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Agrobacterium tumefaciens Mediated Genetic Transformation in Coker-312 (*Gossypium hirsutum* L.) Using Hypocotyls Explants

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A B S T R A C T

In present investigation we performed *Agrobacterium*-mediated transformation in Coker-312 (*Gossypium hirsutum* L.). Freshly pre-cultured (48 hrs.) hypocotyl explants were infected with two different *Agrobacterium tumifaciens* strain EHA105 and LBA4404 carrying *cry*1Ac and *cry*1Acm respectively along with *neomycin phosphotransferase* - II gene as selection marker. Selection of kanamycin resistant embryogenic calli were performed on MS media supplemented 0.1 mg/l 2, 4-D + 0.5 mg/l kinetin with cefotaxime (400 mg/l) and kanamycin (100 mg/l), followed by subculture and maintenance for embryo maturation. Putative transformants were screened for the presence of specific gene and *neomycin phosphotransferase* (*npt*-II) by for its transgene amplification. Further, expression analysis was carried out using Reverse transcriptase-PCR (RT-PCR). Protein expression was checked with *Bt* expression strips in T_0 generation. Further analysis of T_1 plants demonstrated the failure detection of the transgene.

Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most important economic crop and it plays a major role in the world's global economy. Along with its dominance for the production of textile fiber, cottonseed has become an important source of oilseeds in India in last fifteen years (2002-2016).

In case of cotton production major concern world over has been to protect it from bollworm (*Helicoverpa armigera*), a single pest capable of causing damage to the extent of 40%. Extensive adoption of the *Bt* cotton technology by the major cotton producers countries, such as China, India, and Brazil, has notably brought great economic benefits to producers (James, 2015). *Bt* cotton seed oil contributed 15% of total edible oil production in India during 2016-17. This remarkable achievement indicates the demand for low-priced cooking oil in the domestic market. Out of the total *Bt* cotton cultivated area (22.3 million hectares) all over the world during 2016, India alone contains 10.8 million hectares. It indicates the prime importance of the cotton in the Indian economy as well as its role in employment creation.

The main prerequisite for genetic transformation in cotton is standardized transformation protocol. In case of cotton varying types of results had been reported

regarding with effective colonization duration ranging from 5 seconds to 30 minutes infection period (Wilkins *et al.,* 2004 Nandeshwar *et al.,* 2009; Sumitra *et al.,* 2010; Sangannavar, *et al.,* 2016), but in most of the study the transformation efficiency was more when more explants colonized for 10 minutes (Sumitra *et al.,* 2010) which was followed in present investigation.

Although optimal co-cultivation duration was critical for transformation in some crops, in case of cotton it was found uncertain ranging from 36 to 72 hours (Sunilkumar and Rathore, 2001; Leelavathi *et al.,* 2004). Jin *et al.,* (2005) suggested that co-cultivation at 19^0C for 48 hours was effective for the efficient transformation which was confirmed earlier by Dillen *et al.,* (1997) and Sunilkumar and Rathore (2001).

In cotton, so far *in vitro* regeneration or totipotency has been observed in Coker-312, 310, 201 and 315 (*Gossypium hirsutum*) genotypes belong to USA. Therefore in the present study, the possibility of regeneration in Coker-312 cotton (considering the availability of seeds) was tried and plants were successfully regenerated.

Materials and Methods

Gene constructs for genetic transformation

The disarmed *Agrobacterium* strain EHA-105 and LBA4404 harbouring binary vector pBinAR, carrying *cry*1Ac gene and pHS100, carrying *cry*1Acm gene respectively, were used for transformation (Fig. 1). *cry*1Acm is a codon-optimized modified *cry*1Ac gene synthesized by using the codon usage table of tobacco (http://www. kazusa.or.jp/codon) by Mohan T.C. (2008) at Dept. of Biotechnology University of Agricultural Sciences Dharwad, Karnataka, India. The vector harbors an *npt II* gene under the control of nopaline synthase (nos) promoter and terminator was used as a selectable marker. The *cry*1Ac construct was obtained from National Research Centre on Plant Biotechnology, IARI, New Delhi.

Plant material and genetic transformation

Coker-312 seeds available in germplasm pool of *Gossypium hirsutum* cotton maintained at Agricultural Research Station, Dharwad, Karnataka. Seeds were delinted by using (100ml/kg) of concentrated H_2SO_4. Delinted seeds were dipped in 0.2% $HgCl_2$ solution (w/v) for 20 minutes with constant stirring followed by 5-6 washes with sterile water under laminar airflow. Seeds were kept in sterilized distilled water for 48–72 hours to soften the seed coat which was removed before the seeds were placed on half strength MS medium (George *et al.,* 2008) for germination. Hypocotyls were excised from the 7-8 days old seedlings and cut into (5-6 mm) sections were used for transformation experiments. Genetic transformation and *in vitro* culturing were carried out as described earlier (Sangannvar, 2012)**.** Germinated somatic embryos were grown in 500 ml conical flask containing MS salts in a growth chamber at 24/26°C ± 2 day/night temperature, with 2,000 lux light intensity and a 16-h light/8-h dark cycle at a relative humidity of 65~70%. Established plantlets with well-developed roots and leaves were transplanted in small plastic pots (8 x 5 cm) containing sterilized soil: peat mixture (1:1) and placing them in the transgenic greenhouse condition.

Genomic DNA isolation and gene integration

Genomic DNA was isolated from cotton leaves using a modified version of the CTAB method (Sangannavar *et al.,* 2013). A gene integration study was performed by PCR analysis using specific gene (*cry*1Ac and

*cry*1Acm) and selectable marker gene (*npt*-II) primers. PCR amplification condition consisted of 90^0C for 40 sec, annealing at 58^0C, 60^0C, and 58^0C, for *cry*1Ac, *cry*1Acm and *npt*-II, extension at 72 C for 10 min and hold at 4^0C.

Gene expression analysis

Total RNA was isolated from young leaves collected from control and transgenic plants, using the plant total RNA isolation kit (Cat.No.STRN50-1KT) provided by Sigma Aldrich, USA and the protocol was carried out according to manufacturer's instructions. Total RNA 2 µg was reverse transcribed (Agilent, cDNA synthesis kit). The cDNAs were used as templates, and the primers and reaction conditions for RT-PCR were the same as for PCR above. RT-PCR was performed in a total volume of 20µl. Amplified products were resolved on 1.5% agarose gels with ethidium bromide staining.

Transgenic *Bt* plants were evaluated for Bt-protein expression. Bt-strips supplied by Amar immunodiagnostics, Hyderabad were used in this study. Leaf disc of 5 mm diameter from control and transgenic plants was crushed in 600 µl of extraction buffer supplied by the manufacturer and was used as tissue extract for observing the Bt-expression. The strips work on the principle of antigen-antibody specificity.

Results and Discussion

Genetic transformation and regeneration

Over the last three decades, several methods were developed for cotton transformation (particle bombardment, electroporation, pollen-mediated, *Agrobacterium*-mediated). *Agrobacterium* mediated approach remains the method of choice because of single copy transfer of T-DNA is possible with this method compared to other methods where direct DNA transfer is followed and very difficult to get single copy number. To increase the competency of the plant cells to T-DNA delivery explants were pre-cultured for 48 hours before co-cultivation. The mechanism for the influence of preculture treatment has not been understood but some scientists indicated that preculturing of explants enhanced the binding of *Agrobacterium tumefaciens* (Moralejo *et al.,* 1998) and it reported an increase in transformation efficiency (Khan *et al.,* 2010; Sumithra *et al.,* 2010).

Co-cultivation duration was found uncertain in cotton ranging from 36 to 72 hours, mainly fascinated by the genotype variability (Sunilkumar and Rathore, 2001; Leelavathi *et al.,* 2004; Jin *et al.,* 2005). In present report, hypocotyls were co-cultivated in MS media (0.1 mg/l 2, 4-D + 0.5 mg/l kinetin) (Fig. 2) under dark for 48 hours at 24 ± 2^0C with 75-80 % relative humidity which was standardized previously in our laboratory (Sangannavar, 2012). A longer co-cultivation period (60 hours) might cause hypertonic conditions that burst the cell or it might be due to hyperactivation of defense mechanism that might be lethal to cell and hence results in low frequency of transformation. Less infection time period, of course, produces less number of transformed explants.

Transformation of plant cells and regeneration of those transformed cells to fertile plants are the two important regimes for obtaining a large number of successful transformants. A total of 230 hypocotyls were used for each of *cry*1Ac and *cry*1Acm genes transformation (Table 1). After 4-5 weeks of colonization and co-cultivation, out of 230 explants, the emergence of calli was recorded in 126 and 93 hypocotyls of each *cry*1Ac and *cry*1Acm accounting to 54.78 percent and 40.44 percent respectively.

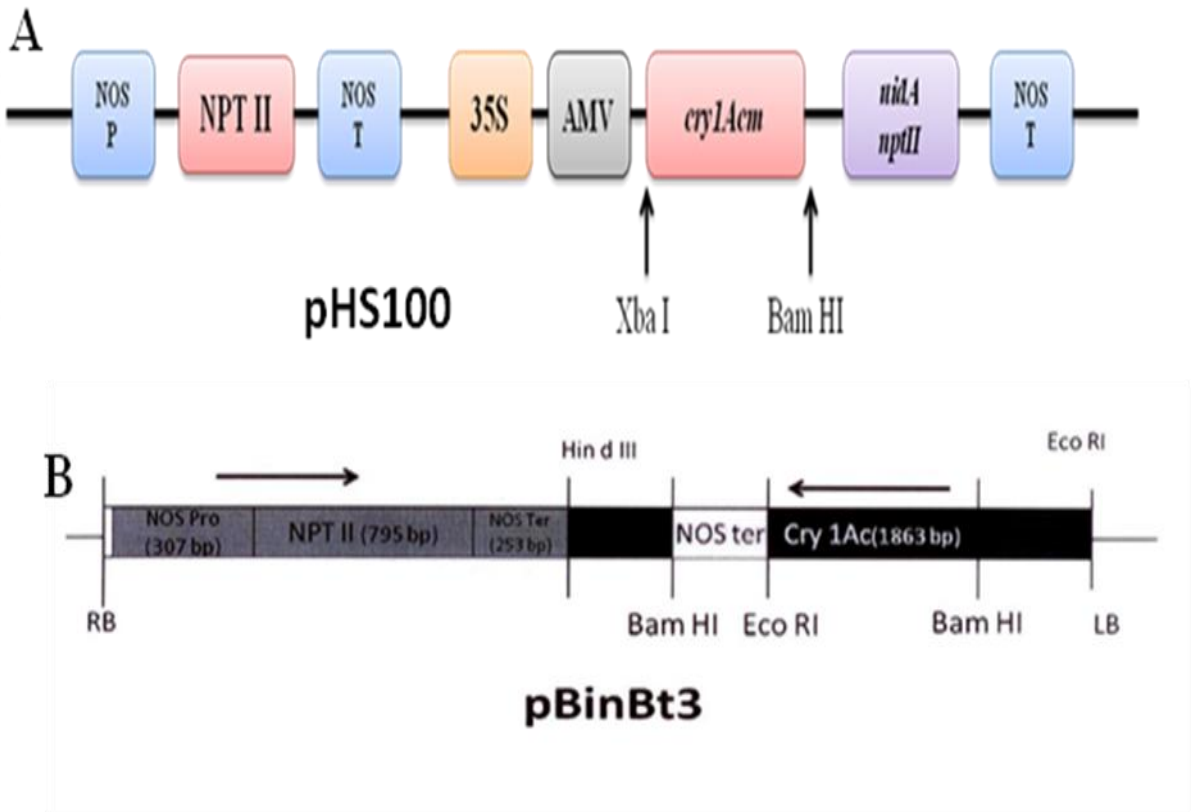
Table.1 Genetic transformation studies in Coker-312

Sl. No	Gene construct	No. of hypocotyl explants co-cultivated	No. of explants including calli survived on kanamycin media (After 4-5 weeks)	No. of calli survived on kanamycin (at embryogenesis)	No. of individual events/plants produced	Transformation percentage (%)
1	*cry*1Ac	230	126	18	8	3.4
2	*cry*1Acm	230	93	8	3	1.3

Table.2 Gene integration studies in T_1 generation

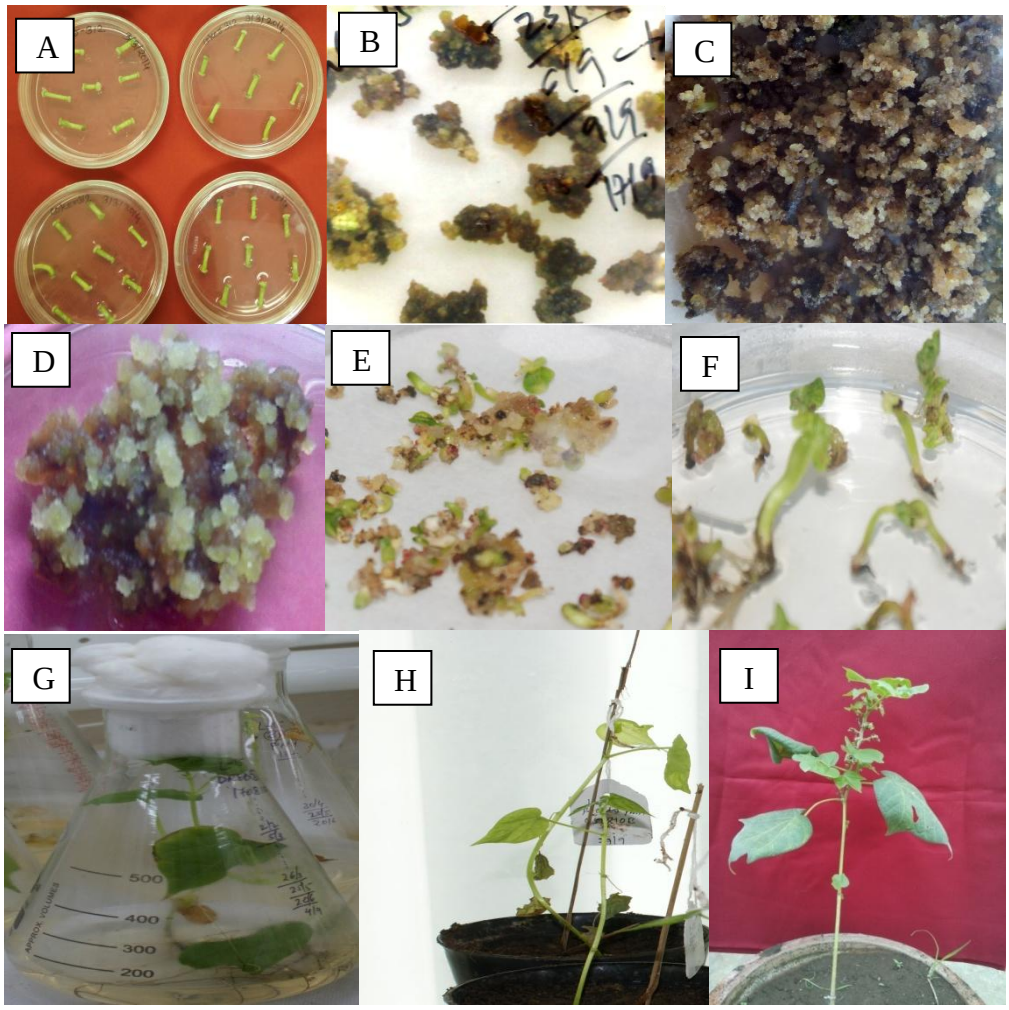
Sl. No.	Gene Construct	Event name	No. of plant established (T_1)	No. of plant PCR positive for Specific gene	No. of plant PCR positive for *npt*-II	RT-PCR positive for specific gene	Bt-expression Strips analysis
1	*cry*1Ac	CKAc-1	14	0	0	-	-ve
2	*cry*1Acm	CKAcm-1	16	0	0	-	-ve
3	Total		30	0	0	-	-ve

Fig.1 Vector maps of the gene construct in Ti Plasmid



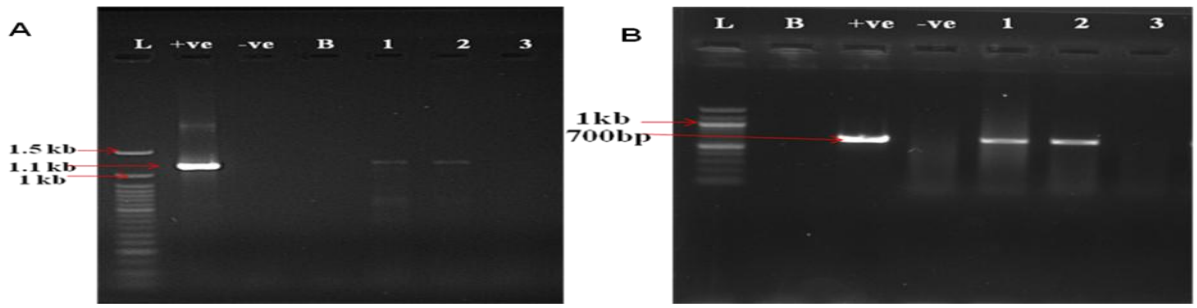
*cry*1Acm (2) *cry*1Ac

Fig.2 Transformation of hypocotyls of *G. hirusutum* cv. Coker-312 by *Agrobacterium* containing *cry*1Ac and *cry*1Acm gene and transgenic plant regeneration



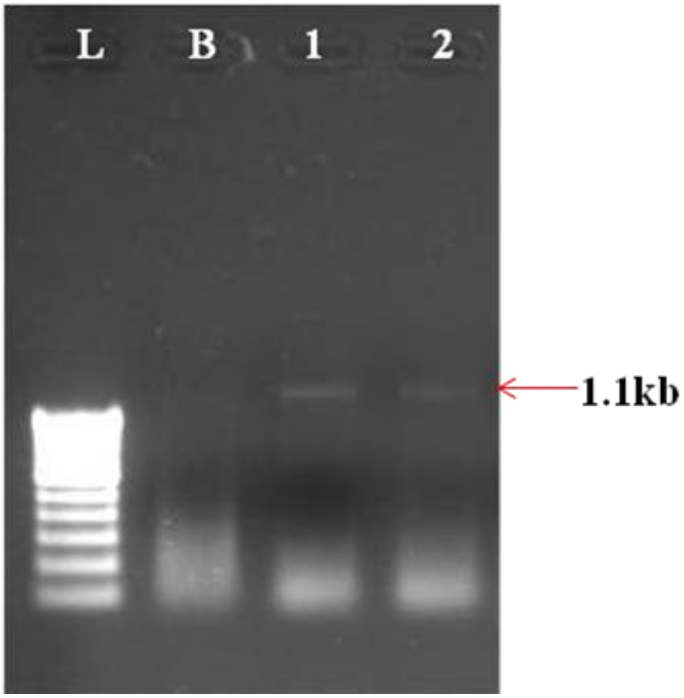
A) Inoculation of hypocotyl explants with *Agrobacterium* culture. B) Selection of the explants and on kanamycin medium. C, D) Regeneration from putative transformed cells on MS medium containing kanamycin. E, F) Somatic embryo formation G, H) Regeneration of transformed plant.

Fig.3 Confirmation of gene integration through PCR for A) *cry*1Ac and B) *npt-II* gene



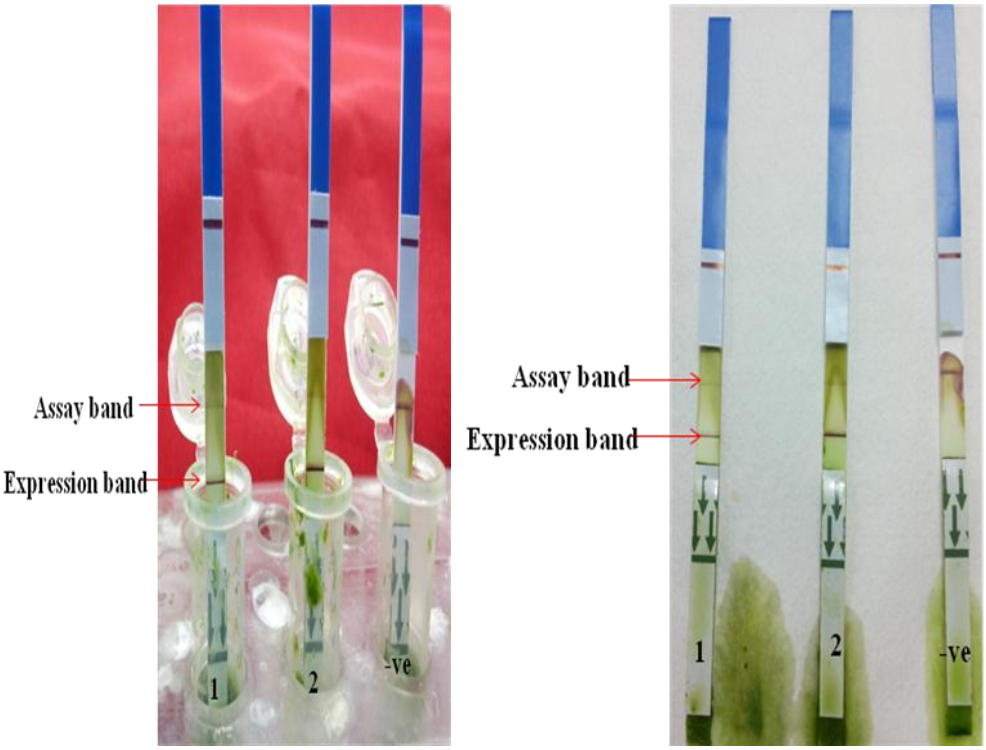
L= 50 bp Ladder, +ve =Plasmid DNA sample, -ve = Untransformed plant sample, B= reaction mixture, 1 to 3= putative transformed plant samples.

Fig.4 *cry*1Ac gene integration confirmation through RT-PCR for gene specific primers



L= 100 bp Ladder B = reaction mixture and 2 = Putative transgenic samples Coker-312

Fig.5 BT- expression Strip analysis of putative transgenics for *cry*1Ac



1 and 2 = Putative transgenic samples of Coker-312
-ve = Untransformed Sample of Coker-312

However, elimination of untransformed calli was done by using 100mg/l kanamycin as selection pressure in subsequent sub-culturing MS medium. Finally at the stage of embryogenesis 18 and 8 calli (Table 1) respectively for *cry*1Ac and *cry*1Acm gene were survived as a kanamycin resistant calli (Table 1). However, out of 25 events (resistant calli remained after 11-12 weeks after colonization and co-cultivation), eight and four plantlets were got established from *cry*1Ac transformed hypocotyls and Calli explants respectively. Based on this percent transformation was 3.4 percent and 1.3 percent respectively for hypocotyls and calli explant (Table 1). This percentage was equal to the previously reported transformation percentage in cotton (Singh *et. al.,* 2016) indicates the sensitivity of the untransformed calli to the kanamycin. In many cases, the production of transgenic plants is prevented due to prolonged culture period leads to secondary metabolites excreted from the explants into the medium, browning of callus, high frequency of abnormal embryo development, low frequency of somatic embryo maturation, low conversion rate of somatic embryos into plants, and a lack of shoot elongation (Kumar and Pental, 1998; Wilkins *et al.,* 2000; Wilkins *et al.,* 2004; Sun *et al.,* 2006).

Gene integration and expression analysis

PCR analysis was carried out in all the T_0 established plants. Out of nine plants (eight and three from *cry*1Ac and *cry*1Acm respectively) established. In case of *cry*1Ac events three plants were amplified with gene specific and *npt*-II specific primers (Fig. 3). However, none of the putative transformant for *cry*1Acm was able to amplifiy neither with gene specific nor with *npt*-II specific primers.

Further, expression of the transgene was analyzed in T_0 established *cry*1Ac transformants through RT-PCR. Specific gene primers for *cry*1Ac were used to generate cDNA through RT-PCR and that cDNA was used as a template for doing further PCR with specific gene primers of *cry*1Ac. The results indicated the expression of the *cry*1Ac gene in both plants which were showed PCR positive (Fig. 4). As it can be made out from the reports in transgene analysis in cotton that with the increase in age (with maturation), the expression of the transgene is drastically reduced. Older leaves of *Bt* cotton do not express the transgene (Wu *et al.,* 2000; Wu *et al.,* 2009). Hence, in present study plants with new sprouts were taken for extraction of mRNA. The *Bt* expression strips designed to recognize the Cry protein in seed and leaf tissue extracts were used to study the expression of Cry protein. According to the instruction of the manufacturers this is known as cross reactivity reaction without causing false positive results. Hence, Cry1Ac sticks were used to detect the expression of the *cry*1Ac carrying cotton transformants. Putative transformats showed positive reaction for Bt-expression strips (Fig. 5), which was not found in control plant. It clearly indicates that positive reaction was due to transformed gene and not a false positive reaction. However, T_1 progeny of both showed consistently negative response for Bt-expression (Table 2).

Characterization of T_1 progenies

Selfed seeds were collected from the events of *cry*1Ac as well as *cry*1Acm and grown on ½ MS medium in a growth chamber. When progenies of these were screened at T_1 generation, none of them were shown as PCR amplification with both gene specific and *npt*-II specific primers (Table 2). From these unexpected results we can conclude that, all the T_1 progenies were found as untransformed either because insufficient seeds were obtained from the T_0 plants or false positive transformed events at T_0 which expressed transiently. Transient expression is a short-term expression of the introduced DNA(s). Directly following the introduction of DNA into the nucleus and start functioning. Transient transformation is not heritable so it cannot be detect into the further generations which was reported in many previous findings (Potrykus *et al.,* 1985; Chyi *et al.,* 1986; Langridge *et al.,* 1992). There may be another possibility that transgenic locus has not

been stably inherited which was find as a major bottleneck in previous studies (Meyer *et al.*, 1992; Hiei *et al.*, 1994).

In conclusion, although we have not get stable transgenic events of cotton, either due to abnormal plants and boll development or instability of the transgene, this study remarks the possible circumstances that constrain the stable transgene integration. Many studies has been devoted and to the standardizing the cotton transformation protocol, still discrepancies are present which need to be rectify. Research should also be focused to investigate the causes for poor boll set using different inoculum densities of *Agrobacterium* used during the course of transformation. Although selection for kanamycin resistant cotton transformants is easy, and highly economic method for screening, there is a lot of discrepancy between the number of kanamycin resistant and PCR positive plants, hence, this gap must be bridged by further investigation.

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