

Development of insect-resistant transgenic cabbage plants expressing a synthetic *cryIA(b)* gene from *Bacillus thuringiensis*

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A synthetic *cryIA(b)* gene coding for an insecticidal crystal protein of *Bacillus thuringiensis* (*Bt*) was transferred to cabbage cultivar 'Golden Acre' by co-cultivating hypocotyl explants with *Agrobacterium tumefaciens*. Transformed plants resistant to kanamycin were regenerated. Hybridization experiments demonstrated gene integration and mRNA expression. Immunoblot analysis revealed high-level expression of *Bt* toxin protein in the transgenic plants. The expression resulted in a significant insecticidal activity of transgenic cabbage plants against the larvae of diamondback moth (*Plutella xylostella*). The results also demonstrated that a synthetic gene based on monocot codon usage can be expressed in dicotyledonous plants for insect control.

BACILLUS thuringiensis (*Bt*) produces a variety of insecticidal crystal proteins (ICPs) upon sporulation. These proteins, called *δ*-endotoxins, are highly toxic to lepidopteran, dipteran and coleopteran insects¹. Different ICP genes of *Bt* have been successfully engineered into many crop-plants to obtain resistance against lepidopteran insects². Poor expression of native *Bt* genes necessitates the use of codon-modified and truncated version of the genes to achieve higher expression³. When expressed in plants, these modified genes confer significant protection against insects to important crops such as cotton, maize, rice, tomato and potato^{2,4}.

Cabbage (*Brassica oleracea* var. capitata) is an important vegetable crop grown extensively throughout the world, including India. One of the major limitations in cabbage production is damage due to insect pests. The most important among them, the lepidopteran diamondback moth (*Plutella xylostella*) alone causes a worldwide damage of up to one billion dollars annually⁵. The larvae bore into the cabbage head and even a little damage into the head reduces marketability of the crop. Synthetic insecticides used to control the pest have raised concerns about food safety and environmental pollution. Evolution of insecticide resistance further adds to the problem of chemical control.

We have transformed a leading cabbage cultivar 'Golden Acre' with a lepidopteran-specific, synthetic *cryIA(b)* gene extensively modified for high expression in plant cells. The transgenic lines were demonstrated to have significant resistance to the larvae of *P. xylostella*.

Plasmids pBT1291 (ref. 1), pBinAR (ref. 6) and derivatives were transformed into *E. coli* strain DH5a (Stratagene). For plant transformation, the binary vector was mobilized into *Agrobacterium tumefaciens* strain GV2260 (ref. 7) by freeze-thaw transformation method⁸. Single transformed colony was transferred into liquid bacterial medium (YEM medium with 50 mg/l rifampicin, 100 mg/l carbenicillin and 50 mg/l kanamycin) and shaken at 240 rpm at 28°C for two days. The suspensions were then mixed with sterile glycerol (1:1 vol/vol) and stored at -80°C until used.

About 5 µg purified DNA of pBT1291 was restricted with *Xba*I. On restriction the 2.4 kb fragment, which includes full length synthetic *cryIA(b)* sequence along with a castorbean intron upstream to structural region, was purified through Wizard DNA Cleanup system (Promega) and ligated into the *Xba*I site of the binary vector pBinAR, which is flanked by CaMV 35S promoter and octopine synthase poly(A) sequences.

The most popular Indian cultivar of cabbage (*B. oleracea* var. capitata) 'Golden Acre' was used. Disinfected seeds were germinated under aseptic conditions on solid MS medium⁹ with 2% sucrose. Hypocotyl segments of approximately 5 mm length from four-day-old seedlings were used for plant transformations. The incubation conditions for germination and *in vitro* culture were 25 ± 1°C and 16 h photoperiod of approximately 28 µEm⁻² s⁻¹.

One ml glycerated culture was inoculated in ABMM broth¹⁰ containing appropriate antibiotics and grown to saturation. A secondary culture, initiated from the saturated primary culture, was grown in the same selective medium supplemented with 50 µM acetosyringone. The culture was grown to OD₆₀₀ = 0.6. Cells from 1.5 ml of culture were harvested by centrifugation at 5000 rpm for 10 min at 22°C and resuspended in 15 ml CI liquid medium (MS basal salts with 2% sucrose + 1 mg/l 2,4-D + 0.5 mg/l kinetin). This suspension was used for infection of hypocotyl explants, preconditioned for one day on solid CI medium (CI liquid medium + 0.8% agar) and co-cultivated for three days at 24°C in dark. The explants were incubated on SI medium (MS basal medium with 2% sucrose + 0.5 mg/l IAA + 2 mg/l BA + 3.5 mg/l silver nitrate + 250 mg/l cefotaxime + 0.25% phytigel) for a delay period of one week, and transferred on S2 selection medium (SI medium + 25 mg/l kanamycin). The regenerated shoots were grown on root induction medium (MS basal medium + 0.1 mg/l IAA + 250 mg/l cefotaxime + 25 mg/l kanamycin). The rooted plants were transplanted into small pots containing vermiculite. After initial establishment, the plants were shifted to earthen pots in the greenhouse.

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Isolation of total genomic DNA was performed by the procedure described by Doyle and Doyle¹¹. DNAs were restricted with *EcoRV*, electrophoresed on 0.8% agarose gel and transferred onto nylon N⁺ membrane (Amersham). Southern hybridization was carried out with a ³²P-dCTP labelled *Xba*I insert (2.4 kb) separated from pBT1291 according to Sambrook *et al.*¹². Total RNA was isolated from young leaf tissues with guanidine thiocyanate-based RNeasy Plant Total RNA kit (QIAGEN) according to the manufacturer's specifications. RNAs were electrophoresed on 1.2% agarose formaldehyde gel and transferred onto nylon membrane by wet-blotting¹². The membrane was hybridized with ³²P-labelled *cryIA(b)* sequence restricted and purified from pBT1291.

The purified CryIA(b) protein and the rabbit anti-CryIA(b) serum were kindly provided by Swapan K. Dutta, IRRI, Philippines. Total cellular protein was isolated following the procedure by Laemmli¹³. Total protein from *Bt*-transgenics and untransformed wild-type plants was fractionated by SDS-PAGE (10% polyacrylamide) and transferred to PVDF (Amersham) as described by Asubel *et al.*¹⁴. The *Bt*-proteins were detected using the rabbit anti-CryIA(b) serum and a goat-anti rabbit IgG coupled to alkaline phosphatase as secondary antibody.

The leaf discs (1.5 cm diameter) from young leaves were cut and placed in small petri dishes containing moistened filter paper. On each leaf disc, five late second-instar (6-day-old) larvae of *P. xylostella* were released and reared at 26–28°C with 60% relative humidity. Each assay consisted of three replicates using larvae originating from the same egg cluster. For each replicate of either transformed or control plants, the percentage of larval mortality was calculated. Leaf area damaged was determined after 24 h of feeding by plotting the total leaf area against the damaged leaf area on a graph paper, and expressed as percentage. Corrected per cent mortality was calculated according to the formula:

$$\text{Per cent corrected mortality} = \frac{[(T - C)/(100 - C) \times 100],}{}$$

where *T* is per cent mortality in transgenics and *C* the per cent mortality in wild-type plants.

Small-scale insect-feeding trials were carried out by releasing 20 to 25 late second-instar larvae of *P. xylostella* on each plant grown in a glasshouse at 28 ± 2°C. Plants were individually covered with three-rings bags, after release of the larvae.

The chimeric gene expression cassette employed in the present study consisted of *CaMV 35S* promoter, a synthetic *cryIA(b)* gene and the 3' polyadenylation signal from octopine synthase gene of *Agrobacterium* (Figure 1). Fifteen plantlets regenerated on S2 medium were subjected to rooting under kanamycin selection. Within two weeks the true transformed shoots produced normal

roots, whereas escapes failed. Total genomic DNA from each of the six normal-looking plants, rooted in presence of kanamycin, was isolated and used for molecular analyses. Presence of the *cryIA(b)* sequence was initially detected through PCR analysis using *cryIA(b)*-specific internal primers (results not shown). The Southern hybridization analysis clearly demonstrated integration of the transgene sequence into the target genome (Figure 2). The DNA from untransformed plant did not show any PCR amplification or signal. Since the cloned transgene *cryIA(b)* does not have any internal site for *EcoRV*, DNA

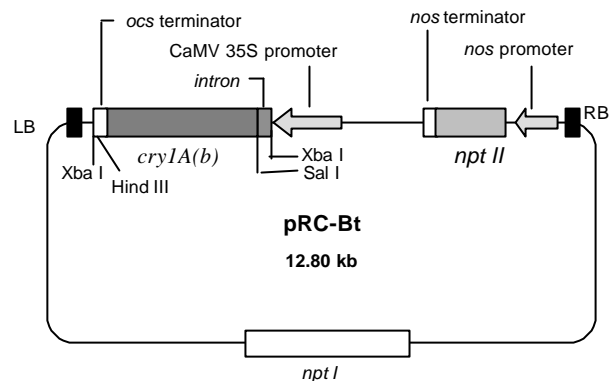


Figure 1. Plant transformation vector pRC-*Bt* containing the ICP gene *cryIA(b)*. *npt*, Neomycin phosphotransferase; LB, Left border; RB, Right border.

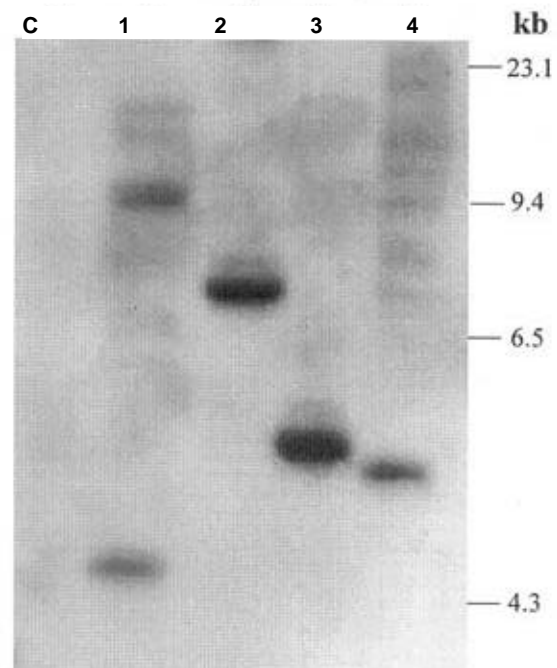


Figure 2. Southern analysis of *Bt*-transgenics probed with *cryIA(b)* sequence. Five µg of total DNA was restricted with *EcoRV*, electrophoresed on 0.8% agarose gel, blotted on nylon membrane and Southern hybridized using ³²P-labelled *cryIA(b)* sequence. Lane C, wild type; lane 1, BT1; lane 2, BT2; lane 3, BT3; lane 4, BT4.

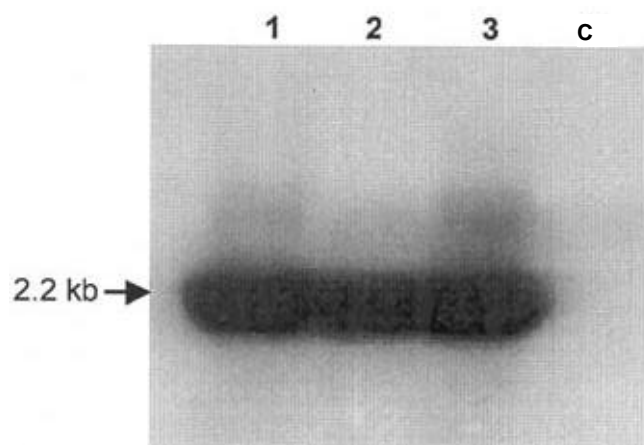


Figure 3. Northern analysis of *Bt*-transgenics. Twenty-five μ g of total RNA from young leaves was electrophoresed on 1.2% formaldehyde-agarose gel and probed with *cryIA(b)* sequence. Lane C, wild type; lane 1, BT2; lane 2, BT3; lane 3, BT4.

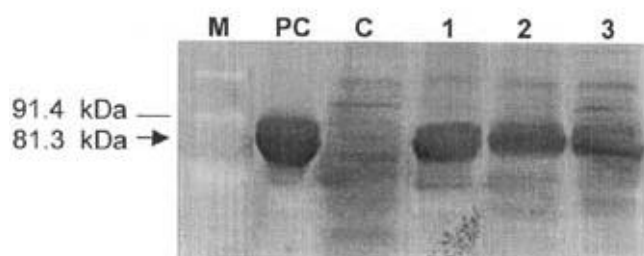


Figure 4. Expression of crystal protein in *Bt*-transgenics. Eighty μ g total protein from leaves was separated on SDS-PAGE and Western-blotted using rabbit anti-CryIA(b) serum. Forty μ g of purified CryIA(b) protein was used as positive control. Lane M, Molecular weight marker; lane PC, Purified CryIA(b) protein (positive control); lane C, Wild-type; lane 1, BT2; lane 2, BT3; lane 3, BT4.

Table 1. Mean percentage of larval mortality of *Plutella xylostella* and leaf area damage in leaf disc bioassay

Plant no.	Per cent mortality (mean \pm SD)	Corrected mortality per cent	Percentage leaf area damage (mean \pm SD)
WT	10 \pm 8.16	–	59.66 \pm 9.10
BT2	76.66 \pm 4.71	74.06	6.0 \pm 0.81
BT3	56.66 \pm 4.71	51.84	26.3 \pm 2.49
BT4	70 \pm 8.16	66.66	18.3 \pm 2.05

The experiments were conducted in three independent sets and each corresponds to 10 larvae independently reared on leaf discs from wild type or transgenic plants.

from putative transgenic plants when restricted with *EcoRV* for Southern blot transfer, is expected to be restricted outside the integrated gene. Consequently, the number of bands obtained in the autoradiogram indicates independent events of transgene-integration. Three independent lines, BT2, BT3 and BT4, showed desirable sin-

gle-copy insertion of the transgene. In Northern analysis of total RNA from Southern-confirmed plants, presence of a single 2.2 kb band in the autoradiogram confirmed the transcription of *cryIA(b)* sequence in BT2, BT3 and BT4 lines (Figure 3). No degradation of the mRNA was detected. BT2 and BT4 showed higher level of transgene expression at the transcriptional level compared to BT3. Appropriate quantities of total cellular proteins from transgenics and untransformed control plants were fractionated by SDS-PAGE, immunoblotted on PVDF membrane and analysed for the presence of *Bt*-protein. A 81.3 kDa Cry protein was detected when rabbit anti-CryIA(b) serum was used as primary antibody (Figure 4).

Bt-transformants, which showed detectable levels of mRNA expression for the transgene and stable presence of Cry protein in immunoblotting, were assessed for their tolerance to late, second-instar larvae of *P. xylostella*. Larval mortality was recorded in order to test the influence of Cry protein ingestion on larvae survival. Extent of leaf damage effected before cessation of larval feeding was measured to assess the *Bt*-mediated resistance. Bioassay on detached leaf discs showed significant larval mortality ranging from 51.84 to 74.06% (Table 1). The highest larval mortality of 74.06% with a minimum leaf damage of 6.0% was obtained on transgenic BT2. The damage on young leaves due to insect feeding was also significantly less on the transformed leaves compared to untransformed wild type (Figure 5). The difference in mortality and leaf area damage observed among the different transgenic lines could be correlated to differences in *Bt* gene expression.

The larvae fed on transgenic leaf discs were severely stunted in growth when compared to larvae fed on wild type leaf (Figure 6). At whole plant level also, transgenic plants showed less insect damage compared to wild type plants (Figure 7). The result demonstrated that *Bt*-mediated resistance was functionally active *in planta*.

It has been convincingly demonstrated by several groups that the genes encoding δ -endotoxins from *Bt* impart resistance to lepidopteran insects pests, when introduced into different crop plants^{15,16}. Improvement in the expression of endotoxin gene through codon modification is crucial for effective *Bt*-mediated resistance against major insect pests. High-level expression was important for our project of imparting pest resistance to cabbage cultivar 'Golden Acre'. A synthetic version of *cryIA(b)* modified extensively on the basis of codon usage in plants¹ was chosen. In this synthetic *cryIA(b)*, the overall G+C content has been changed to 59.2% from the 37.6% of the native gene. This modification was more extensive than that previously described for *cryIA(b)* (ref. 3) and *cryIA(c)* (ref. 17). In constructing plant-expression vector pRC-*Bt*, the coding sequence of synthetic *cryIA(b)* was fused to *CaMV 35S* promoter, the castorbean catalase-1 intron and the polyadenylation signal of the octopine synthase gene. The chimeric pRC-*Bt*

is similar to pBin-*Bt* described by Kumar *et al.*¹⁸. Cabbage is a leafy vegetable and effective control strategy will require that the resistance operates throughout plant growth and development. The constitutive promoter 35S was therefore chosen. The combination of 35S promoter and the castorbean intron has previously been demonstrated to confer high-level expression of foreign genes in transgenic plants^{19,20}. The *Bt*-transformants isolated through the described selection regime of plant transfor-

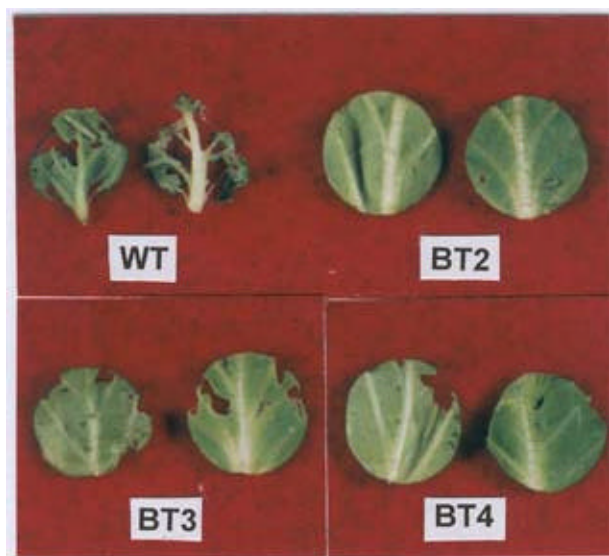


Figure 5. Insect-feeding bioassay of *Bt*-transgenics. Late second-instar larvae of *P. xylostella* were released on leaf-discs of transgenic and wild type (WT) plants. Insect mortality and leaf area damage were recorded at 24 h after release.



Figure 6. Inhibition of larval growth due to feeding on *Bt*-transgenics. Growth of the larvae after 24 h feeding on transgenic and wild type leaf-discs was compared. T, Larvae fed on transgenic leaf-discs; WT, Larvae fed on wild type leaf-discs.



Figure 7. *In planta* bioassay of *Bt*-transgenics. Twenty to twenty-five late second-instar larvae of *P. xylostella* were released on transgenics and wild type plants, grown in a contained environment. C, Wild type; T, transgenic (BT2).

mation showed stable integration of *cryIA(b)* sequence in the genome. Fragment sizes in the Southern blot and detection of expected 2.2 kb mRNA in Northern hybridization indicated integrity of *cryIA(b)*-expression cassette. Northern analysis demonstrated substantially high level of *cryIA(b)* expression. Detection of cry transcripts in transgenic plants is known to be difficult and toxin mRNAs are often present in amounts below the level of detection in Northern blots^{21,22}. The higher expression may be due to extensive codon modification carried out in the synthetic version of the gene. Although the codon modification was originally carried out based on monocot codon usage¹, our study indicated that the same gene could be expressed in dicotyledonous plants also. However, no comparative study was made in this regard. The integrity of *cryIA(b)* transcripts was confirmed by the presence of a corresponding single 81.3 kDa Cry protein in immunoblot analysis. The size of the Cry protein corresponded to the expected size of the truncated CryIA(b). In insect-feeding bioassay conducted on leaf discs, the *Bt*-protein produced in transgenic plants caused rapid cessation of larval-feeding activity and subsequent inhibition of their development. Under controlled laboratory conditions, this provided an estimate of insecticidal effects before larval release could be assessed for damage on control vs transgenic plants. At the whole plant stage, *Bt*-expressing plants were sufficiently protected against damage from diamondback moth attack. The degree of tolerance among different independent transformants varied significantly and the maximum level was obtained in BT2 line.

While the tolerance of transgenics has still to be demonstrated under field conditions, transgenesis in combination with other IPM strategies has the potential of preventing a significant part of the yield losses and quality deterioration caused by diamondback moth.

1. Fujimoto, H., Itoh, K., Yamamoto, M., Kyoizuka, J. and Shimamoto, K., *Bio/Technology*, 1993, **11**, 1151-1155.
2. Kumar, P. A. and Sharma, R. P., *J. Plant Biochem. Biotechnol.*, 1994, **3**, 3-9.

3. Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L. and Fischhoff, D. A., *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 3324–3328.
4. Kumar, P. A., Sharma, R. P. and Malik, V., *Adv. Appl. Microbiol.*, 1996, **42**, 1–43.
5. Talekar, N. S. and Shelton, A. M., *Annu. Rev. Entomol.*, 1993, **38**, 275–301.
6. Hofgen, R. and Willmitzer, L., *Plant Sci.*, 1990, **66**, 221–230.
7. Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. and Schlperoot, R. A., *Nature*, 1983, **303**, 179–180.
8. Hofgen, R. and Willmitzer, L., *Nucleic Acids Res.*, 1988, **16**, 9877.
9. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–497.
10. Chilton, M., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P. and Nester, E. W., *Proc. Natl. Acad. Sci. USA*, 1994, **71**, 3672–3676.
11. Doyle, J. J. and Doyle, J. L., *Phytochem. Bull.*, 1987, **19**, 11–15.
12. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.
13. Laemmli, U. K., *Nature*, 1970, **227**, 680–685.
14. Asubel, F. M., Brent, R., Kingston, R. E., Moore, B. D., Seidman, J. G., Smith, J. A. and Struhl, K., *Current Protocols in Molecular Biology*, Current Protocols, Wiley, 1994.
15. Ding, L. C., Hu, C. Y., Yeh, K. W. and Wang, P. J., *Plant Cell Rep.*, 1998, **17**, 854–860.
16. Mazier, M., Pannetier, C., Tourneur, Jouanin, J. L. and Giband, M., *Annu. Rev. Biotechnol.*, 1997, **3**, 313–347.
17. Sutton, D. W., Havstad, P. K. and Kemp, J. D., *Transgen. Res.*, 1992, **1**, 228–236.
18. Kumar, P. A. *et al.*, *Mol. Breed.*, 1998, **4**, 33–37.
19. Hayakawa, T., Zhu, Y., Itoh, K., Kimura, Y., Izawa, T., Shimamoto, K. and Toriyama, S., *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 9865–9869.
20. Tanaka, A., Mita, S., Ohta, S., Kyojuka, J., Shimamoto, K. and Nakamura, K., *Nucleic Acids Res.*, 1990, **18**, 6767–6770.
21. Cheng, J., Bolyard, M. G., Saxena, R. C. and Sticklen, M. B., *Plant Sci.*, 1992, **81**, 83–91.
22. van der Salm, T. *et al.*, *Plant Mol. Biol.*, 1994, **26**, 51–59.

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