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Transformation and Inheritance of Bt Genes in *Gossypium hirsutum*

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Transgenic plants offer many unique opportunities for managing pest populations. However, the inheritance, integration, and expression of multiple transgenes are prerequisite for maintaining sustainable resistance against insects in crops. We took a gene-pyramiding approach to produce Bt cotton expressing two Bt genes, *cry1Ac* and *cry2A*. Using sonication-assisted *Agrobacterium*-mediated transformation (SAAT), we achieved an efficiency of 6.26%. Putative transgenic plants were confirmed via PCR, Southern hybridization, and western-blotting. Those showing mortality of 75 to 100% for the second instar of *Heliothis armigera* (compared with 0% for the control) were considered Bt-positive. Transgenes were segregated according to a 3:1 Mendelian inheritance pattern in the T1 generation for *Heliothis* resistance. In our insect bioassay, the control plants showed >95% leaf damage, and insects reached the 4th instar stage of larval growth. In contrast, leaf damage on transgenic plants was limited to only a few bites, and insect mortality was 75 to 100%. ELISA confirmed transgene expression, and Bt protein was detected in leaf tissue. This performance was consistent with that of the parent transgenics. PCR and Southern blots verified integration of the *cry1Ac* and *cry2A* genes into the progeny. Therefore, this strategy provides a pathway toward cotton improvement and the development of durable resistance against insect damage.

Keywords: cotton, dual Bt genes, genetic transformation, inheritance, sustainable resistance

Cotton, the silver fiber of Pakistan, is also grown as a source of food and feed. The cotton textile industry provides employment to 38% of the total workforce in the manufacturing sector, accounting for 10.5% of the value added in agriculture and about 2.4% to GDP (Economic Survey of Pakistan, 2004-2005).

Adverse growing conditions and pests can challenge field crops, leading to major losses in yield. Although chemical pesticides are effective, their continuous use is a major cause of resistance and environmental concern (Graves et al., 1999). To achieve target potential yields, growers must adopt modern agricultural practices. Traditional plant breeding techniques have been applied to introduce desirable agronomic traits, e.g., high yield, good quality, and disease resistance, into new breeding lines that can be released after several years of field-testing. Likewise, in less than two decades, the development of technologies that allow the insertion and functional expression of foreign genes in plant cells has been utilized for the production of transgenic plants (Danny et al., 1992).

Bt cotton is an increasingly important tool for farmers around the world. Its main challenge has been from the potential evolution of resistance by insects. Nevertheless, control failures have not yet occurred in any of the current Bt crops (Horner and Dively, 2003). This cotton contains the *cry1Ac* gene, which provides a fairly high degree of resistance to major pests (Stewart et al., 2001). However, reducing the risk of resistance remains a top priority (Tabashnik, 1994). One possible way to delay resistance is by deploying plants that express a mixture of different toxins (Zhao et al., 2003). This strategy can strengthen the adoption of multiple toxins in transgenic crops (Maqbool et al., 2001; Chitkowski et al., 2003; Wu et al., 2003).

Agrobacterium, the model system for cotton transformation (Firoozabady et al., 1987), can also be combined with particle bombardment (Finer and McMullen, 1990; Gould et al., 1991; Zapata et al., 1999; Rajasekaran et al., 2000). Although meristem-based methods have been used successfully in *Agrobacterium*-mediated transformation of cotton (Gould and Magallanes-Cedeno, 1998), efficiencies have been very low and the resultant plants are often chimeric. As described for this current study, we adopted a potentially more efficient method -- Sonication-assisted Agrobacterium-mediated transformation (SAAT) – that involves applying ultrasound to plant tissue for brief periods in the presence of *Agrobacterium*. This produces micro-wounds in the embryonic tissue, allowing the bacteria to penetrate the cells (Trick and Finer, 1998; da-Silva and Fukai, 2002). With such a technical advancement, it has been possible to introduce two insect-resistance Bt genes, *cry1Ac* and *cry2A*, simultaneously into a local variety of cotton. Here, we also used progeny plants to examine the inherent control of *Heliothis armigera* in transgenics.

MATERIALS AND METHODS

Transformation and Plant Regeneration

Vector *pROB5 +cry2A* was digested with *Pvu*II to isolate the *cry2A* gene. The plant expression cassette *pKHG4* was digested with *Xba*I for blunt-end ligation while *cry2A*, under the *CaMV35S* promoter, was digested with *Hind*III. An insert taken from *pIA2* containing the *cry1Ac* gene was also digested with HindIII and cloned in *pKHG4*. This new vector, *pK2Ac*, comprised the *cry1Ac + cry2A* genes driven by the *CaMV35S* promoter as well as the *npt*II (neomycin phosphotransferase) gene (also driven by the *CaMV35S* pro-

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moter), which confers resistance to kanamycin. After *pK2Ac* was transformed into *Agrobacterium tumefaciens* strain LBA 4404, the culture was grown from a glycerol stock for 24 to 48 h at 28°C to attain an A_{600} of 0.5 to 1.0. This bacterial culture was then centrifuged at 3000 xg for 15 min and the pellet was re-suspended in 10 mL of an MS broth (Murashige and Skoog, 1962). Seeds of CIM 482, a local variety of cotton (*Gossypium hirsutum*), were provided by the Central Cotton Research Institute, Multan, Pakistan. A total of 500 mature embryos were isolated (Majeed et al., 2000), then sonicated in a culture tube containing 10 mL of an MS broth. The number of pulses on the Sonicator was controlled by an electronic timer. Afterward, the embryos were immediately co-cultivated very slowly on a rotary shaker for 1 h with an *Agrobacterium* inoculum suspension. The embryos were then cultured for 72 h on an MS + kinetin (1 mg mL^{-1}) medium. Plantlets that developed during this co-cultivation were shifted to a selection medium of MS + kanamycin (100 µg mL^{-1}) and cefotaxime (250 µg mL^{-1}). Non-transgenic plants were maintained on simple MS media as our control. All plants were sub-cultured to fresh selection media at 10-day intervals for 6 to 8 weeks.

Well-developed shoots grown on the selection medium were sub-cultured to a selection-free medium for 4 to 6 weeks. Non-transgenic plants were also carried along as the control. All cultures were maintained at 27°C± 2°C, under a 16-h photoperiod (100 to 120 µmm^{-2} s^{-1}). Finally, putative transgenic plants with well-developed roots were shifted to soil (Rashid et al., 2004). In all, 8,000 mature embryos were used for these transformation experiments.

Based on their performance in molecular analyses and insect bioassays, the first generation of four transgenic parental lines (T8, T11, T19, and T20) were chosen for study in the next generation. A total of 65 seedlings were grown in earthen pots in the greenhouse, where standard agronomic practices were applied.

Molecular Analyses

Cotton genomic DNA was isolated as described by Saha et al. (1997), with some modifications. To amplify *cry1Ac* (0.5 kb) and *cry2A* (0.6 kb), we performed PCR with the following primer pairs:

F, 5′ACAGAAGACCCTTCAATATC3′; R, 5′GTTACCGAGTGAAGTGTTAA3′; and F, 5′AGATTACCCCAGTTCCAGAT3′; R, 5′GTTCCCGA- AGGACTTTCTAT3′, respectively.

Standard Southern blots (Southern, 1975) were conducted with 20 mg of genomic DNA digested with the *Hind*III enzyme. Plasmid *pK2Ac* DNA digested with *Hind*III was run as the positive control while genomic DNA from non-transgenic plants served as our negative control. Two transgenic plants from each parent progeny were selected for PCR and Southern-blotting to confirm the integration and inheritance of Bt genes. Western blots also were done to observe Bt gene expression. Protein extracted from both transgenic and non-transgenic plants was separated by SDS-PAGE and blotted for 1 h onto a nitrocellulose membrane (Hybond-C Amersham), using a Trans-Blot Semi-Dry (Bio-Rad) method. After incubation with primary and secondary antibodies (Enzyme *production* lab CEMB), color was detected with a Sigma Fast NBT/BCIP tablet. Purified Cy1Ac protein was run as the positive control. ELISA was used to screen transgenic progeny for expression of the Cry1Ac and Cry2A proteins; plants were randomly selected according to their growth. Total leaf soluble protein was extracted (Kozeil et al., 1993) and quantified by the assay of Bradford (1976). Briefly, 50 mg of protein from each sample was loaded on a 96-well Corning (#25860) ELISA plate, and the final volume was adjusted to 200 mL with 0.1M carbonate buffer (pH 9.6) before processing according to standard procedures (Crowther, 1995). The intensity of yellow color was recorded with a Bioassay reader (Perkin Elmer, 7000). Bt protein was also determined by a graph of standard BSA curves. Cry protein was expressed as micrograms per gram of total leaf soluble protein. Concentration was calculated as:

$$\mu g \text{ of Bt } g^{-1} \text{ of tissue} = \frac{\text{Conc. on graph x } (1000 \div \text{X mg of tissue taken}) \text{ x dilution factor}}{1000}$$

Insect Bioassay and Segregation of Transgenes

We conducted an insect bioassay to observe the entomocidal activity of *Heliothis armigera* on cotton. Leaves from transgenic and non-transgenic plants were handpicked, then washed with distilled water and kept on moist filter paper in Petri plates. One larva of the second instar was transferred to each plate within 1 h of leaf excision. The plates were held at 27°C±2°C under a 14-h photoperiod, and insect mortality was checked daily for 7 d. Progeny of these transgenic plants were similarly analyzed at both the leaf-level and for the whole plant. The latter was achieved by covering the sides of one-month-old plants (in pots) with polythene sheets and placing muslin cloth on top for ventilation. Each plant was exposed to 15 larvae of the second instar, and percent leaf-area damage was recorded. Transgenic plants showing ≥75% larval mortality were analyzed for transgene segregation; a Chi-square test (χ^2) was performed to observe the inheritance pattern.

RESULTS AND DISCUSSION

Transformation Efficiency

After co-cultivation, the mortality of cotton embryos on culture media was correlated with the number of sonication pulses applied. For example, with three pulses, the survival rate was 42% versus 80% for those embryos (controls) that were neither sonicated nor co-cultivated (Fig. 1). Those that were not sonicated, but only co-cultivated with the bacteria, had a survival rate of 52%, a value that possibly included false positives. Therefore, we selected a pulse rate of 5 for further studies to minimize those false positives and to obtain a sufficient number of transgenic plants. SAAT increases the transient efficiency of transformation in different plant tissues; e.g., GUS expression is highest when immature cotyledons are sonicated for 2 s during *Agrobacterium* co-cultivation (Trick and Finer, 1997, 1998; da Silva and Fukai, 2002; Solís et al., 2003; Jiang et al., 2004). Here, transformation efficiency after 8 weeks was 6.26%, a value that was based on the success of plant formation on a selection

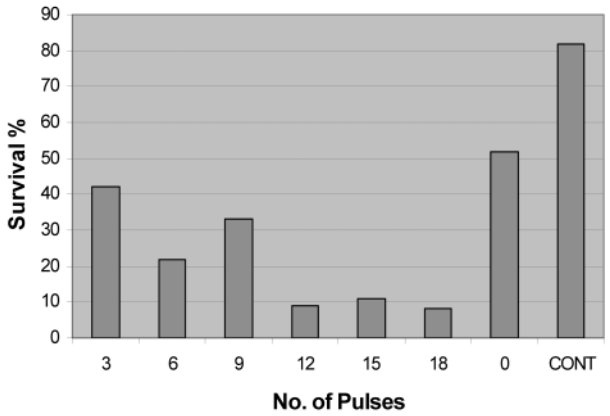

Figure 1. Effect of sonication on mature cotton embryos. **Cont.** Embryos neither sonicated nor cocultivated growing on MS medium

Figure 4. Southern blot of trasgenic cotton plants To showing integration of *cry1Ac* gene. Lane +: pK2Ac plasmid digested with *Hind*III, Lanes 1-4: Transgenic plants (8, 11, 19, 20), Lane 5: Non-transgenic/control plant

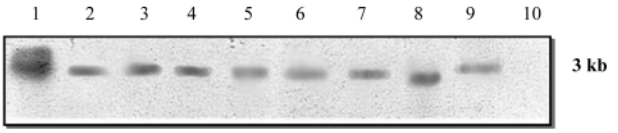

Figure 5. Southern blot of T1 transgenic cotton plants showing integration of *cry1Ac* gene. Lane 1: Plasmid *pK2Ac* digested with *Hind*III, Lanes 2-9: T1 progeny plants (8-5, 8-10, 11-20, 11-34, 19-39, 19-44,20-61, 20-65), Lane 10: Non transgenic/control plant

medium containing antibiotic kanamycin and cefotaxime.

Molecular Analyses

Although multiple gene transfer has been reported (Salm et al., 1994; Cheng et al., 1998; Maqbool et al., 2001; Zhao et al., 2001; Datta et al., 2002), successful transformation depends upon the integration and expression of the target gene into the plant genome as well as on its inheritance to the progeny. Here, we amplified a 0.5-kb fragment of the *cry1Ac* gene in transgenic plants 8, 11, 19, and 20 (Fig. 2A). Detection of a 0.6-kb fragment also confirmed the presence of *cry2A* (Fig. 2B). Genomic DNA from non-transgenic plants (negative control) showed no such amplification. PCR-amplification demonstrated that *cry1Ac* and *cry2A* also were inserted into progeny plants 8-5, 8-10, 11-20, 11-34, 19-39, 19-44, 20-61, and 20-65 (Fig. 3A, B). Southern hybridization further verified integration of the transgene into the genome, with plants 8, 11, 19, and 20 being positive for the *cry1Ac* probe (Fig. 4). No signal was detected in our negative, i.e., non-transgenic, control plants. If the T0 transgenic plants are chimeric, one would expect to find a pattern of transformation in the T1 generation; for Southern blots, it is best to use the T1 or T2 generation (Gould and Magallanes-Cedeno, 1998). Here, the Southern blots of progeny from the same parental plants released a 3-kb *cry1Ac* cassette (Fig. 5); no signal was present in the controls. The same result was reported by Gould and Magallanes-Cedeno (1998), who documented the use of individual progeny (R1, R2, etc.) for Southern-blotting. Transgenic progeny are not chimeric, but reflect the transformation event that was present in the germ line. Our PCR and Southern blots confirmed stable inheritance and integration of the incorporated Bt genes into the progeny plants similar to that found

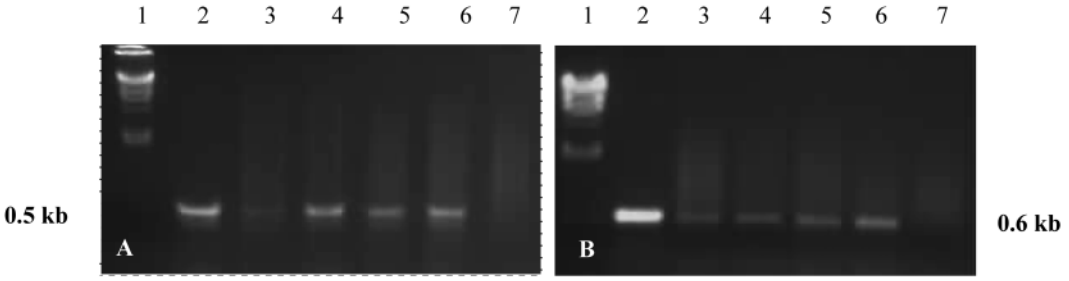

Figure 2. PCR amplification of Bt genes in transgenic cotton To plants. **A.** Amplification of *cry1Ac* gene, **B:** Amplification of *cry2A* gene Lane 1: λ marker, Lane 2: *pK2Ac* plasmid, Lanes 3-6: Transgenic plants (8, 11, 19, 20), Lane 7: Non-transgenic/control plant

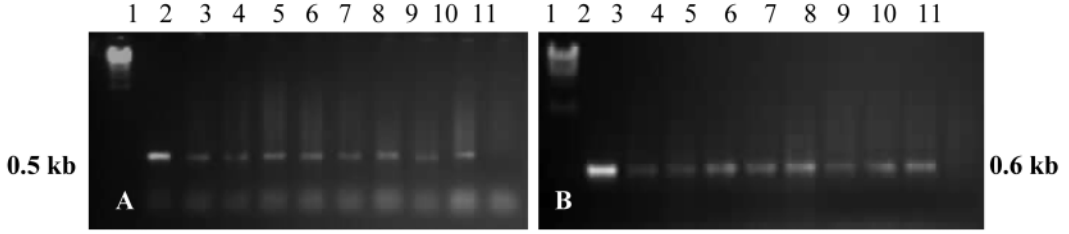

Figure 3. PCR amplification of Bt genes in T1 progeny plants. **A.** Amplification of *cry1Ac* gene **B.** Amplification of *cry2A* gene, Lane 1: λ marker, Lane 2: Plasmid *pK2Ac*, Lanes 3-10: Transgenic plants (8-5, 8-10, 11-20, 11-34, 19-39, 19-44, 20-61, 20-65), Lane 11: Non-transgenic/control plant

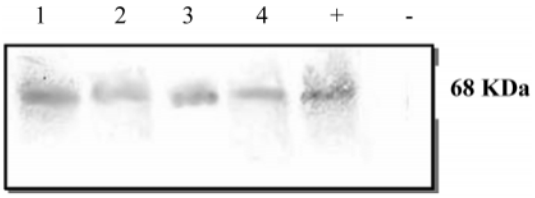

Figure 6. Western blot of plants expressing Cry1Ac protein. Lane1-4: Transgenic plants (8, 11, 19, 20), Lane +: Purified Cry1Ac protein, Lane – : Non-transgenic plant

Table 1. Overall analyses of T0 transgenic cotton plants.

Plant no.	PCR	Southern	Western	Larval mortality (%)	Bt protein (μg g^{-1} leaf tissue)
8	+	+	+	100	16.6
11	+	+	+	80	15.7
19	+	+	+	80	14.9
20	+	+	+	75	13.8
Control/ non-transgenic	-	-	-	0	0.00

by Sanjaya et al. (2005).

Western blot analysis also indicated the presence of a single 68-KDa protein in our transgenics (Fig. 6). Plants 8, 11, 19, and 20 showed positive expression of Cry1Ac compared with no signal in the non-transgenics. Those transformed plants also contained 13.8 to 16.6 μg g^{-1} of Bt protein for Cry1Ac in their leaf tissue (Table 1) whereas no such protein was detected in the non-transgenic plants. ELISA confirmed the inheritance and expression of the Bt genes in the progeny plants, and all transgenics were from their respective parental lines: 8, 11, 19, and 20. However some plants (8-13, 8-15, 11-25, 11-38, etc.) showed little or no expression, perhaps because of chimerism. Similarly, plants 19-39 and 20-65 had much less color detection in the ELISA. This may have been due to protein degradation during their handling. These plants also were negative in other analyses, e.g., PCR. The intensity of color detection showed that Cry1Ac and Cry2A proteins were being expressed in the transgenic leaf

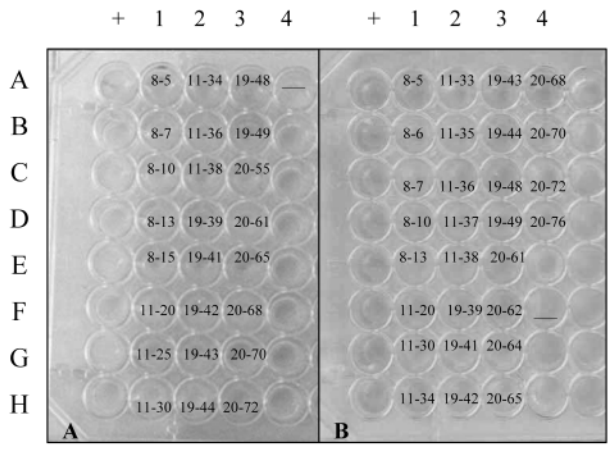

Figure 7. Screening of T1 cotton transgenic plants with ELISA. Samples expressing **A,** Cry1Ac protein or **B,** Cry2A protein.

Table 2. Bt protein in leaves from T1 progeny of transgenic cotton plants

Plant no.	Bt protein[a] (μg g^{-1} leaf tissue)
8-5	16.5
8-10	16.2
11-20	15.5
11-34	15.3
19-39	13.2
19-44	13.9
20-61	13.5
20-65	13.0
Control/non-transgenic	0.00

[a]Bt protein was calculated only for plants considered positive in all other molecular and biological analyses

samples. Authentic Bt proteins Cry1Ac and Cry2A also were run as positive controls and compared with the transgenic plant tissues (Fig. 7A, B). Thus, the ELISA-positive plants manifested the inheritance and expression of the Bt genes into our progeny plants.

The extent of gene expression is defined by the amount of protein that is formed. Here, the level of Bt protein varied slightly among plants, ranging from 13.0 to 16.5 μg g^{-1} leaf tissue (Table 2). However, this fluctuation in quantities was consistent with that observed from the parent plants. Samples 19-39 and 20-65 exhibited negative reactions in their color development during ELISA (Fig. 7A) even though Bt protein was detected in all of them. In contrast, no such protein was detected in the controls. This means that the Bt genes are stably inherited, segregated, and expressed in progeny plants.

Insect Bioassay

Our main study objective was to develop transgenic cotton plants with highly sustainable insect resistance. These lines that expressed the Bt *cry1Ac* and *cry2A* genes were confirmed via bioassay. There, larval mortality ranged from 75 to 100% compared with no mortality on the control plants (Table 1). Larvae consumed 5 to 10% of the total leaf material from transgenic plants, and their mortality started after 36 h of feeding. In contrast, 90 to 100% of the leaf area on control plants was consumed and those larvae grew to the fourth instar stage. Therefore, based on our molecular and entomocidal analyses, we chose four putative transgenic plants (Number 8 with 100% larval mortality on all replicates; and Numbers 11, 19, and 20, with mortality of 75 to 80%) for further study.

Bioassaying with detached plant parts serves, at best, a qualitative purpose. In progeny analysis, leaf damage was >95% on the controls whereas the transgenic plants exhibited only a few bites before those insects died (Fig. 8). Although most transgenics had larval mortalities of 80 to 100%, some had only 40 to 60%. We considered those with rates 75% to be positive for Bt gene expression. Nevertheless, one drawback of such an assessment is the rate of Cry1Ac degradation after tissues are detached from the plant. Therefore, if considerable *in situ* degradation had

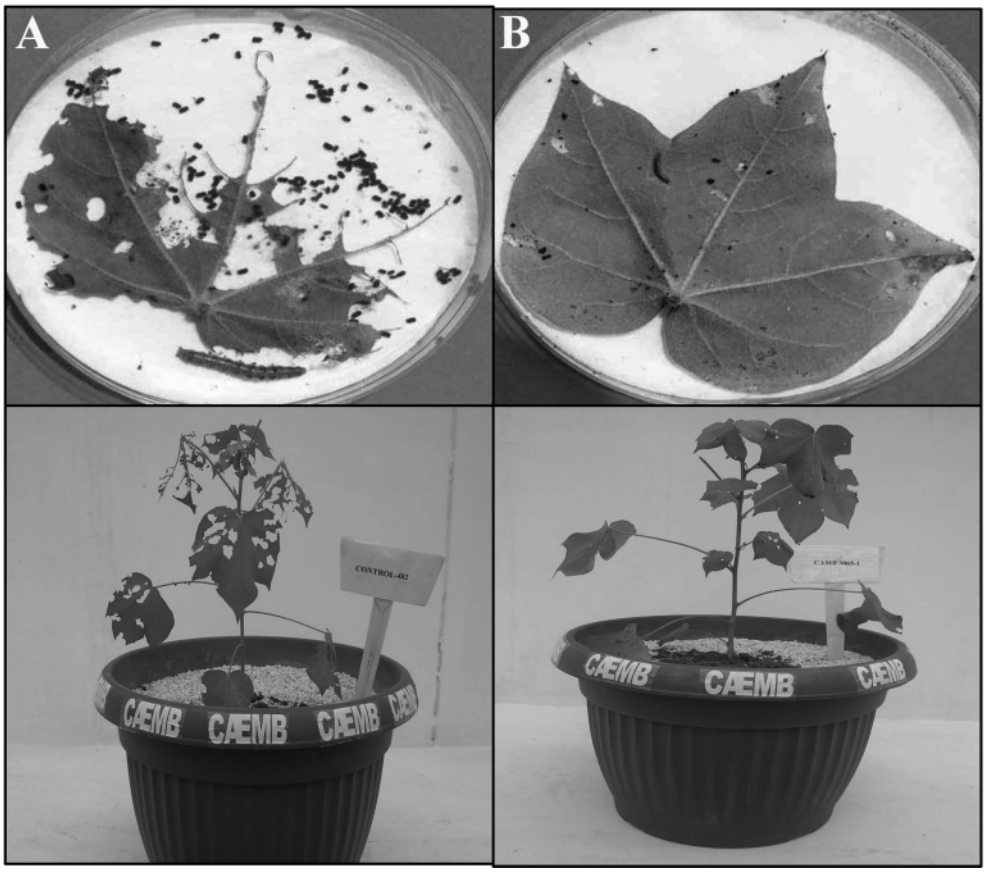

Figure 8. Insect bioassay with *H. armigera* 2nd instar on leaf and whole plant T1 progeny of transgenic cotton. **A.** Non transgenic leaf and plant showing leaf damage and larval, 4th instar growth stage, **B.** Transgenic leaf and plant showing leaf damage and larval mortality

occurred during this assay period, the larvae would have been able to survive even on the tissues from plants with high expression of Cry1Ac. Hence, whole-plant assays, which involve the artificial infestation of plants with one-day-old larvae, can yield more reliable data (Kranthi, 2006). In fact, by utilizing this latter method, we demonstrated that insects damaged 90% of the leaf area on control plants but only 5 to 10% on the transgenics (Fig. 8).

While this whole-plant bioassay was in progress, we observed that larvae were repelled by the transgenic plants and attempted to escape but were prohibited by the surrounding polythene wrap. Furthermore, larval body weights were reduced by 50% on the transgenic plants while those on the controls showed a doubling of their weights as they attained the fourth instar stage. After 7 d, no dead larvae were found on the controls whereas larval mortality was 90% on the transgenics. Thus, our results agree with those reported by Kranthi (2006). Previous research has suggested that plants containing two dissimilar Bt toxin genes can potentially delay resistance more effectively than plants with a single toxin used sequentially or in mosaics (Gould, 2003; Zhao et al., 2003; Chitkowski et al., 2003; Jurat-Fuentas et al., 2003). We compared our results with these earlier studies and can conclude that, although whole-plant insect bioassays indicate 100% insect mortality, the transgenics also must be evaluated very carefully in open field conditions (Ho et al., 2006).

Our T1 progeny plants were not homozygous lines so the variable effect of toxins was observed via larval mortality in the insect bioassay. Transferred genes should segregate in 3:1 pattern (i.e., 75% will be positive). Here, transgenic plants with second instar larval mortality of 75% were considered resistant whereas those with a lower mortality rate were susceptible. Thus, crossing plants that express different Bt toxins is an effective way of delaying the emergence of Bt-resistant pests. This response has also been illustrated in broccoli, where pyramided *cry1Ac* and *cry1C* genes control diamondback moths resistant to either single protein (Cao et

Table 3. Segregation of transgenes on the basis of insect resistance by T1 progeny.

T0 transformant	No. of resistant plants in T1[a]	No. of susceptible plants in T1[b]	χ^{2c}	Expected ratio
8	12	8	0.667	3:1
11	12	4	0	3:1
19	12	4	0	3:1
20	12	5	0.176	3:1

[a]Resistant plants are transgenics with ≥75% larval mortality for *H. armigera* 2nd instar.
[b]Susceptible plants are transgenics with <75% larval mortality for *H. armigera* 2nd instar.
[c]χ^2 Tabulated value is 3.84; p = 0.05.

al., 2002) while significantly delaying the evolution of resistant insects (Zhao et al., 2003). Our Chi square test (χ^2) showed that all parental transgenic lines segregated in a Mendelian inheritance pattern (3:1) for resistance against *H. armigera* (Table 3). Such inheritance of transgenes in the progeny has also been described by Cohen et al. (2000), Maqbool et al. (2001), and Datta et al. (2002).

Because plants grown in pots in the greenhouse were subjected to stress due to the many times their leaves were detached for different analyses, we were unable to record any yield data. However, fertile seeds were collected.

In conclusion, we have now devised an improved technique that enhances the efficiency of *Agrobacterium* infection in plant cells. Moreover, we have successfully introduced two Bt insecticidal genes -- *cry1Ac* and *cry2A* -- into the genome of a local cotton variety. Our results demonstrate that one can use an expression vector carrying two Bt insecticidal genes on the same promoter, making this a valuable candidate in crop breeding programs for delaying the onset of insect resistance.

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