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Expression of Bt Gene in a Dicot Plant Under Promoter Derived from a Monocot Plant

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Abstract: The possibility of using a monocot (maize) derived ubiquitin (Ubi) promoter to express a fully modified insecticidal *cry* gene of *Bacillus thuringiensis* in dicot plant, tobacco was studied. Tobacco (*Nicotiana tabacum* cv. xanthi) plants were transformed with Bt gene *Cry-1Ac* driven by Ubi promoter. Transgenic plants were confirmed for transformation, gene expression and insecticidal activity through PCR, GUS assays, Southern blot, Western blot analyses and insect bioassays. Bioassays with cry-1Ac transformed T_0 and T_1 transgenic plants showed high level of toxicity towards American bollworm (*Heliothis armigera*) giving 100% mortality of the larvae. This paper reported that monocot derived Ubi promoter expresses a Bt gene in a dicot plant in an effective manner to render the transformed plants highly resistant against *Heliothis armigera*.

Key words: Ubiquitin promoter, tobacco, cry-1Ac gene, *Heliothis armigera*, American bollworm.

Introduction

The improvement in transformation technology has resulted in the successful production of a number of transgenic plant species. Insect resistant transgenic crops can be produced which can result in improved yields, environmental safety and pesticide-free produce. The rapidly emerging field of plant biotechnology has shown substantial progress in the development of transgenic plants expressing Bt genes, and there are now several reports available for dicot and monocot species transformed with these genes (Fischhoff *et al*., 1987, Hinchee *et al*., 1988, Perlak *et al.,* 1990, Perlak *et al*., 1993, Nayak *et al*., 1997; Kar *et al*., 1999).

The choice of an appropriate promoter is a very important factor in transformation endeavour. The cauliflower mosaic virus promoter (CaMV 35S) has been most widely used for dicot transformation, especially for the development of *cry* gene containing transgenics (Perlak *et al*., 1990). The maize derived ubiqutine (Ubi) promoter is now being used extensively for the production of Bt gene transformed monocot plants such as maize and rice. The behaviour of Ubi promoter, using reporter gene has been studied well in monocots (Cornejo *et al*., 1993). However, the activity of Ubi promoter in dicots (tobacco) was evaluated to be 10 fold lesser than the expression observed under CAMV 35S promoter (Christensen *et al.,* 1992).

This work was designed to study the possibility of using maize Ubi promoter for the expression of an insecticidal Bt gene in dicot plants to evaluate the strength of the promoter for expressing the Bt gene at the levels high enough to render the transformed plant, resistant to the target insects, as it does in monocots (Nayak *et al*., 1997; Cheng *et al*., 1998). The purpose of present work is to widen the range of promoters that can be used for the expression of transgenes in dicot plants.

Materials and Methods

The work presented here was conducted at the Center as a part of the long-term objective to develop crop plants of agricultural importance, inherently resistant against insect pests.

Construction of an *Agrobacterium* based plant expression vector containing cry-1Ac gene under Ubi promoter: The *Cry1Ac* gene fragment, along with ubiquitin promoter (Ubi pro) and nopaline synthase terminator (nos ter) was excised from plasmid Ubi- *Cry1Ac* (Sardana *et al*., 1996) with *Hin*d III enzyme. This ~4kb fragment was ligated into an *Agrobacterium* based plant transformation vector pKHG4 (Le Gall *et al*., 1994), linearized by the same restriction enzyme. The resultant recombinant construct pKMAC (Fig. 1) was transferred to *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.,* 1983) by electroporation. Same construct has also been prepared and used for rice transformation by Cheng *et al*. (1998).

Plant transformation and regeneration: Tobacco (*Nicotiana tabacum,* cv. xanthi) was used for transformation. Leaf discs of tobacco were inoculated as described by Horsch *et al.* (1985), with the *Agrobacterium tumefaciens* strain LBA4404 containing plasmid pKMAC and pKHG4 (vector transformed negative control) separately. Direct regeneration of the tobacco shoots was taken on medium containing 100mg/l kanamycin for selection and 250mg/l of cefotaxime as a bacteriostatic compound. Rooting of the regenerated shoots was taken on MS medium (Murashige and Skoog, 1962) containing 100mg/l kanamycin and 250mg/l of cefotaxime. Non transformed plants cultured under identical conditions but without selection, served as negative control. Well-rooted plants were shifted to pots for further growth and flowering. The transformed plants were screened for the presence of transgenes by a) GUS assay (Jefferson *et al.,* 1987) b) PCR (Saiki *et al.,* 1988) and c) Southern blot analysis (Southern , 1975).

GUS assays: Transformation of plants was checked using GUS assay (Jefferson *et al.,* 1987). $-glucuronidase (GUS) activity was assayed by incubating leaf sections of two months old plants at 37EC for 24 hours in GUS staining solution (0.889mg/ml X-gluc, 0.1mg/ml Chloramphenicol, 10mM NH_2PO_4, 0.1% triton-X-100, 20% Methanol and pH adjusted at 7-8). The plants transformed with pKMAC (containing

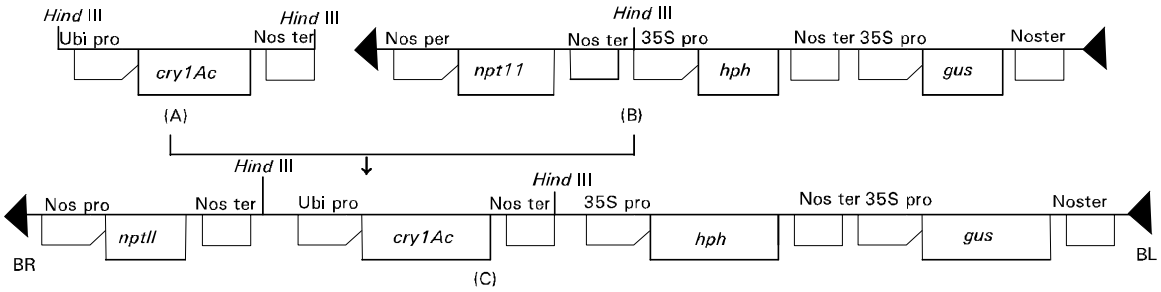

Fig. 1: Construction of plant expression vector pKMAC. A, Ubi-*Cry1Ac*–Nos cassette from plasmid Ubi-*Cry1Ac*. B, region of vector pKHG4 inside T-DNA borders showing *HindIII* site between the *nptII* and *hph* genes. C, positioning of Ubi-*Cry1Ac*-Nos fragment in plasmid pKMAC. Map not drawn to scale. BL, Left boarder BR, Right boarder

Table 1: Insect (*Heliothis armigera*) bioassay of *Cry1Ac* transformed T_o plants (primary transformants) at different growth stages. Vector transformed and non-transformed plants were used as control. All the *Cry1Ac*-transformed plants gave promising larval mortality at all the growth stages tested.

	Mean value of Mortality(%) [a]					
Plants growth stage	Control (non transformed)	Control (Vector transformed)	CM1	CM2	CM3	CM4
E1	0 ± 0	0 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
E2	0 ± 0	0 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
E3	10 ± 10	10 ± 10	90 ± 10	100 ± 0	90 ± 10	100 ± 0

a) Mean ± SE E1, two months old E2, four months old E3, At flowering stage CM, transgenic line

Table 2: Analysis of transgenes expression. Three independent T_1 lines transformed with *Cry1Ac* were bioassayed with *Heliothis armigera* larvae, in five replicates. Non transformed and vector transformed plants were used as controls.

Parent Plants (Primary transformants)	Number of T1 plants tested from each independent line.	Number of plants found resistant.	Number of plants found susceptible	% Mean value of mortality a
Control non transformed)	5	0	5	10 ± 10
Control (vector transformed)	4	0	4	10 ± 10
CM1	10	10	0	100 ± 0
CM2	10	10	0	100 ± 0
CM3	35	34	1	96 ± 0.03

a) Mean ± SE

Fig. 2: Bioassay of transformed T_1 tobacco plants with 2nd instar larvae of *Heliothis armigera.* A and B, larvae actively feeding on the leaves of non-transformed control and vector transformed control plants respectively. C and D, larvae dead after slightly feeding on leaves of *Cry1Ac* transformed plant.

Cry1Ac and GUS genes) and vector pKHG4 (containing GUS gene) transformed plants were checked for transformation with GUS assay using non-transformed plants as a negative control.

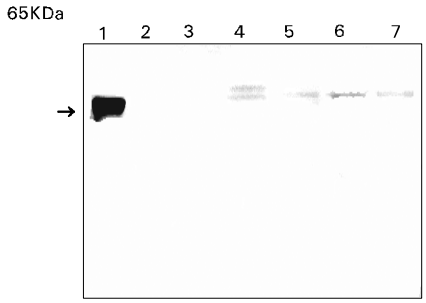

Fig. 3: Western blot analysis to detect expression of 65kDa *Cry1Ac* toxin in total soluble protein from T_o transgenic tobacco plants. All four plants showed the 65kDa band with *Cry1Ac* antibodies (lanes 3-6). Vector transformed plant was used as a negative control (lane 2), and purified, trypsin digested *Cry1Ac* protein was used as a positive control (lane 1).

Genomic DNA Isolation and PCR analysis: The plant DNA was isolated from control and transgenic plants by a modified method of Paterson *et al*. (1993). Briefly, after grinding 300mg of leaves in liquid nitrogen to a fine powder, 1ml of extraction buffer containing 0.35 M glucose, 0.1M Tris-HCl pH8.0, 0.005M EDTA, 2% PVP, 1% ascorbic acid and 0.2%

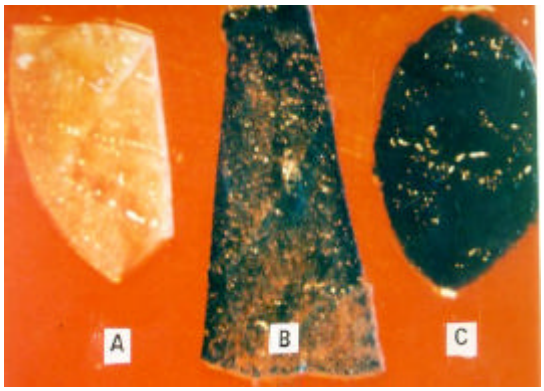

Fig. 4: Gus assay of T_1 plants: A, Non transformed control; B, vector transformed control; C, Transformed with *Cry1Ac*

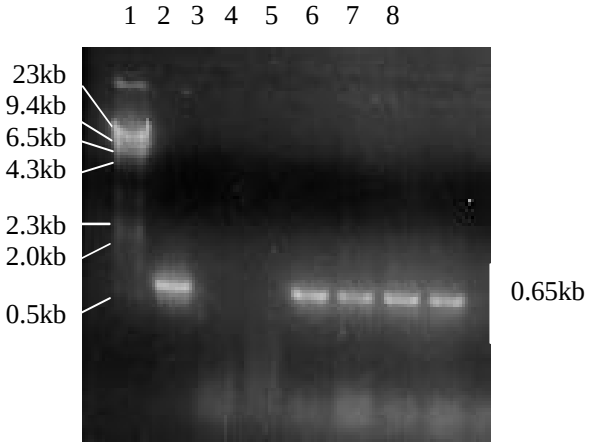

Fig. 5: PCR analyses of T_1 plants. Lane 1, Lambda DNA digested with *HindIII*; Lane 2, Positive control; lane3, Non transformed plant; lane 4, vector transformed plant; Lane 5-8, *Cry1Ac*-transformed plants. All the *Cry1Ac* transformed plants showed the presence of 0.65kb internal fragment of *Cry1Ac* gene.

mercaptoethanol was added to powder. The material was transferred to the eppendorf tubes, mixed well and kept on ice for 10-15 min followed by centrifugation for 20min at 4EC to collect the pellet. To the pellet, 400µl (microliter) of lysis buffer (0.14M Sorbitol, 0.22M Tris-HCl pH 8.00, 0.022M EDTA, 0.8M NaCl, 0.8% C-TAB, 1% Sarkosyl, 50µg/ml Proteinase-K and 1% PVP) was added and mixed well with gentle vortexing. The suspension was then incubated at 65EC for 30min in water bath and extracted once with 500µl of Chloroform:isoamyl alcohol (24:1). The DNA from aqueous phase was obtained by precipitation with an equal volume of Iso-propanol, washed with 70% ethanol and resuspended in TE buffer.

The genomic DNA of T_o (primary transformants) and T_1 (first generation) plants was analyzed for the presence of the *Cry1Ac* gene by performing PCR (Saiki *et al.*, 1988). Non-transformed and vector transformed plants were used as negative controls. The internal fragment of 0.65kb from *Cry1Ac* gene was PCR amplified using following primers:

Forward primer:
5'-ACA GAA GAC CCT TCA ATA TC-3'
(ntd. 1106-1125)
Reverse primer:
5'-GTT ACC GAG TGA AGA TGT AA-3'
(ntd. 1741-1761)

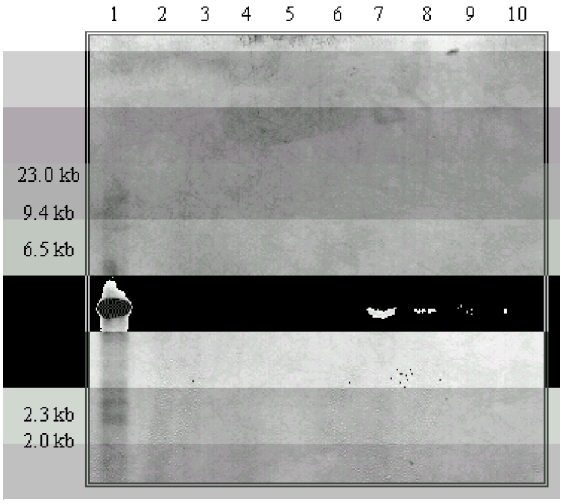

Fig. 6: Southern blot analysis of *HindIII* digested DNA of T1 plants probed with DIG-labeled *Cry1Ac* probe. Lane 1, *HindIII* digested plasmid DNA of construct pKMAC; lane 2, empty; lane 3, *HindIII* digested plasmid DNA of vector pKGH4; lane 4, Non transformed plant; lane 5, vector (pKGH4) transformed plant; lane 6, empty; lane 7, plant CM1.5; lane 8 plant CM2.8; lane 9. CM3.1; lane 10 CM3.27.

For PCR the reaction mixture was subjected to 1 cycle of 93EC for 5 min, 35 cycles of three steps each including 94EC for 45 sec, 52EC for 45 sec, 72EC for 1 min, and one last cycle of 94EC for 45 sec, 52EC for 45 sec and 72 EC for 10 min. The amplified product was analyzed on 1.2% agarose gel.

Southern blot analysis: Genomic DNA was isolated from leaf tissues using osmotic lysis method, adapted from Shure *et al*. (1983) modified by Jychien Chen, Cold Spring Harbor, laboratories, (1986) (Negrutiu and Charti, 1991). To confirm the presence of intact *Cry1Ac* gene cassette, 60µg of genomic DNA and to detect the copy number, 100µg of genomic DNA from each of selected T1 transgenic plants was digested with *HindIII*, fractionated separately on 0.7% agarose gel, transferred onto nylon membrane, and hybridized to digoxigenin (DIG)-labeled probes (*Cry1Ac* and gus probes respectively) according to manufacturer's instructions (Boehringer Mannheim).

Protein expression studies: The transformed plants were studied for the expression of *Cry1Ac* gene through a) Bioassays against *H. armigera* and b) Western blot analysis.

Bioassays: The *Cry1Ac* transformed T_o plants were repeatedly assayed for insect resistance by feeding 2nd instar *Heliothis*

armigera larvae on leaves each from non-transformed control plants, transformed negative control plants and *Cry1Ac* transformed plants. The primary transformants, T_o plants were bioassayed at three different stages: a) two months old, b) four months old. c) Plants at flowering stage. For bioassay, leaves were detached from plants with scissors and placed in petri plates 60 × 15mm^2 (Bibby sterilin, UK) containing filter paper, moistened with few drops of dH_2O to avoid the wilting of leaves during bioassay. Ten replicates were used for each plant per experiment i.e. ten leaves were taken per plant per growth stage, each leaf was separately infested with a single 2nd instar larva and the plates were sealed with parafilm. Sealed plates were kept for bioassay at 12h photoperiod, 27EC temperature and 65-70% relative humidity. Larval mortality was monitored for three days.

Bioassay of T_1 plants from independent lines was conducted by infesting leaves from 4 months old T_1 plants with 2nd instar larvae. Total of 55 T_1 plants from independent lines CM1, CM2 and CM3 were tested to evaluate the potential toxicity of transformed plants. Conditions used for conducting the bioassay of T_1 plants were similar to those used for bioassay of T_o plants.

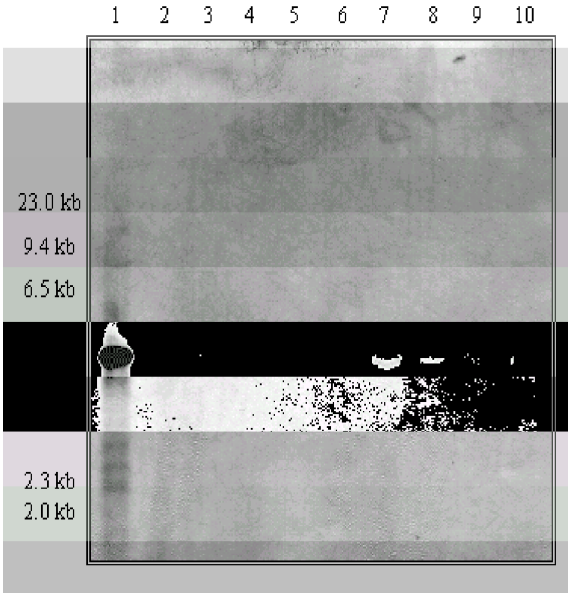

Fig. 7: Southern blot analyses of *HindIII* digested DNA of T1 plants probed with DIG-labeled gus probe to detect the copy number of transgenes. Lane 1, *HindIII* digested plasmid DNA of vector pKGH4; lane 2, *HindIII* digested plasmid DNA of construct pKMAC; lane 3, Non transformed plant; lane 4, pKGH4 transformed plant; lane 5, plant CM1.5; lane 6 plant CM2.8; lane 7, CM3.1; lane 8 CM3.27.

Western blot analysis: Leaf material (weighing 400mg) was taken from each plant and ground to fine powder in liquid nitrogen. To this powder, extraction buffer (10% glycerol, 40mM EDTA pH 8.0, 150mM NaCl, 100mM NH_4Cl, 10mM Tris-HCl pH 7.5, 20mM DTT and 2mM PMSF) was added. The suspension was centrifuged at 14,000 rpm and 4EC for 5 min. The protein concentration was detected by Bio-Rad dye reagent (Bradford, 1976). Approximately 45µg of extracted proteins were resolved on 12% SDS-PAGE. Trypsin digested 65kDa *Cry1Ac* protein was used as a positive control. After electrophoresis, the proteins were electroblotted onto nitrocellulose membrane using semi dry electrotransfer apparatus (Bio-Rad). The membrane was blocked in 5% skimmed milk in TBST buffer (10mM Tris-HCl pH8.0, 10mm NaCl, 0.05% Tween-20) (Sambrook *et al.*, 1989). After washing with TBST the membrane was probed with an anti-serum raised in rabbit against *Cry1Ac* protein. The membrane was washed with TBST and treated with secondary antibody (Alkaline phosphatase-conjugated anti-rabbit IgG) and then washed. The membrane was treated with colour development solution (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt in alkaline phosphatase substrate buffer (Sambrook *et al.*, 1989).

Results and Discussion

The successful expression of a foreign gene in plants depends upon many factors, including selection of an optimum promoter (Perlak *et al.,* 1990). In this study, tobacco was used as a model system to evaluate the potential of Ubi promoter for the expression of Bt gene in dicot plants. The binary construct pKMAC was used for tobacco transformation. The Bt *Cry1Ac* gene under Ubi promoter was fully modified (Sardana *et al.*, 1996) and had 47.7% G + C content, having exactly same sequence of *Cry1Ac* gene reported to be highly expressed in dicots under CaMV35S promoter (Perlak *et al.,* 1990; Perlak *et al.,* 1991). This indicates that the modified *Cry1Ac* gene was equally good for expression in dicot system and an appropriate choice for assessing the efficiency of Ubi promoter for expression of Bt gene in dicot plant.

Ubiquitin promoter has been derived from maize (Christensen *et al.*, 1992) and due to high expression it has been used extensively in transformation of monocots. Rice, in this regard, has been studied well (Cornejo *et al.,* 1993) and the Ubi promoter has been used successfully for the production of number of rice transgenes expressing a variety of Bt genes (Nayak *et al.*, 1997; Cheng *et al.*, 1998).

The studies done on *Cry1Ac* transformed plants included GUS assay, bio-toxicity assay, PCR, Southern blot and Western blot analyses. The T_o tobacco plants were checked for insect toxicity by conducting bioassay on detached leaves at three stages. Clear difference was noted in the feeding behaviours of the larvae for *Cry1Ac* transformed and control plants. Voracious feeding was observed in control plants as compared with *Cry1Ac* transformed plants, where larvae showed an avoidance behaviour after slight initial feeding (Fig. 2). The *Cry1Ac* transformants showed high percentage of larval mortality in all three stages i.e., two months old, four months old and plants at flowering stage, which indicated the persistence of expression throughout the plant life. Whereas on controls, the larval survival was very high (Table 1) as they had entered third instar of their growth. Furthermore, the use of vector (pKHG4) transformed negative control eliminating the chance of toxicity coming from any factor other than *Cry1Ac* gene. Out of 55 T1 plants coming from 3 independent transgenic lines (CM1, CM2 and CM3), 54 exhibited high toxicity towards *H. armigera*. All T_o plants showing toxicity in bioassays were found expressing 65kDa *Cry1Ac* protein in western blot (Fig. 3). The transformation status of transgenic plants was also confirmed by conducting GUS assay of T_1 plants (Fig. 4). Both the control and experimental transformants showed blue coloration upon performing GUS assay (Jefferson *et al.,* 1987) indicating the expression of gus reported gene present in the vector (Fig. 1). The presence of *Cry1Ac* gene was confirmed by PCR

amplification. All the transformed plants analyzed were found containing the *Cry1Ac* gene as indicated by the amplification of a 655 base pair internal fragment of *Cry1Ac* gene (Fig. 5). Further confirmation of the transgenic status of plants was done through Southern blot analysis of T_1 plants indicating the presence of intact gene cassette, as a ~4kb *Hind* III fragment. (Fig. 1 & 6). To study the integration of transgenes/s the blot containing the *Hind* III digested genomic DNAs was subjected to hybridization with DIG-labeled (Boehringer Mannheim) gus probe. Most of the transgenic plants tested, revealed two copies of the *gus* gene represented by two bands in the range of >5kb- 20 kb , in lane 4 - 8 (Fig. 7) indicating insertion at multiple sites, as the minimum estimated size of gus containing fragment between BL (left boarder) and *Hind* III restriction site is 5kb. No plant with single copy was detected among transgenics. The *Hind*III restriction enzyme releases complete Ubi-cryIAc-nos cassette from the construct. Furthermore, as *HindIII* cuts ~5kb upstream of left T-DNA border leaving hph and gus genes attached with the left border, the number of bands appearing in Southern blot with gus probe can be correlated with the copies of the integrated transgenes.

In conclusion, tobacco was successfully transformed with *Cry1Ac* gene driven by Ubi promoter as indicated by positive results of Southern blots. Moreover, the expression of the transgenes in tobacco under the monocot promoter was found to be equitable enough to cause 100% mortality of the pest larvae.

To our knowledge this is the first report regarding the successful use of monocot (maize) derived Ubi promoter for the expression of Bt gene in a dicot system. This observation opens the door to study Ubi promoter for the expression of other foreign genes in other dicots.

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