

Cloning and chloroplast-targeted expression studies of insect-resistant gene with ricin fusion-gene under chloroplast transit peptide in cotton

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Received January 15, 2013 / Accepted August 30, 2013

Published online: November 15, 2013

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Abstract

Background: Transgenic plants inhabiting single Bt gene are prone to develop insect resistance and this resistance has been reported in case of some important yield-devastating insect larvae of commercial crops, such as cotton and rice. Therefore, it has become essential to adapt new strategies to overcome the problem of insect resistance and these new strategies should be sophisticated enough to target such resistant larvae in broad spectrum. Among these, plants may be transformed with Bt gene tagged with some fusion-protein gene that possesses lectin-binding capability to boost the binding sites for crystal protein gene within insect mid-gut in order to overcome any chances of insect tolerance against Bt toxin. Enhanced chloroplast-targeted Bt gene expression can also help in the reduction of insect resistance.

Results: In the present investigation, a combined effect of both these strategies was successfully used in cotton (*G. hirsutum*). For this purpose, plant expression vector pKian-1 was created, after a series of cloning steps, carrying Cry1Ac gene ligated with chloroplast transit peptide towards N-terminal and Ricin B-Chain towards C-terminal, generating TP-Cry1Ac-RB construct.

Conclusions: Efficacy of pKian-1 plasmid vector was confirmed by in-planta *Agrobacterium*-mediated leaf GUS assay in tobacco. Cotton (*G. hirsutum*) local variety MNH-786 was transformed with pKian-1 and the stable integration of TP-Cry1Ac-RB construct in putative transgenic plants was confirmed by PCR; while fusion-protein expression in cytosol as well as chloroplast was substantiated by Western blot analysis. Whereas, confocal microscopy of leaf-sections of transgenic plants exposed that hybrid-Bt protein was expressing inside chloroplasts.

Keywords: chloroplast expression; chloroplast transit peptide; cotton transformation; confocal microscopy; ricin b-chain.

INTRODUCTION

Last decade of twentieth century witnessed cloning and transformation of various full length single Bt genes into higher plants. But insecticidal activity illustrated low levels of protein expression in transgenic plants, mainly due to mRNA degradation. Therefore, truncated Bt genes via deletion of 'non-essential' sequences were introduced (Barton et al. 1987), which enhanced the insecticidal activity by improving the integrity of mRNA. These insect-resistant transgenic crops expressing truncated-Bt genes were regarded as major biotechnological breakthrough (Kim et al. 2009). However, this method proved to be a temporary solution to kill targeted insects as they started to build resistance against single Bt gene (Gatehouse, 2008; Fabrick et al. 2009; Liu et al. 2010).

Another strategy that ensures targeted-expression of Bt genes in green tissues of plants is the chloroplast transformation but this tissue culture-based technique is slow outside the group of

Solanaceous species. Most chloroplast transformation research had been confined to tobacco (Lössl and Waheed, 2011) because of the ease to grow it through callogenesis, quick response to gene-gun transformation, high yield in comparatively less time and less labour required. But chloroplast transformation technique fails in case of recalcitrant crops e.g. cotton, where callogenesis is difficult to achieve.

Nucleus-encoded chloroplast proteins characteristically carry a chloroplast-targeting signal called a transit peptide (TP) as N-terminal extension (Bruce, 2001; Steiner et al. 2005). TP is sufficient to arbitrate the organellar import of proteins and this has been demonstrated in case of green fluorescent protein (GFP) from jellyfish (Lee et al. 2006). Therefore, nuclear transformation of recalcitrant cotton crop might be done using TP attached upstream with Bt gene that would lead Bt protein inside chloroplasts (Kim et al. 2009; Rawat et al. 2011). Plastid-targeted expression, such as in chloroplasts, is an important target for future Bt crops (Bock, 2007). Higher levels of toxin, up to 3-5% of total leaf protein, are accumulated in chloroplasts since the plastid genome is bacterial in origin as are Bt genes (McBride et al. 1995).

The crystal structure of ricin shows that it is comprised of two chains, ricin A-chain and B-chain that are held together by a disulfide bond (Olsnes, 2004). The B-chain of ricin (RB) is non-toxic and possesses three galactose-binding sites (Rutenber and Robertus, 1991; Venkatesh and Lambert, 1997). Ricin binds to the cell via interaction between the B chain and galactose groups on cell-surface receptors in insect midgut cells; then the disulfide bond is cleaved allowing the A chain to enter the cell (Endo et al. 1987). Thus RB bears lectin-binding sites and can facilitate Bt protein as fusion-partner by providing three extra binding sites to Bt in insect midgut. The Cry1Ac and RB have different binding sites in the larval midgut and are considered to be a good combination to deploy in delaying the evolution of resistance (Gatehouse, 2008).

In the present study, a plant expression vector pKian-1 was created carrying TP-Cry1Ac-RB construct. The TP, isolated from *Petunia hybrida* was ligated towards N-terminal; while RB, isolated from *Ricinus communis*, was attached towards C-terminal of the truncated Cry1Ac gene. RB would provide more binding sites to Cry1Ac as Bt protein acts by disrupting the mid-gut cells of insect pests (Bravo et al. 2007). Thus the chances for insects to develop resistance against Bt should be greatly reduced. On the other hand, TP would ensure enhanced chloroplast localized expression of Bt gene.

MATERIALS AND METHODS

Plant Materials

Petunia hybrida cultivar *Grandiflora* and *Ricinus communis* seeds were taken from commercial plant nursery of University of the Punjab, Lahore and grown in CEMB greenhouse at 25°C ± 5°C and 16 hrs photoperiod.

Isolation and TA cloning of chloroplast transit peptide (TP)

Total RNA from *Petunia* leaves was extracted by method of Jaakola et al. (2001) and cDNA library was constructed by using oligo(dT)₁₈ primers and MMLuV-RT enzyme (Fermentas) through reverse-transcription PCR. Primers for TP with NcoI restriction site were designed: forward primer (TTAGCCATGGATGGCACAATTAACAACATGG), and reverse primer (TAAGCCATGGCTGTGCTGTAGCCACTGATGC). A 216 bp fragment of TP of EPSPS gene from cDNA library was PCR amplified. The reaction mixture was heated at 95°C for 3 min, and then 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec with a final step of 72°C for 5 min. The amplified fragment was cloned into TA-vector PCR 2.1 (Invitrogen). Sequencing of TP was done with M13 primers on ABI 310 Genetic Analyzer using BigDye Terminator v3.0 Cycle Sequencing kit (Applied Biosystem). Sequence was edited using software GeneDoc to eliminate the primer and vector sequences.

Isolation and TA cloning of ricin b-chain (RB)

Total RNA from *Ricinus* leaves was extracted and cDNA library was constructed as mentioned above. An 816 bp sequence of RB was PCR amplified from cDNA library using primers: forward primer with XhoI site (AAGCCTCGAGGCTGATGTTTGTATGGATCCTG); reverse primer with XhoI site (GCTACTCGAGACACACTGCAAGAGAGTAATCTGTCTA). The conditions for PCR were (95°C 3 min; [95°C 40 sec, 55°C 30 sec, 72°C 1 min] 35x; 72°C 10 min). PCR product was run on 1% agarose gel, purified and was cloned into TA-vector (Fermentas). Sequencing was done as described above.

Construction of plant expression vector

Because of NcoI restriction site restraint, CaMV35S-Cry1Ac-NOS cassette of 2476 bp was excised from its source plasmid, pk2Ac (Rashid et al. 2008). The excised product was electrophoresed in 0.7% agarose gel, purified and was cloned into pTZ57-RT supercoiled plasmid (Fermentas) to produce pTZCry sub-vector.

NcoI digested TP was then ligated towards N-terminal of Cry1Ac generating TP-Cry1Ac in pTZTPCry sub-vector. Right orientation of ligated TP was confirmed with orientation primers TP2Cry which were designed in such a way that forward primer (ACATGGCTCAAGGGATACAA) was from TP region, while reverse primer (TAGAATCAGGACCCAGACA) was taken from within Cry1Ac gene to give 876 bp product. The construct cassette 35S-TP-Cry1Ac-NOS was then cloned into plant expression vector pBI121 using HindIII site. Orientation of cloned cassette within pBI121 was confirmed by EcoRI, BamHI and SacI enzymes which had unique sites within vector and cloned cassette.

RB digested with XhoI was ligated finally towards C-terminal of Cry1Ac, generating TP-Cry1Ac-RB (name given pKian-1 vector). Right orientation of ligated RB was confirmed through restriction analysis with HindIII and DraI as well as PCR with orientation primers. Orientation primers CryRB were: forward primer (ATTCAGGACCAGGATTCCTACT) and reverse primer (GATGTCGCTGCTAAAAGTAGACT) to give 772 bp amplification product.

Excised cassette (2476 bp) of CaMV35S-Cry1Ac-NOS was cloned directly into pBI121 plasmid vector to generate positive control pKian-0 plant expression vector.

In-planta *Agrobacterium* GUS leaf infiltration assay

The pKian-1 plant expression vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. To check stable transformation of pKian-1 in *Agrobacterium*, in-planta leaf infiltration GUS assay was carried out in tobacco young leaves. The region of the lower side of leaf was gently rubbed with index finger to remove the wax cuticle. *Agrobacterium* samples were taken in 5 mL syringe (no needle), the plastic-tip of the syringe was placed against the underside of the leaf and was pressed down gently on the plunger while directly supporting the upper-side of the leaf with finger. The liquid could be seen diffusing through the leaf as it filled the mesophyllar air spaces. Outline was marked surrounding the infiltrated area with a marker pen for subsequent identification. Infiltrated leaves were tagged and plants were left for 72 hrs in greenhouse at 28°C and 16 hrs light period. The leaves were then subjected to GUS assay.

Detection of GUS activity

GUS activity was histo-chemically detected in leaves by overnight incubation at 37°C in GUS staining solution composed of 0.08% w/v 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) in 0.1M sodium dihydrogen phosphate pH 7.0, 0.2 mM 10% Triton and 20% methanol. After staining in GUS solution, the plant tissues were submerged in fixative solution (5% formaldehyde, 5% acetic acid, 20% ethanol) for 10 min. Chlorophyll was removed from samples by dipping in 70% ethanol for 48 hrs at room temperature. Ethanol was changed 2-3 times. The GUS expression was detected by sight as well as under OLYMPUS SZX7 microscope by distinct blue colour which resulted from the enzymatic cleavage of X-Gluc. Samples were stored at 4°C in 70% ethanol.

Agrobacterium-mediated cotton transformation

Properly delinted sterile seeds of cotton (*G. hirsutum*) variety MNH-786 were grown at 30°C in dark for 48 hrs. Germinating embryos were isolated. Shoot tips of the isolated embryos were injured towards hypocotyl-end a little away from embryonic-axis by using sharp sterile blade held in petri dish, while shoots were highlighted by using light microscope. After making cut, embryos were immediately shifted to the *Agrobacterium* inoculum suspension in MS-medium for co-cultivation with bacterial culture and incubated for 1 hr on a 30°C shaker at slow speed. The embryos were shifted to MS-medium without antibiotic selection for 72 hrs. Healthy germinating embryos were then shifted to MS-medium containing kanamycin antibiotic (100 µg/L) for discriminate selection of transformants. While, antibiotic cefotaxime (250 mg/L) was also added in MS-medium to inhibit the growth of *Agrobacterium* after the cotton embryos had been co-cultivated for 72 hrs.

Root formation

After 6-8 weeks, the developed shoots were sub-cultured in selection-free medium *i.e.* antibiotics were not added. At this stage, MS-medium was supplemented with growth hormones kinetin+IBA (1 mg/L each), for root formation. Plantlets were continued to subculture on rooting-medium for further 4-6 weeks until roots were fully developed. The well-rooted plantlets were then shifted to pots containing sterile soil.

Genomic DNA isolation and polymerase chain reaction

A method of Zhang et al. (2000) was followed for DNA isolation from putative transgenic cotton leaves. In order to confirm the gene integration into cotton genome, polymerase chain reaction was performed using RB as well as Cry1Ac internal primers (mentioned above) to amplify 816 bp and 565 bp regions, respectively.

Extraction of whole-leaf protein

Fresh leaves of transgenic and positive control plants were used. Approximately 300-500 mg weighed leaves were crushed in liquid nitrogen in pre-chilled pestle and mortar. Ground material was transferred to autoclaved 1.5 mL tube and 400 µL of 1X protein extraction buffer was added (Wang et al. 2006). Samples were homogenized by vortex, incubated at 4°C for two hours and centrifuged at 4°C for 10 min at 13000 rpm. Supernatant was taken in another 1.5 mL tube and protein was quantified with Bradford reagent (Bradford, 1976).

Isolation of chloroplast and protein extraction

To isolate chloroplasts from leaves of transgenic cotton plants, midrib and other large veins from cotton leaves were removed and pieces of leaf lamina were homogenized using six 10 sec cycles in blender set at high speed in 20 mL ice-cold chloroplast isolation buffer [Hepes-KOH (pH 7.5) 50 mM; EDTA (pH 8) 2 mM; MnCl₂ 1 mM; MgCl₂ 1 mM; sorbitol 330 mM; ascorbic acid 100 mM; protease inhibitor cocktail 0.05% (Sigma-Aldrich); BSA (w/v) 0.25%] and filtered through two layers of miracloth. The debris that retained in the miracloth was returned to the falcon tube with 20 mL fresh chloroplast isolation buffer and the homogenization step was repeated. The homogenization process was repeated five times. The chloroplasts were then collected by centrifugation for 8 min at 1,000 g at 4°C.

The chloroplast pellet was re-suspended in protein extraction buffer [glycerol 1%; 0.5M EDTA 0.02 M; 5M NaCl 0.2 M; 1M Tris-Cl (pH 7.5) 0.01M; NH₄Cl 100 mM; DTT 3 mg/mL; PMSF 40 mM] and kept at 4°C overnight. Supernatant was taken in another 1.5 mL tube and protein was quantified with Bradford reagent.

Western blot analysis

Chloroplast-enriched as well as whole-leaf protein samples of transgenic plants were separated on 7% SDS-polyacrylamide gel and were blotted onto Nitrocellulose membrane (Hybond-C Amersham).

The blotted proteins were probed using anti-Cry1Ac antibodies. After incubation with HRP-conjugated secondary IgG, colour was developed by DAB as HRP-substrate. The blot was scanned and the *Bt* contents were quantified using Image-Quant TL software (Amersham BioSciences).

Preparation of leaf-sections for microtomy

For fixation and dehydration, small leaf segments of transgenic cotton plants were placed in 50 mL tubes containing 20 mL of 10% formalin and were kept at 4°C for 2 hrs (overnight preferable). Formalin was removed and the leaf segments were placed in 70%, 80%, 90% and 100% ethanol at 4°C for 2-4 hrs successively. At the end of washing steps with ethanol, the leaf segments were dehydrated and chlorophyll was completely removed. Prior to mounting the leaf segments in histochemical wax, the leaf segments were dipped in xylene three times for 30 min each at room temperature for softening. Paraffin wax (Sigma) was warmed slowly on water-bath at 58°C until it melted. The tissues were embedded in paraffin at 58°C in metal specimen block. The leaf segments were held until wax solidified. The wax block was placed at 4°C overnight. Tissue sections 5-15 µm thick were cut using microtome (SORVALL MT-5000).

Immuno-histochemical staining

Prior to immuno-histochemical staining, wax was removed by dipping slides twice in xylene for 2 min each. For rehydration, slides were immersed in 100%, 95%, 70% and 50% ethanol for 5 min each, followed by rinsing in deionized water briefly to remove ethanol and then rehydrated in wash buffer 1X PBS for 10 min. Non-specific binding of primary antibody was blocked by incubating slides in blocking buffer (1% horse serum in PBS) for 30 min at room temperature. Primary antibodies (Monoclonal mouse Anti-Cry1Ac diluted at 1:100 in Incubation Buffer) were applied and incubated overnight at 2-8°C. Incubation Buffer was composed of 1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton® X-100, and 0.01% sodium azide in PBS. This incubation regime allowed for optimal specific binding of primary antibodies to tissue targets and reduced the non-specific background staining. Next day, washed slides 3 times for 15 min each in wash buffer. The slides were then incubated with the secondary antibody (FITC-conjugated anti-mouse IgG diluted 1:100 in Incubation Buffer) for 30-60 min at room temperature. From this step forward, samples were protected from light. Slides were washed 3 times for 15 min each in wash buffer. A 300µL diluted DAPI solution (1 µL of 14.3 mM stock of DAPI for every 5 mL of 1X PBS) was added to each slide and incubated for 2-5 min at room temperature. DAPI binds to DNA and is a convenient nuclear counterstain. Slides were rinsed once with 1X PBS. Slides were mounted with an anti-fade mounting media and were visualized using confocal laser scanning microscope (Zeiss LSM-510).

Insect bioassays

The transgenic cotton plants were subjected to the standard laboratory bioassays with American bollworm (*Heliothis armigera*). For this purpose, five fresh leaves from each plant were taken and placed on wet filter paper in petri plates accommodating one leaf per plate. One 2nd instar *Heliothis* larva, pre-fasted for 4 to 6 hrs, was released in each plate and allowed to feed on the leaf. The data on insect mortality was taken on daily basis up to fifth day.

RESULTS

Isolation and TA cloning of TP and RB

Fragments of TP (216 bp) and RB (816 bp) were amplified from cDNA of *Petunia hybrida* and *Ricinus communis*, respectively (Figure 1 a and b). After confirmation by sequencing, both nucleotide sequences of TP and RB were submitted to NCBI (Genbank accession No. JF499829; and JQ617861, respectively).

Construction of plant expression vector pKian-1

Plant expression vector pBI121 harbours five NcoI sites; therefore, TP could not be cloned using NcoI site. Therefore, CaMV35S-Cry1Ac-NOS cassette was cloned into sub-vector to allow the ligation of TP with Cry1Ac using NcoI site and orientation was checked by TP2Cry orientation primers (Figure 2). Sequencing of amplified fragment established the right orientation of ligated TP towards N-terminal of Cry1Ac. The pTZ57-RT (2.8 kb) plasmid and cloned CaMV35S-TP-Cry1Ac-NOS (2.7 kb) cassette were nearly of same size; therefore, DraI was used to break down pTZ57-RT vector into five pieces to release TP-Cry1Ac-RB (Figure 3), which was cloned into pBI121 vector using HindIII site within T-border region. Orientation of ligation in pBI121 was confirmed by digesting the plasmid with SacI, EcoRI and BamHI restriction enzymes, giving bands of 3.416 kb, 2.9 kb and 2.96 kb (Figure 4). XhoI-digested RB was finally ligated towards C-terminal of Cry1Ac in pBI121 and orientation of RB was confirmed by 710 bp PCR product (Figure 5) with CryRB orientation primers. Restriction analysis with DraI (5.57 kb) and HindIII (3.57 kb) was also performed (Figure 6), which established the ligation of RB in desired orientation. Plant expression plasmid vector pBI121-CaMV35S-TP-Cry1Ac-RB-NOS was given the name pKian-1 vector. The construction pattern of vector has been shown in partial map (Figure 7).

For the construction of positive control plasmid vector, CaMV35S-Cry1Ac-NOS was cloned directly into pBI121 using HindIII site. Successful ligation was confirmed by amplifying 565bp of Cry1Ac with internal primers. This plant expression vector was given the name pKian-0.

Leaf infiltration GUS assay

Both pKian-1 and pKian-0 plant expression vectors were transformed into *Agrobacterium* strain LB4404 and the efficacy of the vectors as well as transformation efficiency of *Agrobacterium* strain was confirmed by *Agrobacterium*-mediated leaf infiltration GUS assay in tobacco. The appearance of blue colour after 72 hrs in GUS solution indicated that both plasmid vectors were intact and *Agrobacterium* cells were efficient (Figure 8).

Cotton transformation

The local variety MNH-786 of cotton (*G. hirsutum*) was transformed using *Agrobacterium*-mediated shoot-apex method of transformation. A total of 5000 embryos were transformed with pKian-1 (TP-Cry1Ac-RB) and pKian-0 (Cry1Ac only). After eight weeks of selection on 100 mg/L kanamycin, 155 plants of pKian-1 and 65 plants of pKian-0 were obtained MS-medium. Finally, 10 plants of pKian-1 and 5 plants of pKian-0 were shifted to the soil-pots; while, three plants of pKian-1 and two plants of pKian-0 were established successfully in the field. The overall transformation efficiency for pKian-1 and pKian-0 remained 0.52% and 0.33%, respectively.

Polymerase chain reaction

The integration of Cry1Ac-RB fusion-gene in pKian-1 putative transgenic plants was checked by PCR of genomic DNA extracted from transformants. The amplified fragment of 816 bp of RB and 565 bp internal region of Cry1Ac showed successful integration of desired genes in genome of cotton plants (Figure 9). All the transgenic plants were phenotypically normal and grew well in field.

Western blot analysis

A single polypeptide of hybrid-protein Bt-RB (98 kDa size) was observed in transgenic cotton plants, K1, K3 and K5 using chloroplast-enriched as well as whole-leaf protein samples in Western blot (Figure 10). Lanes 1-3 contain whole-leaf, while lanes 4-6 contain chloroplast-enriched protein samples of K5, K3 and K1, respectively. Lane 7 shows K0 whole-leaf protein of 67 kDa size and lane 8 carries trypsin-digested HD73 protein isolated from *Bacillus thuringiensis* and used as positive control of Immunoblast. Whole-leaf protein samples were loaded 2.5 fold more as compared to chloroplast-enriched samples, in order to make estimation of protein concentration easier and authentic using ImageQuant software.

The quantification of Bt-RB hybrid protein expressed in chloroplast was estimated to be approximately 3.5% of the total soluble protein (TSP) in pKian-1 transgenic plants, while 0.34% TSP in case of pKian-0 whole-leaf samples.

Confocal laser scanning microscopy

Confocal microscope images showed green fluorescence for FITC at 488 nm and Chloroplast red auto-fluorescence at 580 nm excitation. FITC fluorescence expressed exactly at the same places after merging where red auto-fluorescence of chloroplasts was detected in the case of pKian-1 transgenic leaf samples (Figure 11). This was evident by yellow colour which was produced through overlapping of green and red fluorescence when FITC and chloroplast auto-fluorescence images were merged (Figure 11). Whereas, merged images of pKian-0 positive control leaf samples showed green fluorescence expressing outside chloroplasts after merging FITC and chloroplast auto-fluorescence images indicating that Cry1Ac protein was expressing in cytosol (Figure 11).

Insect bioassays

Fresh leaves from transgenic cotton plants, and NT controls were subjected to standard laboratory bioassays. High mortality rates of insect killing were observed in case of pKian-1 transgenic plants in these bioassays (Figure 12) than controls. All second instar larvae of *Heliothis* were killed on pKian-1 transgenic leaves; while, pKian-0 controls exhibited \approx 60% mortality rates with American bollworm (Figure 12). On the other hand, *Heliothis* larvae grew well on non-transgenic leaves, and increased in size.

DISCUSSION

Under laboratory and field conditions, tolerance against Bt toxins by the insects has been observed (Moar et al. 2008; Tabashnik, 2008); therefore, coordinated application of different crystal proteins appears to be a better choice for pest resistance management. Strategies to minimize the evolution of insect resistance include, expressing multiple Bttoxins at high doses (e.g. chloroplast-targeted expression of Cry proteins) or joining Bt toxins together (e.g. fusions of cry1Ac-cry1C and Cry1Ba-Cry1Ia) or fusing Bt toxin with some fusion-protein gene that has lectin-binding capability in insect midgut (e.g. Ricin B-chain). In the present investigation, combined effect of both the approaches was successfully used in cotton (*G. hirsutum*). The transit peptide of EPSPS gene and Ricin B-chain were ligated with Cry1Ac, generating TP-Cry1Ac-RB and were substantiated by PCR and restriction analysis (Mehlo et al. 2005; Steiner et al. 2005). The TP-Cry1Ac-RB construct was created after a series of cloning and ligation steps in sub-vectors because of the limitation of the unique restriction sites in T-DNA sequence; an topic which has been explained in detail by Ma et al. (2011). After *Agrobacterium*-mediated nuclear transformation, PCR analysis of putative transgenic cotton plants revealed successful integration of desired transgene in the cotton genome (Figure 9); while Western blot analysis with whole-leaf as well as chloroplast-enriched protein samples displayed single polypeptide of Cry1Ac-RB hybrid protein expressed in chloroplasts (Figure 10). The hybrid protein was estimated to be 3.5% of the total soluble protein using ImageQuant software. Chloroplast-localized expression of Cry1Ac-RB fusion-protein was further established through confocal microscopy (Figure 11). In case of transgenic plants, FITC fluorescence excitation at 488 nm was observed exactly at the places where chloroplasts gave red auto-fluorescence at 580 nm in merged images; thus, establishing the chloroplast confined expression of Cry1Ac-RB fusion-protein (Figure 11). These results were in harmony with the findings of Omasa et al. (2009), and Uniacke and Zerges (2009) who portrayed chloroplast localized expressions through confocal microscopy using the same parameters for fluorescence. Thus targeting the recombinant crystal protein with enhanced insect binding domains inside the chloroplasts would bring down the resistant insect larvae. This was obvious in standard laboratory insect-bioassays with American bollworm (*H. armigera*). During these assays, 100% American bollworm larvae were killed in case of transgenic leaves (Figure 12), revealing the efficacy of the hybrid transgene construct and substantiating the hypothesis that insect tolerance to Bt could be hindered by increasing the lectin-binding domains of crystal protein in the midgut of the targeted-insect. This strategy would also open new way to achieve chloroplast-targeted expression in recalcitrant crops (such as cotton), in which chloroplast transformation fails because of poor callogenesis response (Lössl and Waheed, 2011).

CONCLUDING REMARKS

Chloroplast-targeted expression of recombinant insecticidal crystal protein was achieved successfully in recalcitrant cotton crop using nuclear transformation by tagging chloroplast transit peptide and Ricin B-chain with the crystal protein gene. Thus, complex procedures of chloroplast transformation were avoided whose success was difficult to achieve in cotton as callus generation is still challenging in cotton. *Agrobacterium*-mediated nuclear transformation of cotton mediated the expression of single polypeptide of hybrid-transgene inside the chloroplasts under TP and this was validated by Western blot analysis of whole-leaf as well as chloroplast-enriched protein samples of transgenic plants. The chloroplast-localized expression of transgene was also demonstrated by confocal microscopic studies. Higher expression of insecticidal protein in chloroplasts, as verified in Western blot and enhanced binding domains for Cry1Ac-RB in insect-midgut would crack the resistance level of insects that otherwise have been reported to have evolved resistance against single Bt gene.

Financial support: This work was partially financed by Higher Education Commission of Pakistan under Indigenous program.

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How to reference this article:

KIANI, S.; SHEHZAD, K.; MUZAFFER, A. and HUSNAIN, T. (2013). Cloning and chloroplast-targeted expression studies of insect-resistant gene with ricin fusion-gene under chloroplast transit peptide in cotton. *Electronic Journal of Biotechnology*, vol. 16, no. 6. <http://dx.doi.org/10.2225/vol16-issue6-fulltext-2>

Figures

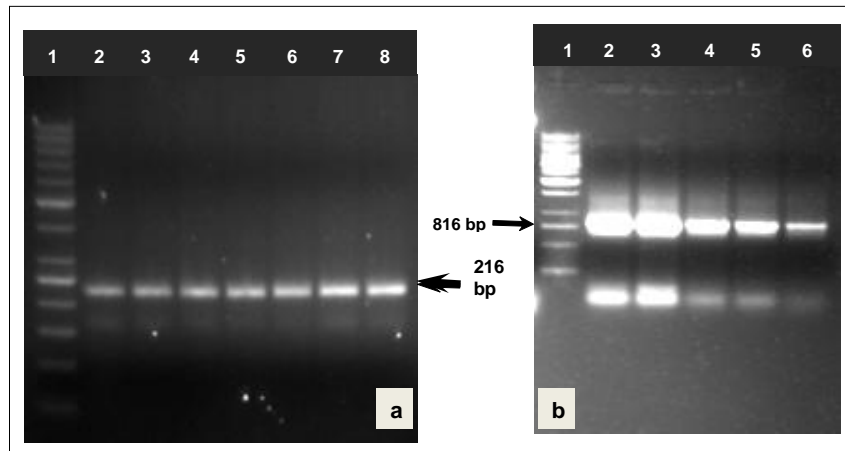


Fig. 1 PCR Amplification of: (a) 216 bp TP of *Petunia hybrida* using cDNA as templates. Lane 1: 50 bp DNA ladder (Fermentas), Lane 2-8: TP from cDNA of *Petunia*. (b) 816 bp RB from *Ricinus communis* using cDNA as templates. Lane 1: 1 kb DNA ladder, Lane 2-6: 816 bp RB from cDNA of *Ricinus*.

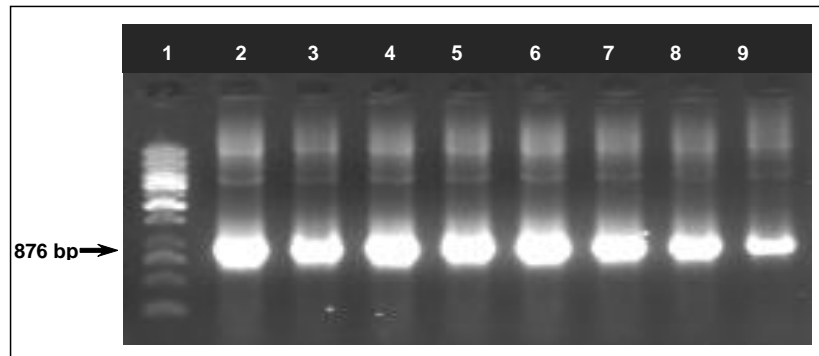


Fig. 2 PCR amplification of 876 bp TP-Cry1Ac region using orientation primers. Lane 1: 1kb DNA ladder (Fermentas). Lane 2-9: 876 bp of TP-Cry1Ac region from pTZ-TPCry vector for orientation analysis.

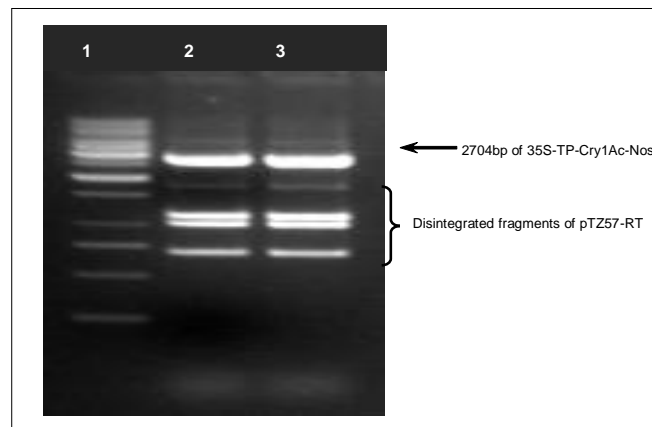


Fig. 3 Digestion of pTZ-TPCry sub-vector with HindIII and Dral enzymes to release CaMV35S-TP-Cry1Ac-Nos cassette. Lane 1: 1 kb DNA ladder (Fermentas). Lane 2-3: Band fragments produced after digestion with HindIII (NEB) and Dral (NEB). 2704 bp band is of 35S-TP-Cry1Ac-NOS with HindIII. While other bands are the fragments of pTZ57-RT plasmid.

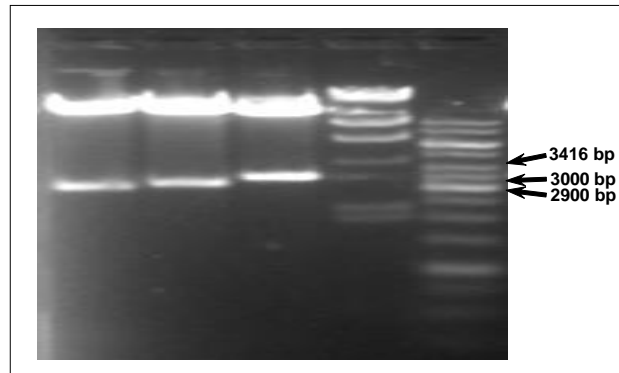


Fig. 4 Restriction analysis of pBI-TP-Cry sub-vector with EcoR1, BamH1 and SacI enzymes. Lane 1: 2900 bp fragment with EcoR1. Lane 2: 2960 bp fragment with BamH1. Lane 3: 3416 bp. fragment with SacI. Lane 4: λ -DNA/HindIII Marker. Lane 5: 1kb DNA ladder (Fermentas).

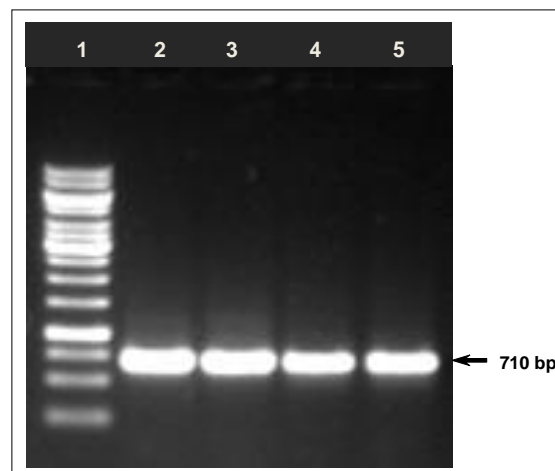


Fig. 5 Orientation PCR of internal region of Cry1Ac-RB in pKian-1 vector. Lane 1: 1 kb ladder (Fermentas). Lane 2-5: 710 bp Cry-RB amplified region in pKian-1 vector.

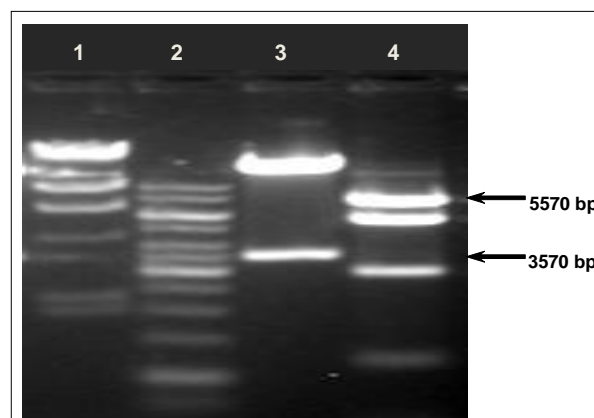


Fig. 6 Restriction analysis of final Plant Expression Vector pBI-TP-Cry1Ac-RB (pKian-1) with DraI and HindIII enzymes. Lane 1: λ -DNA/HindIII Marker. Lane 2: 1 kb DNA ladder (Fermentas). Lane 3: 3570 bp fragment with HindIII. Lane 4: 5570bp fragment with DraI.

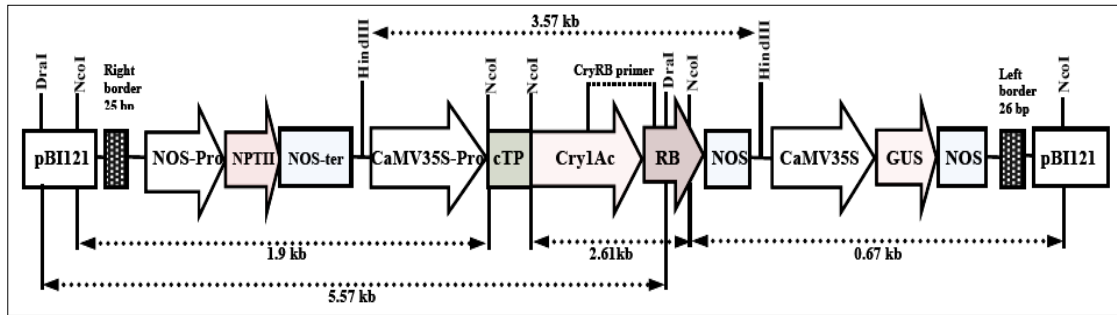


Fig. 7 Partial map of final Plant Expression Vector pKian-1 showing restriction sites.



Fig. 8 *Agrobacterium*-mediated GUS-Infiltration confirmation assay in tobacco leaves using: (I) pKian-1 vector; (II) Positive control pKian-0 vector; (III) Non-infiltrated leaves.

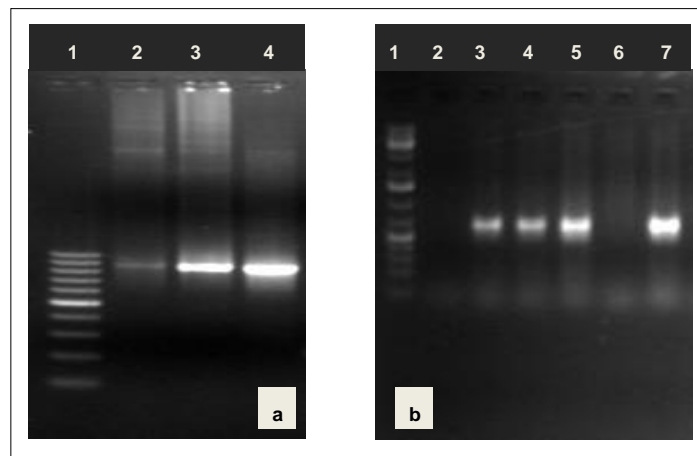


Fig. 9 PCR amplification of: (a) 816 bp RB in pKian-1 transformants. Lane 1: 100 bp DNA ladder (Fermentas); Lane 2-4: 816 bp RB from pKian-1 plants. (b) 565 bp Cry1Ac gene in pKian-1 transformants. Lane 1: 1 kb DNA ladder (Fermentas); Lane 2: Negative control MNH-786; Lane 3-6: 565bp Cry1Ac from pKian-1 plants; Lane 7: Positive control plasmid pKian-0.

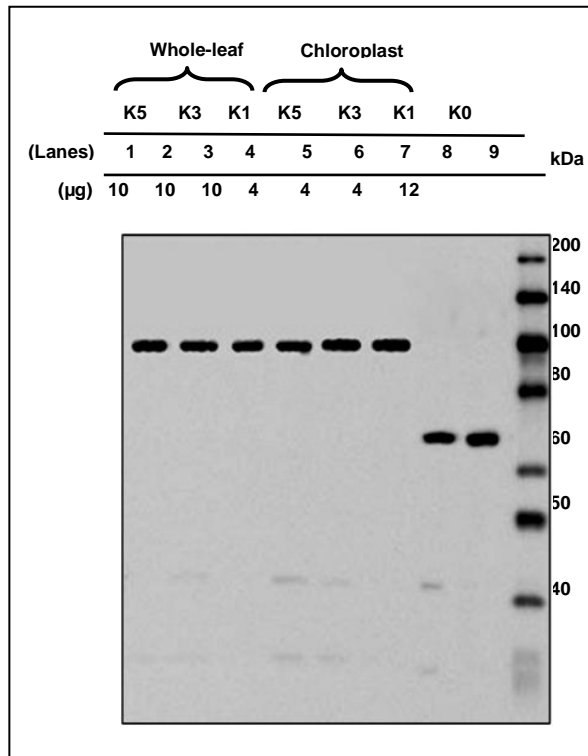


Fig. 10 Hybrid-protein expression. A single polypeptide of hybrid-protein Bt-RB (98 kDa size) was observed in transgenic cotton plants, K1, K3 and K5 using chloroplast-enriched as well as whole-leaf protein samples. Lanes 1-3 contain whole-leaf, while lanes 4-6 contain chloroplast-enriched protein samples of K5, K3 and K1. Lane 7 shows K0 whole-leaf 67 kDa size protein and lane 8 carried trypsin-digested HD73 protein isolated from *Bacillus thuringiensis*. Whole-leaf samples were loaded 2.5 fold more as compared to chloroplast-enriched samples, in order to make estimation of protein concentration easier and authentic using ImageQuant software.

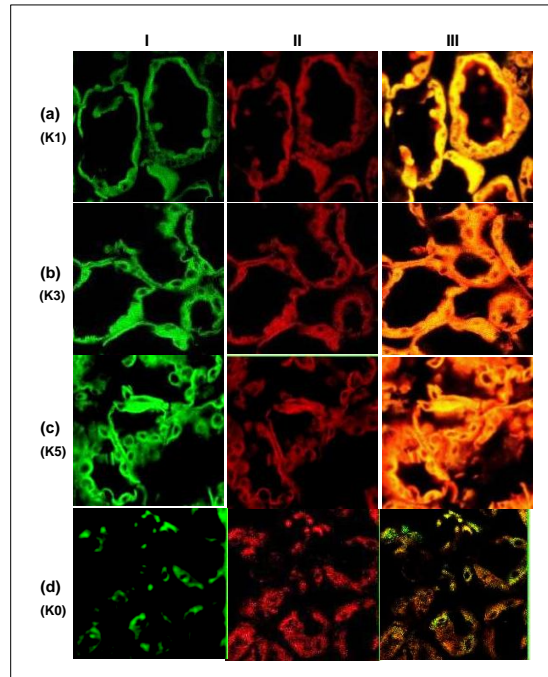


Fig. 11 Confocal Laser Scanning Microscopy of transgenic plants expressing Bt-RB under TP. L.S. of leaf section was labeled with FITC-conjugated IgG and was observed under confocal microscope (Zeiss LSM-510) using appropriate filters. I = FITC green fluorescence at 488nm excitation. II = Chloroplast auto-fluorescence red at 580nm. III = Merged signal of I & II. (a), (b), (c) transgenic plants with pKian-1 (Bt-RB under chloroplast transit peptide); (D) positive control plant with pKian-0 (Cry1Ac without transit peptide). White arrow heads indicate persistent FITC signals; while blue arrow heads indicate persistent chloroplast auto-fluorescence signals.

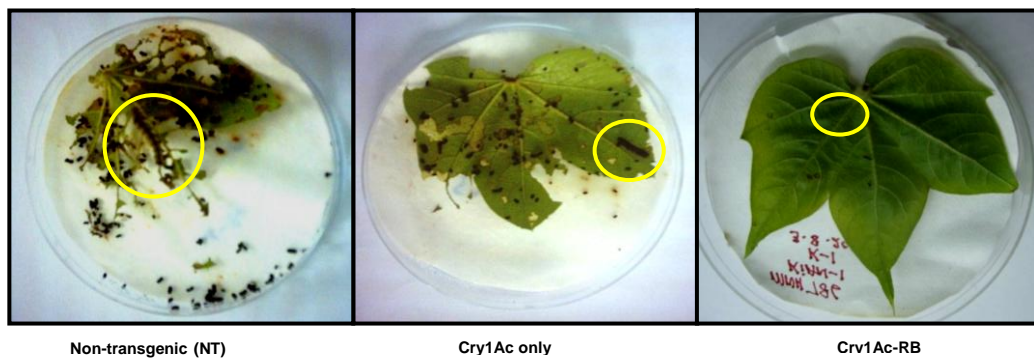


Fig. 12 Standard laboratory insect bioassays with American bollworm: Effects of Bt-RB fusion-protein on average mortality of American bollworm (*H. armigera*) in standard laboratory bioassays of transgenic cotton plants. This representative bioassay shows 100 percent mortality rates of second instar *Heliothis* larvae in transgenic cotton leaves.