Theor Appl Genet (2014) 127:2555–2565
DOI 10.1007/s00122-014-2397-5

ORIGINAL PAPER

Development of pod borer-resistant transgenic chickpea using a pod-specific and a constitutive promoter-driven fused *cry1Ab/Ac* gene

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Received: 24 October 2013 / Accepted: 11 September 2014 / Published online: 25 September 2014

Abstract

Key message **We studied pod-specific *msg* promoter from soybean and developed different transgenic lines of chickpea expressing fused *cry1Ab/Ac* constitutively and pod specifically for resistance against the destructive pest *Helicoverpa armigera*.**

Abstract Crystal (Cry) proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) play an important role in controlling infestation of *Helicoverpa armigera*, which has been considered a serious problem in chickpea productivity. This study was undertaken to overcome the problem by introducing fused *cry1Ab/Ac* insecticidal gene under the control of pod-specific soybean *msg* promoter as well as rice *actin1* promoter into chickpea var. DCP 92-3 by *Agrobacterium*-mediated transformation. Transgenic chickpea lines were characterized by real-time PCR, ELISA and insect bioassay. Expression of fused *cry* gene under constitutive and pod-specific promoter results in increase of 77- and 110-fold, respectively, compared to non-transgenic control plants. Levels of Cry toxins produced under the control of *actin1* and soybean *msg* promoter were also estimated by ELISA in the leaves and pods, respectively. The higher expression of fused *cry* gene caused a lethal effect in larvae. The results of insect bioassay study revealed significant reduction in the survival rate of *H. armigera* reared on transgenic chickpea twigs as well as on pods. Pod-specific promoter-driven fused *cry* gene provides better and significant management strategy of pest control of chickpea without phenotypic cost.

Abbreviations

Bt	*Bacillus thuringiensis*
Cry	Crystal protein
TSP	Total soluble protein
ELISA	Enzyme-linked immunosorbent assay
WT	Wild type

Introduction

Legumes are important crop for human consumption and account for 27 % of the world's primary crop production. *Cicer arietinum* L. (chickpea) holds the third position in food legume crop production in the world. Besides being of high dietary protein value providing 33 % of dietary nitrogen requirement, it also helps in soil fertility management through nodular nitrogen fixation (Abu-Salem and Abou 2011; Maiti 2001). Insect pests are the major limitations to chickpea production. Pod borer *Helicoverpa armigera* is the major insect pest that causes considerable damages to chickpea. Sap-sucking insects act as vectors for spreading viral diseases and also pose a big threat to chickpea production (Romeis et al. 2004). However, the recent years have seen a reduction in its global yield due to infestation by pests and pathogen infection, particularly by *H. armigera*

Communicated by Emmanuel S. Guiderdoni.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2397-5) contains supplementary material, which is available to authorized users.

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(Sharma et al. 2005). Breeding for resistance in chickpea to *Helicoverpa* has remained a serious challenge due to its complex nature and non-availability of good resistance resources (Sharma et al. 2008).

Helicoverpa armigera is a cosmopolitan pest having high mobility, high reproductive rate, short generation time and high polyphagy (Fitt 1989). The insect mainly attacks or infests the young pods and foliages of chickpea causing 20–30 % yield losses in India (Gaur et al. 2010). Development of improved insect pest-resistant varieties of chickpea by conventional breeding is difficult due to its narrow genetic base, limited genetic diversity for this trait, barriers for sexual incompatibility and high degree of autogamy (Van Rheenen et al. 1993; Somers et al. 2003). Hence, limited success of conventional breeding, hazardous chemical means and development of resistance in pest have directed for a better, potential choice of insect resistance by incorporating *cry* gene derived from *Bacillus thuringenisis* into chickpea.

To generate insect-resistant transgenic plants, different strategies have been developed involving constitutive and tissue-specific expression of transgenes. Apart from the seed-specific (Chen et al. 1989; Cho et al. 1995) and nodule-specific promoters (Stougaard et al. 1987; Lauridsen et al. 1993), pod-specific *msg* gene promoter has been characterized from soybean. The *msg* promoter has the potential to direct gene expression in the pod, but not in the mature seeds (Stromvik et al. 1999). The expression of resistant gene under the control of *msg* promoter may protect the developing pods from pest and pathogens.

Different types of *cry* genes have been reported to be used successfully to develop insect-resistant crops. Many of the transgenic crops with *cry* genes have been commercialized such as Bt-cotton in several countries including India, and Bt-corn in USA. *cry* gene has been successfully applied to different crops species, particularly in rice (Datta et al. 1998; Tu et al. 1998; Datta et al. 2002) and cotton (Perlak et al. 2001), to improve resistance against insects. Synthetic *cry 1X* gene comprising different elements of *cry 1Aa, cry 1Ab, Cry 1Ac* and *cry 1I* is found to be effective against lepidopteran insect pests (Asharani et al. 2011). Development of insect resistance through transgenesis with fused *cry* genes has been previously applied to protect cotton from damage to cotton bollworm (Pray et al. 2001). *Cry1Aa* showed improved resistance in cassava against *Helicoverpa armigera* (Duan et al. 2013). Gene pyramiding with *cry1Ac* and *cry1Ab* also showed improved resistance against *H. armigera* (Mehrotra et al. 2011).

In the present study, we have demonstrated the successful introduction of fused *cry1Ab/Ac* gene in chickpea genome governed by two different promoters, i.e. constitutive (*actin 1* from rice) and pod-specific promoters (*msg* from soybean) in separate transformation experiments to develop resistant lines for improved plant protection against insect pest *H. armigera*. The expression pattern of this fused *cry* gene under two different promoters has been analyzed through RT-PCR, real-time PCR and ELISA. Insect feeding assay of the transgenic plants for a period of 96 h indicated effective plant protection against *H. armigera* (100 % mortality) compared to WT non-transformed plants. The agronomic performances of the transgenic chickpea plants were compared with non-transgenic chickpea control plant to highlight the overall performances of the transgenic plants.

Materials and methods

Plant material

The chickpea (*Cicer arietinum* L.) cultivar DCP 92-3 was used in this study as plant material. Seeds were obtained from the Indian Institute of Pulses Research, Kanpur, India.

Insect strain and rearing

Eggs of *H. armigera* were obtained from the Indian Agricultural Research Institute (IARI), New Delhi. After 2 days, the neonate larvae of *H. armigera* were reared on artificial diet as provided by IARI, New Delhi, and maintained at 26 ± 2 °C and 70 ± 5 % RH for 3 days. The 3-day-old larvae were finally used for bioassay.

Cloning and construct preparation of soybean *msg* promoter with reporter gene

A 1.2 Kb fragment of pod-specific promoter P_{msg} was PCR amplified from soybean genomic DNA with primer pair (F-5′-CCAAGCTTGGCTAGATGAACTGCTTTAAGG-3′; R-5′-CGGGATCCCGTCTTGAATTCAAATAATTGC-3′, Accession No: AJ239127.1) containing restriction sites for *Hin*dIII and *Bam*HI enzymes as overhang using HotStar HiFidelity DNA Polymerase (Qiagen, Hilden, Germany). This fragment was cloned into pBluescript II SK+ vector (pBSK+) and sequenced for verification. The fragment was digested with *Kpn*I and *Bam*HI from pBSK+ and subcloned in pCAMBIA1301 upstream of *gus* reporter gene to generate *pCAMBIA-*P_{Msg}*-gus* construct. CaMV35S promoter, which was originally present at the upstream of *gus* gene in pCAMBIA 1301, was eliminated by *Hin*dIII–*Bgl*II digestion and klenow treatment, followed by self-ligation.

Vector construction for insect-resistant transgenic plant development

Fused cry *1Ab/Ac* gene was provided by Yunliu Fan. The *cry1Ab* and *cry1Ac* were fused to prepare *cry1Ab/Ac*. The first 448 amino acids of the fused protein are identical to

the analogous region of CRY1Ab (except C5-R6, D304 and D385, instead of P5-N-I-N-E-C-I11, A309 and Y390, respectively). The remaining 449–615 amino acids are truncated from CRY1Ac without any change (Tu et al. 1998; Ye et al. 2001).

Two different vectors were constructed with this gene under the control of two different promoters, namely constitutive rice *actin1* and pod-specific soybean *msg*. The promoter P_{msg} was cloned in the MCS of pCAMBIA1301 at *Hin*d III–*Bam* HI site. The gene *cry1Ab/Ac* with nos terminator was released from pFHBT1 (Tu et al. 2003) vector and subsequently cloned at the downstream of P_{Msg} promoter at *Bam*HI–*Kpn*I site to generate *pCAMBIA-*P_{msg}*-cry1Ab/Ac-nos* (*msg-Bt*) construct.

Actin1 promoter and *nos* terminator fragment were cloned at *Hin*dIII/*Sac*I and *Sac*I/*Eco*RI, respectively, in the vector pCAMBIA1301. *Sac*I fragment containing the fused *cry1Ab/Ac* gene was released from the vector pFHBT1 and was ligated at *Sac*I site of the vector to generate *pCAMBIA-actin-cry1Ab/Ac-nos* (*actin-Bt*) construct. These two constructs were made reporter gene (*gus*) free by *Bst*EII–*Bgl*II digestion, klenow treatment and self-ligation.

The constructs were finally mobilized to *Agrobacterium tumefaciens* by the freeze–thaw method for plant transformation (An 1987).

Plant transformation

Plant transformation was carried out following the method as described by Sarmah et al. 2004 and Acharjee et al. 2010 with some modifications. The seeds were surface sterilized with sodium hypochlorite solution for 20 min and rinsed thoroughly in autoclaved double-distilled water. Sterilized seeds were germinated on seed germination medium (MS medium supplemented with sucrose 3 % and agar 0.8 %). Embryonic axis obtained from aseptically germinated seedlings and were wounded and infected with *Agrobacterium* strain harbouring the constructs for 30 min (OD_{600} 0.8). After 9 weeks on regeneration medium containing 50 mg l^{-1} of hygromycin, the surviving healthy putative transgenic plants were transferred to a greenhouse.

PCR screening

The transgenic plants were initially verified by PCR with *cry1Ab/Ac* gene-specific primer, using genomic DNA isolated from young leaves (Dellaporta et al. 1983). PCR amplification was performed with 100 ng of genomic DNA as template. The PCR cycle was as follows: 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1 min. The amplification was done for 35 cycles. PCR was performed using the primer pairs (Bt F-5′-CGGATCCGATCTTCACCTCAGCGTGCTT-3′ and Bt R-5′-CGAGCTCGGGCACATTGTTCTGTGG-3′).

Histochemical GUS staining for pod-specific activity of *msg* promoter

Histochemical staining of GUS activity was conducted as described previously (Jefferson 1987). Different parts of plants transformed with the *promoter-gus* construct were used in qualitative gus staining. Flowers and pods were incubated in the solution at 37 °C for 24 h. The pigments and chlorophylls were removed by repeated ethanol treatment.

Qualitative detection of CRY protein using dipstick method

Determination of Bt protein expression by using dipstick as supplied by Maharashtra Hybrid Seed company, Maharashtra, India, was performed with T_1 transgenic plants of actin-Bt plants.

Genomic DNA extraction and Southern blot analysis

Genomic DNA was extracted from the leaf tissues of transgenic and control plants following the method of Dellaporta et al. (1983). Quantification of DNA was performed using Nanodrop spectrophotometer (Thermofisher, USA). Southern hybridization was carried out, following the protocol as described by Sambrook and Russell (2001). About 15 µg of DNA per sample was digested with *Hind*III/*Xba*I restriction endonucleases (Roche, Germany). The digested DNA was separated by electrophoresis on 1 % (w/v) agarose gels. A 800 bp PCR product of fused *cry1Ab/Ac* gene was used as a probe and labelled with (α-^{32}P) dCTP using a DecaLabel™ DNA Labelling Kit (Fermentas). The membrane was exposed for autoradiography.

Semi-quantitative reverse transcriptase (RT) PCR and quantitative real-time PCR analysis

Total RNA was extracted from leaves as well as from pods (100 mg) of transgenic plants and WT control plants, using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA samples were treated with recombinant DNase I (Roche Applied Science, Penzberg, Germany) to remove all traces of genomic DNA from the samples. Five micrograms of RNA was used for cDNA synthesis using the Maxima First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). One microlitre of the cDNA was used as template for semi-quantitative PCR using transcriptor high-fidelity cDNA synthesis kit (Fermentas, Ontario, Canada). The quantitative real-time PCR was performed according to a previously described protocol (Molla et al. 2013). For each reaction, 1 µl of cDNA was used as template in a 10 µl qRT-reaction mixer. Primers were synthesized for quantification of *cry1Ab/Ac* transcript (Bt-rt-F-5′-GACTGCTGG

AGTGATTATCGACAGA-3′ and Bt-rt-R-5′-AGCTCGGT ACCTCGACTTATTCAG-3′; amplicon size 83 bp; Made et al. 2006) and for an internal control gene, *GAPDH* (GF-5′-GGAGTCACATGCTGCCTAAGGTT-3′ and GR-5′-TC ACTGCCA GCTTACGGAGG-3′; amplicon size 65 bp; Accession No. AJ010224). The specificity of the PCR amplification was checked with a heat dissociation curve (65–95 °C) following the final cycle of the PCR and agarose gel electrophoresis of the products. Quantitative variation among different samples was determined using the $\Delta\Delta C_t$ method. All the data were analysed using Bio-Rad CFX manager software (BioRad). The mean value for the expression level of the gene was calculated from three independent experiments.

Enzyme-linked immunosorbent assay (ELISA) for quantitative estimation of Bt-protein

Quantitative estimation of fused insecticidal Cry1Ab/Ac endotoxin protein was performed by the ELISA kit as supplied by the Central Cotton Research Institute, Nagpur, India. Protein was isolated from the leaves of transgenic chickpea plants transformed with *actin-Bt* construct, the young pods of transgenic plants transformed with *msg-Bt* construct and non-transgenic control plants and monitored according to the manufacturer's instructions.

Insect bioassay

Insect bioassays were performed with two trifoliate leaves in case of transgenic plants with *actin-Bt* construct and with healthy green pods in case of transgenic plants with *msg-Bt* construct. Samples were collected from 15 selected transgenic plants as well as WT control plants. At first, the samples for feeding were washed with distilled water and wiped thoroughly to clean off all dirt. About 250 mg fresh twigs and healthy green pods were placed in 35 mm Petri dishes and incubated on top of moist Whatman No. 1 double filter papers. Six neonate larvae were used for each Petri dish. Plates were covered to prevent desiccation and incubated at 25 ± 1 °C for 16 h photoperiod and 70 % relative humidity. Water requirement was checked daily and topped up according to necessity. Feeding was allowed for 4 consecutive days. Larval mortality rate was recorded at 48, 72 and 96 h.

Agronomic performance

Yield performances of the transgenic lines were compared with WT chickpea plants. Several agronomic parameters such as plant height (cm), pod length (mm), pod width (mm), pod thickness (mm) and number of pods per plant were evaluated with both WT and transgenic plants at different developmental stages of their life cycle.

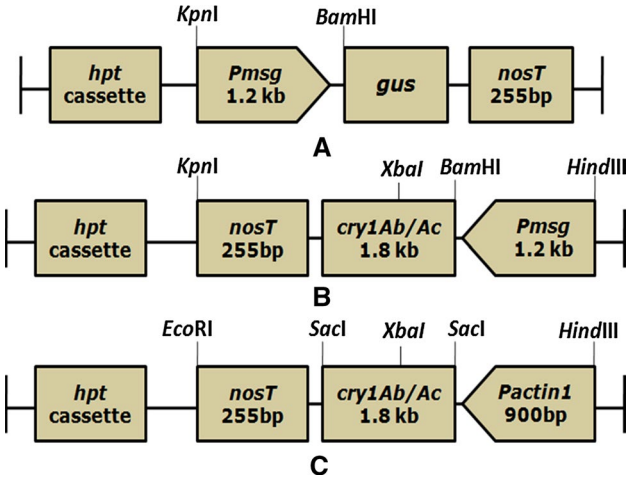

Fig. 1 Schematic representation of the constructs made for chickpea plant transformation. **a** *msg-gus* construct for pod specificity checking of the promoter *msg*. **b** Construct for insect-resistant chickpea development with fused *cry1Ab/Ac* gene under the control of the *msg* promoter and **c** construct for insect-resistant chickpea development with fused *cry1Ab/Ac* gene under the control of the *actin1* promoter

Statistical analyses

The experimental data values were mean value from three independent series, each done with three replicates, and the results presented as mean ± standard error (SE). The statistical significance at $P \leq 0.05$ was calculated. One-way and two-way analyses of variance (ANOVAs) were used to compare the differences between the non-transgenic control and the transgenic plants.

Results

Development of transgenic plants

Plants were transformed with *msg-gus*, (*gus* gene driven by msg promoter), *actin 1-gus* (*gus* gene driven by actin 1 promoter), *pCAMBIA-msg-cry1Ab/Ac-nos* (*msg-Bt*) and *pCAMBIA-actin-cry1Ab/Ac-nos (actin-Bt)* (Fig. 1a, b, c). Forty antibiotic-resistant (hygromycin) DCP92-3 plants transformed with *actin-Bt* construct and 36 hygromycin-resistant plants transformed with *msg-Bt* were generated using *Agrobacterium*-mediated vacuum infiltration method. T_0 putative transgenic plants were initially confirmed by PCR analysis using fused *cry1Ab/Ac* gene-specific primer capable of amplifying the 800 bp product. No PCR product was detected in the non-transgenic chickpea plants. Selected T_0 transgenic lines based on PCR analysis were grown in the greenhouse for further generation until maturity to obtain seeds for further studies. Southern blot showed integration of fused *cry1Ab/Ac* in positive progeny of chickpea selected

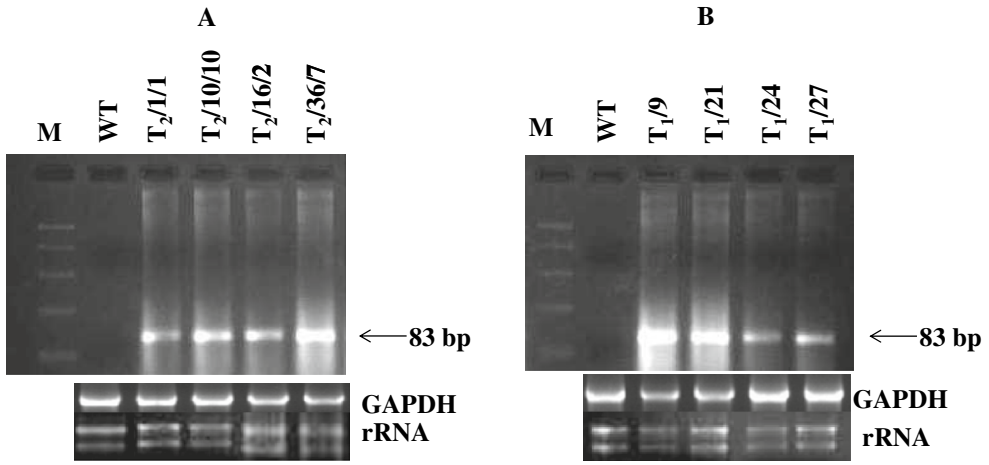

Fig. 2 Semi-quantitative RT-PCR showing mRNA amplification of *GAPDH* (internal control) and fused *cry1Ab/Ac* transcript. **a** Actin-Bt transgenic plants, **b** msg-Bt transgenic plants

by PCR analysis at the T_1 generation in case of *msg-Bt* and T_2 generation in case of *actin-Bt* (Supplementary Figure 1).

Tissue-specific expression of pod-specific promoter

Histochemical gus assay was performed with the plants transformed with *msg-gus* construct to confirm the tissue specificity of the *msg* promoter. A blue stain was observed in the young pods and flower (Supplementary Figure 2). The stain pattern in different parts of the plants clearly demonstrates that the *msg* promoter controls the expression of *gus* gene in the developing pods.

Expression analysis of transgene in the transgenic plants by semi-quantitative RT-PCR and quantitative real-time (qRT) PCR

The expression of *cry1Ab/Ac* fused gene in both types of transgenic plant lines (transformed with *actin-Bt* and *msg-Bt*) was analysed by semi-quantitative RT-PCR. The transgenic lines showed different levels of *cry1Ab/Ac* transcript accumulation, whereas the non-transgenic counterpart did not show any RT-PCR expression band indicative of no transcript accumulation (Fig. 2).

Real-time PCR analysis was also conducted to quantify the transcript of fused *cry* gene in different transgenic lines. The analysis was performed in T_2-transgenic plants transformed with *actin-Bt* and in T_1-transgenic plants transformed with the *msg-Bt* construct. The result revealed substantially higher levels of *cry1Ab/Ac* gene expression in transgenic plants. No expression was detected in WT non-transformed counterpart, as it is not an endogenous gene. Quantitative RT-PCR analysis revealed enhanced level (35- to 77-fold in case of *actin-Bt* and 36- to 110-fold in case of *msg-Bt*) of transcript accumulation when compared with that of the internal control *GAPDH* gene (Fig. 3). Among different transgenic lines, T_2/1/1 in case of *actin1* and T_1/9 in case of pod-specific promoter were selected as the most promising lines in which transcripts of *cry* gene were increased by 77- and 110-fold, respectively.

Bt toxin accumulation in transgenic plants

Initially, the presence of fused insecticidal proteins in different transgenic plants was confirmed by the dipstick method (Supplementary Figure 3). The quantitation of fused Cry endotoxin in different transgenic lines was done by ELISA. Variable amounts of toxin were accumulated in all transgenic plants, whereas non-transformed wild-type plants did not show any accumulation of Cry endotoxin. In case of *actin-Bt* transgenic plants, 21 different T_1-transgenic plant lines and 4 different T_2-transgenic lines (five plants from each of the lines 1, 10, 16 and 36) were analyzed for Cry endotoxin estimation (Table 1). The T_2/1/1 and T_2/1/28 plants showed the highest level of endotoxin accumulation (18 ng mg^{-1} TSP). On the other hand, in the case of msg-Bt transgenic, eight different T_1-transgenic lines were analyzed. The T_1/9 plant showed the highest level of endotoxin accumulation (19 ng mg^{-1} TSP) (Table 2).

Insect bioassay of transgenic plants

The entomocidal activity was evaluated by insect feeding bioassays performed with second-instar larvae of *H. armigera.* The T_2-transgenic plants expressing *actin-Bt* and T_1-transgenic plants expressing *msg-Bt* with moderate to high level of endotoxin were used in the insect bioassay. 21-day-old plant twigs from actin Bt chickpea plants and

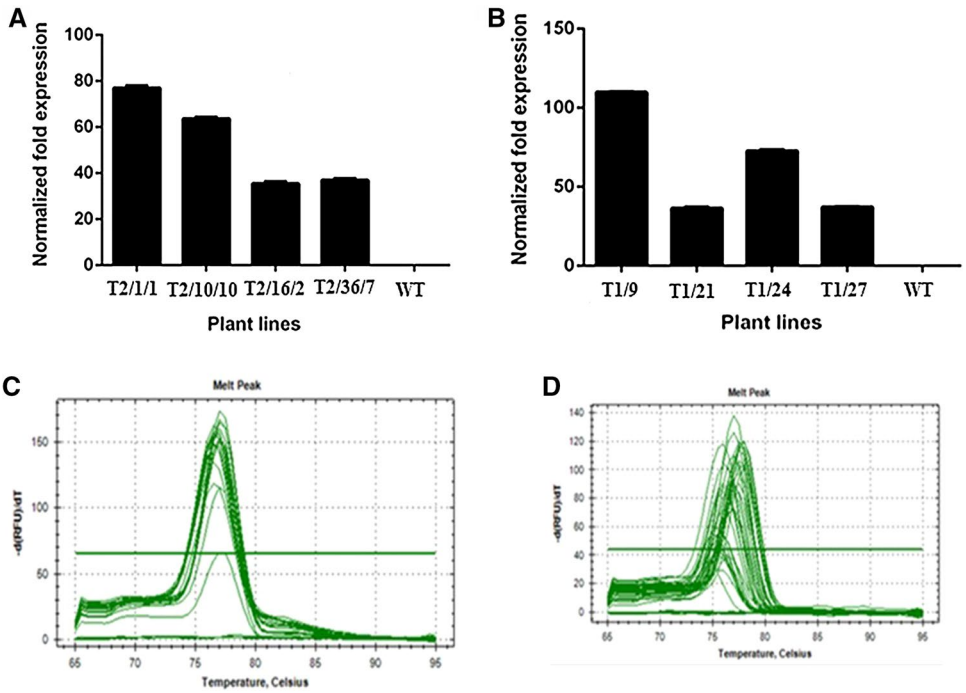

Fig. 3 Relative quantitative estimation of fused *cry1Ab/Ac* mRNA of chickpea transgenic plants and wild-type (WT) plants as determined by real-time polymerase chain reaction (PCR). **a** T_2 lines of *actin-Bt* transgenic and **b** T_1 lines of *msg-Bt* transgenic. **c** Melt curve of the *actin-Bt* and **d** the *msg-Bt* generated by a heat dissociation (65–95 °C) curve, indicating the specificity of real-time PCR product. All the results are the mean ± standard error (SE) of three independent experiments

Table 1 Expression of Bt toxin in T_1- and T_2-transgenic plants of DCP92-3 (*actin-Bt*) by ELISA in leaf twig

Plant line no. (T_1)	Bt toxin expression (ng mg^{-1} TSP)	Plant line no. (T_2)	Bt toxin expression (ng mg^{-1} TSP)
WT	–	WT	–
T_1/1	18 ± 2	T_2/1/1	18 ± 1
T_1/9	8 ± 1	T_2/1/16	17 ± 1
T_1/10	17 ± 2	T_2/1/28	18 ± 2
T_1/16	9 ± 1	T_2/1/29	17 ± 2
T_1/17	5 ± 2	T_2/1/32	17 ± 1
T_1/24	6 ± 1	T_2/10/4	17 ± 2
T_1/27	13 ± 1	T_2/10/6	16 ± 1
T_1/28	14 ± 2	T_2/10/10	17 ± 2
T_1/30	9 ± 1	T_2/10/14	16 ± 2
T_1/32	15 ± 2	T_2/10/17	17 ± 2
T_1/36	11 ± 1	T_2/16/2	11 ± 2
T_1/58	4 ± 2	T_2/16/6	10 ± 2
T_1/66	14 ± 1	T_2/16/7	8 ± 1
T_1/72	8 ± 2	T_2/16/11	8 ± 2
T_1/79	9 ± 1	T_2/16/12	9 ± 1
T_1/83	10 ± 1	T_2/36/1	13 ± 2
T_1/88	9 ± 2	T_2/36/7	10 ± 1
T_1/92	11 ± 1	T_2/36/8	11 ± 1
T_1/99	11 ± 1	T_2/36/14	10 ± 1
T_1/104	9 ± 1	T_2/36/15	11 ± 2
T_1/107	10 ± 2		

young healthy green pods of *msg-Bt* transgenic plants were infested with six neonate larvae of *H. armigera* in a Petri dish (Fig. 4). The mortality rate after feeding for 4 days was found to reach 100 % in three promising transgenic chickpea lines in case of *actin-Bt* (Table 3). Two promising *msg-Bt* (Table 4) lines were identified based on 100 % mortality, whereas the mortality in WT untransformed plants was found to be nil. The *H. armigera* larvae that fed on transgenic chickpea plants were observed to have died, with shrinking and darkening of body colour, whereas larvae fed on control plants reached an advanced stage of development after 96 h of feeding assay (Supplementary Figure 4). A wide range of variation in the mortality rate was observed in different transgenic lines ranging from 67, 83 to 100 % in T_2/27/8, T_2/28/2 and T_2/1/1, respectively.

Pods from non-transformed control plants were found to be extensively bored by *H. armigera* larvae, whereas the pods from transgenic chickpea plants with msg-Bt showed less infestation (Fig. 4b). Transgenic pods in case of msg-Bt plants were not bored in 72 h of infestation by the larvae and the seeds inside the pod were intact.

Agronomic performance study

Agronomic characters of the T_2-transgenic plants in case of actin-Bt and T_1 in case of msg-Bt were compared with those of non-transgenic plants to observe any phenotypic

Table 2 Expression of Bt toxin in T_1-transgenic plants of DCP92-3 (*msg-Bt*) by ELISA in pods

Plant line no. (T_1)	Bt toxin expression (ng mg^{-1} TSP)
WT	–
T_1/3	9 ± 2
T_1/9	19 ± 1
T_1/21	13 ± 2
T_1/24	18 ± 1
T_1/27	16 ± 2
T_1/30	13 ± 1
T_1/33	14 ± 1
T_1/41	11 ± 2

alteration due to incorporation of *Bt* gene (Supplementary Table-1). All the transgenic and non-transgenic plants were of similar morphological nature. There were no significant differences ($P < 0.05$) in plant height, grain length, grain width and number of pods per plant. Seed viability of transgenic and WT chickpea seeds were tested by germination percentage analysis. It was observed that both transgenic and wild-type seeds exhibited 100 % germination in normal growth condition. Growth and fertility of the plants were compared with the respective wild-type plants. All of these independent primary transgenic plants showed a normal phenotype and were fertile. No significant difference was found in growth and fertility status of the transgenic plants in comparison to their respective wild type.

Discussions

Helicoverpa armigera causes considerable damage in chickpea production all over the world. The use of chemical pesticide to reduce the yield loss due to the infestation

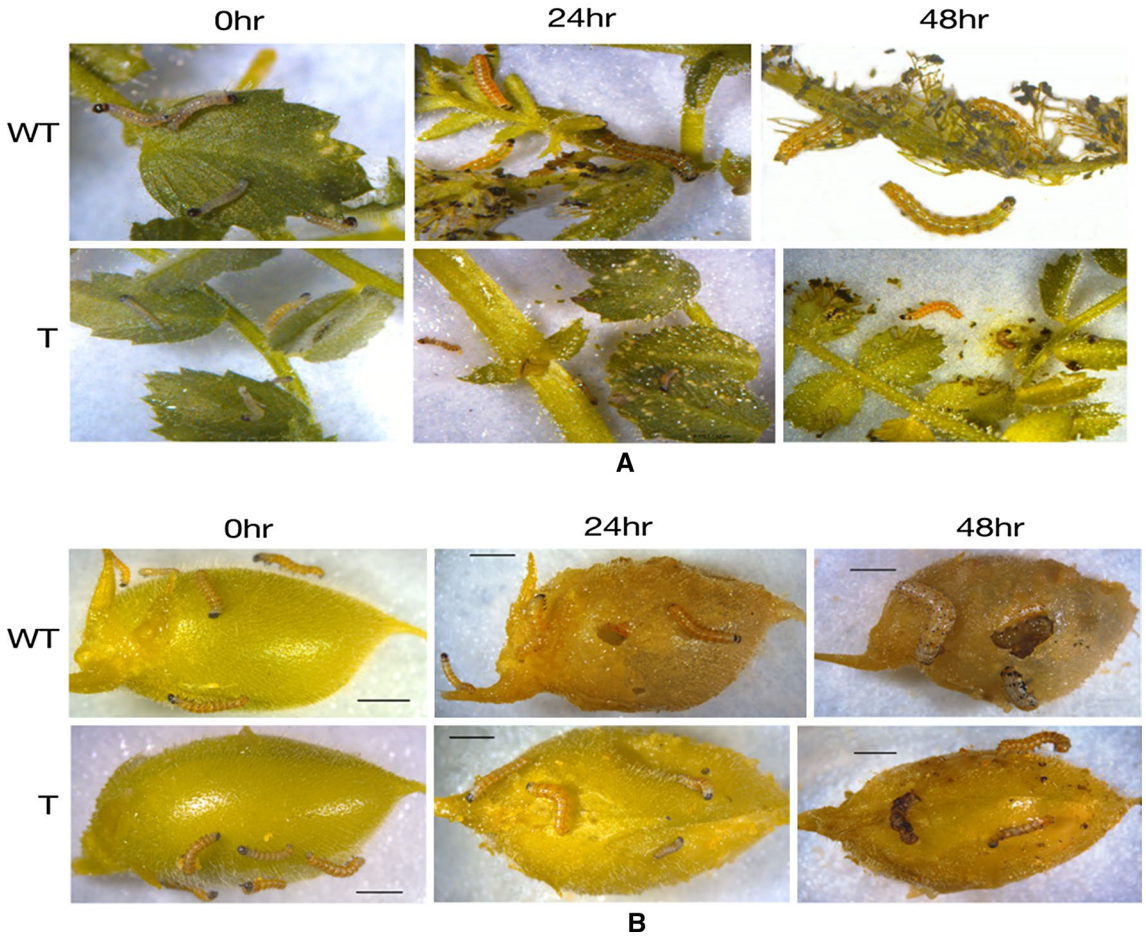

Fig. 4 Representative image of insect bioassay performed on transgenic chickpea with second-instar larvae of *Helicoverpa armigera*. (A) WT twigs at 0, 24 and 48 h of time interval. The WT chickpea plants were challenged with *H. armigera* that resulted in extensive consumption of twigs of chickpea. Extra twigs (200 mg) were added to maintain the larvae for 96 h. Insect bioassay of transgenic line (T) no. T_2/1/1 in case of *actin-Bt* at 0, 24 and 48 h of time interval. A maximum number of larvae were paralysed after 48 h and most of them were found dead at 72 and 96 h time interval. **b** WT and transgenic pod at 0, 24 and 48 h time interval used in the insect bioassay of young pod of transgenic line no. T_1/9 of *msg-Bt* at 0, 24 and 48 h time interval

Table 3 Bioassay chart of T_2-transgenic chickpea plants transformed with *actin-Bt* in leaf twig

Plant line no	No. of larvae released	No. of larvae survived after 48 h	No. of larvae paralysed after 48 h	No. of larvae dead after 72 h	No. of larvae dead after 96 h	Leaf tissue fed	Mortality % after 96 h
WT (1)	6	6	0	0	0	All fed	0
WT(2)	6	6	0	0	0	All fed	0
T_2/1/1(1)	6	2	4	6	6	Not fed	100
T_2/1/1(2)	6	1	5	6	6	Not fed	100
T_2/10/10(1)	6	0	5	6	6	Not fed	100
T_2/10/10(2)	6	1	5	6	6	Not fed	100
T_2/20/4(1)	6	4	2	4	6	Fed very less	100
T_2/20/4(2)	6	3	3	5	6	Fed very less	100
T_2/27/8(1)	6	3	3	2	4	Fed well	67
T_2/27/8(2)	6	3	3	4	4	Fed well	67
T_2/28/2(1)	6	1	5	3	5	Fed well	83
T_2/28/2(2)	6	1	4	4	5	Fed well	83
T_2/32/1(1)	6	2	4	5	6	Not fed	100
T_2/32/1(2)	6	1	4	6	6	Not fed	100
T_2/36/5(1)	6	2	3	4	5	Not fed	83
T_2/36/5(2)	6	1	4	4	5	Not fed	83
T_2/16/9(1)	6	4	2	2	4	Fed well	67
T_2/16/9(2)	6	5	3	3	4	Fed well	67
T_2/24/3(1)	6	4	2	2	4	Fed well	67
T_2/24/3(2)	6	5	1	4	4	Fed well	67

Table 4 Bioassay chart of T_1-transgenic chickpea plants transformed with *msg-Bt* in healthy green pods

Plant line no	No. of larvae released	No. of larvae survived after 48 h	No. of Larvae paralysed after 48 h	No. of Larvae Dead after 72 h	No. of Larvae Dead After 96 h	Mortality % after 96 h
WT (1)	6	6	0	0	0	0
WT(2)	6	6	0	0	0	0
T_1/9(1)	6	2	4	6	6	100
T_1/9(2)	6	2	4	6	6	100
T_1/21(1)	6	1	3	4	4	67
T_1/21(2)	6	4	2	3	3	50
T_1/24(1)	6	2	4	5	6	100
T_1/24(2)	6	1	5	5	6	100
T_1/30(1)	6	4	2	4	5	83
T_1/30(2)	6	3	3	5	5	83
T_1/41(1)	6	3	3	2	4	67
T_1/41(2)	6	3	3	4	4	67

of the insect is one of the major strategies followed by farmers worldwide may cause severe harm to the environment and to the non-target beneficial insects, soil annelids, arthropods and microorganisms. On the other hand, the development of insect-resistant variety through breeding programme is a sustainable strategy. The availability of the draft genome sequence of chickpea has proved to be an additional benefit to look for suitable target genes that can be employed for selected gene manipulation to achieve biotic/abiotic stress tolerance in chickpea (Jain et al. 2013; Varshney et al. 2013). To date, no known resistance gene has been identified from the chickpea germplasm that can be readily introduced through breeding programmes into elite chickpea cultivars.

Different insecticidal crystal proteins (ICP) from *B. thuringiensis*, expressed in transgenic crop plants, have shown significant resistance to important insect pests in the agricultural field (Koziel et al. 1993; Sardana et al. 1996;

Perlak et al. 2001). However, there is always a risk that field insects could develop resistance to these toxins after prolonged and consistent exposure (Bates et al. 2005).

Fusion or pyramiding of two dissimilar *cry* gene/genes may delay resistance development in insects more effectively than single *cry* gene transformants, even if different single *cry* gene plants were deployed sequentially (Zhao et al. 2003). Hence, the use of pyramided *cry* genes construct to develop transgenic plants is predicted to cause a great delay in the evolution of resistance (Roush, 1998). In a previous study, the safety assessment of Cry1Ab/Ac fusion protein was done and it was concluded that there was a reasonable certainty of no harm resulting from the inclusion of the Cry1Ab/Ac protein in human food or animal feed (Xu et al. 2009).

In this study, we successfully incorporated the fused gene *cry1Ab/Ac* in chickpea to develop resistance against the pod borer insect. The gene was controlled by two different promoters, constitutive promoter (rice *actin1*) and pod-specific promoter (soybean *msg*). Initially, we investigated the expression pattern of a 1.2 kb fragment of the pod-specific *msg* promoter element cloned from soybean through histochemical *gus* staining. The images clearly indicated that the promoter element was strongly expressed in developing pods. The *msg* promoter can express the *gus* gene in different parts of flowers, nectarines, and young pods in both soybean and *Arabidopsis* as shown in a previous study (Stromvik et al. 1999). Cloning of pod-specific promoter and construction of expression vector for soybean pod borer were also demonstrated by Shuyin et al. 2008. So expression of the fused *cry1Ab/Ac* gene under the control of this pod-specific promoter can effectively and precisely protect the young pod from the infestation of the pod borer. In a previous study, it was demonstrated that rice actin 1 works well in chickpea (Husnain et al. 1997). A green tissue-specific expression of the fused genes in rice has been successfully reported to confer resistance against rice leaf folder (Qiu et al. 2010).

All transgenic plants showed variable levels of increased mRNA expression compared with the non-transformed wild type, as analysed by semi-quantitative RT PCR and quantitative real-time PCR. The transgenic plants exhibited high levels of *cry* gene expression with less variation of resistance to the feeding larvae in transgenic chickpea plants, which is in accordance with a previous study (Tu et al. 1998). Genomic analysis of 40 plants in case of *actin-Bt* and 36 plants in case of *msg-Bt* confirmed the integration of *Bt* gene in transgenic chickpea plants. The increased expression was with two distinct genes, *cry1Ab* and *cry1Ac*. Codon optimization for plant expression may even result in 10- to 100-fold more *cry1Ab* translational efficiency and subsequent protein synthesis (Perlak et al. 1991). The production of fused protein in transgenic plants was qualitatively determined by the dip-strip test. Quantitative estimation of the protein in transgenic plants showed a level of accumulation of 4–18 ng mg^{-1} TSP in case of *actin-Bt* and 9–19 ng mg^{-1} TSP in case of *msg-Bt* transgenic. The maximum expression of Cry1Ab and Cry1Ac toxins in transgenic chickpea was up to 40 ng mg^{-1} TSP (Mehrotra et al. 2011). The accumulation of the toxin protein was lower in our study than in earlier reports (Stewart et al. 1996; Tu et al. 2000).

Transgenic Bt plants exhibited enhanced resistance to *H. armigera* as evident from insect bioassay. The results of the bioassay study revealed a significant reduction in the survival of *H. armigera* reared on transgenic chickpea leaves as well as pods. In insect feeding bioassay, transgenic plants showed significant and effective resistance against the larvae of *H. armigera* and larval mortality ranged to 100 % in two promising transgenic chickpea lines, viz., T_2/1/1, T_2/10/10 in case of *actin-Bt* and T_1/9 in case of *msg-Bt*. The transgenic plants showed notable resistance to larvae compared to non-transformed WT plants. After 48 h of incubation, the surviving larvae exhibited drastic reduction in body weight as compared to those fed on control non-transformed plants, were phenotypically severely stunted, paralysed in growth and became inactive after feeding on transgenic leaves, similar to the results obtained by Tu et al. (1998). The larvae showed different feeding pattern on control and transgenic leaf twigs, in accordance with Chakrabarty et al. (2002). Extra feed was added to control WT Petri dishes to make the larvae survive for 96 h after infestation. CRY toxins present in chickpea leaves provide protection from extensive damage caused by *H. armigera.* This clearly reveals the capability of transgenic plants to resist insect pest, and the use of fused *cry* gene will definitely help in the integrated pest management system to expand the options of farmers to protect their harvest from insect destruction.

The chickpea variety chosen in this study was also selected on the basis of high yield and short duration of growth and development. The morphological features of the transgenic plants were comparable to the non-transgenic plants in case of both the promoters under greenhouse conditions, without any significant phenotypic changes in growth, flowering pattern and seed setting. All the transgenic chickpea plants produced fertile pods. There were no morphological abnormalities in insect-resistant chickpea plants when both constitutive and pod-specific promoters were employed to express the fused *cry1Ab/Ac* gene.

In summary, we have developed chickpea lines expressing the fused *cry 1Ab/Ac* gene under the control of both *actin1* and pod-specific promoter *msg*. As the pod-specific promoter expressed the gene only during the reproductive phase of the chickpea plants, i.e. during pod formation, this promoter can effectively be used to control the damage of

H. armigera during the onset of the damage process. The developed lines showed enhanced resistance to insect pests. In addition, these lines did not have any negative impact on agronomically important traits.

As per our knowledge, this is the first report on the generation of a transgenic chickpea variety using fused *cry 1Ab/Ac* gene governed by two different promoters. Moreover, the use of pod-specific promoter to express the insecticidal protein in chickpea in the present study has proved to be a promising strategy in insect-resistant legume developmental programme for the future. Thus, our studies reveal the potentiality of fused/modified *cry* gene in conferring a relatively high degree of tolerance to chickpea plants against insect pests particularly *H. armigera*.

Author contributions SKD and KD designed the experiments. MG, KM and SK planned and performed the experiments. MG, KM, SK, KD and SKD analysed the data. MG and KM prepared the manuscript. KD and SKD edited the manuscript. All authors discussed the results and commented on the manuscript.

Acknowledgments The financial support from the Department of Biotechnology (DBT), Govt. of India, in the form of DBT Programme Support [Sanction no. BT/COE/01/06/05] is thankfully acknowledged. The fellowship from the Council of Scientific and Industrial Research (CSIR), Govt. of India [Sanction no. -09/028(0749)/2009-EMR-1] to K. Ali Molla is highly acknowledged. We thankfully acknowledge Yunliu Fan for providing the *Bt* construct. The authors thank the Department of Biochemistry, University of Calcutta, for the ELISA analysis. The authors are also grateful to Prof. Kailash Chandra Bansal, Director, National Bureau of Plant Genetic Resources, India, and Dr. V. V. Ramamurthy and Dr. S. Subramanian, Division of Entomology, Indian Agricultural Research Institute, New Delhi, for arrangement of insect larvae of *H. armigera* for the bioassay.

Conflict of interest The authors declare that they have no conflict of interest.

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