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SHORT COMMUNICATION

AGROBACTERIUM MEDIATED *IN PLANTA* TRANSFORMATION OF *GOSSYPIUM HIRSUTUM* CV. G. COT. 10

V.H. SOLANKI, VIKAS KHANDELWAL, D.H. PATEL AND M.K. MAHATMA

Department of Biotechnology, Navsari Agricultural University, Navsari-396 450, Gujarat

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An improved protocol of using simple *in planta* transformation methods (Injection method and Cut method) were developed for *Gossypium hirsutum*. The frequency of transformation was 1.16 % for injection method and 0.83 % for cut method. The effect of different factors like age of explants, pH of co-cultivation medium, effect of kanamycin and co-cultivation time period of *Agrobacterium tumefaciens* on the efficiency of regeneration and transformation was studied. The combined result of this study shows that three days old seedlings inoculated with *Agrobacterium* and co-cultivated for three days in half strength MS medium at pH 5.8 had highest frequency of transformation.

Key Words*: *Agrobacterium tumefaciens*, *cry*IF gene, *Gossypium hirsutum

Cotton remains one of the world's most important economic crop species, providing raw material for textile manufacturing, mulch and cattle feed, as well as vast array of consumer based product. Genetically modified cotton is one of the first transgenic crops in commercial production on large scale, now accounts for vast majority of cotton acreage in India and increasingly so in cotton producing countries around the world. Cotton is recalcitrant to regeneration and regeneration is strongly genotype dependent. The developed protocol overcomes the problem related to somatic embryogenesis, somaclonal variation and genotype dependent regeneration. *Agrobacterium*-mediated transformation has several advantages, such as higher transformation efficiency, the ability to transfer large pieces of DNA, minimal re-arrangement of transferred DNA, integration in low copy numbers and low cost, etc. (Zhu *et al.* 2006 and Tyagi *et al.* 2007).

To optimize the transformation protocol, it is important to optimize several parameters like age of the explants, concentration, infection and co-cultivation time of the *Agrobacterium* as these factors affects the efficiency of transformation (Atichart *et al.* 2007). Therefore, this study was carried out to optimize various factors affecting transformation condition of cotton using *Agrobacterium tumefaciens* strain containing binary vector. The conditions optimized in this study can be exploited later to transform cotton in order to enhance the insect, pest and disease resistance.

The seeds of cotton breeding line G. Cot.10 from Cotton Research Station, Surat, have been used for present research work. Overnight presoaked cotton seeds were surface sterilized first with 1% Bavistin (BASF, India) for 10 minutes and later with 0.1% $HgCl_2$ (Hi-media Laboratories, Mumbai, India) for few seconds and washed thoroughly with distilled water. The washed seeds were germinated aseptically on half strength of MS medium till the age of one, three and five days (Fig. 1) and subsequently infected with *Agrobacterium tumefaciens* strain LBA 4404 containing *cry*IF gene (Fig. 2) by injection and cut method. Infected seedlings were co-cultivated in dark for one, two, three and four days. *Agrobacterium* was inoculated into plumule, cotyledonary node and adjacent region of the meristem

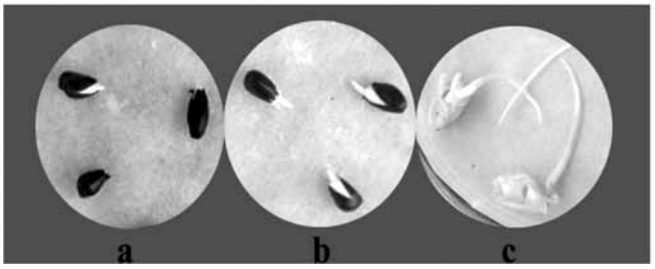

Fig. 1. Age of the seedlings a. One day old seedlings b. Three days old seedlings c. Five days old seedlings

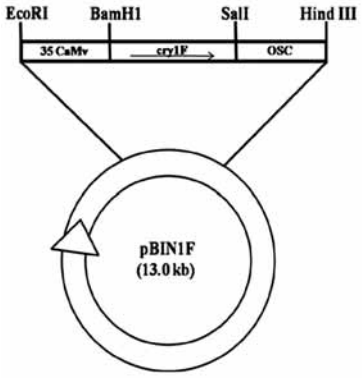

Fig. 2. Diagram of the construct

by injection method (Fig. 3a). While in cut method seed coats and one of the two cotyledons of each germinated seed was removed and the half seed with the plumule, cotyledonary node and adjacent cotyledon tissue was inoculated with culture of the *A. tumefaciens* (Fig.3b).

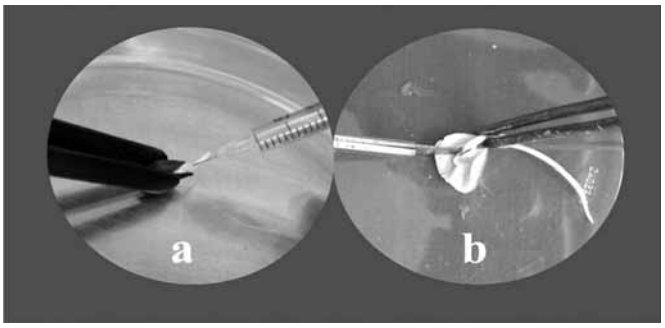

Fig. 3. a. Infection through injection method, b. Infection through cut method

Co-cultivation duration has significant effect on survival percentage of the seedlings and frequency of transformation. Co-cultivation duration for three days gave highest survival with 73.61% and 62.18% of the seedlings in injection and cut method respectively. Increasing co-cultivation duration above three day drastically reduced the survival percentage in both the method of transformation. Maximum frequency of transformation was also found highest in three days of the co-cultivation duration with 3.33 % and 2.66 % for injection method and cut method, respectively. Results obtained were in conformity with the Bayley *et al.* (1991), Gould and Cedeno (1998), Mondal *et al.* (2001), Katageri *et al.* (2007) and Ganesan *et al.* (2009). The differential requirement of co-cultivation period depends on *Agrobacterium* strain used for co-cultivation or medium for bacterial culture. Wilkins *et al.* (2004) recommended co-cultivation for one to three days until the light halo of bacterial growth observed around the explants. Similarly, Zhao *et al.* (2006) co-cultivated hypocotyl for two days with *Agrobacterium* which gave 1.98% frequency of transformation. Increasing *Agrobacterium* concentration did not always increase the transformation rate. Our results also supports that increasing the co-cultivation above three days will also increase the problem associated with the overgrowth of the *Agrobacterium* that behaves as pathogen with plants (explants) and decrease nutrient supply (Rohini and Sankara Rao 2000, Wilkins *et al.* 2004, Ganesan *et al.* 2009 and Mannan *et al.* 2009). When we consider the interaction effect between age of seedlings and co-cultivation duration three days old seedlings and three days of co-cultivation duration gave highest frequency of transformation in both method of transformation (injection method and cut method). For injection method and cut method, maximum frequency of transformation 6% and 4% was obtained for three days old seedling and three days of co-cultivation duration, respectively.

For the optimization of pH of co-cultivation medium, pH range from 5.0 to 6.6 with pH interval of 0.2 was used (Mannan *et al.* 2009) because in transformation protocol considerable effect of pH was observed on frequency of transformation. Among the selected pH range from 5.0 to 6.6 with pH interval of 0.2, pH 5.8 proved best for *Gossypium hirsutum* (cv.G.Cot.-10) transformation, where as both decrease and increase in pH showed lower transformation efficiency (Fig. 4). The pH of the co-cultivation medium also effects expression

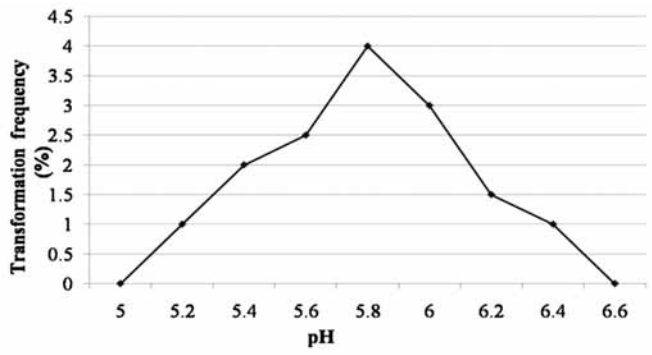

Fig. 4. Effect of pH on efficiency of transformation

of *vir* genes and T-DNA transfer. Acidic pH considered optimum for this purpose depending upon type of plant and explants. Hie *et al.* (1994) obtained high efficiency of gene transfer through *Agrobacterium* strain at pH 5.2 in rice. In the same way Mannan *et al.* (2009) found pH 5.8 is most effective for transformation of *Artemisia absinthium*. Age of the seedlings also affects the frequency of transformation. In this study, survival percentage was highest for three days old seedlings which was 69.17% for injection method and 69.82 % for cut method as compared to one day and five days old seedlings. In addition, when we consider the frequency of transformation, three day old seedlings gave higher frequency of transformation 2% and 1.5% for injection and cut method, respectively. The identical age of the seedlings were used by Chee *et al.* (1989) and Keshamma *et al.* (2008). Likewise in sunflower, Sankara Rao and Rohini (1999) had taken two days old germinating seedlings with one cotyledon removed for transformation. Age of the seedlings is the key factor often affective in gene transformation. It was observed that with the increase in age of the seedlings, decrease in the frequency of gene transformation occurs. Result gained for the three days old seedlings may be due to seedlings tissue or cells were competent enough to withstand bacterial infection and other related stress. Three days old seedlings exhibited higher frequency of transformation may be attributed to actively dividing competent cell which readily uptake T-DNA from *Agrobacterium* (Rohini and Sankara Rao 2000).

After co-cultivation, the germinated seedlings were washed with sterile water containing 500 mgL^{-1} cephotaxime (Sigma Aldrich, St. Louis) for 5 minutes followed by 4-5 washing with sterile water and transferred to petridishes containing half strength liquid MS medium for eight to ten days. Plants were then transferred to pots containing soilrite and grown to maturity in greenhouse. These plants were selected for further characterization of transgene using polymerase chain reaction for the presence of *npt*II marker and *cry*IF genes. Approximately 0.55 kb fragment of *npt*II gene was amplified using specific primers 5'-AAGAACTCGTCAAGAAGGCGATA-3' and 5'-ATGGGGATTGAACAAGATGGATT-3' as forward and reverse primers, respectively. The 1.0 kb fragment of *cry*IF gene was amplified using 5'-ATGGAGAACAACATCCAGAAT-3' and 5'-CAGTTTGTTGGAAGGCAACT C-3' as forward and reverse primers, respectively. Each PCR reaction was performed in 50 µl (total volume), consisting of 1x reaction buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs, 3 pmol of each primer, 0.3 units of Taq DNA polymerase (Genei, Bangalore) and 50 ng of plant genomic DNA. The amplification reactions were carried out in a PCR Cycler under the following conditions: 94°C for 5 minutes followed by 35 cycles at 94°C for 1 min, 56°C (*npt*II) or 53°C (*cry*IF) for 1 min, 72°C for 1 min 25 seconds with a final extension at 72°C for 5 min. The PCR product was fractionated by electrophoresis on a 0.8% agarose gel and visualized under UV light.

For preliminary evaluation of transgene from the seeds of T_0 plants root lateral assay was performed using selection medium (Hoagland with Kanamycin 50 mg L^{-1}, Sigma Aldrich, St. Louis). The plant that grew root laterals were scored as positive while those without root laterals were negative (Kumar *et al.* 2009).

About 960 transformants were tested by PCR amplification of genomic DNA using a set of gene specific primers for both *cry*IF and *npt*II genes. PCR analysis resulted in the expected sizes for both *npt*II (0.55 kb; Fig. 5a) and *cry*IF gene (1 kb; Fig. 5b) which corresponded to that of the plasmid control. No amplified product was detected in the sample containing genomic DNA isolated from nontransgenic control plant. Seeds obtained from transgenic T_0 plants were screened for the presence of root lateral. Plantlet germinated on

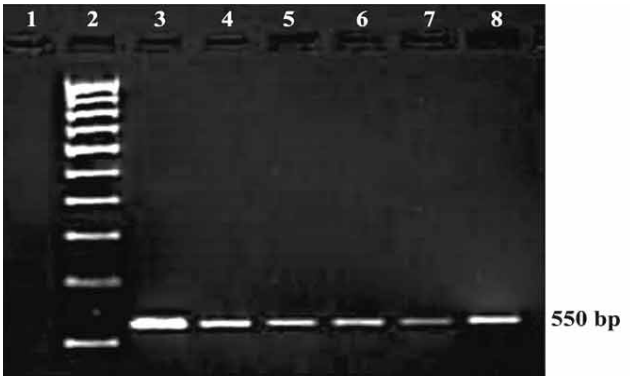

Fig. 5a. PCR verification of transgenic plants using primer for *nptII* gene
Lane 1: Negative control (untransformed plant)
Lane 2: 500 bp ladder
Lane 3: Positive control, pBIN1F plasmid
Lane 4, 5, 6, 7 and 8: Genomic DNA of transformed plant

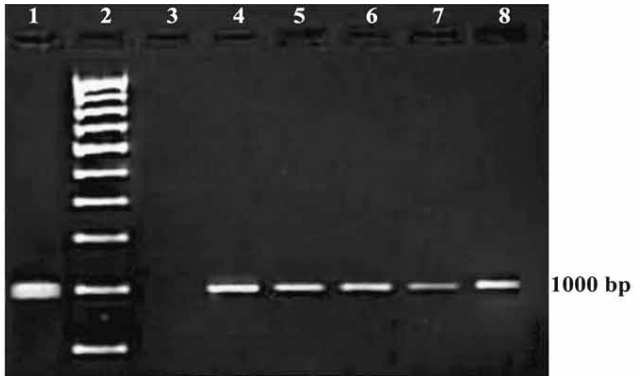

Fig. 5b. PCR verification of transgenic plants using primer for *cry1F* gene
Lane 1: Positive control, pBIN1F plasmid
Lane 2: 500 bp ladder
Lane 3: Negative control (untransformed plant)
Lane 4, 5, 6, 7 and 8: Genomic DNA of transformed plant

Hoagland media containing (50 mg L^{-1}) kanamycin shows the development of root lateral (Fig. 6) which indicates expression of the marker gene for *npt*II and the presence of the *cry*IF gene. Similar type of assay was performed by Kumar *et al*. (2009) for selection of the transgenic cotton seeds from the T_0 transgenic cotton plants.

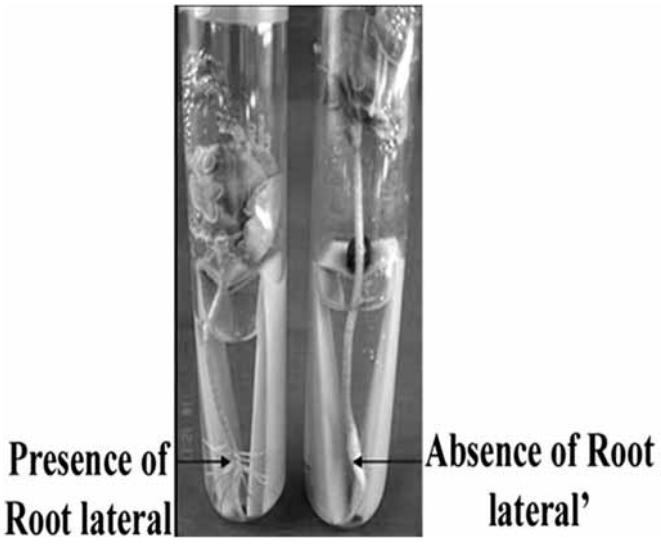

Fig. 6. Root lateral assay showing the expression of *nptII* gene

Insect bioassay was performed to determine the expression of *cry*IF gene using *Spodoptera litura*. *Spodoptera* was raised from the larvae collected from the castor field, Department of the Plant Breeding, NAU, Navsari. Second instar larvae of *S. litura* were used for insect bioassay. Feeding assay (Fig. 7) with 12 T_0 plants confirmed that the CryIF protein produced in the transgenic lines were highly toxic to *Spodoptera litura*. The larvae began to die one or two days after feeding on the transgenic leaf tissues. Most of the larvae abandoned feeding after two days on leaves of A1, A2, A3, A4, A5, A7, A8, A9, A11, and A12, plants, exhibited significant reduction in weight and showed 60.00-96.00 % mortality (Table 1). A mortality of 100 % reached

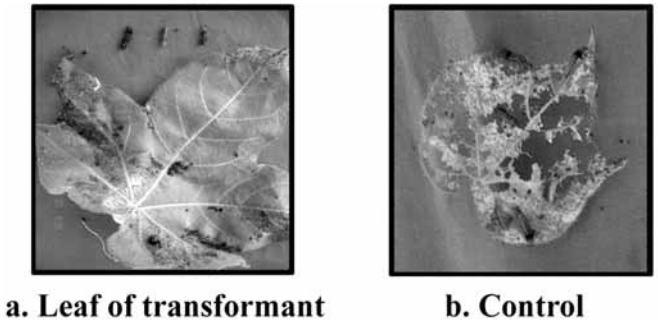

Fig. 7. Insect bioassay on to plants

Table 1. Average weight of the larvae, weight loss and larval mortality after two day of infestation on leaves of T_0 transgenic plants.

Transgenic Plants	Average weight of the larvae (mg) (mean ± SD)	Weight loss (%) (mean ± SD)	Larval mortality (%) (mean ± SD)
A1	7.34 ± 0.87	90.70 ± 0.70	83.33 ± 5.77
A2	36.78 ± 4.25	55.09 ± 0.62	60.00 ± 0.00
A3	6.69 ± 3.00	93.83 ± 0.37	86.67 ± 5.77
A4	N.D.	N.D.	100.00 ± 0.00
A5	7.91 ± 3.26	97.03 ± 0.34	96.67 ± 5.77
A6	34.12 ± 5.54	69.21 ± 0.68	70.00 ±10.00
A7	20.00 ± 2.00	75.62 ± 0.56	73.33 ± 5.77
A8	32.12 ± 3.78	61.19 ± 0.22	63.33 ± 5.77
A9	55.23 ± 4.95	34.37 ± 0.35	46.67 ± 5.77
A10	N.D.	N.D.	100.00 ± 0.00
A11	19.47 ± 0.99	78.02 ± 0.29	76.67 ± 5.77
A12	62.72 ± 2.58	42.42 ± 1.23	53.33 ± 5.77
Control (Non-transgenic)	81.22 ± 2.86	-	-

N.D. – Not determined due to mortality

two to three days after infestation. Similarly, 100% of mortality was observed in transgenic peanut by Tiwari *et al.* (2008) using *cry*IEC gene against *Spodoptera*. Thus, insect bioassay proved, the *Bt* protein produced in transgenic cotton plants caused rapid cessation of larval feeding activity and subsequently inhibit their development.

Our study revealed, *in planta* transformation of cotton variety G. Cot. 10 using *Agrobacterium* strain LBA 4404 containing pBIN1F (13.0 kb) plasmid harboring *cry*IF gene construct presents excellent protocol for transformation. Three days old seedlings co-cultivated for three days and regenerated at pH 5.8 yielded higher survival percentages as well as the frequency of transformation in both injection and cut method.

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