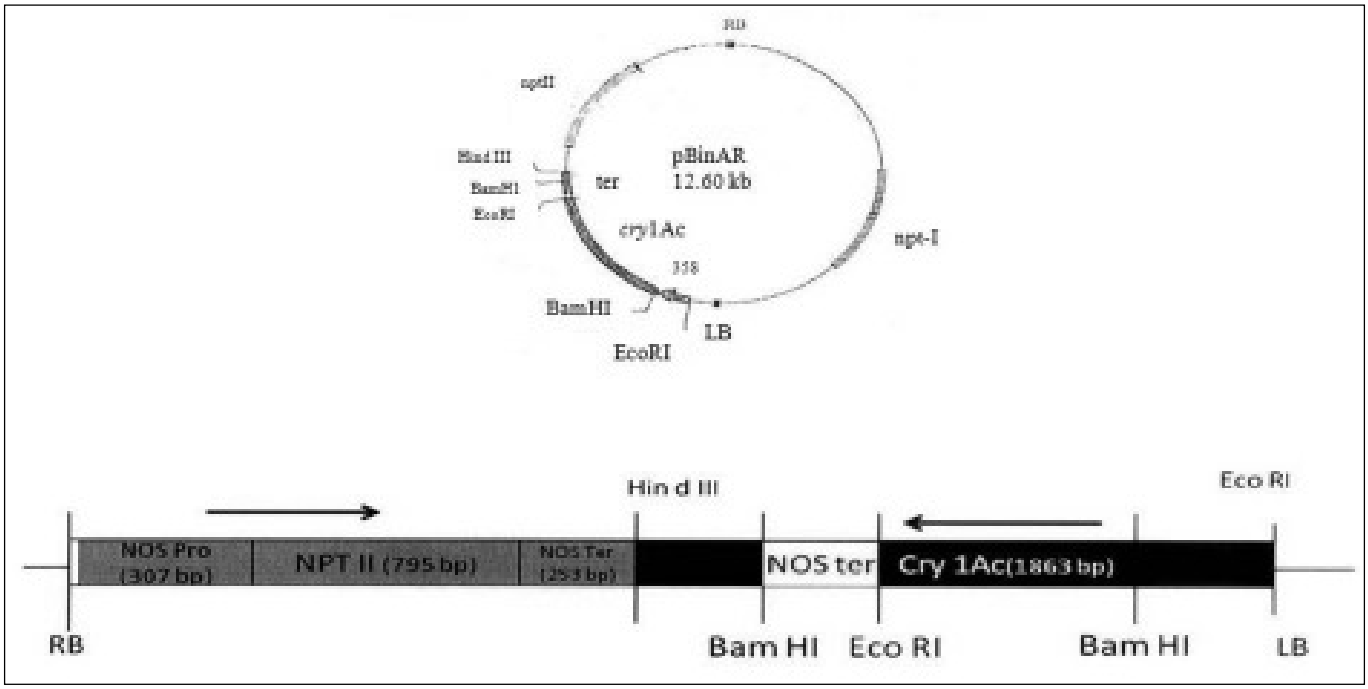


Fig. 1. Vector map of the *cry*1Ac gene construct in Ti Plasmid

T_0 generated putative transformants were subjected for PCR confirmation using *cry*1Ac and *npt*-II gene specific primers followed by inheritance study in T_1 generation. The PCR conditions were as follows as 94°C for 5 min, followed by 32 cycles of 94°C for 40 sec, 58°C for 40sec, and 72°C for 40 sec, cycling was followed with a final extension step at 72°C for 10 min.

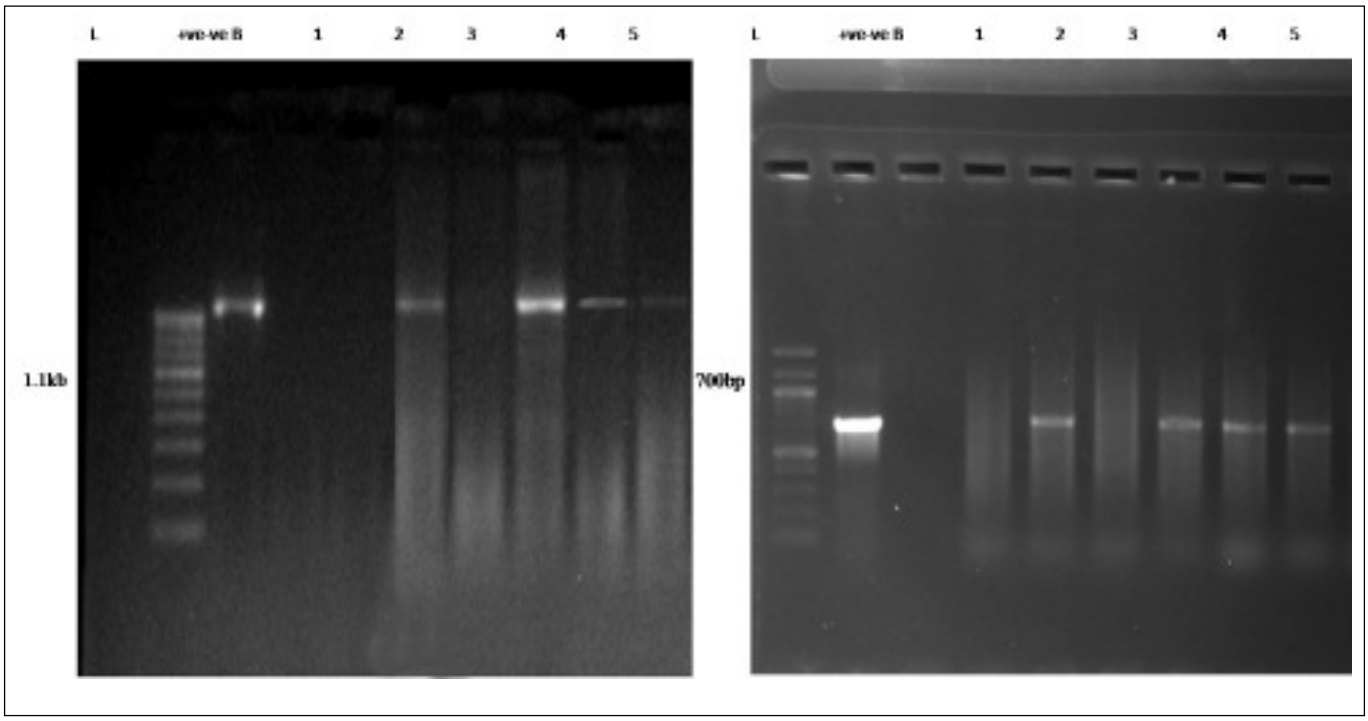
Results and discussion

Advances in transgenic technology increases feasibility of gene transfer and express various genes in agriculturally important crops like cotton. The rapid development provides an opportunity to improve important agronomic traits of plants, as well as to study of gene function and regulation. However, transformation rate has not been significantly improved since the first report of success in the transformation of cotton (Firoozabady *et al.,* 1987), it mainly constrained because of genotype dependency. Different approaches like vaccum infiltration transformation (Tague and Mantis, 2006), seed transformation (Supartana *et al.,* 2005), pollen tube mediated transformation (Wang *et al.,* 2007; Liu *et al.,* 2013), pistil transformation (Chumakov *et al.,* 2006), prickling apical meristem (Keshamma *et al.,* 2008; Alkuddsi *et al.,* 2014) and floral-dip method (Desfeux *et al.,* 2014) are attempted in various crops. Most of these methods have been difficult to reproduce and have not gained widespread acceptance. However, by considering future thrust this emerging *in planta* technique was tried.

Eight to ten days old seedlings co-cultivated with solid Agrobacterium culture by damaging the apical meristematic tip with sharp sterilized knife. The number of seedlings co-cultivated, number of seedlings established and the number of seedlings showing transformed status are presented in Table 1. Presence of transgene confirmation was done by PCR amplification study using *npt*-II and *cry*1Ac specific primers. Totally 90 and 70 seedlings were used for the transformation in sahana and BCS23-18-7, four and five plants shown amplification with both the primers respectively (Fig. 2). Percent transformation was calculated as 4.4 and 7.1 percent for Sahana and BCS 23-18-7 respectively. To study the inheritance of the transgene in T_1 generation seeds were collected from each branch of the positive T_0 events and sown in transgenic greenhouse. After one month of sowing DNA was isolated from all the T_1 generation plants and subjected to the PCR. All the plants were failed to amplify with *npt*-II and *cry*1Ac specific primers.

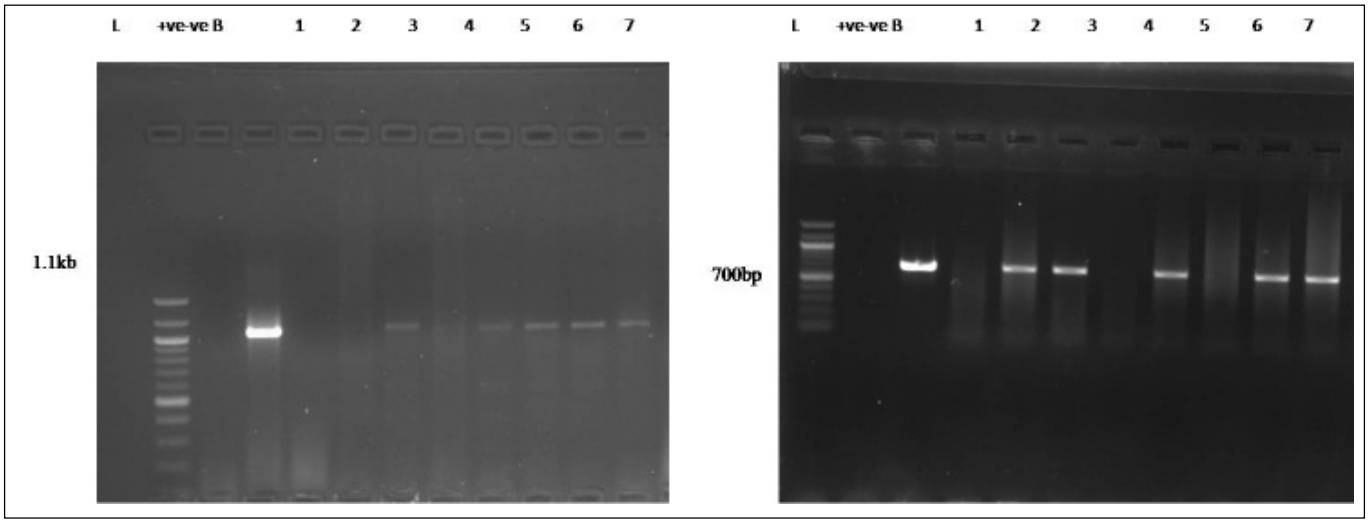
Table 1. In *planta* genetic transformation studies in Sahana and BCS 23-18-7

Sl. No.	Cultivar Name	No. of seedlings co-cultivated	No. of seedlings established	No. of plants attained maturity in green house	No. of plants PCR positive for *npt*-II and specific gene	Transformation percentage
1	Sahana	30	17	14	2	4.4%
		30	10	6	0	
		30	13	10	2	
2	BCS23-18-7	25	12	8	2	7.1%
		25	16	10	2	
		20	11	9	1	



a) Sahana transformants

L= 100 bp DNA Ladder, +ve =Plasmid sample, -ve = Untransformed plant sample,
B= Blank reaction mixture, 1-5=Plant sample Sahana



b) BCS 23-18-7 transformants

L= 100 bp DNA Ladder, +ve =Plasmid sample, -ve = Untransformed plant sample,
B= Blank reaction mixture, 1-7=Plant sample BCS 23-18-7

Fig. 2. PCR analysis of T_0 transformants using the cry1Ac and npt II primers

In present study the tissue targeted for gene transfer was diverse cells which are already destined to develop into specific organs and the meristematic cells still to be differentiated. This results in the primary transformants (T_0) being chimeric in nature. Hence the analysis of the transgenic plants was to be carried out in the T_1 generation. A rationalization of this result would be to suppose that the transferred gene was not stably integrated into the plant genome and was lost as the seedling developed. Similar findings were reported by Langridge *et al.,* 1992 in case of *Arabidopsis* transformation by *in planta* method. It may because of instability of nuclear genome integration or a possibility of T-DNA integration into the plastid genome.

Table 2. Gene integration studies in T_1 generation in Sahana for *cry*1Ac gene

SI. No.	Gene construct	Events name	Boll No.	No. of seeds collected	No. of plants established and specific gene	No. of plant PCR positivefor *npt*-II
1	*cry*1Ac	Ac 010814-1	1	25	15	0
			2	24	16	0
			3	26	14	0
			4	22	13	0
2		Ac 010814-2	1	25	20	0
			2	27	21	0
			3	28	20	0
3		Ac 010814-3	1	25	18	0
			2	26	16	0
			3	23	15	0
			4	24	18	0
			5	19	10	0
4		Ac 010814-4	1	20	14	0
			2	21	17	0
			3	22	15	0
			4	23	16	0
	Total		16	380	258	0

Table 3. Gene integration studies in T_1 generation in BCS 23-18-7 for *cry*1Ac gene

SI. No	Gene construct	Events name	Boll No.	No. of seeds collected	No. of plants established	No. of plantPCR positivefor *npt*-II and specific gene
1	*cry*1Ac	Ac101214-1	1	21	15	0
			2	22	14	0
			3	24	17	0
			4	23	17	0
2		Ac101214-2	1	25	16	0
			2	25	18	0
			3	23	14	0
3		Ac101214-3	1	26	19	0
			2	27	19	0
			3	18	12	0
			4	15	8	0
4		Ac101214-4	1	23	18	0
			2	24	14	0
			3	21	14	0
			4	25	18	0
5		Ac101214-5	1	22	14	0
			2	16	9	0
		Total		380	256	0

Transgene deletion or chimerism may be the possible cause of the gene instability. The main cause of chimera formation is either transformation takes place in L1 or L2 layer. L1, a single layer undergoes division and forms epidermis. L2 is also a single layer which gives rise to ground tissue, while the innermost L3 layer generates the body of new tissues, including vasculature and germline tissue. Transformation events that occur only in the L3 layer result in germline transformation. The possible cause for this is transformations take place in the L1 Layer leading to chimerism at the organ level. Juturu *et al.,* 2014 suggested that screening for transgenics at T_0 generation would lead to false results, screening in T_1 generation would be ideal.

Finally, it does not appear that Agrobacterium was able to transform in cotton via this approach. Nevertheless, DNA is transferred from the *Agrobacterium* host and was able to persist in the plant for one generation. A possible explanation for our results is that the T-DNA was transferred from the Ti plasmid to an endophytic bacterium that was prevalent in the glasshouse at the time of these experiments. It is not possible to generalize from these results to those of other workers. However, the data shown here indicate that one must carried out thorough analysis of 'transgenic' plants generated by using such kind of protocols. However, it was one step towards finding the major technical and scientific barriers that need to be overcome in order to bring the technology to its full potential. It is clear, however, that utilization of this technology opens the way for the transformation of tissues and species that are not otherwise accessible to genetic modification using tissue culture technique.

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