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

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Abstract
Transgenic rice plants obtained through the incorporation of Bt cry1Ac gene. Initially putative transformants were screened for 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 primers 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 fragment from the genomic DNA of putative transformants. Selection based on PCR and initial bioassays, two transgenic plants designated as CAMB 1148 and CAMB 1150 were further confirmed for toxin expression by ELISA, Western blot analysis and comparative biotoxicity assay. ELISA and Western blot were performed with polyclonal antibody to Cry1Ac toxins that showed the presence of active ingredient of Bt toxin in both plants. Biotoxicity assays also confirmed the stability and activity of Cry toxins in both transgenic rice plants, eventually aiming for homozygosity with enhanced resistance to rice leaf folder.

Introduction
*Bacillus thuringiensis* crystalline inclusion ranging in molecular weight from 27-140 KDa are protoxins that are proteolytically converted into smaller toxic polypeptide in larval midgut of Lepidoptera, Diptera, Coleoptera, lepidopters and other insects (Peferoen, 1997). Midgut processes the protoxin to form a stable 55-70 KDa toxin core (Bietlot et al., 1990). Activated toxins subsequently binds to epithelial cells lining causing lysis (Bravo et al., 1992a, 1992b). Electrophysiological and chemical evidence adjusts that the toxins generate pores in the cell membrane thus disturbing the osmotic balance (Knowles, 1994). Consequently, the larvae stops feeding and eventually dies (Manthaven et al., 1989). Commercial formulation of *Bacillus thuringiensis* containing the spore crystal 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 reports on transgenic plant production with expression of cry1Ac genes in plants that are highly toxic to certain lepidopterous pests, killing all of the larvae of target insects (Stewart et al., 1996), yet they have little or no toxicity to man, most beneficial insects and other non-target organisms (Hilbeck et al., 1998). The production of commercial crop varieties producing these pesticidal proteins is expected to reduce environmental pollution from synthetic insecticides, increase worker safety and improve grower profitability (Gasser and Fraley, 1989).

A loss of paddy yield due to direct insect damage is estimated to at least several billion dollars per year (Wyniaensen, 1996). Past successes in conventional breeding for resistance, the ability of plants to compensate for types of damage and biological control by natural enemies combine to limit many pests problems to a stable level (Khush, 1995). Most insects had developed resistance 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 cry1Aa, cry1Ab, cry1Ac and cry3A (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* (Gueneu) (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 cry1Ac gene in the rice genome is effective against rice leaffolder, *Cnaphalocrocis medinalis*.

Materials and Methods
Plasmid construct and Rice transformation: Putative transformed plants harboring cry1Ac, 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 Cry1Ac, Pin II and Hygromycin gene in the ratio of 1.5:1:5:1.0 exploiting biologic procedure of transformation as described by Riazuddin et al. (1996). The construct was described by Altosan 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 (Cnaphalocrosis 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 Cnaphalocrosis 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 per a formula as given below.

\[
\% \text{ of leaf area consumed} = \frac{\text{leaf area consumed by larvae}}{\text{Total leaf area}} \times 100
\]

\[
\text{Larval growth retarded} = \frac{\text{Larval weight}(\text{cont}) - \text{Larval weight}(\text{trans})}{\text{Larval weight}(\text{cont})} \times 100
\]

DNA isolation and agarose gel electrophoresis: Isolation of plant DNA was carried out following the modified method of Metter (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 13 and 3′ GTC CTT ACC GAG TGA AGA TGT AA 5′ for amplification of a 656bp fragment from the cry1Ac 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 Cry1Ac 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 of 31 cycles each, 95 °C for 1 minute (denaturing), 52 °C for 2 minutes (annealing) and 72 °C for 1 minute (extension). Last cycle of three more steps, 95 °C for 1 minute (denaturing), 52 °C for 2 minutes (annealing) and 95 °C for 10 minutes (extension). PCR products were then analyzed by agarose gel electrophoresis.

Extraction of proteins from rice plant: Leaves of non-transformed and transformed plants (plant # CAMB1148, CAMB 1148, and CAMB 1150) were washed with deionized sterilized water and then dried. The leaves from four plants were weighed separately (200-300 mg each) and then were ground in liquid Nitrogen. NE buffer was added in each ground material (50 ul NE buffer 200mg leaves immediately before thawing. This material was kept at 4 °C for 24 hours and then centrifuged at 12K at 4 °C for 5 minutes. Protein concentration was determined using dye-binding method (Bradford, 1976). Supernatants were collected and stored at -20 °C for further use in molecular analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE (10%) was done using a modified procedure (Laemmeli, 1971). Plant proteins were mixed in 1:1 ratio with 2X denaturing sample buffer and heated for 5 minutes in boiling water bath. Plant extracts were electrophoresed in BIO-RAD minigel apparatus using a constant current (20mA). After completing electrophoresis, the gel was stained with Coomassie brilliant blue R-250 for two hours and destained in Destaining solution.

![Fig. 1: Insect bioassay of Cry transformed rice plants to rice leaf folder, (a) Control plant, happy CAMB 1148 plant showing resistance and CAMB 1150 also showed resistance.](Image)
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 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: Entomocidal 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 (Cnaphalocrosis medialis). 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 Cnaphalocrosis medialis 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 folder (C. medialis).

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

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

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

<table>
<thead>
<tr>
<th>Plant No.</th>
<th>% Damage</th>
<th>% Mortality Growth retardation(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMB 1150</td>
<td>69</td>
<td>60</td>
</tr>
<tr>
<td>CAMB 1113</td>
<td>23</td>
<td>75</td>
</tr>
<tr>
<td>CAMB 115</td>
<td>53</td>
<td>20</td>
</tr>
<tr>
<td>CAMB 1148</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>65</td>
<td>9</td>
</tr>
</tbody>
</table>

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 cry1Ac 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 1115, and # CAMB 1113 did not amplify. To confirm genomic DNA of # CAMB 1150, DNA of # CAMB 1150 was digested with restriction endonuclease, HindIII and amplified it with two sets of primers one cry1Ac and other any cry1Ac with ubiquitin promoter and terminator. The amplification product of 656 bp in (Fig. 2) lane 5 and 2kb in lane 1 clearly showed that cry1Ac gene has been integrated the genome of # CAMB 1150.

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

Western Blot Analysis: Western blot analysis indicated presence of single band of 65KDa in plant # CAMB 1148 and CAMB 1150 (Fig. 3B). However, negative control, if the protein extract of non-transformed plant as well as protein extract of plant # CAMB 1113 did not give a signal for Cry1Ac toxin.

Enzyme Linked Immunosorbent Assay (ELISA): In ELISA, the desired protein expressed in a target plant was detected by binding with specific polyclonal antibodies. Employed ELISA, it was seen that rabbit anti-Cry1Ac antibody exclusively, recognized Cry1Ac expressed in transform plant # CAMB 1148 and CAMB 1150. Whereas, no reaction was observed with transformed plant #: CAMB 1113, non-transformed plant and secondary antibody alone (Fig. 4). ELISA reconfirmed our previous results of western blot analysis.

Discussion

The use of Bt sprays had demonstrated their specificity on safety. the few Bt crystal protein known at that proved to be very active against certain important agronomic pests like rice yellow stem borer, Scirpophaga incertulas and leaf folder, Cnaphalocrosis medialis; crystal proteins were encoded by single gene and diverse programme indicated that Bt was an excellent source for protein with improved and new pesticidal activities. Cry protein genes are becoming available to the gene (Krattiger, 1997). Preliminary biotoxicity assays for resistance showed that drug selected plants expressed cry1Ac genes had increased resistance to C. medialis. The only constraint in our biotoxicity assay was the growth of rice plants in green house. Comparative results as listed in Table 1 and 2, for the biological activity expressed Cry1Ac toxin in the rice plants shows cry1Ac gene is a useful tool to control rice pest. An developmental response to the toxin present in transgenic plants was evident within 72 hrs of feeding and this result is in agreement with previously published by Ghareyaze et al., 1997. In the transgenic rice harboring Bt gene, the damage to the leaf area was also considerably less as compared to control plants. Within assay, mortality increased over time of exposure to the plants. CAMB showed a resistance up to 100 percent towards target. This is consistent with our observation that Cry1Ac is toxic (has a lower LC50) to rice leaf folder than other (Karim and Riazuddin, unpublished data). Polymorphism.

action analysis was further done to check the presence of γ1Ac gene in the genome of selected plants. The results showed an amplification of positive control, a smear in the CAMB 1150. CAMB 115 and CAMB 1113 did not amplify. An amplified product of 656 bp in Fig. 2 Lane 5) and 2 Kb Lane 10, clearly indicated the presence of cys1Ac gene in the genome of CAMB 1150. Data was further confirmed using ELISA. ELISA results (Fig. 4) showed the presence of Crys1Ac toxin in CAMB 1148 and CAMB 1150 rice plants. The results of proteins profile and western blot analysis of transgenic and control plants as mentioned in Fig 3A and 3B. Western blot analysis indicated the presence of a single sharp band of 66 KDa protein in CAMB 1148 and CAMB 1150. Biototoxicity assays, PCR amplification, ELISA, SDS-PAGE and western blot analysis showed the integration and expression of desired gene in the transformed CAMB 1148 and CAMB 1150 rice plants.

Several groups have introduced genes encoding Bt δ-endotoxin, or insecticidal fragments of δ-endotoxin at selectable levels (for review, see Koziel et al., 1993a). The native gene from Bt encoding the δ-endotoxins are not expressed in rice. There are some confusions concerning the possible toxic effects on plant cells by proteases and chitinase. Western blot analysis revealed crystal protein gene mRNA species which were too short to code toxic proteins (Huang et al., 1987. Barton et al., 1987). It has also been calculated that the high A + T-content of the native toxin genes results in a number of fortuitous processing signals recognized by plant cell. These putative processing signals could initiate polyadenylation, mRNA splicing, mRNA instability or, most likely by, some combination of these. Expression of high levels of the δ-toxins has been effect in plant by introducing synthetic genes encoding δ-endotoxins or insecticidal fragments of δ-toxins (Koziel et al., 1993b). These synthetic genes all contain a higher G+C content than the native gene, thus reducing the fortuitous processing signals. In this study, rice optimized synthetic gene encoding cry1Ac derived from Bacillus thuringiensis var. kurstaki HD73 by using the best preferred codon from rice for each amino acid (Murray et al., 1991) and increasing the G+C content from 37% in the native gene to 45% in the synthetic gene (Altosaar et al., 1996). In cotton, the modified genes were expressed in an increase in crystal protein levels with high levels of cotton bollworm control (Perlak et al., 1990). In the field, cotton leaves with a truncated modified cry1Ab gene having high levels of protection from pink bollworm, cotton leaf perforator and beet army worm larvae (Wilson et al., 1992). Hundreds of transgenic plants must be treated in order to select those lines which not only have a phenotype as encoded by the transgene but also have expected agronomic performance in the field. Our data from molecular analysis and biotoxicity assays indicates a number of independent transgenic lines are required to select one line having enhanced resistance to rice folders.

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References


