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CLONING OF *cadherin* GENE INTO PLANT BINARY VECTOR AND RETRANSFORMATION INTO *cry1Ac* TRANSGENIC TOBACCO

K. MOMENA[1], P.A. KUMAR[2], H. MEHRAJ[3] AND A.F.M. JAMAL UDDIN[3]*

[1]Advanced Seed Research and Biotech Center, ACI Limited, Dhaka, Bangladesh; [2]National Research Center on Plant Biotechnology, IARI, New Delhi, India; [3]Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh.

*Corresponding author & address: A.F.M. Jamal Uddin, E-mail: jamal4@yahoo.com
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ABSTRACT

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Cadherin belongs to the families of animal glycoproteins which is responsible for calcium-dependent cell-cell adhesion. Recent literature showed that the cadherin like in mid gut of several insects served as the receptor of the Bt toxin *cry1A* and the variation of cadherin-like is related to insect resistance to *cry1A*. The interaction between *Bacillus thuringiensis* insecticidal crystal protein *cry1A* and cadherin receptor in Lepidopteran insects induces toxin oligomerization. In this study to prove this hypothesis that *Helicoverpa armigera* cadherin fragment (*HaCad1*) containing its toxin binding region was cloned into binary vector and retransformation has done into the *cry1Ac* plants through *Agrobacterium* mediated plant transformation. Insect bioassay was carried out to compare the toxicity enhancement between *cry1Ac* and *cry1Ac* with *HaCad1* transgenic plants. Based on this insect bioassay, retransformed plants were showed equal resistant to the *cry1Ac* transgenic plants; it may be the reason for protein expression level of transgenic events. Development of more transgenic events with high protein expression level will be proven the hypothesis of enhancement of insecticidal activity of *cry1Ac* with cadherin.

Key words: *Helicoverpa armigera, Bt-receptor, cadherin, cry1Ac, insecticidal crystal protein, toxicity enhancement, oligomer formation*

INTRODUCTION

Biological pesticides based on *Bacillus thuringiensis* (Bt), can provide a valuable alternative to synthetic insecticides that suffer some disadvantages related to environmental damage and health hazards. Biotechnology is rapidly producing a suite of new corps with enhanced insect resistance. *Bacillus thuringiensis* (Bt) is a Gram-positive bacterium which produces insecticidal crystal proteins. Insecticidal proteins of *Bacillus thuringiensis* have been used for developing insect resistance in plants. These insecticidal crystal proteins (ICPs) display insect specific toxicity and harmless to human and non target animals. Consequently they have been widely used in biopesticides and transgenic plants to control agricultural pests (Schnepf *et al.* 1998; Pigott and Ellar, 2007). The development of resistance to *Bacillus thuringiensis* toxin is now considered the major threat to the long-term effectiveness of *Bacillus thuringiensis* products (Bravo and Soberon, 2008). *Helicoverpa armigera* is a serious pest of cotton, chickpea and many other crops and it has already become resistant to many kinds of chemical pesticides (Wu and Guo, 2005). Cadherin belongs to one of the families of animal glycoproteins responsible for calcium dependent cell-cell adhesion. They are dependent on calcium (Ca^{2+}) ions to function, hence their name. The variation of cadherin is related to insects' resistance to *cry1A* (Wang *et al.* 2005). The interaction between *Bacillus thuringiensis* insecticidal crystal protein (ICPs) *cry1A* and cadherin receptors in lepidopteran insects induces toxin oligomerization, which is essential for membrane insertion and mediates *cry1A* toxicity. In this study cadherin receptor binding portion was cloned into binary vector and retransformed to *cry1Ac* transgenic tobacco plants. Molecular analysis and insect bioassay was carried out to check the expression and enhancement of *cry1Ac* toxicity.

MATERIALS AND METHODS

Experiment was conducted at Bt Laboratory of National Research Centre on Plant Biotechnology (NRCPB), Indian Agricultural Research Institute (IARI), New Delhi, India.

Designing of primers for amplification of *HaCad1* from *Helicoverpa armigera*: Based on published *HaCad1* (Genebank no.DQ973295 - Peng *et al*. 2009) gene sequence, a set of specific primers were used to amplify the receptor binding portion of cadherin from the cDNA sample of *Helicoverpa armigera* midgut portion.

Insect total RNA isolation: Total RNA was isolated from the mid gut portion of the 4th instar larvae of the *Helicoverpa armigera*. RNA isolation was done by using TRizol reagent (Invitrogen). Quality and quantity of the total RNA was checked by gel electrophoresis and Nanodrop1000 spectrophotometer reading respectively.

cDNA synthesis from the total RNA: Single stranded cDNA was prepared by using AffinityScript QPCR cDNA Synthesis Kit. (Stratagene). The following reaction components were added in microcentrifuge tube in order:

RNase-free H_2O	5.0 µl
Total RNA (1 µg)	1.0 µl
First strand master mix (2×)	10.0 µl
Oligo (dT) primer (0.1 µg/µl)	3.0 µl
AffinityScript RT/RNase Block enzyme mixture	1.0 µl

The above reaction was kept in thermal cycler and followed the condition according to the manufacturer's instruction. After cDNA synthesis the quality and quantity of the cDNA was checked on 1.2% agarose gel and Nanodrop respectively.

Amplification of the *cadherin* gene fragment by PCR: Gradient PCR was performed to find out the annealing temperature for amplification of *cadherin* receptor region at 50-60ºC by using Taq DNA polymerase enzyme (Bangalore Genei). Pfu DNA polymerase was used to amplify the final product and avoid the mismatch of the nucleotide sequences. Amplification was performed in an eppendorf thermal cycler (Eppendorf AG, Germany).

Analysis and purification of PCR products: After amplification of cDNA, it was checked in 1% agarose gel electrophoresis and purified the cDNA using QIAquick PCR purification kit (QIAGEN, Germany).

Cloning of PCR amplified *cadherin* fragment into CaMV35S cassette: 732bp *cadherin* gene fragment was purified and restricted with *Bam*HI and *Sac*I then ligated with corresponding sites of CaMV35S cassette under 35S promoter and 35S terminator. The clone was further confirmed by multiple restriction digestion analysis. Routine laboratory protocols were followed for ligation, transformation and screening the transformants (Fig. 1).

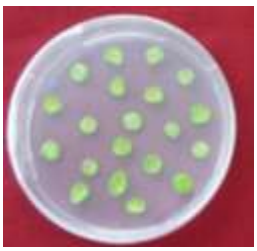
Cloning of *cadherin* cassette into binary vector pGreenII0000-35S-*HPT* cassette: 1407bp *cadherin* cassette was restricted out from CaMV35S-*cadherin* cassette using *Eco*RV site and ligated into *Sma*I site of pGreenII000+35S *hpt*II cassette. 1407bp of *cadherin* cassette was inserting out with *Eco*RV and 5160bp pGreen-35S *hpt*II cassette was restricted with *Sma*I then eluted the respective fragments and purified by using QIAGEN gel extraction kit, ligation was done with the vector and insert based on molar ratio. The clone was further confirmed by multiple restriction digestion analysis. Routine laboratory protocols were followed for ligation, transformation and screening the transformants (Fig. 2).

Mobilization of binary plasmid into *Agrobacterium tumefaciens* strain *EHA*105 by electroporation: Electroporation was used to mobilize the binary plasmid into *Agrobacterium* strain EHA105. 500ng-1.0µg of binary vector pGreen-*hptII* and *cadherin* cassette and helper plasmid pSoup was added to the electrocompetent cells, total mixture was transferred to a pre-chilled electroporation cuvette (Eppendorf). Electroporation was performed as per the recommendation of the manufacturer (Eppendorf AG, Germany). The *Agrobacterium* colony harbouring the binary plasmid was confirmed by colony PCR with *hpt*II and *cadherin* specific primers (Fig. 3).

***Agrobacterium* mediated genetic retransformation of binary plasmid into *cry1Ac* transgenic tobacco**

Co-cultivation: Leaf disc was cut from six independent tissue culture grown *cry1Ac* tobacco plants (from Bt Laboratory of NRCPB, IARI New Delhi,) Cry1AC and incubated with brush border membrane vesicles (BBMV) (Hofmann *et al.* 1988) for two days in pre-culture medium. After two days infection was done to the pre-cultured leaf disc and kept in the MS medium containing 2.0 mg/l BAP and 0.1 mg/l NAA and it was kept for co-cultivation for two days in the dark.

Selection and regeneration: After two days the explants were shifted to the selection medium [BAP (2 mg/l), NAA (0.1 mg/l), Cephotoxime (500 mg/l), Hygromycin (50 mg/l)]. Within 5-6 weeks small shoots were obtained from the callus, excised and transferred to the rooting medium containing Cephotoxime (500 mg/l) and Hygromycin (50 mg/l). The putative transgenic plants were maintained for the molecular analysis and bioassay.



Pre-incubation

Callus & Shoot induction 50mg/l Hygromycin and 500mg/l Cephotaxime

Putative transgenic plantlet

Plate 1. Pre-incubation, Callus & Shoot induction and transgenic plantlet of tobacco

Molecular analysis of putative transgenic plants

Isolation of genomic DNA and PCR analysis: Total genomic DNA was isolated from retransformed putative transgenic tobacco plants using CTAB method (Doyle and Doyle, 1987) and it was analysed by 0.8% agarose gel for checking. PCR analysis was done in the retransformed putative transgenic plants by using *nptII*, *hptII*, *cry1Ac* and *HaCad* gene specific primers to detect the presence of transgene. The PCR reaction was performed in eppendorf thermal cycler (Eppendorf AG, Germany) with 94^{o}C for 5 min, followed by 30 cycles 94^{o}C for 1min, Annealing temperature for *nptII*; *cry1Ac* is 58ºC and *hptII*; *HaCad* 55ºC and 72^{o}C for 1min and 72^{o}C for10min final extension. The PCR samples were analysed in 1% agarose gel electrophoresis (Fig. 4a and 4b).

Antibiotic assay for transgenic leaf disc: To analyse and confirm the integration of plant selectable marker gene, antibiotic assay was performed with all transgenic lines. Leaf discs from transgenic plants were kept in MS media containing kanamycin 100mg/l and hygromycin 50mg/l. Callus induction was observed after 10-15 days. Experiment was performed with respective controls (Fig. 5).

Insect bioassay of putative transgenic tobacco plants: To study the expression and toxin enhancement of retransformed of *cry1Ac* transgenic tobacco plants was performed by insect bioassay. It was done in triplicate and mortality of the larvae and leaf area consumed were served after 3days with respective control plants of transgenic *cry1Ac* and wild type (Fig. 6).

RESULTS

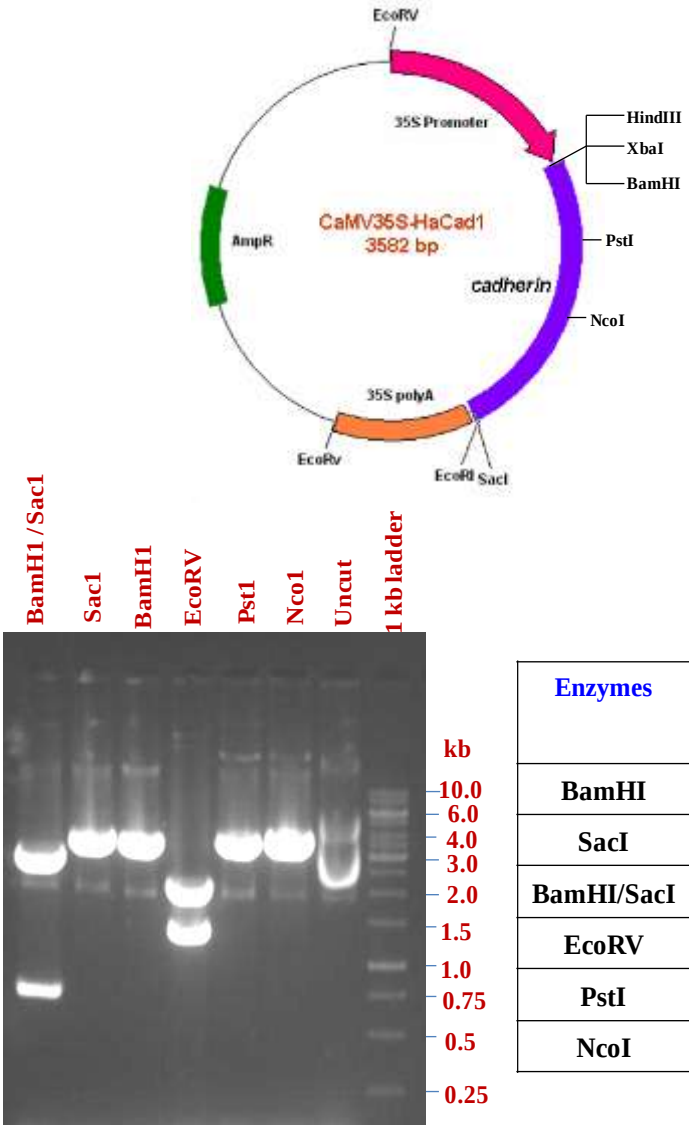
Cloning of c*adherin* gene fragment into CaMV35S cassette and plant transformation vector: Total RNA was isolated from the mid gut portion of *Helicoverpa* larvae then cDNA was synthesised from the total RNA using Affinity Script QPCR cDNA Synthesis Kit (Stratagene). A set of gene specific primers were designed based on published *HaCad1* (Gene bank no. DQ973295) and then obtained the 732bp gene fragment. In order to clone the gene, the PCR product was gel eluted and purified using QIAGEN gel extraction kit. The purified product was restrict with *Bam*HI and *Sac*I and cloned in corresponding site of CaMV35S cassette (Fig. 1) then 1407 bp cadherin cassette was restricted out with EcoRV and cloned into *Sma*I site of binary vector pGreenII0000-35S hpt. The binary plasmid was further confirmed by multiple restriction digestion analysis (Fig. 2). As we got the exact bands for all the restriction sites of the vector construct except EcoRV. That is why we ignored the result of the restriction site of EcoRV and considered the other site of the vector construct.

Mobilization of binary plasmid into *Agrobacterium tumefaciens*: The *Agrobacterium* colony harbouring the binary plasmid was confirmed by colony PCR with *hpt*II and *cadherin* specific primers (Fig. 3).

PCR confirmation of putative transgenic tobacco plants: The binary plasmid was mobilized into *Agrobacterium* strain EHA105 and plant transformation was carried out using *cry1Ac* transgenic leaf disc. PCR analysis was performed in all *cry1Ac* and *cry1Ac* + *HaCad* plants using *npt*II, *cry1Ac*, *hpt*II, *HaCad* gene specific primers to detect the presence of transgene. All the plants gave expected size of 750 bp, 1068 bp, 900 bp and 732 bp respectively (Fig. 4a).

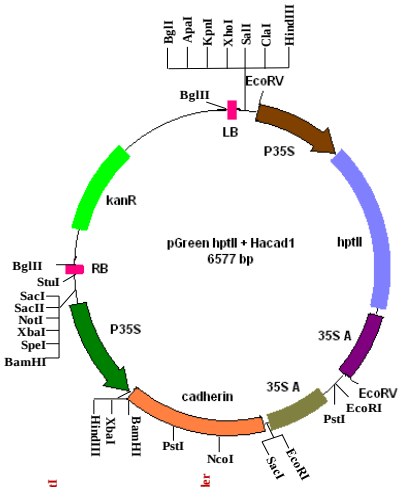
Leaf disc antibiotic assay: Antibiotic assay was performed in transgenic leaf disc for checking the expression of antibiotic resistance gene (Plant selection marker). Leaf discs were taken from control, *cry1Ac* and *cry1Ac* retransformed with *cadherin* tobacco plants and kept on the MS medium containing 2.0 mg/l BAP, 0.1 mg/l NAA, 100mg/l Kanamycin and 50mg/l Hygromycin. The plates were kept in the tissue culture room for 16:8 hrs light and dark periods for 10-15 days. Callus induction was observed in after 10-15 days. control and *cry1Ac* leaf discs kept in the selection media containing both antibiotics Kanamycin and Hygromycin were not giving any response and leaf discs became yellowish, but in case of *cry1Ac* retransformed with *HaCad1* leaf discs proliferate the callusing similar to control leaf discs (Fig. 4b).

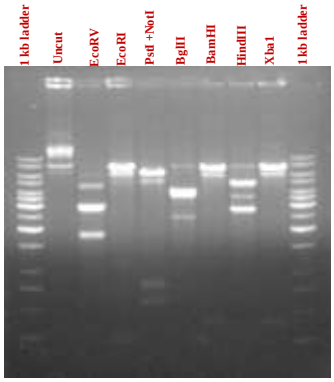
Insect bioassay: After molecular analysis of the retransformed *cry1Ac* plants were evaluated for their effect on neonate larvae of *Helicoverpa armigera*. Insect bioassay was performed with *cry1Ac* and *cry1Ac* retransformed with *HaCad1* plants. The main purpose of the bioassay was whether the activity of *cry1Ac* toxin was increased after retransformation with the *HaCad1* or not. Observation was taken after three days of insect bioassay it was found that the expression of retransformed *cry1Ac* plants activity was equal to *cry1Ac* transgenic plants. After 72hrs it was found that one of the retransformed plants showed relative mortality compare to respective *cry1Ac* plants. But other lines showed similar expression level (Table 1).



Enzymes	Expected Fragments (kb)
BamHI	3.58
SacI	3.85
BamHI/SacI	2.8, 732 bp
EcoRV	2.1, 1.4
PstI	3.58
NcoI	3.58

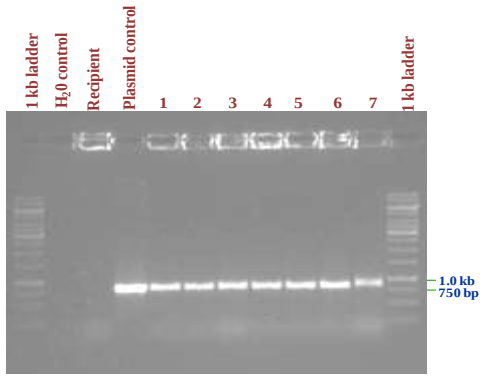
Fig. 1. Construction and Restriction digestion analysis of CaMV35S-*HaCad1* cassette



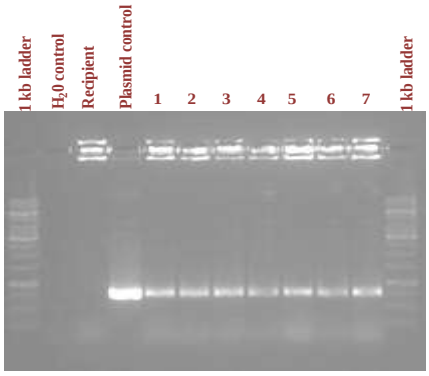


Enzymes	Expected Fragments (Kb)
EcoRV	1.8, 3.2, 4.7
EcoRI	0.232, 5.43
PstI-NotI	0.6, 0.8, 5.1
BglII	2.5, 4.0
BamHI	0.42, 6.1
HindIII	2.5, 4.2
XbaI	0.42, 6.1

Fig. 2. Construction and Restriction digestion analysis of *pGreen hpt*II *HaCad1*

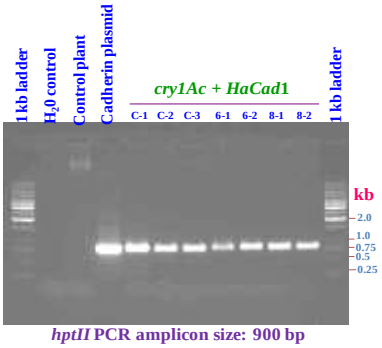


hptII PCR amplicon size: 900 bp

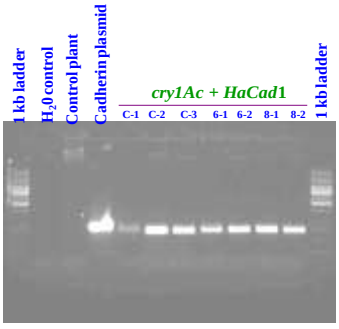


***Cadherin* gene specific PCR amplicon size:735bp**

Fig. 3. Colony PCR confirmation of A*grobacterium* strain *EHA*105 harboring Binary vector pGreen-*hpt*II, *Hacad1* cassette with helper strain pSoup

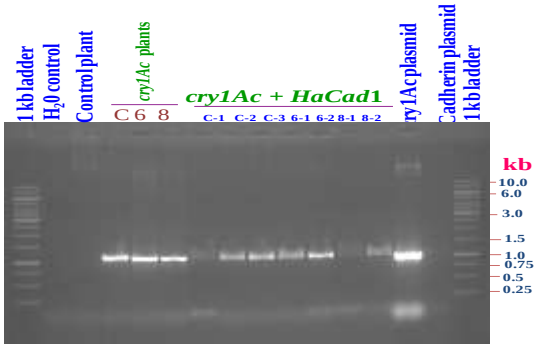


***hptII* PCR amplicon size: 900 bp**

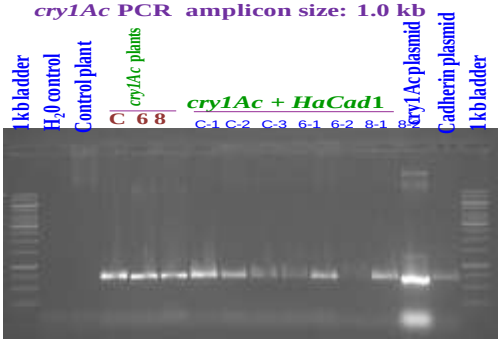


***Cadherin* (Gene specific) PCR amplicon size: 732bp**

Fig. 4a. PCR analysis of 35S::*cry1Ac* transgenic tobacco retransformed with 35S:: *HaCad1*

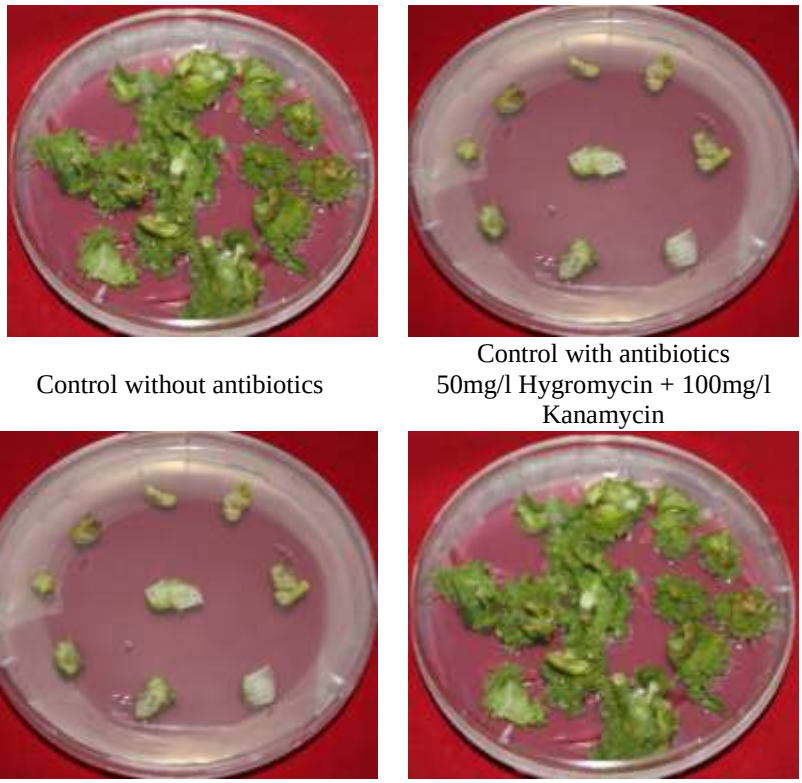


***cry1Ac* PCR amplicon size: 1.0 kb**



***nptII* PCR amplicon size: 750 bp**

Fig. 4b. PCR analysis of 35S::*cry1Ac* transgenic tobacco retransformed with 35S:: *HaCad1*



Control without antibiotics

Control with antibiotics 50mg/l Hygromycin + 100mg/l Kanamycin

Cry1Ac leaf disc 50mg/l Hygromycin + 100mg/l Kanamycin

Cry1Ac + *HaCad1* leaf disc 50mg/l Hygromycin + 100mg/l Kanamycin

Fig. 5. Antibiotic sensitive assay of transgenic leaf disc

control tobacco

cry1Ac tobacco

cry1Ac retransformed with *HaCad1*

Fig. 6. Insect bioassay of transgenic plants

Table 1. Insect bioassay on neonate larvae of *Helicoverpa armigera*

S.N	Sample	No of larvae	Mortality at 24 hrs	% of Insect Mortality	Mortality at 48 hrs	% of Insect Mortality	Mortality at 72 hrs	% of Insect Mortality
1	Control	5	0/5	0	0/5	0	0/5	0
2	Control	5	0/5	0	0/5	0	0/5	0
3	Cad c	5	0/5	0	0/5	0	5/5	100
4	Cad c	5	0/5	0	0/5	0	3/5	70
5	Cad 6	5	0/5	0	2/5	40	3/5	70
6	Cad 6	5	0/5	0	1/5	20	4/5	80
7	Cad 8	5	0/5	0	0/5	0	0/5	0
8	Cad 8	5	0/5	0	0/5	0	0/5	0
9	Ac c	5	0/5	0	1/4	25	3/4	75
10	Ac c	5	0/5	0	1/4	25	2/4	50
11	Ac 6	4	0/4	0	1/4	25	3/4	75
12	Ac 6	4	0/4	0	1/4	25	2/4	50
13	Ac 8	4	0/4	0	1/4	25	1/4	25
14	Ac 8	4	0/4	0	1/4	25	1/4	25

DISCUSSION

Bacillus thuringiensis is a ubiquitous gram-positive, spore- forming bacterium which forms insecticidal crystal proteins (ICPs). These insecticidal crystal proteins display insect specific toxicity and these are harmless to humans and non-target animals. They have been also used as biopesticides and transgenic plants to control agricultural pests (Schenpf *et al.* 1998; Pigott and Ellar, 2007). *Cadherin* belongs to one of the families of animal glycoproteins which are responsible for calcium dependent cell to cell adhesion. Recent literature showed that the cadherin-like in mid gut of several insects served as the receptor of Bt toxin *cry1A*. The interaction between *Bacillus thuringiensis* insecticidal protein *cry1A* and cadherin receptors in lepidopteran insects induces toxin oligomerization. *Helicoverpa armigera*, *cadherin* fragment (*HaCad*) containing its receptor binding region, expressed in *E. coli* enhanced *cry1Ac* activity against *Helicoverpa armigera* larvae.

Based on all this previous concepts the present study has done to compare the toxicity level of *cry1Ac* protein and the combination of *cry1Ac* and *HaCad* protein against *Helicoverpa armigera*. PCR amplified 732 bp of *cadherin* gene was cloned into CaMV35S cassette and further cloned into plant transformation binary vector pGreenII 0000-35S*hpt*II, utility of any insecticidal gene in agricultural biotechnology can be determined by its performance in the plant system. For this purpose *cry1Ac* tobacco plants were retransformed with the cadherin gene through *Agrobacterium* mediated plant transformation. Tobacco is a model plant system to study the expression of various insecticidal genes (Vaeck *et al.* 1987; Barton *et al.* 1987; Warren *et al.* 1992; Sutton *et al.* 1992; Van der Salm *et al.* 1994; McBride *et al.* 1995; Selvapandiyan *et al.* 1998; Gleave *et al.* 1998; Kota *et al.* 1999; De Cosa *et al.* 2001). Highly expressed *cry1Ac* plants were taken and retransformed with *Agrobacterium* strain EHA105 harbouring the binary plasmid pGreenII-35S*hpt*II-*HaCad1*. Hygromycin was a plant selection agent to select transgenic plants because *cry1Ac* plants carrying the kanamycin resistant gene *nptII* for plant selection. Antibiotic leaf disc assay was performed with transgenic *cry1Ac* and retransformed *cry1Ac* plants along with control plants on the MS medium containing BAP 2.0 mg/l, NAA 0.1 mg/l, Kanamycin 100 mg/l and Hygromycin 50 mg/l. Callus proliferation was observed in retransformed *cry1Ac* plants. Leaf disc was completely yellowish and no callus proliferation was observed in *cry1Ac* transgenic plants respective to control plants. For molecular analysis, PCR was performed using *nptII*, *hptII* and gene specific primers. Seven plants were positive with *nptII*, *hptII* and gene specific primers; it gives amplification of 750 bp, 900 bp and 732 bp respectively. Insect bioassay was carried out to study the expression of *cry1Ac* toxin and *cry1Ac + HaCad* expression to compare the efficiency of *HaCad1* with *cry1Ac*. After 72hrs it was found that one of the retransformed plants showed relative mortality compare to respective *cry1Ac* plants. But other lines showed similar expression level, it may needs further proteomics analysis or more transgenic lines with highly expressing cadherin protein.

CONCLUSION

The aim of the present study was to compare the toxicity enhancement level in *cry1Ac* plants and retransformed *cry1Ac* plants with *HaCad1*. Insect bioassay was showed equal resistance to both the transgenic plants; further proteomic level study is needed to prove the strategy.

REFERENCES

Barton KA, Whiteley HR, Yang NS (1987) *Bacillus thuringiensis* s-endotoxin expressed in transgenic *Nicotiana tabacun* provides resistance to Lepidopteran insects. *Plant Physiol.* 85, 1103-1109.

Bravo A, Soberon M (2008) How to cope with insect resistance to Bt toxins? *Trends Biotechnol.* 26, 573–579.

De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt *cry2Aa2* operon in chloroplasts leads to formation of insecticidal crystals. *Nat. Biotechnol.* 19, 71−74.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19, 11-15.

Gleave AP, Mitra DS, Markwick NP, Morris BAM, Beuning LL (1998) Enhanced expression of the *Bacillus thuringiensis cry9Aa2* gene in transgenic plants by nucleotide sequence modification confers resistance to potato tuber moth. *Mol. Breed.* 4, 459-472.

Hofmann C, Vanderbruggen H, Hofte H, Van Rie J, Jansens S, Van Mellaert H (1988) Specificity of Bacillus thuringiensis delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc. Natl. Acad. Sci. USA* 85, 7844–7848.

Kota M, Daniell H, Varma S, Garczynski SF, Gould F, Moar WJ (1999) Overexpression of the *Bacillus thuringiensis* (Bt) *Cry2Aa2* protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proc. Natl. Acad. Sci. USA*. 96, 1840-1845.

McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P (1995) Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Biotech.* 13, 362-364.

Peng DH, Luo Y, Guo SX, Zeng H, Ju SY, Yu ZN, Sun M (2009) Elaboration of an electroporation protocol for large plasmids and wild-type strains of *Bacillus thuringiensis*. *J. Appl. Microbiol.* 106, 1849-1858.

Pigott CR, Ellar DJ (2007) Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev.* 71, 255-281.

Schnepf E, Crickmore N, van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal proteins. *Microbiol. Mol. Biol. Rev.* 62, 775-806.

Selvapandiyan A, Reddy VS, Kumar PA, Tewari KK, Bhatnagar RK (1998) Transformation of *Nicotiana tabacum* with a native *cry1Ia5* gene confers complete protection against *Heliothis armigera*. *Mol. Breed.* 4, 473-478.

Sutton DW, Havstad PK, Kemp JD (1992) Synthetic *cryIIIA* gene from *Bacillus thuringiensis* improved for high expression in plants. *Transgenic Res.* 1, 228-236.

Vaeck M, Reynaerts A, Hofte H, Jansens S, DeBeuckeleer M, Dean C, Zabeau M, Van M, Montagu M, Leemans J (1987) Transgenic plants protected from insect attack. *Nature*. 328, 33-37.

Van der Salm T, Bosch D, Honee G, Feng L, Munsterman E, Bakker P, Stiekema WJ, Visser B (1994) Insect resistance of transgenic plants that express modified *Bacillus thuringiensis CryIA*(b) and *CryIC* genes: a resistance management strategy. *Plant Mol. Biol.* 26, 51-59.

Wang G, Wu K, Liang G, Guo Y (2005) Gene cloning and expression of cadherin in midgut of Helicoverpa armigera and its Cry1A binding region. *Sci China C Life Sci.* 48(4), 346-56.

Warren GW, Carozzi NB, Desal N, Koziel MG (1992) Field evaluation of transgenic tobacco containing a *Bacillus thuringiensis* insecticidal protein gene. *J. Econ. Entomol*. 85, 1651-1659.

Wu K, Guo Y (2005) The evolution of cotton pest management practices in China. *Annual Review of Entomology*. 50, 31–52.