Comparative Toxicity of Chimeric Cry2AX1 Bt Protein Isolated from Recombinant Bt and E. coil Hosts against Rice Leaf Folder (Cnaphalocrosis medinalis)

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ABSTRACT

A novel chimeric protein of Bacillus thuringiensis was expressed in two different hosts viz., Bt (4Q7) and E. coli (BL21). The Cry2AX1 protein was expressed in recombinant Bt and E. coli cells harboring cry2AX1 gene driven by cry2Aa and T7 promoter, respectively. The recombinant Cry2AX1 protein from Bt and E. coli at 13.33 and 20 ng/µl showed mortality of rice leaf folder (RLF) 86.70 and 56.67 per cent, respectively. These results indicated that the Cry2AX1 protein isolated from Bt was more toxic than that of E. coli, which could be probably due to variation in organization of protein folding between the two hosts. Since the Cry2AX1 protein is effective against RLF even at a very low concentration, further efforts can be made for developing transgenic rice with cry2AX1 gene to import insect resistance.

Keywords cry2AX1, Rice leaf folder, Insect resistance, Bacillus thuringiensis.

Rice (Oryza sativa) is the most widely consumed food crop worldwide. Increased rice production by 60 % in 2025 is must to meet the needs of the growing population (Khus, 1997). Rice productivity is adversely obstructed by several biotic and abiotic factors. Among biotic stresses, insect pests continue to be a major threat for increased rice production. Rice leaf folder (Cnaphalocrosis medinalis and Marasmia patnali) is one among the major pests of rice that causes severe yield loss in most rice producing countries. Effective control of these pests has mainly depend on the use of chemical insecticides, leading to substantial environmental pollution, damage to the ecosystem and increase the cost of pro-duction. Producing insect resistant varieties are the most economical and effective approach to protect rice from insect attack (Wu, et al., 1997).

Bacillus thuringiensis is a spore-forming gram-positive bacterium produces the intracellular insecticidal crystal proteins (Cry proteins) as inclusions during sporulation stage. These proteins are toxic to insect larvae of different orders Lepidoptera, Diptera, and Coleoptera (Beegle, and Yamamoto, 1992). These endotoxins are actually protoxins activated by proteolytic cleavage in the insect mid gut after ingestion. The activated toxins destroy mid gut epithelial cells, killing sensitive insects within a day or two of ingestion (Bietlot, 1990 and Federici, 2007). Formulations of B. thuringiensis have been used as one of the most successful biological agents in agriculture control insect pests. As a good alternative to synthetic insecticides, these formulations are safe to the user and environment. With the development of plant genetic engineering, genes expressing Bt insecticidal crystal protein can be introduced into plants for insect control. Transgenic plants expressing Bt genes will be more effective on insect pest than Bt formulations (Chen, et al., 2005). Bt genes were first introduced and expressed in tobacco (Barton, 1987 and Vaeck, 1987) and tomato (Fischhoff, 1987). Since then, development of insect-resistant crops containing Bt genes are rapid.

The first transgenic rice plant with insect resistant Bt toxin was reported by Fujimoto, et al., 1993. Since then, considerable research efforts have been invested to introduce insecticidal crystal protein genes into rice by transgenic approaches. The predominantly used Bt gene in rice are cry1A genes (Cheng, et al., 1998, Shu, et al., 2000, Tu, et al., 2000 and Ye, et al., 2001). However, other classes of Bt genes (cry2A, cry1C and cry9C) have also been reported in Bt rice (Alcantara, et al., 2004). The Bt gene cry2A produces crystal protein that is active against both lepidopteran and dipteran insects. Besides it also has unique binding sites in the mid gut of targeting insects as compared to other cry toxins (Lee, et al., 1997). The difference in the structural and insecticidal mechanism of Cry2A protein leads it as a promising candidate for insect resistance management (Morse, et al., 2001). Insect bioassay of Cry2A protein confirmed its toxicity to the lepidopteran pests of rice includes yellow stem borer, striped stem borer, and rice leaf folder when these insects fed the toxin in an artificial diet (Karim, and Dean, 2000). Transgenic rice plant containing cry2A gene has also been reported to exhibit toxicity towards rice leaf folder (Maqbool, et al., 1998, Karim, and Dean, 2000, Bashir, et al., 2004 and Chen, et al., 2005). In this study a novel chimeric Bt
gene cry2AX1 consisting of sequences from cry2Aa and cry2Ac (Udayasuriyan, et al., 2010) cloned in an acrystalliferous Bt strain 4Q7 and in E. coli BL21 were expressed. The recombinant Cry2AX1 protein derived from Bt and E. coli were tested for toxicity against rice leaf folder.

MATERIALS AND METHODS

Plasmids, bacterial strains and culture conditions:

Acrystalliferous B. thuringiensis strain 4Q7 harboring pHT2AX (pHT3101-cry2AX1) driven by cry2Aa promoter and E. coli strain BL21 harboring pET2AX (pET28a-cry2AX1) driven by T7 promoter were obtained from Bt lab, Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore. The antibiotic concentration used for culturing of recombinant Bt and E. coli was 50µg of erythromycin and 100µg of kanamycin per ml, respectively. B. thuringiensis culture was grown on T3 medium (Martin, and Travers, 1989) at 30ºC at 200 rpm for 3 days and the bacterial sporulation was monitored through phase contrast microscope for 3 days. E. coli was grown on LB medium for 24 hours at 37ºC.

Isolation of recombinant Cry2AX1 protein:

The Cry2AX1 protein expressed in recombinant Bt was isolated from 20ml of sporulated broth containing more than 90 % of lysed cells as described by Lenin, et al., (2001). Sporulated broth culture was transferred to 4ºC, at least half-an-hour before harvesting and centrifuged for 10 min at 10000 rpm at 4ºC. The pellet was washed once with 20 ml of ice cold Tris-EDTA buffer [Tris 10 mM, EDTA 1mM, pH 8.0 with 1mM phenyl methyl sulphonyl fluoride (PMSF)], once with 20ml of ice-cold 0.5 M NaCl followed by two more washes with 20 ml of Tris-EDTA buffer with 1 mM PMSF by centrifuging at the same speed and time. Finally, the spore-crystal pellet was suspended in 500 µl of sterile distilled water containing 1 mM PMSF and stored at -20ºC.

On the other hand, the Cry2AX1 protein expressed in E. coli BL21 harboring pET2AX was isolated as followed by Shantanu Kumar, et al., 2004. E. coli, BL21 harboring pET2AX grown in 5 ml of LB broth at 37ºC overnight and used as mother culture. One per cent of mother cultures (250 µl) was used for inoculating 25 ml LB broth and allowed to grow at 37ºC until OD600 reaches ~0.6, then the culture was induced with 1mM Isopropyl â-D-thiogalactopyranoside (IPTG) for 6 hrs at 30ºC on a shaker maintained at 225 rpm. Cells were harvested by centrifugation, washed once in 1X TE buffer and then the pellet was suspended in 20 ml TE buffer containing 1 mM phenyl methyl sulfonyl fluoride (PMSF). E. coli cells were sonicated by ultra-sonic liquid processor (Sonic and Material, Inc., USA) till more than 90 per cent of cells were broken. Sonication was carried out at 20 amplitude for 60 seconds, 4 times with a time interval of 1 min. Broken cells were centrifuged at 7000 rpm for 15 min at 4ºC. The pellet was suspended in the TE buffer containing 0.1 per cent Triton X-100 and washed twice in the same buffer. Finally, the pellet was dissolved in 200 µl of sterile double

Fig. 1. SDS–PAGE analysis of Cry2AX1 protein isolated from recombinant strains of Bt and E. coli. M Genei Protein marker (Higher Range #105977), Lane 1 Cry2AX1 protein from recombinant Bt. Lane 2 Inclusion protein from acrystalliferous strain of Bt 4Q7; Lane 3 inclusion protein from BL21-pET28a; Lane 4 Cry2AX1 protein from recombinant E. coli BL21.
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distilled water containing 2 mM PMSF. Protein was isolated from acrystalliferous strain of Bt 4Q7 and BL21 carrying empty vector pHT3101 and pET28a to serve as control. An aliquot of Bt and E. coli Cry2AX1 protein were analyzed on SDS-PAGE and concentration of the protein was estimated by densitometry scanning method in comparison with the known concentration of bovine serum albumin (BSA) band.

Insect Bioassay:

Adults of rice leaf folder were (C. medinalis) collected from the rice field at Paddy Breeding Station, TNAU, Coimbatore and they were released on the TN1 rice plants maintained in insect cage for culturing. After two generations the neonates of C. medinalis larvae were used for the bioassay. Insect bioassay was carried out to determine the efficacy of Cry2AX1 protein against rice leaf folder by leaf coating method. Healthy leaves of uniform diameter (about 1cm) from rice plants (ASD16) grown in greenhouse were cut into pieces of 5cm length. They were kept in water to prevent drying. The leaf bits were washed with 0.02% triton x-100 solution followed by rinsing with sterile distilled water and blot dried. Thirty micro liter of Cry2AX1 protein of desired concentration was coated on the upper surface of the 5 cm² leaf-bits using glass rod and they were air dried. Three treated leaf-bits were placed in a petri dish containing moist filter paper to maintain turgidity of leaves. Ten freshly hatched neonates of rice leaf folder were released to each petri dish. The leaf-bits coated with sterile distilled water and with 0.02 % Triton x-100 were used as controls. The experiment was carried out at 25 ± 1°C, 80 % RH and three replications were maintained. Larval mortality was recorded for 5 days.

RESULTS AND DISCUSSION

Rice leaf folder is a major lepidopteran pest causing severe loss in most rice producing countries. Among the Bt genes, cry1Ab, cry1Ac and a fusion gene cry1Ab/1Ac are the most commonly used in transgenic crops including rice for protection against lepidopteran pests. In addition, cry1C, cry2A and cry9C have also been reported to impart resistance towards rice lepidopteran pest (Sikha, and Sharmistha, 2010). Use of novel Cry protein with unrelated target sites and modes of action may have vast significance in their toxicity.

The Bt gene cry2A has limited homology to other cry genes (Hermann, and Whiteley, 1989), which gives difference in protein structure (Morse, et al., 2001) and insecticidal action (English, et al., 1994) and hence Cry2A proteins are promising candidates for management of resistance-development in insects against the first generation Bt-gene such as cry1Ab or cry1Ac. The insects that were resistant to both Cry1Ac and Cry1Ab toxins were susceptible to Cry2Ab toxin. Hence, the combination of Cry1Ac and Cry2Ab is used in the second version of Bt-cotton, Bollgard II (Perlak, et al., 2001). However, the Cry2Ab is relatively less toxic than Cry1Ac against the H. armigera (Qiong, et al., 2013). Variation of a single amino acid residue at certain positions of Cry proteins can remarkably influence the level of toxicity (Udayasuriyan, et al., 1994 and Rajamohan, et al., 1996). Therefore, efforts were made to improve the level of toxicity of Cry2A by constructing a chimeric cry2AX1 gene consisting sequences of cry2Aa and cry2Ac genes which were isolated from two different indigenous Bt isolates. The chimeric Cry2AX1 protein is found to be more effective against H. armigera than Cry2Aa, Cry2Ab and Cry2Ac in artificial

![Fig. 2. Coated leaf bioassay with Cry2AX1 protein isolated from Bt and E. coli against rice leaf folder a, b & c Comparison of feeding by rice leaf folder on leaf bits coated with Bt-Cry2AX1, E. coli-Cry2AX1 and 0.02 % Triton X 100 (control). d, e & f Microscope view of survivors from Cry2AX1 treated and control, g, h & i Size of the surviving larvae from Cry2AX1 treated and control.](image-url)
Table 1. Toxicity of Cry2AX1 protein against rice leaf folder

<table>
<thead>
<tr>
<th>S. No</th>
<th>Treatment</th>
<th>Concentration of Cry2AX1 protein ng/µl</th>
<th>Mortality (%) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cry2AX1 from Bt</td>
<td>13.33 ng/µl</td>
<td>86.70 ± 4.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.67 ng/µl</td>
<td>70.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.33 ng/µl</td>
<td>46.67 ± 4.71</td>
</tr>
<tr>
<td>2</td>
<td>Cry2AX1 from E. coli</td>
<td>20 ng/µl</td>
<td>56.67 ± 4.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ng/µl</td>
<td>36.67 ± 4.71</td>
</tr>
<tr>
<td>3</td>
<td>Control (Triton X 0.02 %)</td>
<td>--</td>
<td>00</td>
</tr>
<tr>
<td>4</td>
<td>Control (Distilled water)</td>
<td>--</td>
<td>00</td>
</tr>
</tbody>
</table>

diet based bioassay (Udayasuriyan, et al., 2010).

In the present study, recombinant strain of Bt and E. coli harboring cry2AX1 gene were confirmed by PCR. In SDS-PAGE analysis recombinant Cry2AX1 proteins harvested upon expression in Bt and E. coli showed a molecular mass of ~65kDa, whereas no prominent band of 65kDa was obtained in control lanes (Fig. 1). The Cry2AX1 protein expressed in both Bt and E. coli were isolated in micro-inclusion form. Based on the densitometry quantification of CBB (Coomassie brilliant blue) stained protein in comparison with BSA, concentration of Cry2AX1 was determined to use in the insect bioassay. The recombinant Bt-Cry2AX1 protein at the concentration of 13.33, 6.60 and 3.33 ng/µl recorded 86.70, 70 and 46.67 per cent mortality, respectively in rice leaf folder. Whereas, the recombinant E. coli-Cry2AX1 protein showed 56.67 and 36.67 per cent mortality in RLF at the concentration of 20 and 10ng/µl, respectively. No mortality has been observed in control leaf bits coated with water as well as with triton X 100 (Table 1). During the bioassay period, significant tissue damages and normal development of larvae were seen in leaf bits coated with water as well as triton x 100. On the other hand, the leaf bits coated with Cry2AX1 protein showed very less damage and surviving larvae showed severe growth inhibition (Fig. 2). The differences in the toxicity of Cry2AX1 protein expressed in E. coli and Bt system may be due to the differences in organization of protein folding property. Earlier, Donovan, et al., (1988) also found that CryIAA-transformed B. megaterium was much more toxic to H. virescens and Lymantria dispar (L.) than the E. coli expressed CryIIA, this variation in toxicity was due to changes in the protein structure, or other components associated with B. megaterium. In addition, suitable physicochemical conditions are required in the bacterial cells to ensure correct assembly of the Cry protein molecules to form insect gut- soluble and active inclusion for the toxicity against pest (Du, et al., 1994).

In the present study, the RLF larval mortality and reduced leaf tissue damage indicates the potential of Cry2AX1 protein towards leaf folder larvae even at the concentration of 3.3ng/µl. More potent Bt proteins will be effective even at a lower level of expression in transgenic plants. This leads to the suggestion that deployment of transgenic rice with cry2AX1 will be an effective tool in the control and management of rice leaf folder populations. In general, the native prokaryotic Bt genes express poorly in eukaryotic plants. Modification of coding sequences to match codon preferences in plants is essential for higher level expression of Bt gene (Perlak, et al., 2001). Earlier workers have achieved high level expression of Cry2A protein in transgenic rice ranging from 1 to 10µg/g fresh weight. Hence, codon optimization of cry2AX1 gene is essential to develop a transgenic line with high level of insect resistance.

In addition, managing evolution of resistance in target insect is a challenge in adapting this technology. Developing transgenic lines with different toxin combinations which recognize unique binding site are useful in implementing strategies that decrease the rate of pest adaptation (Lee, et al., 1997). Studies showed that cry1Ac with cry2A are good toxin combination for lepidopteran insects (Fiuza, et al., 1996 and Chen, et al., 2005). From this study the potency of cry2AX1 against rice leaf folders have been revealed and hence it can be used alone or in combination with any other Cry toxin to delay the development of resistance in insects. In conclusion, the chimeric cry2AX1 is proved to be effective against rice leaf folder. Further efforts to make synthetic cry2AX1 gene with plant preferred codon and development of transgenic rice line will be helpful for insect resistance management in rice.
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LITERATURE CITED


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