

## Vacuolar Targeting of Cry1Ac and its Effects on Expression and Stability in Tobacco

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### Abstract

Increasing heterologous expression of delta endotoxins of *Bacillus thuringiensis* in transgenic plants is being actively pursued as a means to increase their efficacy and to delay insect resistance. To examine if vacuoles could be used as alternate localization sites of delta endotoxins we developed binary vectors with a chimeric vacuole targeting signals and verified its localization efficiency by creating GFP fusions of Cry1Ac. Transgenic tobacco plants expressing Cry1Ac localized either to cytosol and vacuoles were generated and confirmed by PCR, QPCR and ELISA. Comparative protein expression analysis by quantitative ELISA showed that maximum, percentage total soluble protein of Cry1Ac was 0.64 and 1% in cytosol and vacuole targeted plants, respectively. However, detailed protein expression analysis showed that there are no significant differences in expression of Cry1Ac between cytosol and vacuole targeted plants. These results were further corroborated by immunoblot analysis as well as insect bioassays. Nevertheless, our study demonstrated that delta endotoxins could be targeted to vacuoles and expressed successfully which is of importance when gene stacking is being pursued where alternate localization sites are employed for different genes.

**Key words:** Cry1Ac, delta endotoxin, Vacuole targeting, *Helicoverpa armigera*

### Introduction

The economic and environmental costs of insect control in agriculture and the losses incurred in spite of different measures are very high. As an alternative strategy, generation of exogenous *Bt* gene expressing insecticidal proteins in the transgenic plants has shown promise for increasing plant resistance to insects and reducing the use of traditional chemical insecticides (1). The delta endotoxin arsenal of *Bacillus thuringiensis* (*Bt*) possesses a wide array of insecticidal proteins that are active against different groups of insects. *Bt* proteins are specific to certain species of insects and are non-toxic to beneficial insects on crops, birds or mammals, including humans (2). This biotechnological approach of generation of *Bt* transgenic insect resistant plants is a major breakthrough in protecting crops from damaging infestation (1, 3).

*Bacillus thuringiensis* is a gram-positive spore forming bacterium that forms parasporal crystals during its sporulation. These parasporal crystals consist of one or more delta-endotoxins or crystal (Cry) proteins that makes *Bt* an effective insect pathogen. Insect midgut having an alkaline environment causes the crystal to dissolve and release the protoxins. Subsequently protoxin converts to 65-70 kDa N-terminal truncated form, the activated toxin through the action of midgut proteases (4). Sequential binding events of activated toxin with the different insect gut

proteins leads to membrane insertion through specific binding to insect midgut epithelial cell receptors (4,5) and forms lytic pores in the microvilli of the apical membrane that kill the epithelial cells and eventually the insect (4, 6-9). Initial efforts in generation of viable transgenic crops carrying *Bt* genes resulted in failure due to the low expression of the transgene due to the prokaryotic nature of the gene and protein degradation. Innovations by using codon optimized genes (modifying coding sequences), deleting the mRNA destabilizing sequences, truncated genes (via deletion of C-terminal domain) have significantly increased the transgene expression levels in plants (10-14) providing viable insect resistant plants. However, with the wide spread use of different *Bt* crops in the field, the field evolution of insect resistance to delta endotoxin is an area of huge concern (15, 16). Insect resistance to *Bt* genes are reported to occur through different mechanisms that include reduced binding of Bt proteins to the midgut receptors, reduced activation of protoxins by midgut proteases, increased immune response and detoxification of Bt toxins and enhanced esterase removal (15, 17-20). However, different approaches have been proposed to curtail/delay this insect resistance. These include use of refugia, high protein dosage and gene stacking (21, 22). Of these, the use of refugia is carried out in the field. It is necessary to increase the expression of *Bt* genes to the maximum level possible without affecting the normal physiological state of the plant.

Targeting of the protein to different sub-cellular organelles has been envisaged to enhance the stability of the foreign proteins in the cells there by increasing the quantity of the heterologous proteins. This was proved to be right for a wide array of proteins (23-26). Similar strategy was adapted to Bt proteins but only the chloroplasts were tested (27-31). Vacuole acts as an alternate site for the storage of proteins in large quantities and is a promising candidate for the storage of heterologous proteins. The protein storage vacuoles are endoplasmic reticulum-

derived cisterns that are specialized in stable protein storage and accumulation. They are characterized by absence of amino peptidases and since these organelles do not undergo membrane fusion with lytic vacuoles protein degradation are avoided. The vacuoles provide an attractive system for targeting heterologous proteins and an excellent sub-cellular location for long-term protein storage (32, 33). This approach has been successfully applied for different proteins such as chloramphenicol acetyl-transferase (34), human insulin (35), barley lectin (36), human lysozyme (37) to name a few. In addition, when phaseolin was expressed in transgenic tobacco under a constitutive promoter, it was sorted to the vacuole in leaf cells and was stored as protein body-like aggregates (38, 39). Therefore, we used codon optimized, truncated version of Cry1Ac and expressed it in cytosol as well as targeted it to vacuoles, to verify if expression of Bt proteins in plants could be increased by targeting them to vacuoles.

#### Materials and Methods

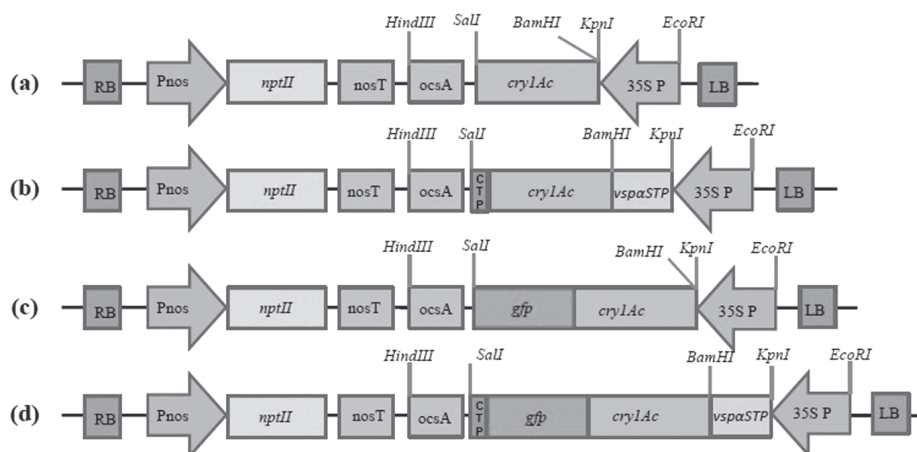
**Construction of vector:** *cry1Ac* gene was excised from pBinBt3 (40) using *Bam*HI and *Sal*I restriction enzymes and cloned in corresponding sites present between CaMV35S promoter and OCS terminator of pBinAR (41) to obtain *pBinAc* and was used for cytosol targeting of the Cry1Ac protein. For targeting of the protein to the vacuole a 69 bp N-terminus peptide targeting sequence from soybean vegetative storage protein (VSP $\alpha$ S) (42) was amplified from total DNA isolated from soybean, using forward primer (5'-GGGGTACCATGAAAATGAAGG-3') and reverse primer (5'-CGGGATCCTCCATGGCATT-3') tagged with *Kpn*I and *Bam*HI restriction sites respectively along with breathing nucleotide sequences to facilitate restriction digestion. This fragment was then cloned into *Kpn*I-*Bam*HI sites of pBinAR binary vector containing *cry1Ac* sequence. 27 bp C-terminal vacuolar targeting sequence of tobacco chitinase A sequence (43) was added to the end of *cry1Ac* by extension PCR using a single forward primer (5'-GGGGTACCATGAAAATGAAGG-3') tagged with *Kpn*I

restriction site along with four sets of reverse primers, RP-I (5'-TCCATTTTCCTT CAGCCTC GAGTGTTC-3'), RP-II (5'-GACT AAAAGT CCAT TTCCTTCAGC CTC-3'), RP-III (5'-CATAGTATCG ACTAAAAGTCCATT TCC-3') and RP-IV (5'-ACGCGTCTGACTC ACATAGTAT CGACTAA -3') that are having overlapping sequences of the targeting signal. Fragment was amplified first with forward primer containing *KpnI* site with breathing nucleotide and 'I' reverse primer. Amplified product obtained from the 'I' primer were again amplified with forward primer and 'II' reverse primer, product obtained from 'II' reverse primer has been PCR amplified with forward primer and 'III' reverse primer. Product obtained from the 'III' reverse primer were further amplified with forward primer and IV reverse primer containing *SalI* restriction site with breathing nucleotides to obtain the final product containing C-terminal 27 bp tobacco chitinase A sequence. After sequencing confirmation of the product this fragment was cloned in *KpnI* and *SalI* restriction sites of the pBinAR binary vector to generate *pBinARVac-Ac* vector. To confirm the targeting of Cry1Ac to the sub-cellular compartments, *cry1Ac-GFP* fusions with respective targeting signals were constructed by

cloning the *GFP* gene at the 3' end of the *cry1Ac* gene along with or without targeting signals using a similar strategy (Fig. 1).

**Agroinfiltration, transient expression and microscopy:**

The cytosolic and vacuole targeted *cry1Ac-GFP* fusion constructs were mobilized into *Agrobacterium tumefaciens* EHA105 by freeze thaw method. *Agrobacterium* harboring the vector was inoculated in YEM media with appropriate concentrations of rifampicin and kanamycin. An overnight culture of *Agrobacterium* was harvested at 0.6 to 0.8, OD<sub>600</sub> and sedimented at 4,000 xg for 20 minutes, and washed in 25 ml of infiltration medium. Finally, *Agrobacterium* cells were suspended in 100 ml of infiltration medium supplemented with 10 mM MgCl<sub>2</sub>, 25 mM MES-KOH (pH 5.6), and 200 μM acetosyringone. The bacterial solution was incubated at room temperature for four hours with gentle shaking in dark before infiltration. *Nicotiana tabacum* var. Petit Havana plants were grown for 4-5 weeks in the soil. *Agrobacterium* infiltration medium was applied using blunt-tipped plastic syringe and was forced into abaxial epidermis of fully expanded leaves of tobacco (44). After 72 hr of incubation, the leaves were washed twice with water and cut



**Fig. 1.** T-DNA map of binary vectors (a) pBinAc (b) pBinVacAc (c) pBinAc-gfp (d) pBinVacAc-gfp. In all four vectors *cry1Ac* gene was driven by CamV35S promoter and selectable marker gene (*nptII*) was driven by nos promoter (Pnos). *vspαSTP* is N-terminal vegetative storage protein signal transit peptide from soyabean and CTP is C-terminal transit peptide from Chitinase A of tobacco

into small sections with a surgical blade. The cut sections were observed under confocal microscope from Leica, TCS SP6 (Germany).

**A. tumefaciens-mediated transformation:** Fully expanded tobacco leaves were excised from axenically grown wild-type plants and cut into 1-2 cm leaf discs. The explants were placed for 10 min in a culture of *A. tumefaciens* carrying the plasmid of interest, and then blotted on to sterile filter paper to remove excess *Agrobacterium* culture. Leaf discs were incubated at 25 °C in 16 h of light on shoot-inducing medium (MS salts containing 3 % sucrose, 2 mg/l 6-benzylamino purine and 0.1 mg/l  $\alpha$ -naphthalene acetic acid). After 2 days, the leaf discs were transferred to MS agar containing 2 mg/l 6-benzylaminopurine, 0.1 mg/l  $\alpha$ -naphthalene acetic acid, 300 mg/l kanamycin and 500mg/l cefotaxime for the selection of transgenic shoot growth. Elongated shoots were excised from calli and transferred on to 3 % sucrose MS agar, 300 mg/l kanamycin and 250 mg/l Cefotaxime. Transformed plants were grown at 25 °C for 16 hr in light in axenic conditions in the presence of kanamycin and were sub-cultured every 5-6 weeks.

**ELISA:** A double-antibody sandwich enzyme-linked immunosorbant assay (ELISA) was used to detect the presence of the Cry1Ac protein expressed in the leaves of transgenic plants. Experiments were performed with double sandwich quantitative Cry1Ac/Ab ELISA plates from Envirologix, Portland, USA. Proteins from leaf samples of targeted, untargeted and wild type tobacco plants were extracted using the protein extraction buffer provided in the kit. Leaf extract was diluted to fit in the linear range of the provided Cry1Ac standards and steps were performed essentially according to manufacturer's instructions. Halo MPR-96 microplate reader (Dynamica, Ottawa, Canada), was used to read the plate at 450 nm.

**Western blotting:** Sample proteins from young leaves of transgenic and wild type plants, was extracted using a protein extraction buffer (pH 9.5,

50 mM Na<sub>2</sub>CO<sub>3</sub>, 150 mM NaCl, 1 mM PMSF,  $\beta$ -mercaptoethanol). The total extracted crude proteins were quantified using Bradford reagent (Bio-Rad, Hercules, USA), and protein concentrations were determined against a standard of bovine serum albumin. Crude protein samples were then run on 12 % SDS-PAGE gel according to the method described by Laemmli (45) with a Dual Mini Slab Chamber (Bio-Rad). This protein gel was transferred to Immobilon-P membrane (Millipore, Billerica, USA) using a Mini Trans-Blot electrophoretic cell (Bio-Rad) by applying 40 V for 3 h and transferred to a blocking solution containing 5 % skimmed milk powder in phosphate buffered saline (PBS). This was followed by incubation in primary antibody (mouse anti-Cry1Ac antibody, Envirologix) at 1:5000 dilutions, and secondary antibody (goat anti-mouse IgG Alkaline phosphatase conjugate, GeNei, Bengaluru, India) at 1:5000 dilutions for 1 h each at room temperature. The signal was detected using BCIP/NBT substrate (Sigma-Aldrich, St Louis, USA) after developing for 5-10 min.

**QRT PCR:** Total RNA, from the transgenic and wild type plant leaves was isolated with the use of Plant RNA Isolation Kit (Sigma-Aldrich) following the manufacturer's instructions. It was visualized in 0.8 % agarose gel to confirm its quality and quantified using nanodrop (Thermo scientific). cDNA was synthesized with 1 $\mu$ g of total RNA using oligodT (18mer) primers using SuperScript-III cDNA Synthesis System (Invitrogen, Carlsbad, USA). Real-time PCR was performed in a 96-well reaction plate by using Stratagene Mx3005P unit, using VeriQuest SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, USA). For specific amplification of *cry1Ac*, oligonucleotides 5'-TTCTTGACGGAA CAGAGT TCG CCT-3', and 5'-TGGGTGGC ACATTGTTG TTCTGTG-3' were used as forward and reverse primers. Results were analyzed and calculated by comparative threshold cycle method according to the manufacturers' instructions for data normalization. L25 ribosomal protein from tobacco was used as a reference gene (46).



**Northern blotting:** Thirty micrograms of the total RNA extracted from the leaf sample of the tobacco plant was separated on a 1.5 % formaldehyde agarose gel in 1X MOPS buffer and transferred to a positively charged microporous (0.45- $\mu$ m pores) nylon immobilon membrane (Millipore, India) by capillary method and fixed through ultraviolet cross-linking. The membrane was probed with 1 kb *cry1Ac* probe labeled with  $\alpha$ - $P^{32}$ -CTP using Mega Prime DNA labelling kit (GE Healthcare, Buckinghamshire, UK), and hybridized in UltraHyb buffer (Ambion, Austin, USA) at 55°C. After hybridization, the membranes were washed with different stringency buffers and developed after 2 days.

**Insect bioassay:** Cotton bollworm (*Helicoverpa armigera*) insects were obtained from the Division of Entomology, IARI, New Delhi; and were reared on a semi-synthetic diet (47). Hatched eggs were maintained up to second instar larvae. Five healthy neonate larvae of cotton bollworm were released onto leaf discs placed on a moist filter paper. Leaf discs from non-transformed plants were used as controls. Each bioassay was repeated twice. These cups were stored at 27 $\pm$ 1°C and larval mortality data was recorded at 24, 48, 72 and 96 hours post release of the larvae.

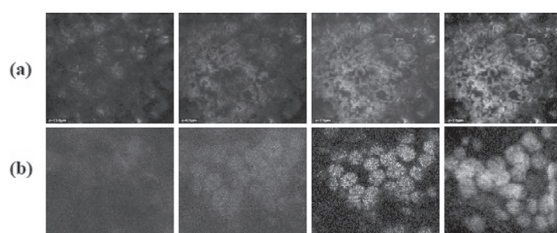
## Results

**Construction of vectors:** Different vectors were constructed to target Cry1Ac protein either to cytosol or vacuole and also *Cry1Ac-GFP* fused vectors to verify the same (Fig. 1). The green fluorescent protein (GFP) coding region was fused in frame to the C-terminus of synthetic *cry1Ac* under the control of the 35S promoter, generating the vector *pBinCry1Ac-GFP* which is used as control in form of cytosol localization. The N-terminal leader sequence of soybean vegetative storage protein (VSP $\alpha$ S) that is responsible for targeting the protein to endoplasmic reticulum (42) and C-terminal signal sequence (GNLLVDTM) from tobacco chitinase A that is responsible for targeting the protein to vacuole (43) were fused with *Cry1Ac-GFP* towards N-terminus and C-terminus respectively

to target the protein into the plant vacuole; generating the *pBinARCry1Ac-GFPVac* (Fig. 1). Binary vectors that were devoid of GFP were used for stable transformation of tobacco plants and compared.

### **Agrobacterium mediated transient expression and localization of Cry1Ac-GFP fusion protein:**

Laboratory grown four to five week old tobacco plants were used for transient expressions studies. An infiltration medium containing the fusion construct *pBinCry1Ac-GFP* and *pBinARCry1Ac-GFPVac* was applied to abaxial epidermis of full-expanded leaves of the tobacco plant using blunt-tipped plastic syringe. Three days after the infiltration, the infiltrated portions of the leaves were examined for the GFP expression. Portions of leaves infiltrated with vector devoid of targeting signals showed a homogenous green fluorescence throughout the cells demonstrating that the GFP fused Cry1Ac is spread throughout the cytoplasm. Whereas the portions infiltrated with *Agrobacterium* harboring *Cry1Ac-GFPVac* vector showed localized green fluorescence in form of concentric rings confirming the vacuolar targeting GFP fused Cry1Ac and the ability of the targeting signals to localize the Cry1Ac protein to the vacuoles (Fig. 2).



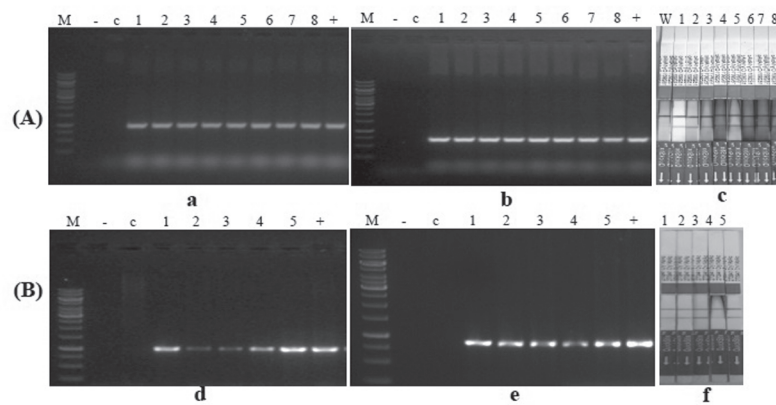
**Fig. 2.** Merged images of Ac-GFP fusions transient expression in tobacco leaf. (a) Cytosol localization (b) Vacuole localization

### **Agrobacterium mediated stable transformation of tobacco:**

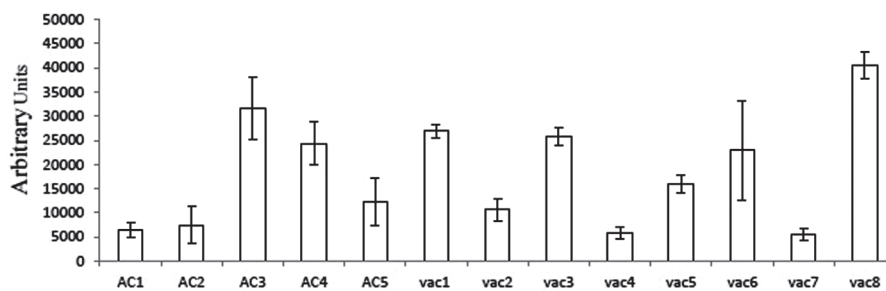
Transformation of tobacco leaf discs was carried using *Agrobacterium* harboring *pBinAc* and *pBinVac-Ac* to obtain putative transgenic plants with Cry1Ac expressed

in cytosol and localized in vacuoles respectively. Initially, 15 independent plants regenerated from *Agrobacterium* mediated stable transformation of tobacco leaf discs with these two constructs were confirmed with PCR analysis for gene integration, Cry protein ImmunoStrip and qualitative ELISA for protein expression. Of these a total of 5 and 8 morphologically similar independent transgenic lines obtained with *pBinAc* (untargeted) and *pBinVac-Ac* (vacuole targeted) were selected and analyzed comparatively to determine the efficiency of vacuole localization to attain higher protein expression. The untargeted putative transgenic plants were confirmed by *cry1Ac* gene

specific primers and the putative vacuole targeted *cry1Ac* plants were confirmed by PCR with a forward *cry1Ac* specific primer and a reverse primer specific to the C-terminal vacuole targeting signal (Fig. 3). Transgenic plants confirmed with PCR analysis were subjected to protein analysis using ImmunoStrips specific for *cry1Ab/Ac* gene products. Two bands appeared on each ImmunoStrip that are immersed into the extracts from transgenic plants as compared to the control where a single band appeared (Fig. 3). Also, expression analysis of mRNA was carried out with qRT-PCR for different transgenic lines to confirm the successful expression of the transgene. The



**Fig. 3.** Confirmation of putative transgenic plants. Panel A: confirmation of cytosol targeted tobacco transgenic plants. Panel B: confirmation of vacuole targeted tobacco transgenic plants. a and d: PCR analysis with *cry1Ac* specific primers. b and e : PCR analysis with *nptII* specific primers. C and f: ImmunoStrip assay



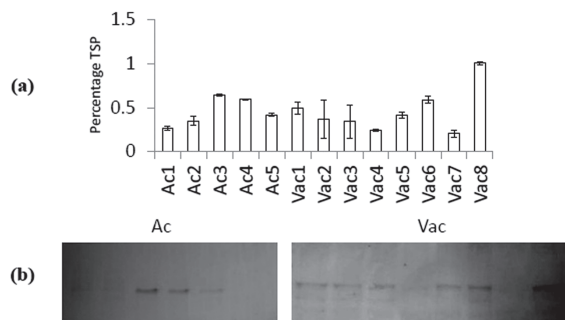
**Fig. 4.** qRT-PCR analysis. Ac1-Ac5 are cytosol targeted samples and vac1-vac8 are vacuole targeted samples

results showed that the lines that were selected were expressing the transgene and the expression levels were varied (Fig. 4).

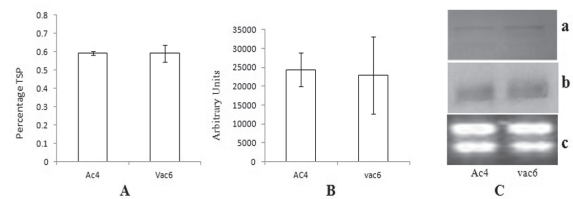
**Cry1Ac protein expression analysis:**

Quantitative ELISA was performed to determine the protein concentrations in independent transgenic lines belonging to both the groups. The

average expression levels in terms of percentage of total soluble protein (% TSP) of Cry1Ac protein in the untargeted group of transgenic lines was 0.453 whereas for the vacuole targeted transgenic lines it was 0.458. Minimum and maximum levels of protein expression found in this study were 0.24 and 0.64 % TSP for the untargeted group and 0.2 and 1 % TSP for the vacuole targeted group respectively (Fig. 5). Immunoblot analysis of total soluble protein from the transgenic tobacco leaves was also performed to confirm the results of ELISA. The pattern of expression detected in the immunoblot analysis was similar to the results obtained with ELISA. The results obtained from ELISA experiments showed that there were no significant differences between the untargeted and vacuole targeted groups in terms of expression levels of Cry1Ac protein.

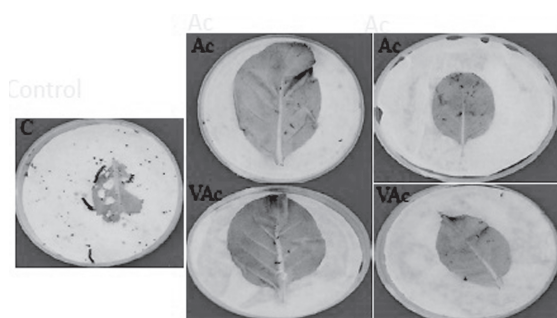


**Fig. 5.** Protein expression profile of different targeted groups. (a) quantitative ELISA (b) Western analysis. Ac, and Vac represents different lines of untargeted group and vacuole targeted samples



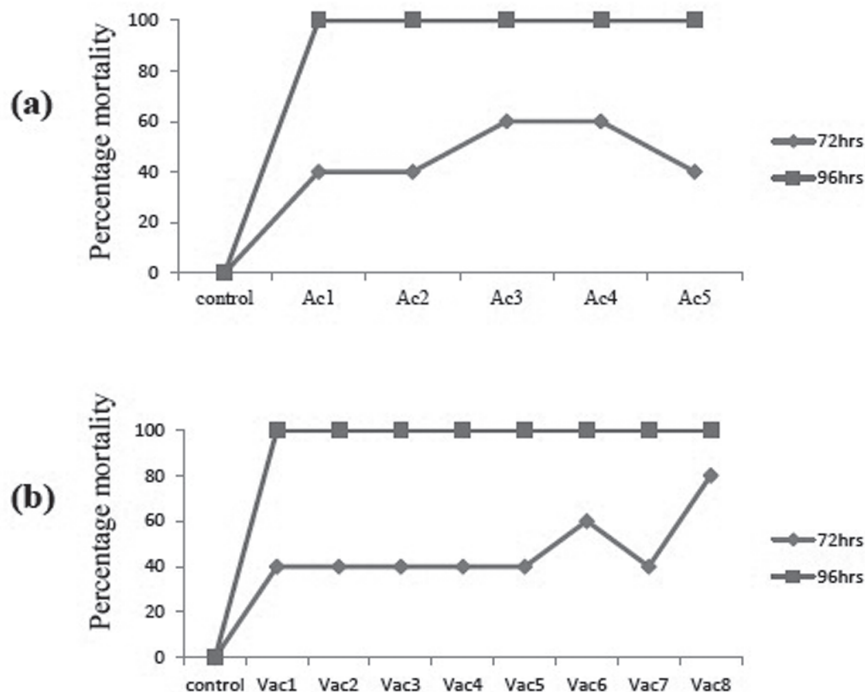
**Fig. 6.** Comparative expression profiling. A: ELISA, B: QRT-PCR, C: Blot analysis. a, b and c are Western Blot, Northern blot and loading control for the northern blots respectively

However, to further validate these results we selected a single independent transgenic line from each group showing approximately equal levels of *cry1Ac* mRNA as assessed by qRT-PCR results (Ac4 and vacAc6) and performed comparative mRNA expression profiling by qRT-PCR and Northern analysis and also protein expression profiling by quantitative ELISA and Western analysis. QRT-PCR analysis and Northern analysis of the samples indicated approximately equal expression of *cry1Ac* mRNA, while Western analysis and quantitative ELISA demonstrated that approximately equal levels of protein is expressed in the two different lines (Fig. 6).



**Fig 7.** Leaf disk bioassay of wild type cytosolic *cry1Ac* and vacuole targeted *cry1Ac* transgenic lines against *H. armigera*

**Insect bioassay:** Transgenic lines expressing Bt-toxin Cry1Ac in the cytosol, as well as vacuole were also evaluated for entomocidal activity by insect feeding bioassays performed with second instar larvae of *H. armigera* to study if there are any differences in the biological activity of the expressed Cry1Ac protein among the two groups. Leaves from the young tobacco plants were fed to the larvae and mortality was monitored. Insects fed on the leaves of the transgenic lines showed retarded growth and also the leaves were less damaged when compared to the control non-transgenic leaves. A 40-60 % mortality rate was



**Fig. 8.** Percentage mortality rate of *H. armigera* challenged with the leaf of different *cry1Ac* targeted lines of tobacco. (a) cytosol targeted *cry1Ac* transgenic lines (b) vacuole targeted *cry1Ac* transgenic lines. 72 and 96 hrs are the time intervals for mortality observation

observed in different transgenic lines of both the groups at 72hr. Also, the mortality rate corresponded to the Cry1Ac protein levels in both groups. At 96hrs 100 % mortality was observed in nearly all the lines (Fig. 7, Fig. 8).

### Discussion

Different approaches were followed in increasing the transgene expression especially of *Bt* genes that lead to the development of viable transgenic events and thereby their commercialization. However, to sustain the success novel approaches to increase the transgene expression as well as stability are to be developed. Targeting of proteins to different cellular compartments of the plant cells has become an important and effective method to increase the production and stability of recombinant proteins. Targeting peptide

sequences obtained from either plant or other organism have been utilized to target the protein in the various specialized organelle compartments such as apoplast, ER lumen, and vacuole, leading to a higher level of protein accumulation (48). In the present study, we comparatively analyzed the efficacy of delta endotoxin targeting to tobacco vacuole to find whether this is an ideal localization site for not only obtaining higher protein expression but also to increase biological efficacy.

In this study, we constructed a vacuole targeting vector with a chimeric targeting signal having an N-terminal ER targeting signal from Soybean vegetative storage protein (VSP<sub>v</sub>) (42) and a C-terminus vacuole targeting signal from tobacco chitinase A (43) and tested the localization efficiency using GFP localization. Transgenic plants were developed with both the



untargeted and vacuole targeted vectors and the integration and expression of the transgene was confirmed with PCR, qRT-PCR and ELISA. We observed that there were no differences among the groups with regard to Cry1Ac expression. The average expression of Cry1Ac among the targeted and untargeted groups was approximately 0.45 %. Also, comparative analysis of lines showing similar levels of mRNA also supported that there were no differences in the expression of the transgene. Although, different reports demonstrated that higher expression of heterologous proteins was possible when targeted to vacuoles (34-39), present study shows that no added advantage can be obtained by targeting Bt proteins or at least truncated Cry1Ac to the vacuoles. The reasons behind the inability to increase the levels of Bt proteins could be multifold like the natural resistance of Bt proteins to different proteases, one of the main reason for increased heterologous protein expression when targeted to vacuoles, the acidic nature of the vacuole environment as against the alkaline environment required to solubilize the Bt proteins. The cytosol is having a neutral environment, which is also not ideal for the Bt proteins. Since the same constraints are present for the expressed Cry1Ac protein in the cytosol and vacuole, it may be the probable reason behind no added advantage of targeting Bt proteins to the vacuole. However, our present study shows that the vacuole localization can still be used for expressing of Bt proteins on par with the cytosol and this could be especially useful when adopting the gene pyramiding strategy.

### Conclusion

In summation, we have created vectors that can efficiently target Cry proteins to the vacuole and generated transgenic tobacco plants with Cry1Ac localized to cytosol and vacuoles. Detailed protein expression analysis showed that there are no significant differences in the expression of Cry1Ac between cytosol and vacuole targeted plants. This result was further corroborated by immunoblot analysis as well as insect bioassays. However, our study

demonstrated that delta endotoxins could be targeted to vacuoles and expressed successfully albeit with no added advantage over cytosol expression.

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