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# Entomocidal Activity of *Bacillus Thuringiensis* Transgenic Rice Plants Against Rice Leaffolder, *Cnaphalocrocis Medinalis* (Lepidoptera: Pyralidae).

Rizwana Anwar, Shahid Karim and S. Riazuddin
National Centre of Excellence in Molecular Biology, University of the Punjab, Canal Bank Road, Lahore-53700, Pakistan.

## bstract

ransgenic rice plants obtained through the incorporation of Bt *cry*1Ac gene. Initially putative transformants were screened r pesticidal activity against rice leaf folder larvae. Selected putative transformants showed a range of larval mortality from 0-100 percent to *C. medinalis*. The integration of the Bt *cry* gene was checked by Polymerase Chain Reaction (PCR), using imers 5' ACA GAA GAC CCT TCA ATA TC 3' and 3' GTT ACC GAG TGA AGA TGT AA 5' for amplification of a 656 bp agment of DNA from the genomic DNA of putative transformants. Selection based on PCR and initial bioassays, two ansgenic plants designated as, CAMB 1148 and CAMB 1150 were further confirmed for toxin expression by ELISA, Vestern blot analysis and comparative biotoxicity assay. ELISA and Western blot were performed with polyclonal antibody f *Cry*1Ac toxins that showed the presence of active ingredient of Bt toxin in both plants. Biotoxicity assays also econfirmed the stability and activity of *Cry* toxins in both transgenic rice plants, eventually aiming for homozygosity with nhanced resistance to rice leaf folder.

## ntroduction

*acillus thuringiensis* crystalline inclusion ranging in olecular weight from 27-140 KDa are protoxins that are roteolytically converted into smaller toxic polypeptide in he larval midgut of Lepidoptera, Diptera, Coleoptera, ematodes and other insects (Peferoen, 1997). Midgut roteases process the protoxin to form a stable 55-70 KDa oxin core (Bietlot *et al.*, 1990). Activated toxins ubsequently binds to epithelial cells lining causing lysis Bravo *et al.*, 1992a, 1992b). Electrophysiological and iochemical evidence adjusts that the toxins generate cores n the cell membrane thus disturbing the osmotic balance Knowles, 1994). Consequently, the larvae stops feeding nd eventually dies (Manthavan *et al.*, 1989). Commercial formulation of *Bacillus thuringiensis* containing the spore rystal complex, can effectively control economically important pests in agriculture and forestry. In fact, *Bacillus thuringiensis* is at present the most widely used microbial control agent for insect pests (Karim and Riazuddin, 1997). The development of built in insect resistant transgenic lines through genetic engineering in crops figured as a potential strategy for crop protection (Fischhoff, 1996). There are eports on transgenic plant production with expression of ry1A genes in plants that are highly toxic to certain epidopterous pests, killing all of the larvae of target insects Stewart *et al.*, 1996), yet they have little or no toxicity to umans, most beneficial insects and other non-target rganisms (Hilbeck *et al.*, 1998). The production of ommercial crop varieties producing these pesticidal roteins is expected to reduce environmental pollution from nthetic insecticides, increase worker safety and improve ower profitability (Gasser and Fraley, 1989).

he loss of paddy yield due to direct insect damage is timated to at least several billion dollars per year oenniessen, 1996). Past successes in conventional eeding for resistance, the ability of plants to compensate r many types of damage and biological control by natural emies combine to limit many pests problems to a erable level (Khush, 1995). Most insects had developed istance to chemical pesticides (Georghiou, 1990), which often permits minor pests to become major pests (Bull *et al.*, 1979). It is widely distributed in paddy growing areas. Pest management through transgenic plant technology directly avoids the complicated issues of production, formulation, handling and application of insecticides. Insect resistant transgenic plants have been obtained through the introduction and expression of genes *cry* 1Aa, *cry*1Ab, *cry*1Ac and *cry*3A (Bennet *et al.*, 1997; Cheng *et al.*, 1998).

Rice leaf folder is an important pest of rice in several Asian countries (Islam and Karim, 1997; Karim and Riazuddin, 1999). *C. medinalis* which was formerly a minor pest but during the past decade, the pest have become increasingly important threat to rice production (Shepard *et al.*, 1991). In rice growing areas of Asia, *C. medinalis* became a serious pest with the intensification of rice production. It presently causes the loss of about 1 million tons of rice per year and control of this species accounts for about half of the synthetic insecticide applied to rice (Riley *et al.*, 1995). Of the eight species composing the leaf folder complex of rice, the one most widespread and important species is *Cnaphalocrocis medinalis* (Guenee) (Pathak and Khan, 1994). In rice growing areas of Pakistan, where only one rice crop is grown per year, *C. medinalis* is the only leaffolder species because it can survive on weeds in rice fields between rice crops. The neonatal larvae, after hatching crawl to the leaf and fold it, feed by scraping the leaf surface tissues. When infestation is high, each plant may contain many folded leaves. Heavily damaged leaves become dry and fields appear scorched and heavy yield affected.

The present communication deals with the results of our tests for expression of transformed cry1Ac gene in transgenic rice cultivars. We show that the integrated *cry*1Ac gene in the rice genome is effective against rice leaffolder, *Cnaphalocrocis medinalis*.

## Materials and Methods

**Plasmid construct and Rice transformation:** Putative transformed plants harboring *cry*1Ac, Pin II and Hygromycin

genes were the generous gift of Drs. Tayyab Husnain and Abdul Bari, CAMB Plant Tissue Culture Laboratory. Meristem tissues of local aromatic commercial variety Bas 370 and IR6 (IRRI based variety) were transformed with three genes *Cry*1Ac, Pin II and Hygromycin gene in the ratio of 1.5:1.5:1.0 exploiting biolistic procedure of transformation as described by Riazuddin *et al.* (1996). The construct was described by Altosaar *et al.* (1996); Sardana *et al.* (1996) were selected on the basis of initial Hygromycin selection. The Hygromycin resistant rice plants were established in MS medium then hydroponics and finally in the soil.

**Biological activity of the putative transgenic rice plants to leaf folder (*Cnaphalocrocis medinalis*) larvae:** The leaves of six months old plants were taken for larval feeding to study the resistance. One leaf piece was cut from each transformed and control plant, washed extensively with autoclaved distilled water to remove any microbial or fungal contamination, air dried and cut into 5 cm long pieces. Two leaf pieces of 5 cm each were placed in a plastic disposable petriplates lined with moist filter paper to keep green rice larvae fresh for maximum time. Second instar larvae of *Cnaphalocrocis medinalis* were taken from CAMB Insect Rearing Laboratory, starved for four hours and weighed individually on Saratorius Analytical Balance before infesting the leaf pieces. Two larvae were used to infest two leaf pieces. To prevent the larval escape from petriplates, both plates were tightly closed with parafilm "M". Petriplates were kept at 28 ± 2 C, photoperiod 14/10 day/night. The fate of the larvae was monitored after 120 hours of larval infestation. The resistance in the plants was studied by recording the mortality, weight gain/loss of larvae, leaf area damaged by larvae. The percentage of leaf damage was calculated by a formula as given below.

% of leaf area consumed = leaf area consumed by larvae / Total leaf area X 100

Larval growth retarded = Larvalweight(cont)-Larval weight(trans)/ Larval weight(cont) X 100

**DNA isolation and agarose gel electrophoresis:** Isolation of plant DNA was carried out by following the modified method of Metler (1987) and electrophoresis was carried out as described by Sambrook *et al.*, (1989).

**Polymerase Chain Reaction:** PCR analysis employed the following primers 5` ACA GAA GAC CCT TCA ATA TC `3 and 3` GTT ACC GAG TGA AGA TGT AA `5 for amplification of a 656bp fragment from the *cry*1Ac synthetic gene. PCR analysis was carried out as described by Saiki *et al.*,(1988) with some modifications. PCR reaction cocktail contained template 1ng of genomic DNA of transformed and non transformed plants 2 pm of primer 5 and primer 3 each, 0.2mM dNTPs, 1X PCR buffered 1 unit of Taq polymerase I. Total volume of PCR reaction cocktail was kept 25 µl by adding autoclaved double distilled water. Following are the denaturing, annealing and extension conditions for the amplification of Cry1A(c) gene. The DNA was subjected to 1 cycle of three steps, 95 C for 10 minutes (denaturing), 52 C for 2 minutes (annealing) and 72 C for 2 minutes (extension). Again three steps 31 cycles each, 95 C for 1 minute (denaturing), 52 C 2 minutes (annealing) and 72 C for 1 minute (extensio Last cycle of three more steps, 95 C for 1 min (denaturing), 52 C for 2 minutes (annealing) and 95 C 10 minutes (extension). PCR products were then analy by agarose gel electrophoresis.

**Extraction of proteins from rice plant:** Leaves of n transformed and transformed plants (plant # CAMB11 CAMB 1148, and CAMB 1150) were washed w deionized sterilized water and then dried. The leaves fr four plants were weighed separately (200-300 mg) a then were ground in liquid Nitrogen . NE buffer was add in each ground material (50 ul NE buffer\200mg leav immediately before thawing. This material was kept at 4 for 24 hours and then centrifuged at 12K at 4 C for minutes. Protein concentration was determined using dye-binding method (Bradford, 1976). Supernatants w collected and stored at -20 C for further use in molecu analysis.

**Sodium dodecyl sulfate polyacrylamide gel electrophores** SDS-PAGE (10%) was done using a modified procedu (Laemmeli, 1971). Plant proteins were mixed in 1:1 ra with 2X denaturing sample buffer and heated for 5 mini in boiling water bath. Plant extracts were electrophoresed in BIO-RAD minigel apparatus using a constant current mA). After completing electrophoresis, the gel was stai with Coomassie brilliant blue R-250 for two hours and t destained in Destaining solution.


**Fig. 1:** Insect bioassay of Cry transformed rice plan rice leaf folder, (a) Control plant, happy o CAMB 1148 plant showing resistance a CAMB 1150 also showed resistance.

**Western Blotting:** Western blotting was done modification of the method as described (Towbin 1979). The SDS-PAGE after electrophoresi equilibrated with transfer buffer for 15 minutes a placed in BIO-RAD semidry transblot cell and pr

according to the procedure outlined in the equipment manual. Electrophoresed proteins were transferred onto 0.45 µm nitrocellulose paper using constant voltage (15V) for one and half hour in transfer buffer. Immuno-staining on nitrocellulose membranes were done as follows:- The

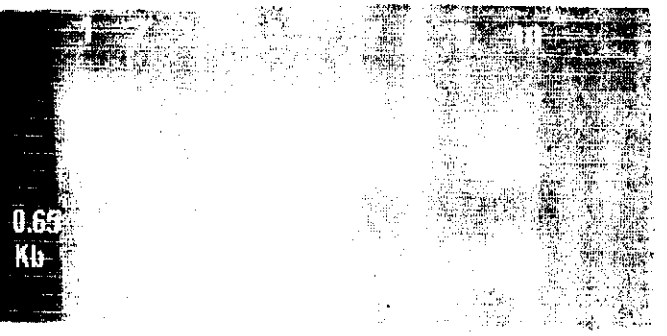
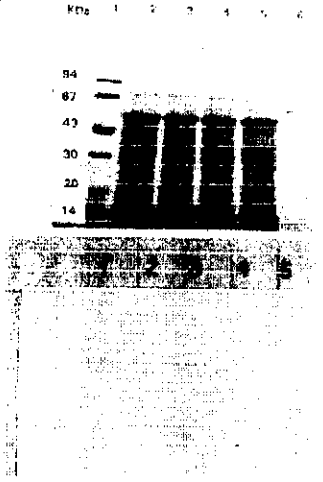



Fig.2: PCR analysis of transgenic rice plants transformed with *Cry*1Ac gene, Lane 1; λ HindIII molecular weight DNA marker, Lane 2: *cry*1Ac positive control amplified DNA, Lane 4; negative control, Lane 5; CAMB 1150 amplified DNA, Lane 7; positive control ( DNA amplified from cry1Ac with ubiquitin), Lane 9; negative control, Lane 10; CAMB 1150 amplified DNA and Lane 3, 6 and 8; empty.




**g. 3A:** Protein profile of putative rice transformed plants on 10 percent SDS-PAGE, Lane 1; Low molecular mass marker, Lane 2-5: Proteins from 1113, 1148 and 1150 plants and Lane 6; purified activated Cry1Ac toxin. **Fig. B:** Western blot analysis of rice transgenic plants, Lane 1: Purified and activated Cry1Ac toxin, a positive control, Lane 2-3; CAMB 1150 and CAMB 1148 expressed Cry1Ac toxin, Lane 4; CAMB 1113 and Lane 5; protein extracted from control plant.

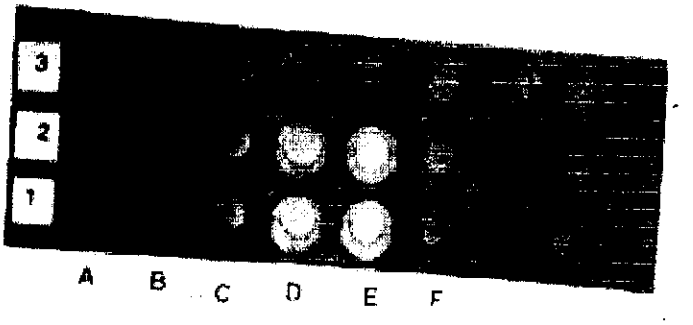



Fig. 4: Enzyme linked immunosorbant assay of transformed rice plants, 1A and 2A, control plants, 1B and 2B, CAMB 1113, 1C and 2C, CAMB 1148, 1D and 2D, CAMB 1150 expressed proteins, 1E and 2E, activated Cry1Ac toxin, only secondary antibody and 3A, 3B, 3C, 3D and 3E, protein of control plant, CAMB 1113, CAMB 1148, CAMB 1150, positive control of Cry1Ac, all well contain secondary antibody excluding primary.

non-specific binding sites were blocked by incubating the blot in blocking buffer for one hour. These were then exposed to rabbit anti-Cry1Ac antibodies diluted (1:1000 dilution) in blocking buffer for one hour. The blots were washed 6 times in TBST (10 minutes per wash). Primary antibodies bound to antigens were then allowed to react with AP-conjugated goat anti-rabbit IgG secondary antibody diluted (1:2000 dilution) in blocking buffer for 45 minutes. Blots were again washed 6 times with TBST. Color reaction was developed using alkaline phosphatase substrates (BCIP and NBT).

**ELISA:** The expression of the introduced gene was also studied by ELISA. 50 µl of each sample was mixed with 50 µl of 50 mM carbonate buffer pH 9.6 separately. A 24-well ELISA plate was coated with samples and incubated overnight at 4°C. After 24 hours, all wells were washed with TBST and the non-specific binding sites were blocked with blocking buffer for one hour at 37°C. After five washes with TBST, 0.1 ml of Rabbit anti-Cry1Ac antibody diluted as 1:1000 ratio in 3 percent BSA was added per well and antigen-antibody reaction was allowed to develop for one hour at 37°C. The plate was washed 5 times with TBST and primary antibody bound to antigen was then allowed to react with 0.1 ml of alkaline phosphatase conjugated goat anti-rabbit IgG antibody diluted (1:2000 dilution) in blocking buffer for 30 minutes at 37°C. Finally, after 6 washes with TBST, a color reaction was developed using 0.1 ml of alkaline phosphatase substrate buffer containing pNPP as substrate. Negative control assays were performed with protein of non transformed plant and secondary antibody alone.

## Results

**Evaluation of Biological activity of toxin in transgenic rice plants:** Entomodcidal activity of the toxin expressing in the tissues of 16 putative transgenic plants was assayed after 120 hrs as listed in Table 1 and 2 through a feeding assay of second instar larvae of rice leaf folder (*Cnaphalocrocis medinalis*). Adverse developmental response to the toxin present in the transgenic plants was evident within 72 hrs of feeding as shown in Fig. 1. Leaf cut pieces of transformed and untransformed were offered to 2nd instar larvae of *Cnaphalocrocis medinalis* larvae fed on untransformed leaf pieces grew well (Table 1). However, larvae fed on transgenic plant tissues suffered from severe deleterious effect (Fig. 1). Mortality ranged between 20 percent to 100 percent and surviving insects suffered reduction in body weight from 43 percent to 100 percent. Leaf damage area was calculated (Table 1).

**Table 1:** Biological activity of putative transformed rice plants against rice leaf folders (*C. medinalis*).

| Plant No. | Leaf area damage (%) | Toxicity* |
|---|---|---|
| CAMB 115 | 33 | + |
| CAMB 1113 | 35 | + |
| CAMB 1117 | 79 | +/- |
| CAMB 1130 | 55 | +/- |
| CAMB 1111 | 30 | +/- |
| CAMB 1125 | 40 | +/- |
| CAMB 1129 | 80 | - |
| CAMB 1127 | 77 | - |
| CAMB 1139 | 83 | - |
| CAMB 1138 | 77 | - |
| CAMB 1134 | 66 | - |
| CAMB 1150 | 40 | + + |
| CAMB 1137 | 44 | - |
| CAMB 1148 | 23 | + + |
| CAMB 1149 | 100 | - |
| Control | 80 | - |

*Toxicity: -No mortality, + <50% mortality, +/-<20% mortality, + + >50% mortality

**Table 2:** Larval mortality of *C. medinalis* after 120 hrs feeding on putative transformed rice plants.

| Plant No. | % Damage | % Mortality | Growth retardation(%) |
|---|---|---|---|
| CAMB 1150 | 69 | 60 | 75 |
| CAMB 1113 | 23 | 75 | 89 |
| CAMB 115 | 53 | 20 | 43 |
| CAMB 1148 | 18 | 100 | 100 |
| Control 65 | 9 | 0 | |

**PCR Amplification:** Rice genomic DNA was extracted from the leaves of transgenic and non transgenic plants as documented in experimental procedure section. Purified DNA was used as template to amplifying a 656bp *cry*1Ac internal sequences of the gene by using two primers of said gene. Results showed amplification of positive control, a smear in the lane #CAMB 1150 plants while # CAMB 115, and # CAMB 1113 did not amplify. To confirm genomic DNA of # CAMB 1150, DNA of # CAMB 1150 was digested with restriction endonuclease, *Hind*III and amplified it with two sets of primers one *cry*IAc and other any *cry*IAc with ubiquitin promoter and terminator. The amplificatio product of 656 bp in (Fig. 2) lane 5 and 2kb in lane 1 clearly showed that *cry*1A(c) gene has been integrated the genome of # CAMB 1150.

**SDS-polyacrylamide Gel Electrophoresis:** The protein pattern of putative transformed plants and non-transforme plants were analyzed by 10 percent SDS-polyacrylamide g electrophoresis. Staining of SDS-polyacryiamide gel wit Coomassie brilliant Blue R-250 showed that plant # CAM 1148 and CAMB 1150 had a band of about 65KDa whi plant # CAMB 1113 and non-transformed had no such ban (Fig. 3A).

**Western Blot Analysis:** Western blot analysis indicated t presence of single band of 65KDa in plant # CAMB 11 and CAMB 1150 (Fig. 3B). However, negative control, i the protein extract of non-transformed plant as well protein extract of plant # CAMB 1113 did not give a signal for Cry1Ac toxin.

**Enzyme Linked Immunosorbent Assay (ELISA):** In ELIS the desired protein expressed in a target plant was detect by binding with specific polyclonal antibodies. Employi ELISA, it was seen that rabbit anti-Cry1A(c) antibod exclusively. recognized Cry1A(c) expressed in transform plants # CAMB 1148 and CAMB 1150. Whereas, reaction was observed with transformed plants # CA 1113, non-transformed plant and secondary antibody al (Fig. 4). ELISA reconfirmed our previous results of west blot analysis.

## Discussion

The use of Bt sprays had demonstrated their specificity safety, the few Bt crystal protein known at that t proved to be very active against certain import agronomic pests like rice yellow stem borer, *Scripoph incertulas* and leaf folder, *Cnaphalocrocis medinalis*, crystal proteins were encoded by single gene and disco programme indicated that Bt was an excellent sourc protein with improved and new pesticidal activities. Cry protein genes are becoming available to the gro (Krattiger, 1997). Preliminary biotoxicity assays for in resistance showed that drug selected plants expres *cry*1Ac genes had increased resistance to *C. medi* The only constraint in our biotoxicity assay was the growth of rice plants in green house. Comparative re as listed in Table 1 and 2, for the biological activit expressed Cry1Ac toxin in the rice plants suggest *cry*1Ac gene is a useful tool to control rice pest. Ad developmental response to the toxin present in transgenic plants was evident within 72 hrs of feeding 1) and this result is in agreement with previously publ by Ghareyazie *et al.*, 1997. In the transgenic rice harb Bt gene, the damage to the leaf area was also conside less as compared to control plants. Within assay, mor increased over time of exposure to the plants. CAMB showed a resistance upto 100 percent towards target This is consistent with our observation that Cry1Ac is toxic (has a lower $LC_{50}$) to rice leaf folder than other (Karim and Riazuddin, unpublished data). Polymerase

action analysis was further done to check the presence of y1Ac gene in the genome of selected plants. The results PCR amplification are documented in Fig. 1. Results owed an amplification of positive control, a smear in the AMB 1150, CAMB 115 and CAMB 1113 did not amplify. n amplified product of 656 bp in Fig. 2 (Lane 5) and 2 Kb Lane 10, clearly indicated the presence of cry1Ac gene the genome of CAMB 1150. Data was further confirmed doing ELISA. ELISA results (Fig. 4) showed the esence of Cry1Ac toxin in CAMB 1148 and CAMB 1150 ants. The results of proteins profile and western blot alysis of transgenic and control plants as mentioned in g. 3A and 3B. Western blot analysis indicated the esence of a single sharp band of 65 KDa protein in CAMB 148 and CAMB 1150. Biotoxicity assays, PCR nplification, ELISA, SDS-PAGE and western blot analysis s showed the integration and expression of desired gene the transformed CAMB 1148 and CAMB 1150 rice ants.

everal groups have introduce genes encoding Bt δ-dotoxin, or insecticidal fragments of δ-endotoxin at secticidal levels (for review, see Koziel *et al.*, 1993a). ne native gene from Bt encoding the δ-endotoxins are not pressed in rice. There are some confusions concerning e possible toxic effects on plant cells by protoxins and rthern blot analysis revealed crystal protein gene mRNA ecies which were too short to encode toxic proteins dang *et al.*, 1987. Barton *et al.*, 1987). It has also been eculated that the high A + T-content of the native dotoxin genes results in a number of fortuitous ocessing signals recognized by plant cell. These rtuitous processing signals could initiate polyadenylation, ron splicing, mRNA instability or, most likely by, some mbination of these. Expression of high levels of the δ-dotoxins has been effect in plant by introducing synthetic ne encoding δ-endotoxins or insecticidal fragments of δ-dotoxins (Koziel *et al.*, 1993b). These synthetic genes all ntain a higher G+C content than the native gene, thus moving the fortuitous processing signals. In this study, rice optimized synthetic gene encoding *cry*1Ac derived rm *Bacillus thuringiensis* var. *kurstaki* HD73 by using the ost preferred codon from rice for each amino acid (Murray *al.*, 1991) and increasing the G+C content from 37 ercent in the native gene to 45 percent in the synthetic ne (Altosaar *et al.*, 1996). In cotton, the modified genes so resulted in an increase in crystal protein levels with gh levels of cotton bollworm control (Perlak *et. al.*, 1990). the field, cotton leaves with a truncated modified *cry*1Ab ne should high levels of protection from pink bollworm, otton leaf perforator and beet army worm larvae (Wilson *al.*, 1992). Hundreds of transgenic plants must be nerated in order to select those lines which not only have e phenotype as encoded by the transgene but also have e expected agronomic performance in the field. Our data sed on molecular analysis and biotoxicity assays indicate t a number of independent transgenic lines are required select one line having enhanced resistance to rice ffolder.

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## References

Adang, M. J., E. Firoozabady, J. Klein, D. Deboer, V. Sekar, J. D. Kemp, E. Murray, T. A. Rocheleau, K. Rashka, G. Staffield, *et al.*, 1987. Application of a *Bacillus thuringiensis* crystal protein for insect control. In: Arntzen, C. J. and Ryan, C. (Eds.), Molecular strategies for crop protection, NY: Alan R. Liss, pp: 343-353.

Altosaar, I., R. Sardana and H. Kaplan. 1996. Synthetic Bt toxin genes for insect resistance and their rapid bioassay using maize endosperm suspension cultures. In: Rice genetics III. Proceedings of the 3rd International Rice Genetics symposium, 16-20 October, 1995, Manila, Philippines, IRRI, pp: 737-742.

Barton, K., H. Whiteley and N. S. Yang, 1987. *Bacillus thuringiensis* δ-endotoxin in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol., 85: 1103-1109.

Bennett, J., M. B. Cohen, S. K. Katiyar, B. Ghareyazie and G. S. Ghush, 1997. Enhancing insect resistance in rice through biotechnology. In: Carozzi, N. and Koziel, M. (eds.), Advances in insect control: the role of transgenic plants, pp: 75-93, Taylor and Francis.

Bietlot, H. P. L., I. Vishnubhatla, P. R. Carey, M. Pozsgay and H. Kaplan, 1990. Characterization of the cysteine residues and disulphade linkages in the protein crystal of *Bacillus thuringiensis*. J. Biochem., 267: 309-315.

Bradford, M. M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein using the principle of protein dye binding. Annal Biochem., 72: 248-254.

Bravo, A., K. Hendickx, S. Jansens and M. Peferoen, 1992a. Immunocytochemical analysis of specific binding of *Bcaillus thuringiensis* insecticidal crystal proteins to lepidoptera and coleoptera midgut membranes. J. Invert. Pathol., 60: 247-253.

Bravo, A., S. Jansens and M. Peferoen, 1992b. Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. J. Invert. Pathol., 60: 237-246.

Bull, D. C., U. S. House, J. R. Ables and R. K. Morrison, 1979. Selective methods for managing insect pests of cotton. J. Econ. Entomol., **78**: 841-846.

Cheng, X., R. Sardana, H. Kaplan and I. Altosaar, 1998. *Agrobacterium*-transformed rice plants expressing synthetic cry1A(b) and cry1A(c ) genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA. 95: 2767-2772.

Fischhoff, D. A., 1996. Insect-resistant crop plants. In: Persley, G. J. (Ed.), Biotechnology and Integrated Pest Management, pp: 214-227, CAB International, Wallingford, UK.

Gasser, C. S. and R. T. Fraley, 1989. Genetically engineered plants for crop improvement. Science (Washington, DC), 244: 1293-1299.

Georghiou, G. P.,1990. Overview of insecticide resistance. In: Green, M. B., Le-Baron, H. M. and Moberg, W. K. (eds.), Managing resistance to Agrochemicals, American Chemical Society, Washington, DC, USA, pp: 18-41.

Ghareyazie, B., F. Alinia, C. A. Menguito, L. G. Rubia, M. J. de Palma, A. E. Liwanag, M. B. Cohen, G. S. Khush, and J. Bennett. 1997. Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cry*IA(b) gene. Mol. Breeding, **3**: 401-414.

Hillbeck, A., M. Baumgartner, M. P. Fried and F. Bigler, 1998. Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). Environ. Entomol., 27: 480-487.

Islam, Z. and A. N. M. R. Karim, 1997. Leaf folder behavior of *Cnaphalocrocis medinalis* (Guenee) and Marasmaia patnalis Bradley, and the influence of rice leaf morphology on damage incidence. Crop Protection 16: 215-220.

Karim, S. and S. Riazuddin, 1999. Rice insect pests of Pakistan and their control: A lesson from past for sustainable future integrated pest management. Pak. J. Biol. Sci:, 2: 261-276.

Karim, S. and S. Riazuddin, 1997. *Bacillus thuringiensis* δ-endotoxins: Molecular mechanism of action and pest management. Proc. Pak. Acad. Sci., 34: 135-155.

Khush, G. S. 1995. Modern varieties-their real contribution to food supply and equity. GeoJournal, 35: 275-284.

Knowles, B. H., 1994. Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. Advances in insect Physiology, 24: 275-308.

Koziel, M. G., G. L. Beland, C. Bowman, N. B. Carozzi, R. Crenshaw, L. Crossland, J. Dwason, N. Desai, M. Hill, S. Kadwell, *et al.*, 1993a. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology, **11**: 194-200.

Koziel, M. G., N. B. Carozzi, T. C. Currier, G. W. Warren and S. V. Evola, 1993b. The insecticidal crystal proteins of *Bacillus thuringiensis*: past, present and future uses. Biotechnol.Genet. Engin. Rev., 11:171-228.

Krattiger, A. F., 1997. Insect resistance in crops: A case study of *Bacillus thuringiensis* (Bt) and its transfer to developing countries. ISAAA Briefs No. 2, ISAAA: Ithaca, NY, pp: 1-42.

Laemmli, U. K., 1971. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), **227**: 680-685.

Manthavan, S., P. M. Sudha and S. M. Pechimuthu, 1989. Effect of *Bacillus thuringiensis* on the midgut cells of Bombyx mori larvae: a histopathological and histochemical study. J. Invert. Pathol., 53: 217-227.

Metler, I. J. 1987. A simple and rapid method for mini preparation of DNA from tissue cultured plant cells. Plant Mol. Biol. Rept., **5**: 346-349.

Murray, E. E., T. Rocheleau, M. Eberle, C. Stock, V. Sekar, and M. Adang, 1991. Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. Plant Mol. Biol., 16: 1035-1050.

Pathak, M. D. and Z. R. Khan, 1994. Insect pests of pp: 33-36, IRRI, Philippines.

Peferoen, M., 1997. Insect control with transgenic p expressing *Bacillus thuringiensis* crystal proteins Advances in Insect control, the role of transgenic pl N. Carozzi and M. Koziel (eds.), pp: 21-48.

Perlak, F. J., R. W. Deaton, T. A. Armstrong, R. L. Fu S. S. Sims, J. T. Greenplate and D. A. Fischhoff, 1 Insect resistant cotton plants. Bio/Technology, **8**: 943.

Riazuddin, S., T. Husnain, E. Khan, S. Karim, F. Khanu Makhdoom and I. Altosaar, 1996. Transformati indica rice with Bt pesticidal genes. In: Rice genetic Proceedings of the 3rd International Rice Ger symposium, 16-20 October, 1995, Manila, Philipp IRRI, pp: 730-734.

Riley, J. R., D. R. Reynolds, A. D. Smith, A. S. Edwar X. Zhang, X. N. Cheng, H. K. Wang, J. Y. Cheng a P. Zhai, 1995. Observations of the autumn migrati the rice leaf roller *Cnaphalocrocis medinalis* (Lepidop Pyralidae) and other moths in eastern China. Bull. Res., 85: 397-414.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scha Higuchi, G. T. Horn, K. B. Mullis and H. A. Eh 1988. Primer-directed enzymetic amplification of with a thermostable DNA polymerase. Sci., 239: 491.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A laboratory manual (Cold S Harbor Laboratory, Cold Spring Harbor, N. Y.).

Sardana, R., S. Dukiandjiev, M. Giband, X. Cheng Cowan, C. Sauder and I. Altosaar, 1996. Constru and rapid testing of synthetic and modified toxin sequences *cry*1A(b and C) by expression in m endosperm culture. Plant Cell Reports, 15: 677-68

Shepard, B. M., Z. R. Khan, M. D. Pathak and E Heinrichs, 1991. Management of insect pest of ri Asia. In: handbook of Pest Management in agricul 2nd editions, D. Pimentel (ed.) CRC Press, pp: 225-

Stewart, C. N., J. M. Adang, J. N. All, P. L. Rayme Ramachandran and W. A. Parrott, 1996. Insect co and dosage effects in transgenic Canola containi synthetic *Bacillus thuringiensis cry*1Ac gene. P Physiol., 112: 115-120.

Toenniessen, G. H., 1996. Progress in Biotechnology 5th Annual meeting of National Rice Biotechno network, pp: 1-11, New Delhi, India.

Towbin, H., T. Staehelin and J. Gordon, 1 Electrophoretic transfer of proteins from polyacryla gels to nitrocellulose sheets: Procedures and applications. Proc. Natl. Acad. Sci., USA 76: 4 4354.

Wilson, F. D. H., H. M. Flint, W. R. Deaton, Fischhoff, F. J. Perlak, T. A. Armstrong, R. L. Fuc A. Berberich, N. J. Parks and B. R. Stapp, Resistance of cotton lines containing a B thuringiensis toxins to pink bollworm (Lepid Gelechiidae) and other insecst. J. Econ. Entom 1516-1521.