
Detrimental effect of expression of *Bt* endotoxin Cry1Ac on *in vitro* regeneration, *in vivo* growth and development of tobacco and cotton transgenics

PREETI RAWAT^{1,†}, AMARJEET KUMAR SINGH^{1,†}, KRISHNA RAY^{1,†}, BHUPENDRA CHAUDHARY¹,
SANJEEV KUMAR¹, TARU GAUTAM¹, SHAVETA KANORIA¹, GURPREET KAUR¹, PARITOSH KUMAR¹,
DEEPAK PENTAL^{1,2} and PRADEEP KUMAR BURMA^{1,*}

¹Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

²Centre for Genetic Manipulation of Crop Plants, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

*Corresponding author (Fax, +91-11-24112761; Email, pburma@south.du.ac.in)

[†]These authors contributed equally to the work.

High levels of expression of the *cry1Ac* gene from *Bacillus thuringiensis* cannot be routinely achieved in transgenic plants despite modifications made in the gene to improve its expression. This has been attributed to the instability of the transcript in a few reports. In the present study, based on the genetic transformation of cotton and tobacco, we show that the expression of the Cry1Ac endotoxin has detrimental effects on both the *in vitro* and *in vivo* growth and development of transgenic plants. A number of experiments on developing transgenics in cotton with different versions of *cry1Ac* gene showed that the majority of the plants did not express any Cry1Ac protein. Based on Southern blot analysis, it was also observed that a substantial number of lines did not contain the *cry1Ac* gene cassette although they contained the marker gene *nptII*. More significantly, all the lines that showed appreciable levels of expression were found to be phenotypically abnormal. Experiments on transformation of tobacco with different constructs expressing the *cry1Ac* gene showed that *in vitro* regeneration was inhibited by the encoded protein. Further, out of a total of 145 independent events generated with the different *cry1Ac* gene constructs in tobacco, only 21 showed expression of the Cry1Ac protein, confirming observations made in cotton that regenerants that express high levels of the Cry1Ac protein are selected against during regeneration of transformed events. This problem was circumvented by targeting the Cry1Ac protein to the chloroplast, which also significantly improved the expression of the protein.

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1. Introduction

The δ -endotoxin contained in *Bacillus thuringiensis* is highly toxic to many insect species. With the advent of genetic transformation techniques in plants, successful attempts have been made to introduce and express the toxin encoding genes in various crop species. Transgenic cotton

containing the *cry1Ac* gene that confers resistance to *Helicoverpa* sp. was one of the first transformed crops to be commercialized. Currently grown in more than 50 countries, the transformation events that have been used to introgress *cry1Ac* gene into different cotton cultivars and hybrids are limited to six to seven original transgenic events (www.agbios.com). One of the major breakthroughs in the

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development of *cryIAc* transgenics has been the designing of synthetic versions of the gene with codon modification to remove the putative polyadenylation sequences and to bring in the codon usage favourable for high expression in dicotyledonous plants (Perlak et al. 1990). This leads to expression levels that were toxic to the target insect pests. In fact, the expression of the wild-type *cryIAc* gene in plants has been a model to understand the reasons that can limit the expression of a foreign gene in plants (Diehn et al. 1996; Rocher et al. 1998). In a series of studies it was reported that the expression of the full-length (3.5 kb) wild-type gene could not be achieved mainly due to the presence of sequences that could lead to premature transcription termination and consequent transcript instability (Perlak et al. 1990). These difficulties were circumvented by the use of a synthetic truncated version (1.8 kb) of the gene that encoded the toxin portion of the Bt protein (Perlak et al. 1990). In this truncated synthetic gene, the sequences detrimental to the expression of the *cryIAc* gene were removed by synonymous codon changes. However, the first line to be by the same group contained the modified version of the full-length gene rather than a truncated gene and was reported to provide reasonable protection from *Heliothis virescens* (tobacco budworm). The same event has been used to develop *Bt* transgenics resistant to damage by *Helicoverpa armigera*, the major target pest in India, China and Australia.

In several studies it has been observed that the expression of the *cryIAc* gene is not sustained uniformly during the life cycle of the plant. For example, Kranthi et al. (2005) observed that in eight *Bt*-cotton Bollgard hybrids, the CryIAc protein expression was found to be highly variable in different plants, and more importantly, it declined progressively over the life cycle of the plant. The lowest expression was observed in the ovary of flowers and rind of the young green bolls, which are the most favoured sites of bollworm feeding. As a result the crop could be most vulnerable to insect attack at the time of flowering, which in turn could cause huge losses in crop productivity. Thus, it is important to develop transgenics with high, uniform levels of CryIAc toxin during the entire period of the plant growth and development.

In spite of the several modifications reported in literature to improve expression of the *cryIAc* gene in plants, achieving high expression does not seem to be a routine affair. While there has been extensive work on how to improve the expression of the *cryIAc* gene, no attempt seems to have been made to find out why lines with very high levels of expression cannot be routinely achieved or why the kind of expression spread that is observed with other kinds of transgenes is not observed in the case of the *cryIAc* gene. Some passing references (Diehn et al. 1996) have been made to the fact that the transcripts of the *cryIAc* gene could still be unstable, but no direct evidence seems to have been generated.

In this work we present evidence that the problem in achieving high levels of expression is due to detrimental

effects of the CryIAc protein on the processes of *in vitro* plant regeneration and *in vivo* development of the plants. High expression of *cryIAc* gene in a way acts as a negative selection, and regenerated plants with low or no expression of the gene are selected for. Further, expression beyond certain levels causes morphological abnormalities in transgenic plants.

2. Materials and methods

2.1 Constructs

For transformation of cotton three codon-modified, truncated versions of *cryIAc* gene, viz. *cryIAb+Ac*(Perlak), *cryIAc*(UDSC) and *cryIAc*(PG) were used in the present study. The *cryIAb+Ac*(Perlak) gene is as described by Perlak et al. (1990). The *cryIAc*(UDSC) gene was similar in codon usage to the *cryIAc* used by Perlak et al. (1990) and is described in Kumar et al. (2005), while *cryIAc*(PG) represents the modified sequence as reported by Rocher et al. (1998). The first two genes were synthesized in the laboratory while *cryIAc*(PG) gene was synthesized commercially (Bio Basic Inc., Canada). These genes were cloned downstream of various promoters, viz. double enhancer versions of *Cauliflower Mosaic Virus* 35S (CaMV 35Sde; Kay et al. 1987), *Mirabilis Mosaic Virus* (MMVde; Dey and Maiti 1999) and *34S Figwort Mosaic Virus* (FMVde; Maiti et al. 1997). To develop the different constructs as summarized in table 1. To construct the 35Sde promoter, the enhancer region (−90 to +343) was cloned upstream of the 35S promoter available in the laboratory (Bhullar et al. 2007). The 330-bp-long full-length promoter of MMV was synthesized in the laboratory by recursive PCR, and the double enhancer version was developed by amplifying and cloning the −38 to −297 enhancer region upstream of the MMV promoter. The double enhancer version of the FMV promoter was similarly constructed wherein the −249 to −197 enhancer region of the promoter was placed upstream of the 314-bp-long (−249 to +65) full-length promoter. The polyadenylation signal (pA) of 35S was used in all the above expression cassettes.

The *cryIAc* constructs for genetic transformation of tobacco (table 2) carried either *cryIAc*(UDSC) or *cryIAc*(PG) under the transcriptional control of CaMV 35Sde promoter. A construct with β -glucuronidase (*gus*) gene instead of *cryIAc* was used as a control in all the tobacco transformation experiments. Apart from the passenger gene, each construct also carried a neomycin phosphotransferase (*nptII*) expression cassette for selection of transformed plants. In the constructs 35Sde-*cryIAc*TP(UDSC) and 35Sde-*cryIAc*TP(PG), the *cryIAc* gene is translationally fused at its N-termini to the chloroplast-targeting (TP) sequence of a *rbcs 1b* gene isolated from *Gossypium hirsutum*. The primers CF (5'-GATCCATGGCCTCCTCCATGATC

Table 1. Analysis of cotton transgenic lines developed with cry1Ac expression cassette vis-à-vis phenotype of the line and expression of Cry1Ac protein

Expression cassette	Number of lines		
	Developed	Showing normal phenotype	Expressing Cry1Ac protein*
35Sde::cry1Ab+Ac(Perlak)	26	9	1(9)
35Sde::cry1Ac(UDSC)	208	63	17(111)
MMVde::cry1Ac(UDSC)	56	15	1(21)
FMVde::cry1Ac(UDSC)	35	11	1(9)
Total	325	98(30%)	20(150)=13%

*Numbers in parantheses indicate the number of lines analysed.

TCATCGGCA-3') and CR (5'-GATCCATGGCCTGCATGCATTGCACTCTCCCACCG-3') were used to amplify the chloroplast-targeting sequence from cDNA prepared from RNA isolated from the leaf tissue. The amplicon was cloned, sequenced to confirm the fidelity of the amplified product and placed in frame at the 5' end of the *cry1Ac* genes.

The expression cassettes of *cry* genes were cloned in the binary vector pPZP200 (Hajdukiewicz *et al.* 1994) modified in the laboratory (Arumugam *et al.* 2007) to contain a neomycin phosphotransferase (*nptII*) expression cassette as a selection marker cloned between *lox* sites for the eventual removal of the marker genes. In all the binary vectors, the marker gene was towards the left border of the T-DNA while the passenger gene was placed towards the right border.

Binary vectors with different expression cassettes were introduced into *Agrobacterium tumefaciens* strain GV3101 and GV2260 by electroporation for genetic transformation of cotton and tobacco, respectively.

2.2 Production of transgenic plants

The genetic transformation of cotton (*G. hirsutum* L. cv Coker 310FR) was carried out according to the method

described by Chaudhary *et al.* (2003). This method involved the inoculation of cotyledonary explants with *A. tumefaciens* strain GV3101, and the selection of transformed plants using kanamycin. All the transgenics developed were maintained under controlled environmental conditions in the green house [32±2°C, 16/8 h (light/dark) photoperiod, 70% relative humidity]. The primary transgenic (T₀) and their backcross (BC₁) progenies were used for morphological analysis and molecular characterization.

Genetic transformation of tobacco (*Nicotiana tabacum* cv. Xanthi) was carried out using *Agrobacterium* mediated transformation of leaf explants following the protocol described by Svab *et al.* (1995). Transgenic plants obtained were maintained as shoot cultures in controlled environmental conditions [28±2°C, 16/8 h (light/dark) photoperiod, 70% relative humidity] in a culture room.

2.3 Genomic DNA isolation and Southern blot analysis

Genomic DNA from leaves of transgenic cotton plants was extracted using a modified version of the CTAB method described by Rogers and Bendich (1994). In this method 1 gm of leaf tissue was crushed in liquid nitrogen in the presence of 100 mg of PVPP (Sigma Chemical Co., USA) before the extraction. The extraction buffer also contained 1% PVP

Table 2. Constructs used for transformation of tobacco

Name of the construct	Passenger gene	Promoter used
35Sde::cry1Ac(UDSC)	<i>cry1Ac</i> (UDSC)	CaMV double enhancer
35Sde::cry1Ac(PG)	<i>cry1Ac</i> (PG)	CaMV double enhancer
35Sde::gus	<i>β-glucuronidase</i>	CaMV double enhancer
35S::cry1Ac(UDSC)	<i>cry1Ac</i> (UDSC)	CaMV single enhancer
35Sde::cry1AcTP(UDSC)	<i>cry1Ac</i> (UDSC) fused to a transit peptide*	CaMV double enhancer
35Sde::cry1AcTP(PG)	<i>cry1Ac</i> (PG) fused to a transit peptide*	CaMV double enhancer

*In two of the constructs the *cry1Ac* gene was translationally fused at the N-terminal with a transit peptide isolated from an *rbcS* gene of cotton to target the protein to chloroplast.

(Sigma Chemical Co., USA). Approximately 10 µg of DNA isolated from each transgenic was digested with *EcoRI* enzyme. The digested samples were electrophoresed on a 0.8% agarose gel and blotted on nylon membranes (Hybond N+; Amersham). The λ DNA digested with *HindIII* was used as a size marker. Junction sequences toward the left and right border were analysed using appropriate probes. The probe DNA was labelled with α-[³²P]-dCTP using a Megaprime DNA labelling kit (Amersham Pharmacia Biotech, UK). Standard procedures were followed for hybridization and washing. Prior to reprobing, blots were deprobed for 40 min in 0.4 N NaOH at 42°C followed by treatment with a neutralization solution (0.2 M Tris pH 8.0, 0.1× SSC, 0.5% SDS for 40 min at 42°C). The membrane was subjected to autoradiography for 36–48 h at –80°C.

2.4 Estimation of Cry1Ac protein by ELISA

The Cry1Ac protein levels in the transgenic plants were analysed by Enzyme Linked Immunosorbent Assay (ELISA) kit from Envirologix, USA, as per the manufacturer's instructions. Protein extracts were made by grinding approximately 500 mg leaf tissue in 1 mL extraction buffer (provided in the kit) using chilled pestle and mortar on ice. The extracts were centrifuged at 12000 rpm for 15 min at 4°C in 1.5 mL micro-centrifuge tubes. After centrifugation, the supernatant was transferred to a fresh tube and the total protein concentration of the supernatant was estimated by Bradford's method (Bradford 1976). Estimation of Cry1Ac protein by ELISA was generally carried out using 0.5 µg and 1 µg of total protein. Depending on the objective of the experiment in some cases, higher amounts of total protein, viz. 2 µg and 4 µg, were also taken. The amount of Cry1Ac protein was calculated based on Cry1Ac protein standards provided in the kit.

2.5 RNA extraction and RT-PCR

For RT-PCR total RNA was isolated from the leaves of plants using the Spectrum Plant RNA isolation kit from Sigma following the manufacturer's instructions. The RNA isolated from the leaf tissue was treated with DNase (DNA-free™ kit, Applied Biosystems, USA) and cDNA synthesis was carried out from total RNA using MuLV reverse transcriptase and Oligo d(T)₁₆ primers (GeneAmp® RNA PCR, Applied Biosystems, USA). cDNA synthesis was carried out in a reaction volume of 100 µL, the reaction mix containing 1 µg of RNA, 10 µL reaction buffer, 20 µL of 500 µM dNTP, 22 µL of 25 mM MgCl₂, 2 µL of RNase inhibitor, 5 µL of 25 pmol/µl poly d(T) primer and 1 µL (50 U) of reverse transcriptase. This cDNA pool was used to carry out gene-specific amplification using two sets of primers. Primer set C(F) 5'-ATGGACAACAACCCAAA

CATC-3' and C(R) 5'-GGCGCTGTTCATGTCGTTGAA-3' was designed to amplify a part of the *cry1Ac* transcript, while N(F) 5'-ATGGATTGCACGCAGGTTCT-3' and N(R) 5'-TTCGCTTGGTGGTCAATG-3' would amplify the *nptII* part of the transcript. The PCR reaction was carried out using the above primers in a total volume of 25 µL, which contained 5 µL of cDNA, 2.5 µL reaction buffer, 2.5 µL of 500 µM dNTP, 1 µL of 25 pmol/µL forward and reverse primers for each and 0.2 µL *Taq* polymerase (1 U). The initial denaturation at 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. The final elongation step was carried out at 72°C for 7 min. Amplified PCR product were analysed by gel electrophoresis on 1.0% agarose gel.

2.6 Histochemical analysis of gus gene expression

Leaf sections from one month old tobacco transgenics were analysed for GUS activity using 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid (X-Gluc) as the substrate (Jefferson et al. 1987). Transgenics were scored for GUS activity based on the development of blue colour.

3. Results and discussion

3.1 Transformations in cotton: high expression plants have abnormal phenotypes

The major aim of the study in the beginning was to generate transgenics in cotton with high levels of *cry1Ac*-encoded δ-toxin throughout the developmental cycle of the cotton plant. To this end, several plasmid constructs described in material and methods and table 1 were developed containing modified versions of the *cry1Ac* gene [viz., *cry1Ab+Ac* (Perlak) and *cry1Ac*(UDSC)] under the transcriptional control of different viral promoters, viz. double enhancer versions of CaMV 35S, MMV and FMV.

We made three unusual observations (table 1) in our attempts to develop transgenics in cotton with the above constructs. About 70% of the 325 independent T₀ lines showed abnormalities in the phenotype of the regenerated plants. These abnormalities ranged from extreme retardation in the growth of the plant, to no flowering, abscission of the flowers following crossing and no setting of bolls. Abnormal plants were mainly of two types: one, in which the plants showed abnormalities in their vegetative growth of which only a small percentage set seed on selfing or backcrossing, and the other, in which the plants showed normal vegetative growth but did not set seeds either on selfing or on backcrossing.

Of the 147 lines analysed by Southern blot analysis for the presence of the *cry1Ac* and the *nptII* gene, 29% of the lines did not show hybridization signals when probed with the *cry1Ac* gene although they showed the presence of the

nptII gene (figure 1). In order to confirm that this was not due to experimental error, Southern hybridization of many of the samples was repeated and washing of the blots was carried out at low stringency. In almost all the cases, no

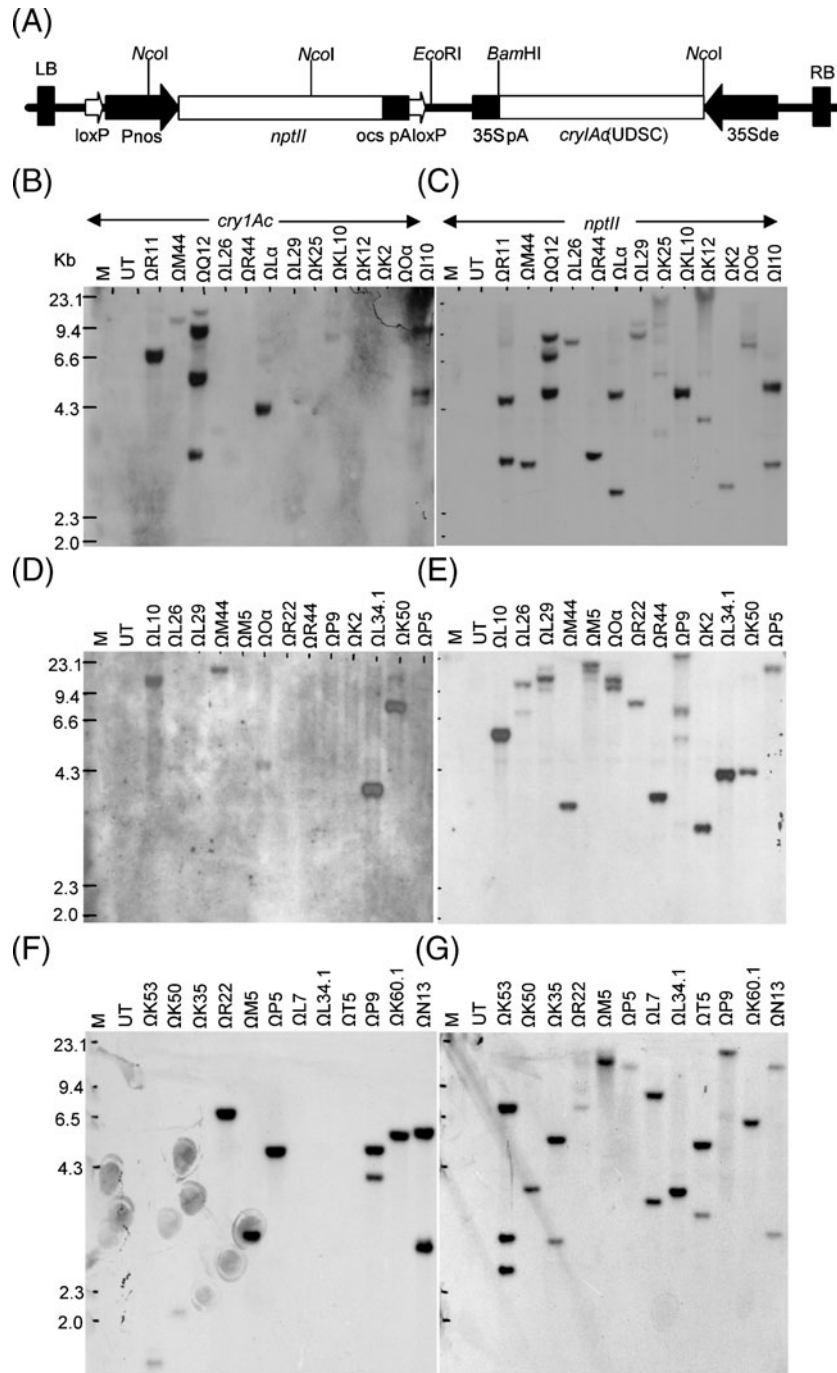


Figure 1. Southern blot analysis of *EcoRI* digested genomic DNA of transgenic events in cotton. These lines were developed with a binary vector carrying the *cry1Ac*(UDSC) gene under 35Sde promoter towards the right border of the T-DNA and a *nptII* selection cassette towards the left border. The *EcoRI* site is present once within the T-DNA and is located between the two expression cassettes (A). The blot was probed with *cry1Ac* gene in (B), (D) and (F) and was re-probed with *nptII* in (C), (E) and (G). No hybridization signal was observed with *cry1Ac* probe in several events, many of which (shown in bold) were repeated a second time. In the case of (B) and (D), the blots were exposed for a longer period of time. λ DNA digested with *HindIII* was used as a size marker. The second lane in all the blots contained *EcoRI*-digested DNA from untransformed cotton Coker310FR line (UT).

hybridization signal was observed with the *cry1Ac* probe, while the presence of the *nptII* gene could be confirmed. This suggested that these lines were not escapes in transformation experiments but specifically lacked the *cry1Ac* cassette. This was an unusual observation as the *cry1Ac* cassette was placed towards the right border of the T-DNA, while the selection marker was placed towards the left border. It is well established that T-DNA transfer

proceeds from the right to the left border, and although incomplete transfers can take place, the placement of the marker gene towards the left border ensures the presence of the passenger gene in most of the cases (Lee and Gelvin 2008). Although rearrangements during T-DNA integration can lead to the loss of the passenger gene placed towards the right border (Rai et al. 2007; Zhang et al. 2008), to the best of our knowledge there has been no earlier work that has

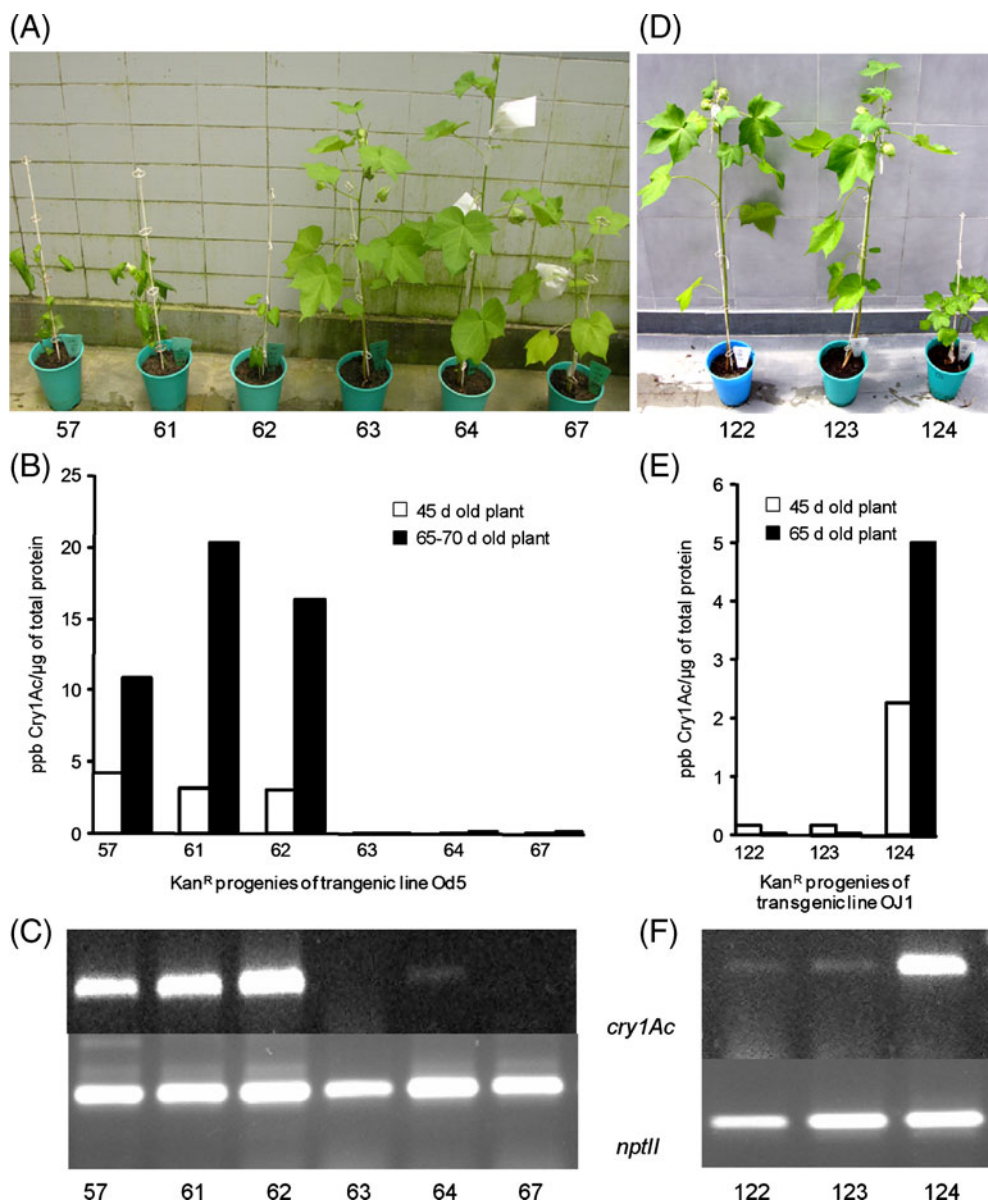


Figure 2. Phenotype of progenies of two cotton transgenic lines D5 (A–C) and ΩJ1 (D–F) and expression analysis of the transgenes in them. Progenies of lines D5 and ΩJ1 were selected on a medium containing 100 mg/L of kanamycin. The kanamycin-resistant (kan^R) progenies were transferred to soil and their phenotype (A, D) vis-à-vis the expression of transgenes were observed. The expression of *cry1Ac* gene was analysed at the levels of protein (B, E) and its transcripts (upper panel in C and F) by ELISA and RT-PCR, respectively. The expression of the *nptII* gene used as a selection marker in the binary vector was analysed by RT-PCR (lower panel in C and F).

reported loss of the passenger gene at such a high frequency. A similar situation was observed in our laboratory when developing transgenics with the *barnase* gene, which encodes for RNase, a cytotoxic product (Jagannath *et al.* 2001).

Independent of the Southern blots, 150 transgenic lines were analysed for the levels of Cry1Ac protein by ELISA. The protein could not be detected in a majority (87%) of the lines (table 1). More importantly, all the lines that showed appreciable levels of expression were found to exhibit phenotypic abnormalities. In some events that showed appreciable levels of expression but exhibited abnormal phenotype, we were able to collect only a few seeds either by selfing or backcrossing. Interestingly, when we observed the progenies of such events (figure 2), there was a strong correlation between the expression level of the Cry1Ac protein and phenotypic abnormalities. For example, in six of the kanamycin-resistant progenies of a multicopy transgenic line (D5), three progenies, viz. 57, 61 and 62, that showed expression of the Cry1Ac protein (figure 2A–C) were observed to be stunted in their growth and did not flower, while the remaining three that did not show any detectable amount of Cry protein showed a normal phenotype. The expression of the *cry1Ac* as well as that of the *nptII* gene was analysed in these progenies by RT-PCR. While expression of *nptII* was observed in all the kanamycin-resistant progenies, transcripts for *cry1Ac* were absent in the three progeny plants with normal phenotype. Another similar example (Ω J1) has been presented in figure 2D–F.

These observations and our continued failure to identify transgenic lines with a normal phenotype with reasonable levels of Cry1Ac protein led us to hypothesize that expression of Cry1Ac protein was detrimental to the regeneration and normal development of the transgenics. In essence, during

in vitro regeneration, lines that did not express the Cry1Ac protein either due to deletion of the transgene (as evidenced by Southern blot analysis; figure 1) or its inactivation were being preferentially selected for. Further, the events that escaped the negative selection imposed by the expression of the *cry1Ac* gene in *in vitro* regeneration showed abnormalities in later plant development.

Although the majority of the papers reporting development of transgenics with *cry* genes have been success stories, one report (Rocher *et al.* 1998) has briefly commented on the detrimental effects of using a codon-modified *cry1Ac* gene for developing transgenics in tobacco. The codon usage pattern in the gene synthesized in this study is, however, different from that reported earlier by Perlak *et al.* (1990).

Based on our observations, we felt that the expression of *cry1Ac* gene is detrimental to regeneration and development of cotton. As designing *in vitro* regeneration experiments in cotton is cumbersome due to the long period taken for developing transgenics (approximately nine months) and the overall low frequency at which transgenics are recovered, we carried out transformation experiments in tobacco with different constructs to critically test the observations made with cotton.

3.2 Transformations in tobacco: high levels of Cry1Ac proteins is detrimental to *in vitro* regeneration

A set of constructs as summarised in table 2 and essentially similar to that represented in figure 1A was developed for transformation of tobacco as described in materials and methods. The constructs used two different versions of the *cry1Ac* gene, one developed in the present study *cry1Ac* (UDSC) as described above and the second *cry1Ac*(PG) as

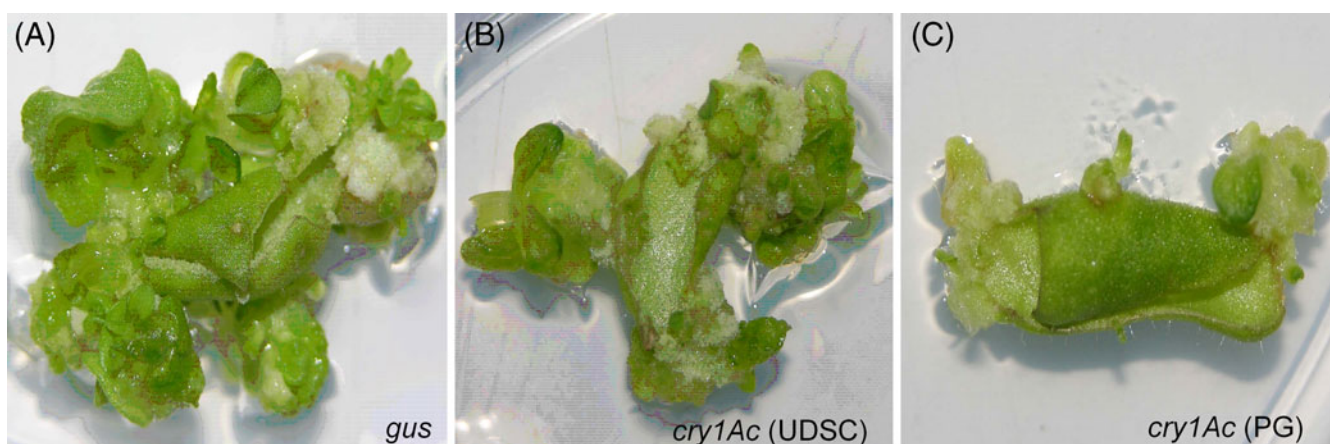


Figure 3. Regeneration of tobacco leaf explants. The panel represents the extent and nature of regeneration ~20 days after transformation with constructs containing (A) *gus*, (B) *cry1Ac*(UDSC) and (C) *cry1Ac* (PG) genes.

described by Rocher *et al.* (1998). The two genes differ in their codon usage patterns but code for proteins with similar amino acid sequences. The *cry1Ac*(PG) gene has a higher (80%) percentage of optimal codon in comparison to that in the *cry1Ac*(UDSC) gene (56%). The optimal codons were identified on the basis of the relative codon usage patterns of genes that are highly expressed in dicotyledonous plants (Duret and Mouchiroud 1999). These genes were cloned downstream of the 35Sde promoter. In one of the constructs a single enhancer version of the 35S promoter regulates the *cry1Ac*(UDSC) gene. A construct with β -glucuronidase (*gus*) under the control of a 35S double enhancer was used as a control in the transformation experiments.

The first set of transformations were carried out with the constructs 35Sde::*cry1Ac*(UDSC) or 35Sde::*cry1Ac*(PG). The construct 35Sde::*gus* was used as a control. In order to rule out experimental variations, transformations with constructs to be compared were carried out at the same time in each experiment. Three to four independent experiments were carried out with each of the construct for a comparison between 35Sde::*cry1Ac*(UDSC) and 35Sde::*gus* as well as between 35Sde::*cry1Ac*(PG) and 35Sde::*gus*. It was observed that the presence of the *cry1Ac* gene led to suppression in regeneration in terms of the extent (figure 3) as well as the number of explants regenerating (figure 4) when observed after about 15 days of *in vitro* growth. The

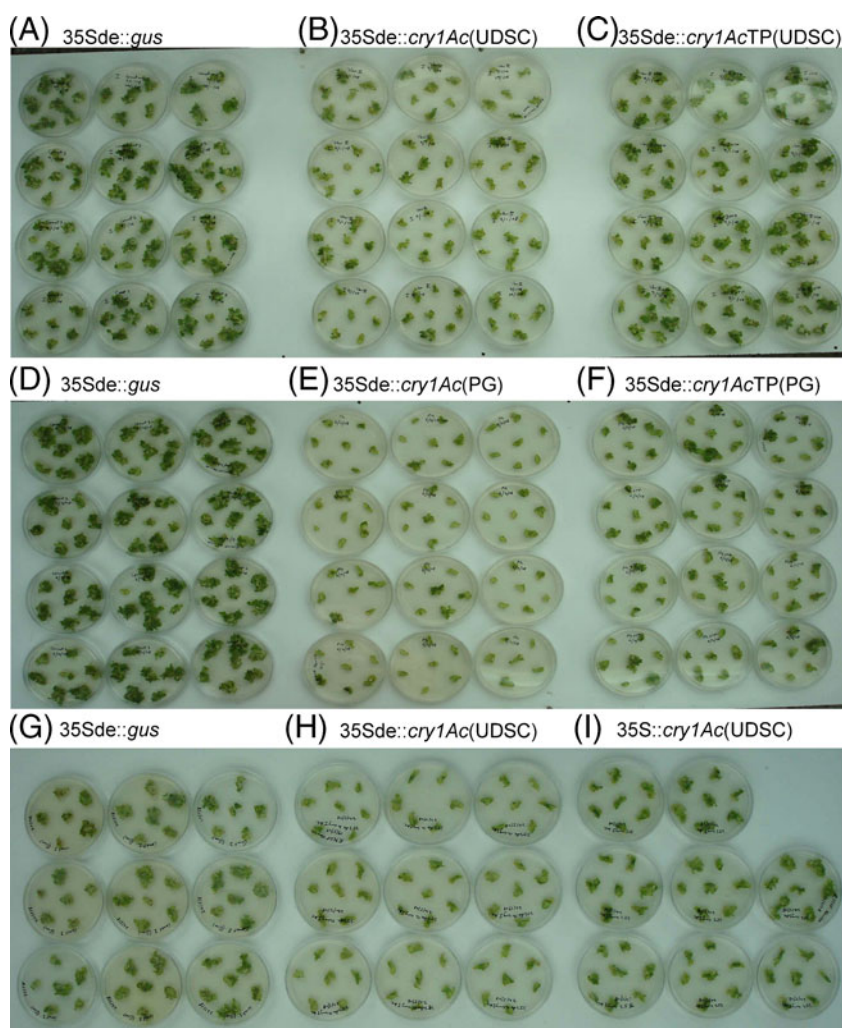


Figure 4. State of regeneration of tobacco leaf explants after ~15 days of transformation on medium containing 100 mg/L of kanamycin. Transformation experiments represented in each panel have been carried out simultaneously. In one set of experiments (A–C), the effect on regeneration following transformation with *cry1Ac*(UDSC) gene with (B) and without (C) the transit peptide sequence has been analysed, while in the second experiment (D–F), the same has been observed for the *cry1Ac*(PG) gene. In a third experiment (G–I), a comparison of the influence of *cry1Ac* gene under a strong (H, 35Sde) and a comparatively weaker (I, 35S) promoter has been analysed. In each of the experimental sets a binary vector carrying the 35Sde::*gus* expression cassette was used as a control (A, D and G).

overall suppression of regeneration when genetic transformation was carried out with constructs containing the *cry1Ac* gene vis-à-vis *gus* gene construct is shown in figure 4 and summarized in table 3. Figure 3A represents the kind of regeneration that was observed in transformants with *gus* gene, while figure 3B–C represent the extent of regeneration with *cry1Ac*(UDSC) and *cry1Ac*(PG) genes, respectively. The extent of regeneration seemed to pick up after the initial phase of block and no significant difference in terms of regenerating explants was observed after about 30 days due to adventitious regeneration from the transgenic tissues. Further, the number of shoots that were obtained and those that formed roots in the presence of kanamycin was observed to be similar to transformations with *cry* gene or the *gus* gene (control).

A total of 80 independent transgenic events (only one shoot from each initial explant) in the case of transgenics developed with the 35Sde::*cry1Ac*(UDSC) construct and 65 events in the case of the 35Sde::*cry1Ac*(PG) construct were analysed for the presence of the Cry1Ac protein. Similarly, 139 lines developed with the 35Sde::*gus* construct were histochemically analysed for the expression of the *gus* gene (table 3). We observed that the number of independent transformants showing detectable levels of Cry protein was significantly small (only ~15%) as compared with those showing GUS activity (~93%). Further, the level of Cry1Ac protein detected in transgenic lines developed with the 35Sde::*cry1Ac*(PG) construct was significantly lower than that observed in lines transformed with the construct 35Sde::*cry1Ac*(UDSC) (figure 5A–B), in spite of the former gene being better codon-optimized than the latter. This in all probability reflects the relationship between expression levels and the detrimental effects of the *cry* genes.

In order to further substantiate the above observations, we compared the effect of transforming tobacco

with the *cry1Ac*(UDSC) gene when expressed under the control of promoters with varying strengths, viz. 35S and 35Sde. The 35Sde promoter is known to lead to ~10-fold higher levels of expression as compared with the 35S promoter (Kay *et al.* 1987). Two independent transformation experiments were carried out with the 35Sde::*cry1Ac*(UDSC), 35S::*cry1Ac*(UDSC) and 35Sde::*gus* constructs. As shown in figure 4H–I, explants showing regeneration are substantially higher in transformation experiments with the 35S::*cry1Ac*(UDSC) construct as compared with those with the 35Sde::*cry1Ac*(UDSC). This was also reflected in the number of transgenics recovered which show the expression of the Cry proteins (figure 6). It was also observed that although the 35Sde promoter is a stronger promoter than the 35S promoter, the level of expression achieved by the latter promoter is higher than that by the former. This again indicates that the regenerants that express high levels of the Cry1Ac protein are selected against.

Most of the tobacco regenerants showed less than 4 ppb Cry1Ac/μg of total protein and were phenotypically normal. However, one of the events (1.5) developed with the 35Sde::*cry1Ac* (UDSC) construct, which showed ~9.01 ppb Cry1Ac/μg of total protein, exhibited an extremely abnormal phenotype. We conclude that the expression of the *cry1Ac* gene is detrimental to *in vitro* regeneration and, beyond a threshold level, detrimental to the *in vivo* growth and development of the transgenic plants.

3.3 Targeting Cry1Ac protein to chloroplast improves regeneration in tobacco

In reports where the expression of a foreign protein in the cytosol leads to phenotypic and developmental abnormalities, targeting of the protein to chloroplast has been found to

Table 3. Summary of results following transformation of tobacco with different constructs

Construct	No. of experiments	No. of explants transformed	Percent (%) explants showing regeneration after		Percent explants (%) from which shoots were obtained	Percent (%) shoots which rooted on Kanamycin	No. of plants showing expression/total plants analysed
			~15 days	30 days			
35Sde:: <i>gus</i>	3	243	73.7	99.6	76.1	94.0	129/139
35Sde:: <i>cry1Ac</i> (UDSC)		249	26.9	92.1	74.1	88.3	12/80
35Sde:: <i>cry1AcTP</i> (UDSC)		250	49.2	96.4	79.3	92.8	22/80
35Sde:: <i>gus</i>	3	208	98.6	99.6	70.8*	86.3*	NA
35Sde:: <i>cry1Ac</i> (PG)		209	21.1	56.3	60.9	90.4	9/65
35Sde:: <i>cry1AcTP</i> (PG)		210	41.9	83.3	81.4	86.0	28/70

*In these cases the number of explants transferred for shoot regeneration and root formation was 72.

NA = not analysed.

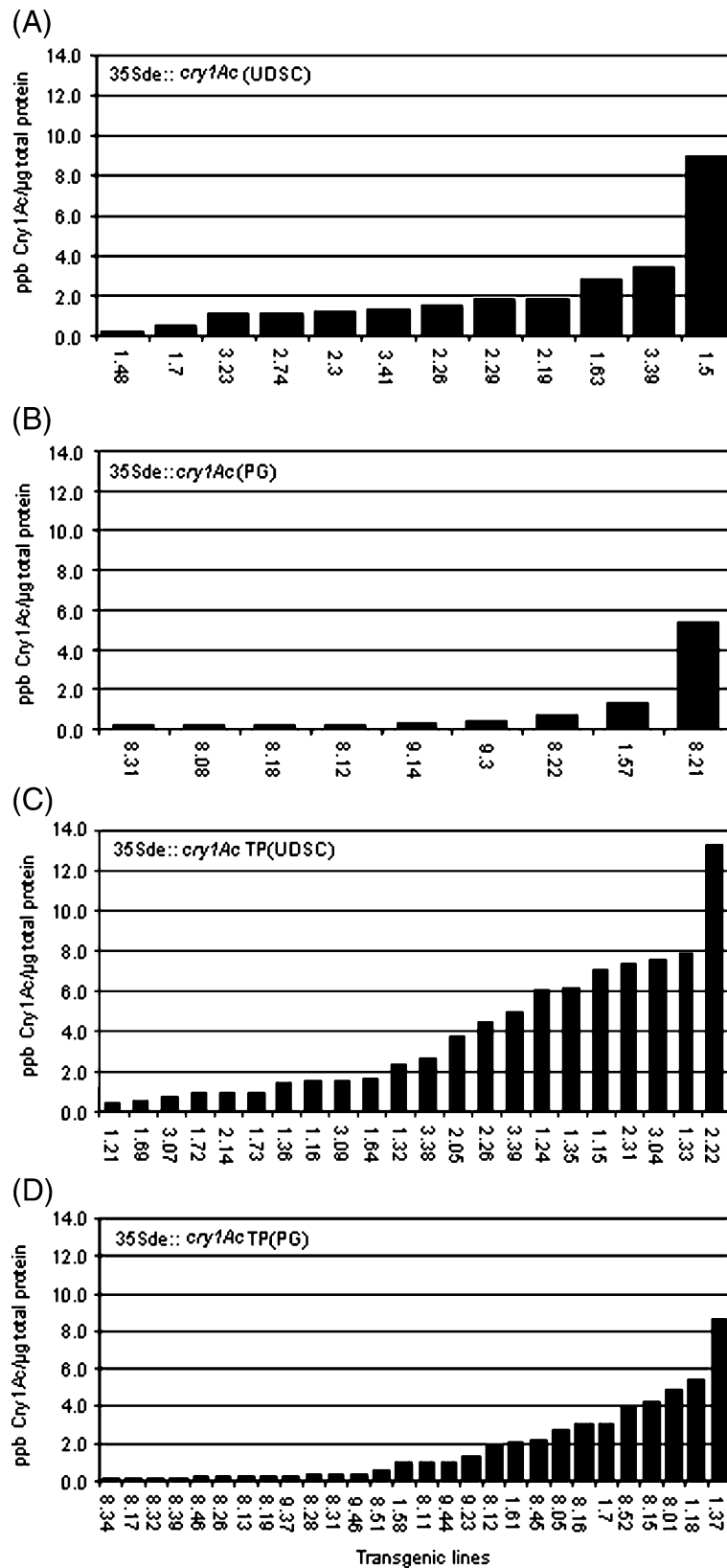


Figure 5. Levels of Cry1Ac protein in T_0 transgenics of tobacco. Distribution of the expression profile in lines transformed with constructs (A) 35Sde::cry1Ac(UDSC), (B) 35Sde::cry1Ac(PG), (C) 35Sde::cry1AcTP(UDSC) and (D) 35Sde::cry1AcTP(PG), where Cry1Ac protein could be detected by ELISA, has been presented.

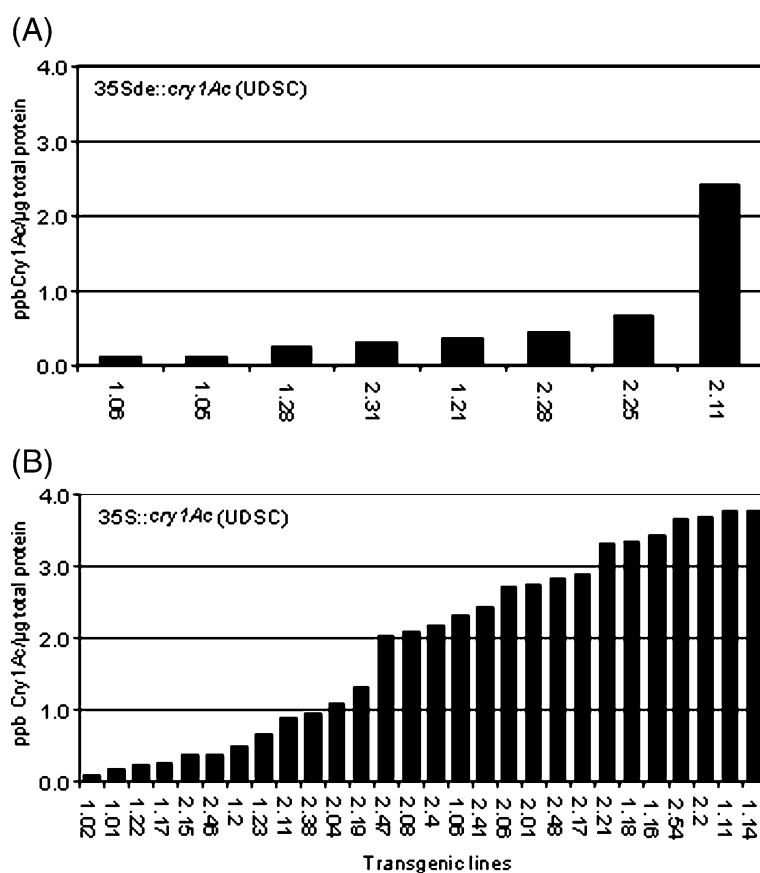


Figure 6. Levels of Cry1Ac protein in T_0 transgenics of tobacco. Distribution of the expression profile in lines transformed with constructs (A) 35Sde::cry1Ac(UDSC) and (B) 35S::cry1Ac(UDSC) where Cry1Ac protein could be detected by ELISA has been presented.

circumvent the problem (Corbin *et al.* 2001; Dai *et al.* 2005; Karim *et al.* 2007). We transformed tobacco with the constructs 35Sde::cry1AcTP(UDSC) and 35Sde::cry1AcTP(PG) (table 2). In these constructs, the cry1Ac gene has been translationally fused at its N-termini to a 63-amino-acid-long chloroplast-targeting peptide (TP) sequence of a *rbcS* gene isolated from *G. hirsutum* in our laboratory. The peptide (supplementary figure 1) shows approximately 80% sequence identity to the TP of *ats1A* SSU protein of *Arabidopsis thaliana*, reported to target heterologous proteins to the chloroplast (Wong *et al.* 1992; Corbin *et al.* 2001; Karim *et al.* 2007).

As represented in figures 4C and F, the inclusion of a TP upstream of the Cry1Ac protein led to an overall improvement in the regeneration of transformed tobacco. A significant increase in the number of explants showing regeneration was observed. This improvement was also reflected in the number of transformants expressing the cry1Ac gene (table 3) as well as in their levels of expression (figure 5C and D). A large number of transgenic events

showed Cry1Ac protein levels higher than 4 ppb/μg of total protein with the 35Sde::cry1AcTP(UDSC) construct and were phenotypically normal. However, one of the events '2.22', developed with 35Sde::cry1AcTP(UDSC) construct showing Cry1Ac levels at 13.27 ppb/μg of total protein, had delayed flowering.

3.4 Phenotypic abnormalities are due to expression of the cry1Ac gene

Our observations in cotton and tobacco demonstrate that accumulation of high levels of Cry1Ac protein is detrimental to plant regeneration and development. Such observations have been probably overlooked earlier as most of the studies were focused on identifying a useful line with 'reasonable' levels of expression for field application. However, there have been two earlier reports (Rocher *et al.* 1998; Barton *et al.* 1987) that mention phenotypic and developmental abnormalities associated with transgenics developed with the cry1Ac gene.

Barton *et al.* (1987) observed that the expression of the intact δ -endotoxin was lethal to tobacco tissue 'since transformants that contained the toxic protein soon died and no regenerated healthy transformant contained the toxin'. In the second report (Rocher *et al.* 1998) the toxic effects of a highly expressed active *Bt* toxin in regenerating tobacco cells has been recorded. In order to understand the cause for poor expression of the wild-type *cry1Ac* gene in plants, the authors developed a codon-modified synthetic *cry1Ac* gene. They introduced a codon bias typical for maize genes. As observed in our analysis, this codon bias also leads to an improvement in codon usage in dicotyledonous plants. The authors failed in their attempts to generate transgenics in tobacco with a construct expressing this codon-modified gene. That the expression of the protein was detrimental to regeneration was demonstrated by carrying out transformations with a *cry1Ac* gene with a frame-shift mutation by insertion of a C base in the original synthetic gene that resulted into the synthesis of a truncated non-functional protein. The synthetic gene reported by Perlak *et al.* (1990), and the one used in the present study, is less optimized for its codon usage as compared with that of Rocher *et al.* (1998), and therefore is expected to provide lower levels of the Cry1Ac protein. However, as observed in this study, such levels also lead to detrimental effects in terms of the extent of regeneration.

That such detrimental effects have been overlooked by groups developing commercially viable transgenic lines is also reflected in Chakrabarti *et al.* (2006) although they have used a different *cry* gene. In this work, describing the development of transgenic tobacco expressing the *cry9Aa2* gene in plastids, the authors observed a delay in plant development. These authors also point out that several groups who have earlier reported the development of plastid-expressed Bt proteins have not recorded any adverse affects of the Cry proteins.

In another study (Sachs *et al.* 1998) involving the inheritance of the *cry1Ac* gene in MON 249 event in cotton, it has been hypothesized that the reduced fitness of some of the transgenic lines may be a result of direct insertion effects leading to the down-regulation of one or more native genes or the result of a linked somaclonal variation. Keeping in view the polyploid nature of the *G. hirsutum* genome, it is hard to envisage that the phenotypic and developmental abnormalities observed in the present study are due to insertional inactivation of the host genes. In case these are somaclonal variations, one should be able to eliminate all but very tightly linked genetic or epigenetic mutations. In the present work on cotton, the question of somaclonal variation can be ruled out as the backcrossed progenies of the transgenics segregated for normal versus abnormal phenotype that was linked to the levels of the Cry1Ac protein in these progenies.

In spite of these detrimental effects, one is still able to recover insect resistant plants, but these may not be the best possible lines. This is reflected in studies on the released transgenics of cotton that do not have sustained levels of *cry1Ac* expression (Kranthi *et al.* 2005). It has also been reported that *H. armigera*, the major target pest in India, China and Australia is 10-fold more tolerant to the Cry1Ac protein compared with the tobacco budworm (Kranthi *et al.* 2005). As a result the crop could be most vulnerable to insect attack at the time of flowering, which in turn could cause huge losses in crop productivity. Thus, higher levels of Cry1Ac expression are needed to control the insect pest. Furthermore, sustained high levels of expression throughout the life cycle of the plant are essential to delay the evolution of resistance to *Bt* toxins in the insect population.

These earlier reports strengthen our observations that expression of the *cry1Ac* gene is detrimental to plant growth and development. To the best of our knowledge, such extensive analysis recording the negative effects of the *Bt* toxin on plant regeneration and development has not been reported earlier. Although the reasons for this detrimental affect need to be analysed, the study shows that in the current scenario for developing transgenics that express optimal levels of *cry* gene is not routine, due to the detrimental effects of high expression of these genes.

Targeting the protein to the chloroplast alleviates the problem to a certain extent. Improvements in Cry1Ac expression by targeting the protein to the chloroplast in transgenic tobacco and rice has been reported earlier (Wong *et al.* 1992; Kim *et al.* 2009). It has been speculated that this improvement in expression is due to stability of the corresponding mRNAs and the subsequent translation efficiency (Kim *et al.* 2009). However, our observations show that the purported improvement in expression is probably because of the improvement in the ability of transformants with higher levels of Cry1Ac protein to regenerate when the protein is targeted to the chloroplast rather than improvements in mRNA stability and translation. In this context it is interesting to note that the second-generation insect-resistant cotton developed by Monsanto (Bollgard II®) has a chloroplast-targeted Cry2Ab protein. Although targeting the Cry1Ac protein to chloroplast circumvents the problem to a certain extent, strategies need to be designed to realise the full potential of the toxicity of such genes without cost to the fitness of such transgenic lines. Further, if a sustained high level of *cry1Ac* expression is achieved during the entire phase of growth and development of a plant's life cycle, the problems of development of insects resistant to *Bt* toxin can be circumvented much better than in the present situation in which prophylactic insecticidal sprays are recommended to avoid building up of resistance in target *Helicoverpa* species. Lastly, the regeneration system of

tobacco can be used to check the detrimental effects, if any, of other *cry* genes.

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