Comparative performance of modified full-length and truncated Bacillus thuringiensis-cry1Ac genes in transgenic tomato

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Abstract

Background: Bt-cry1Ac gene has been reputedly effective against Helicoverpa armigera a notorious lepidopteran pest. Reports on the expression of full-length and truncated cry1Ac genes in plants for effective resistance against Helicoverpa sp. have been documented however, their performance is still ambiguous. Moreover, the question remains to be addressed that truncation of 3′ end of the native gene was documented and suggested for active insecticidal toxin production while the most successful transgenic event(s) of commercialized-cotton are based on full-length of the cry gene. Therefore, we performed a comparative study on the efficacy of the two versions of cry1Ac genes (full-length: 3,510 bp and truncated: 1,845 bp) in T0 and T1 transgenic tomato plants and analyzed the extent of protection against H. armigera and also compared the results with our previous findings related to a successful transgenic tomato line Ab25E, expressing cry1Ab gene. The integration of cry1Ac gene(s) in T0 transgenic plants and its inheritance in T1 progeny was observed by PCR, RT-PCR and Southern blot hybridization analysis while, the toxin integrity, expression and toxicity was monitored by Western immunoassay, DAS-ELISA and insect bioassay respectively.

Results: An average transformation frequency and Bt-Cry protein content of 16.93 ± 2.10 and 0.0020–0.0128% of total soluble protein (TSP) was obtained with pRD400 vector (Trcry1Ac) while, a much lower value of 9.30 ± 2.041 and 0.0001–0.0026% of TSP was observed with pNBRI-1 vector (Flcry1Ac), respectively. The promising Trcry1Ac T0 transgenic plants and their T1 progeny gave full protection from H. armigera. Although Flcry1Ac gene showed lower transformation frequency and lower expression, it showed higher toxicity to H. armigera when compared with truncated Trcry1Ac gene.

Conclusions: The full-length cry1Ac gene can be redesigned for higher expression and performance in dicots or a hybrid gene could be designed having a blend of strong receptor binding and stable expression characteristics for enhanced efficacy and toxicity to the susceptible insects.

Keywords: Agrobacterium tumefaciens; Cry1Ac toxin; Tomato transformation; Leaf-disc; Helicoverpa armigera; Insect mortality

Background

By the year 2050, the global population is expected to rise above nine billion. While, at the same time it is sure that the existing arable land is expected to decrease significantly due to anthropogenic activities related to urbanization and neglecting agricultural crop losses due to insect pests. The worldwide pre-harvest crop losses have been estimated to be 13.8% from insects and other arthropods, 11.6% from disease and 9.5% from weeds (Chrispeels and Sadava 1994). The conventional methods of crop protection rely mainly on the use of synthetic agrochemicals but, they have a significant drawback of environmental contamination and toxicity to non-target organisms, including humans themselves. It is the moral duty of environmentalists and molecular biologists to judiciously introduce healthier strategies to cope with the problem of insect pests and resistance management, for sustainable agricultural productivity.

The most widely used and well documented approach in this context is the insecticidal crystal protein (cry) genes of Bacillus thuringiensis (Bt) coding different
insecticidal δ-endotoxins specific to different group of insects (Schnepf et al. 1998). These toxins are highly specific to the target insects, non-toxic to animals and human beings, non-hazardous and eco-friendly (Schnepf et al. 1998; Gatehouse 2008). Therefore, these are potent “biopesticides” (Sharma 2010; James 2012). The Cry1A group of toxin(s) are effective against lepidopteran insects which are the major group infecting several agricultural crop plants in field. The mode of action of Cry1Ac toxin can be best explained by the ‘Jurat-Fuentes model’ which suggests that cytotoxicity is due to the synergistic effect of osmotic lysis and cell signaling process, and involves the features of both the ‘Bravo model’ and the ‘Zhang model’ of toxin mode of action (Jurat-Fuentes and Adang 2006; Pardo Lopez et al. 2013).

Cotton transgenics developed by the transfer of modified Bt-cry1Ac and Bt-cry1Ac + 2Ab genes and commercialized as Bollgard I and II respectively, are the great success stories in agricultural biotechnology, for providing protection against lepidopteran insects, thereby increasing the crop yield (Perlak et al. 2001; Purcell et al. 2004; Sanahuja et al. 2011). The major limitation in the development of transgenic plants with Bt-cry1A genes is the low expression of native gene in plants, which has been attributed to instability of transcript, poor stability of the toxin protein in plant environment and altered codon usage in plants (Murray et al. 1989). However, it is not possible to use the complete toxin encoding genes in plants because protoxins are not sufficiently soluble in plant cells due to low pH of 7.6, since higher pH above 9.5 is required for solubility of the protoxins (Pefeoreon 1997; Shrivastava 2012). This problem is circumvented by using cry genes with 3’ truncation which produce fully activated toxin molecules and remain in solubilized form in plant cell. Two approaches have been tried to increase the expression of Cry1A toxins in genetically modified plants, (1) selective removal of deleterious DNA sequences by site-directed mutagenesis and (2) completely modified sequences of cry genes with plant-optimized codon usage for enhanced expression in plants. The later approach has led to 100 fold increase in the expression levels of cry1Ab and cry1Ac genes in tobacco, tomato and other crop plants (Perlak et al. 1991; Koziel et al. 1993). The modified cry gene had about 65% nucleotide homology with the native gene while G + C content was increased from 38% to 65% along with codon optimization for over expression in higher plants. Perlak et al. (1990) had reported that the truncated cry gene of B. thuringiensis var. kurstaki HD-73 strain showed detectable expression compared to full-length cry gene in transgenic cotton (Perlak et al. 1990). The transgenic cotton plants harbouring truncated B. thuringiensis cry1Ac gene showed high level of activity against Manduca sexta resulting in 100% mortality. The laboratory trials were further confirmed and demonstrated with field trials. However, the same group reported that full-length gene rather than a truncated gene provides reasonable protection from Heliothis virescens (tobacco budworm) (Perlak et al. 2001; Rawat et al. 2011). They came up with an event that was used to develop Bt-transgenics resistant to damage by Helicoverpa armigera, the major target pest in India, China and Australia.

Reports on the expression of both the full-length and truncated cry1Ac genes for effective resistance against insects have been documented but, unfortunately the performance of Cry1Ac toxin encoded by full-length and truncated cry1Ac gene in plants is still not clear (Kranthi et al. 2005; Rawat et al. 2011). The routine recovery of transgenic events expressing high-level of Cry1Ac toxin is a rare, random and vexatious issue (Rawat et al. 2011). Also, the question remains to be addressed that truncation of 3’ end of gene was documented and suggested for active insecticidal toxin production while the most successful transgenic event(s) of cotton for field performance and commercialization are based on full-length of the cry gene. Therefore, we performed a comparative study on toxicity and performance of two versions of cry1Ac gene using tomato as a model system, for development of H. armigera resistant, stable transgenic plants.

Results

Development of transgenic tomato plants

Agrobacterium-mediated tomato transformation was performed with vector constructs (Figure 1A and B) pRD400 harbouring truncated cry1Ac gene (Trcry1Ac) and pNBRI–1 harbouring full-length cry1Ac gene (Fcry1Ac) using the modified procedure. The explants which regenerated during kanamycin selection cycle (responding explants) were putative transformants. The percentage transformation frequency was determined as independent transgenic events received after the second selection divided by total number of explants, multiplied by 100. An average transformation frequency of 16.93 ± 2.10 and 9.30 ± 2.04 was observed with pRD400 and pNBRI–1 respectively, as shown in Additional file 1: Table S1. The results of molecular characterization of T₀ and T₁ transgenic tomato plants of the respective constructs have been discussed consecutively under respective heading: [A] Truncated cry1Ac gene (vector pRD400) and [B] Full-length cry1Ac gene (vector pNBRI–1). To avoid confusion, the transgenic events obtained with Trcry1Ac were designated as Ac 1–Ac 30 and that with Fcry1Ac as FAc 1–FAc 30.

The T₀ transgenic plants developed with pRD400 vector harbouring Trcry1Ac gene showed normal growth and flowering (43 ± 1.83 days), except the fruit size. The size of fruits was reduced compared to the control and the average weight of the fruits per plant was calculated.
to be 18.64 ± 0.98 gm. It was interesting to note that although the seed count was reduced with 35.4 ± 7.5 seeds (1.9 seeds per gram of tomato pulp) compared to the control tomato bearing 62.6 ± 12.60 seeds per tomato (2.57 seeds per gram of tomato pulp), but the shape and size of the seeds were normal as the control. Seed germination was not affected by the cry1Ac gene and the T1 plants attained an average height of 109.54 ± 20.80 cm with 43.20 ± 4.08 average number of leaves and 5.0 ± 2.10 average number of fruits per plant (Table 1). The T0 plants developed with vector pNBRI–1 harbouring Flcry1Ac gene did not show any abnormality in their growth, and flowering. These transgenic plants attained an average height of 116.4 ± 6.10 cm with 56.31 ± 11.52 average number of leaves (Table 1). The size of the fruits was markedly reduced compared to the control untransformed tomato plants while the fruit number was found to be similar to that of control. The average number of fruits per plant was observed to be 16 ± 7.5 and the average weight of tomatoes was found to be 18.5 ± 1.34 gm with 29 ± 2.82 average numbers of seeds per tomato (1.56 seeds per gram of tomato pulp).

[A] Truncated cry1Ac gene (vector pRD400)

Molecular characterization of transformants

A total of thirty independently selected T0 transgenic tomato plants were screened for the presence of cry1Ac and nptII gene by PCR amplification using the specific set of primers. These plants showed expected amplicon of 995 and 678 bp for cry1Ac and nptII genes respectively (Figure 2A–C). The presence of both the genes (cry1Ac and nptII) indicated intact integration of the transgene and the selection marker. Out of thirty T0 transformants RT-PCR analysis of ten randomly selected plants was performed to confirm the formation of cry1Ac and nptII gene transcript. The first strand cDNA was amplified using specific set of primers which showed expected amplicons of 995 and 678 bp for cry1Ac and nptII genes respectively (Figure 2D,H).

Determination of transcript level by real-time PCR

The transcript level was analyzed by real time PCR in seven randomly selected T0 transgenic tomato plants expressing cry1Ac gene. The expression level of plant ID Ac1 was very low and was therefore taken as a reference which was denoted as TC (transformed control). The comparative transcript level ranged from 2.18 (Ac 9), 0.61 (Ac 11), 3.2 (Ac 16), 2.4 (Ac 21), 3.7 (Ac 25) and 5.1 (Ac 26) folds higher to Ac 1 respectively (Figure 2I). No amplification was observed in non-transformed control plant and on an average the transgenic lines showed 2.9 folds enhanced expression over the reference (Ac 1).

Southern blot hybridization analysis and Western immunoassay

The transformants confirmed by PCR and RT-PCR were further analyzed by Southern blot hybridization. The genomic DNA from transformed and untransformed plants was digested with EcoRI as there is a unique site for this restriction enzyme in the T-DNA region of the binary vector pRD400. Southern blot hybridization of eight T0 plants with 1,845 bp BamHI and EcoRI fragment of cry1Ac gene probe revealed that they were independent transgenic events. Most of the promising T0 transgenic plants showed single and double copy insertion while only one plant (Ac 26) showed triple copy insertion of the transgene and the hybridizing fragments ranged from 4.5–15.5 kb (Figure 3A). Whereas, genomic
Table 1 Comparative assessment of growth parameters of T₀ plants developed with vectors pRD400 and pNBRI–1

| Vector construct | Cry toxin (% of tsp) | Average plant height | Average number of leaves | bOnset of flowering (days after hardening) | bAverage number of fruits/plant | Number of seeds/fruit | cDry weight yield (gm) | cNumber of seeds/gm of fruit | % germination of T₁ seeds |
|------------------|---------------------|----------------------|-------------------------|-------------------------------------------|-------------------------------|------------------------|-----------------------|-----------------------------|--------------------------|
| Control          | –                   | 106.4 ± 6.87         | 42.00 ± 7.21            | 30 ± 1                                     | 14.0 ± 1.93                  | 626 ± 12.60            | 520 ± 8.832           | 2.57                        | 75 ± 2.95                 |
| pRD400           | 0.0020–0.013        | 109.54 ± 20.8 (0.373)| 43.20 ± 4.08 (0.3767)  | 43 ± 1.83 (0.00054)                       | 5.0 ± 2.10 (0.000014)        | 35.4 ± 7.5 (0.00257)   | 470 ± 5.47 (0.00204)  | 1.9                         | 49 ± 2.58                 |
| pNBRI–1          | 0.0001–0.003        | 116.40 ± 6.10 (0.0205)| 56.31 ± 11.52 (0.010)  | 36 ± 1.41 (0.0079)                       | 16.0 ± 7.5 (0.2256)          | 290 ± 2.32 (0.000006)  | 496 ± 10.95 (0.0047)  | 1.57                       | 32 ± 3.00                 |

aOverall average of the days of onset of flowering, in 30 transgenic tomato plants of each group.

bAverage number of fruits per plant in 30 transgenic tomato plants of each group.

cAverage dry weight of 30 transgenic tomato plants of each group of plants.

dAverage number of seeds (per gram of fruit) in eight fruits of individual transgenic plant, in 30 plants of each group.

Values in parenthesis indicate the probability associated with a student's paired t-test with a two tailed distribution. When P < 0.05 the difference of the individual parametric value to that of the value of the control was significant.
DNA from untransformed control tomato plants did not show any hybridization signal with cry1Ac gene probe. Western immunoassay of ten transgenic plants including those 8 plants which passed the Southern blot hybridization analysis was performed using the cell-free extract of leaf tissues. The protein blots showed a light band of approximately 65 kDa which was similar to the positive control (Figure 3B).

**Protein expression and corresponding insect mortality**

The expression level of cry1Ac gene in T0 and T1 transgenic tomato plants was analyzed through DAS-ELISA and calculated as percentage of Bt-protein of total soluble protein (TSP). Cry1Ac toxin content in thirty independent T0 plants ranged between 0.0020–0.0128% of TSP. The resistance bestowed against fruit worm H. armigera corresponding to the Cry1Ac toxin is shown in Additional file 2: Table S2 and Figure 4A.

Few T0 plants expressing Cry1Ac toxin above 0.02% of total soluble protein (TSP) with plant ID Ac 9 (0.0240%), Ac 16 (0.0380%), Ac 21 (0.0255%), Ac 25 (0.0386%) and Ac 26 (0.1259%), were selected for further analysis of their T1 progeny. The T1 seeds of these transgenics plants (plant ID Ac 9, Ac 11, Ac 16, Ac 21, Ac 25 and Ac 26) were grown on kanamycin selection media to study the segregation pattern and further molecular characterization.

**Figure 2** Screening of T0 and T1 Trcry1Ac transgenic tomato plants. A–C PCR amplification of 995 bp of cry1Ac gene. D–F 678 bp of nptII gene using gene specific primers in thirty T0 transgenics. G, H RT-PCR analysis of ten randomly selected T0 transgenic tomato plants of cry1Ac gene using 995 bp cry1Ac gene and 678 bp nptII gene transcripts. M – 100 bp DNA ladder (NEB, USA). -C – non-transgenic control plant; +C – plasmid DNA positive control. I Real-time analysis for Bt-cry1Ac transcript levels in six T0 transgenic tomato plants. TC – Transgenic plant with low expression of Bt-toxin used as control. J–O PCR amplification of 995 bp of cry1Ac gene and 678 bp of nptII gene using gene specific primers inT1 progeny of promising T0 parents. M – 100 bp DNA ladder (NEB, USA). -C: non-transgenic control plant; +C: plasmid DNA positive control.
of the segregated kanamycin resistant population. These highly expressing T₁ progeny of respective T₀ parents gave PCR amplification of the expected amplicon of 995 and 678 bp for cry1Ac and nptII genes, respectively (Figure 2J–L,M–O). The average Cry1Ac toxin content of the progeny of Ac 9, Ac 16, Ac 21, Ac 25 and Ac 26 parents showed 0.01 ± 0.002, 0.015 ± 0.002, 0.009 ± 0.001, 0.01 ± 0.004 and 0.018 ± 0.002% of TSP respectively (Figure 4B) causing 100% mortality of H. armigera after 72 h of feeding on detached vegetative leaves. The control non-transgenic leaves suffered heavy damage due to voracious feeding by the insect. (Figure 4A,B).

Segregation of nptII gene in T₁ progeny was studied in these five highly expressing ELISA positive, independent transformants as shown in Additional file 3: Table S3. It was observed that the segregation ratio was Mendelian in nature with a minimum Chi-square value of 0.19 for Ac 25 and maximum value of 3.34 for Ac 16.

**[B] Full-length cry1Ac gene (vector pNBRI−1)**

**Molecular characterization of the T₀ transgenic plants**

A total of thirty T₀ transgenic plants were taken for the population study and were screened for the presence of cry1Ac gene through PCR. All the plants were PCR positive and showed an expected amplicon of 768 bp gene specific primers (Figure 5A–C). RT-PCR analysis of these plants was performed and the cDNA was amplified using gene specific primers, showing an expected amplicon of 768 bp (Figure 5D–F). These T₀ plants were also screened for the presence of nptII gene and PCR amplification gave an expected amplicon of 678 bp (Figure 5G–I). Similarly, RT-PCR analysis for the nptII gene transcript also showed 678 bp amplicon of nptII gene (Figure 5J–L).

**Protein expression and corresponding insect mortality**

Bt-ELISA of all the PCR and RT-PCR positive thirty T₀ transgenics was performed using pathoscreen cry1Ab/AC ELISA kit (Agdia, USA). Interestingly, only two plants, FLAC 7 and FLAC 11 showed detectable expression of Bt-protein content which was estimated to be 0.0015 and 0.0026% of total soluble protein (TSP), respectively (Figure 6A). Detached leaf bioassay of FLAC 7 and FLAC 11 was performed with second instar larvae of Helicoverpa armigera. Both these plants gave 100% mortality to the larvae in repeated bioassays (Additional file 2: Table S2 and Figure 6A). The leaves of FLAC 7 and FLAC 11 were also subjected to second instar larvae of Spodoptera litura and caused 20% and 30% insect mortality to the larvae, respectively (data not shown). This result indicates the specificity of cry1Ac gene for H. armigera midgut receptors. These two plants FLAC 7 and 11 were selected for further analysis and segregation pattern of the kanamycin selection marker. Segregation of nptII gene in T₁ seeds of the two ELISA positive independent transformants FLAC 7 and FLAC 11, was analysed, as shown in Additional file 3: Table S3. The segregation ratio was Mendelian in nature with a minimum Chi-square value of 0.82 for FLAC 11 and maximum value of 1.25 for FLAC 7.

Fiftyfive T₁ plants of FLAC 7 and fifteen plants of FLAC 11 were screened through DAS-ELISA. As expected, all the T₁ transgenic tomato plants were found to be ELISA positive. The Bt-protein content of few highly expressing plants FLAC 7.2, 7.4, 7.19, 7.34, 7.37, 7.48, 11.8 , 11.9, 11.11 and 11.14 was 0.003%, 0.0055%, 0.0026%, 0.0021%, 0.0025%, 0.0062%, 0.0178%, 0.0015%, 0.001 and 0.0015% of TSP, respectively (Figure 6B,C). These plants were also subjected to insect bioassay with H. armigera and varying degrees of protection corresponding to the expression level of Bt-protein was observed. Only FLAC 7.48 and FLAC 11.8 gave 100% mortality to second instar larvae of H. armigera while FLAC 7.2, FLAC 7.4, FLAC 7.19, FLAC 7.34, FLAC 7.37 and FLAC 11.4 bestowed 80%, 90%, 80%, 60%, 70% and 50% protection respectively, against the insect (Figure 6B,C).
Determination of transcript level by real-time PCR

The two T₀ transgenic tomato plants, FLAc 7 and FLAc 11 were also subjected to quantitative real-time PCR. The expression level of T₀ transgenic tomato plant ID FLAc 9 was very low and was therefore taken as a reference which was denoted as TC (transformed control). The comparative transcript level in transgenic plants ranged from 6.7 (FLAc 7) and 9.6 (FLAc 11), folds higher to FLAc 9 respectively (Figure 7A). No amplification was observed in non-transformed control plant and on an average the transgenic lines showed 8.1 folds enhanced expression over the reference (FLAc 9).

Molecular characterization of the T₁ progeny of promising T₀ transgenic plants

All the ELISA positive T₁ plants FLAc 7.2, FLAc 7.4, FLAc 7.19, FLAc 7.34, FLAc 7.37, FLAc 7.48, FLAc 11.8 and FLAc 11.14, expressing Bt-Cry1Ac toxin above 0.003%, were subjected to RT-PCR analysis using gene specific primers. All the plants showed expected amplicon

Figure 5 Molecular characterizations of T₀ Flcry1Ac transgenic tomato plants. Upper Panel A-C PCR amplification of 768 bp of Flcry1Ac gene using specific primers in thirty T₀ transgenics. D-F RT-PCR analysis of T₀ transgenic tomato plants showing 768 bp amplicon of cry1Ac gene. M : 100 bp DNA ladder (NEB, USA). -C : non-transgenic control plant, +C : plasmid DNA positive control. Lower Panel G-I PCR amplification of 678 bp nptⅡ gene using specific primers in T₀ transgenics. J-L RT-PCR analysis of T₀ transgenic tomato plants showing 678 bp amplicon of nptⅡ gene. M : 100 bp DNA ladder (NEB, USA). -C : non-transgenic control plant, +C : plasmid DNA positive control.
Figure 6 Expression of Flcry1Ac gene in transgenic plants. A Thirty T0 transgenics. B, C T1 progeny of FlAc 7 and 11. The gray shaded vertical bars represents the average content of Bt-Cry1Ac protein and squares (green square) represent the average of the % mortality status of H. armigera, when subjected to the leaves of T0 transgenic plants.

Figure 7 Molecular characterizations of T0 and T1 progeny of FlAc7 and FlAc11 transgenic tomato plants. A Comparative real-time PCR analysis of transcript in T0 Flcry1Ac transgenic plants showing fold change in expression with respect to FlAc 9 (low expressing transgenic plant taken as reference). Control : non-transgenic control. B, C RT-PCR and cDNA amplification of 678 bp nptII gene and 768 bp Flcry1Ac gene of T1 progeny using specific primers. M : 100 bp DNA ladder (NEB, USA). -C : non-transgenic control plant, +C : Flcry1Ac gene plasmid DNA as positive control. D Southern blot probed with 3,510 bp BamHI radiolabelled frament of Flcry1Ac gene. E Western immunoblot assay performed with crude leaf protein extract, lane 1 purified Cry1Ac toxin protein, lane 12 : untransformed control, lane 2–11 : leaf protein extracts from progeny of T0 FlAc7 and FlAc11. A protein band of ~130 kDa in transgenic plants showed hybridization with Cry1Ac antibodies, similar to positive control.
cry1Ac gene showed a lower progeny of FlAc 7 and FlAc 11 revealed seeds of Tr plants and their respective T cry1Ac Sal, 0 1 1 H. armigera and was finally selected and modified Fl–Bt cry1Ac–cry1Ac transgenics, the gene cry1Ac–cry1Ac Bt–cry1Ac H. armigera transgenic tomato were sub- genes ranged between transgenics developed with and modified Fl–Bt–cry1Ac–cry1Ac–cry1Ac gene. Tr–cry1Ac–cry1Ac–cry1Ac gene. Although highly expressing transgenic plants expressing Tcry1Ac gene showed protection against H. armigera whereas, only two low expressing transgenic events harbouring Flcry1Ac gene, (FlAc7 and FlAc11) repeatedly showed full-protection against H. armigera. This observation made us to realize that although Flcry1Ac gene showed a lower transformation frequency and expression but the promising events were highly toxic to H. armigera. Certainly, of 678 bp for nptII gene and 768 bp for Flcry1Ac gene (Figure 7B,C). Southern blot hybridization was performed with six T1 plants (FLAC 7.2, FLAc 11.8, FLAc 7.48, FLAc 11.14, FLAc 7.19 and FLAc 7.37) using 3,510 bp Flcry1Ac gene fragment as probe. The genomic DNA from transformed and untransformed plants was digested with Sal I as there is a unique site for this enzyme in the T-DNA regions of the binary vector pNBRI–I. The hybridizing fragments ranged between 6.5–10.0 kb and showed single copy integration of the transgene (Figure 7D). Western immuno- blot analysis of T1 progeny of FlAc 7 and FlAc 11 revealed a specific but fuzzy band of approximately 130 kDa, in all the plant samples. The non-transgenic control lane did not show any colour signal (Figure 7E). The T1 transgenic plant FlAc 11.8 showed complete protection against second in-star larvae of Helicoverpa armigera and was finally selected as a promising event (Figure 6C).

Discussion

The first transgenic crop that was commercialized in the USA was Bt-cotton. It was developed to express full-length synthetic cry1Ac-like Bt-gene sequence and its worldwide acceptance has grown exponentially since its introduction. This product has significantly reduced cotton production costs and the recurrent use of pesticides by providing a promising alternative for the control of Heliothis virescens, Helicoverpa zea, and Pectinophora gossypiella. Bt-cotton ensures considerable agronomic, economic and environmental benefits to its growers. The information on Cry-toxin receptors, the stability and efficacy of different Cry-toxins and the resistance mechanisms developed by the target pests is crucial to maintain the utility of Bt-transgenic technology.

There are few reports available in order to prove the efficacy and the stability of native full-length cry1Ac gene and its expression, in transgenic plants (Barton et al. 1987; Vaeck et al. 1987; Perlak et al. 1990; De Rocher et al. 1998). In most of the reports the research groups have tried to depict the performance of the Bt-genes in T0 primary transformants or did not perform a population study in subsequent progenies to confirm the stability of cry gene expression in terms of growth characteristics and inheritance. Our results obtained from PCR, Southern blotting and RT-PCR analyses of transgenic tomato plants have confirmed the stable integration of Tcry1Ac and Flcry1Ac genes in T0 plants and their respective T1 progeny. The different sizes of hybridizing genomic DNA fragments (~4.5–15.5 kb) of transgenic tomato plants with the respective cry gene-probe indicated that they resulted from the independent stable T-DNA integration event into the plant genome. The Bt-toxin content of T0 transgenic population of tomato developed with modified Tcry1Ac and modified Flcry1Ac genes ranged between 0.0024–0.126%, and 0.0001–0.0026% of TSP, respectively. Although, all the T0 transgenics were PCR and RT-PCR positive however, a remarkable difference was observed in the number of true transgenics (transgenics with detectable expression) obtained with equal number of transformation experiments and overall transformation frequencies obtained with both vector constructs. A higher transformation frequency of 16.93% ± 2.10 was observed with vector harbouring modified Tcry1Ac gene and a much lower value of 9.30 ± 2.041 with vector harbouring Flcry1Ac gene. The level of transgene expression in plants is generally unpredictable and may vary with different vector-constructs and also independent transformants with same vector-construct.

The T1 seeds of Tcry1Ac transgenic tomato were subjected to kanaycin selection. It was observed that the average Cry1Ac toxin content of the progeny of Ac 9, Ac 16, Ac 21, Ac 25 and Ac 26 were less than their respective parents. After segregation of the Bt-transgene very few candidate plants retained expression level similar to their respective parents. There are certain factors which do influence transgene expression and its stability in transgenic plants and may lead to highly variable expression within populations of plants from individual parents, developed in the same transformation experiment. The position effect being the most important factor reflects the significant role of genomic DNA adjoining the site of transgene integration (Wilson et al. 1990). Another factor is the copy number, intactness of the transgenes and their relative arrangement, which influences the probability of physical interactions, recombination within the locus and the induction of gene silencing (Hobbs et al. 1993; Heinrichs 2008). The probable cause for low expression of the Trcry1Ac–T1 population is, during segregation the transgenic locus is directed to or near a heterochromatin rich region thereby producing variants with a lower and varied Bt-content. But in case of Flcry1Ac transgenics, the gene shows single copy integration and is segregated along the gene rich euchromatin region of the chromosome.

Our results also suggest that Flcry1Ac gene is comparatively poorly expressed than Tcry1Ac, despite the optimal conditions of transformation and the overall transformation frequency was low. An interesting observation was noticed when T0 transgenics developed with pRD400 and pNBRI–I vector-constructs were subjected to insect bioassay with H. armigera. Although highly expressing transgenic plants expressing Tcry1Ac gene showed protection against H. armigera whereas, only two low expressing transgenic events harbouring Flcry1Ac gene, (FlAc7 and FlAc11) repeatedly showed full-protection against H. armigera. This observation made us to realize that although Flcry1Ac gene showed a lower transformation frequency and expression but the promising events were highly toxic to H. armigera. Certainly,
there is a scope for more improvement of Flcry1Ac gene for higher expression in transgenic plants. These results can be co-related to the most successful transgenic event Monsanto 531 which was developed with full-length modified cry1Ac-like gene and commercialized as Bollgard cotton, for complete protection against bollworm complex (Perлak et al. 2001; Purcell et al. 2004).

There are not many reports concerning the development of promising transgenic plants over-expressing variants of Cry1Ac toxin (native truncated and native full-length or modified and truncated or modified full-length). It may be attributed to toxic or suppressive response of Cry1Ac toxin during early stage of plant development (Barton et al. 1987; De Rocher et al. 1998; Rawat et al. 2011). It was reported by Rawat et al. 2011 that high expression of cry1Ac gene acts as a negative selection and regenerated plants cells with low expression of the gene are the ones which actually proliferate. The same group reported that certain morphological abnormalities occur in in vitro regeneration of explants after transformation with cry1Ac gene and in the growth of the transgenic plants. Our results of transformation frequency with Flcry1Ac gene can be compared with it. In the present study, although the transformation frequency was reduced but we did not come across morphological abnormalities in the regenerating explants transformed with cry1Ac genes. The only growth related abnormality which was observed in few T₀ plants during glass house-stage was their delayed flowering and fruit setting. This problem was confirmed after observing the floral morphology of pRD400 transformants where the stigmatic apparatus was longer than the filaments and projected outside the unopened flower. As tomato is strictly self pollinated crop because of cleistogamous nature of flowers, the stigma and style should reside within the unopened flower. But, to our surprise the T₁ generation did not face the same problem. In the study of Sachs et al. (1998) involving the inheritance of the cry1Ac gene in MON249 event in cotton, it has been hypothesized that reduced fitness of some of the transgenic lines may be a result of direct insertion effects leading to the down-regulation of one or more native genes or the result of a linked somaclonal variation (Sachs et al. 1998).

It was interesting to note that apart from a low Bt-toxin protein content Flcry1Ac-transgenics gave full protection to H. armigera which was comparable to Trcry1Ac-transgenics expressing higher levels of Bt-toxin. In a recent study of Gomez et al. (2014) on Manduca sexta insect bioassay, they have reported that the insecticidal activity of modified Cry1Ab toxin (active toxin) was 8-fold lower than the modified Cry1Ab protoxin. Their experimental data show protoxin molecules trigger the formation of pre-pore structures and supports the pore-formation model involving sequential interaction with different mid-gut receptor which culminates to pore formation in the gut membrane and insecticidal activity. These findings could be related to our study and the question as to why the full-length Cry1Ac toxin at a lower concentration is effective than the truncated Cry1Ac toxin can be answered. The modified full-length Cry1Ac toxin, although at a lower expression levels, efficiently induces oligomerization, pre-pore formation and insecticidal activity compared to modified truncated Cry1Ac toxin at higher expression levels. These results suggest the importance of modified full-length cry1Ac gene for stability and integrity of the insect-resistance trait compared to truncated version of cry1Ab or cry1Ac genes alone (Koul 2013). Thus, the functional role of protoxin segments in the pore formation is yet to be analysed.

The truncated cry1Ab gene bears >80% homology with truncated cry1Ac gene. In our previous findings, we raised Bt-cry1Ab transgenic tomatoes and performed similar tests. A maximum transformation frequency of 28.20% was obtained with binary vector pBIN200 harbouring the modified truncated 1,845 bp cry1Ab gene (Koul 2014b). The best transgenic line Ab25 E, expressing 0.47 ± 0.01% Cry1Ab toxin of total soluble protein (TSP) was finally selected in the T₁ generation from the segregating population showing 100% mortality to the second instar larvae of H. armigera and S. littura and minimal damage to leaves and fruits (Additional file 4: Figure S1). The success of transgenic event Ab25E expressing modified truncated cry1Ab may be attributed to possible incorporation of cry1Ab transgene in euchromatin hot spot region of the genome. The position effect on transgene expression probably reflects pre-existing features of the insertion site, such as proximity to genome enhancers and degree of chromatin condensation (Beaujean et al. 1998). In tomato Cry1Ab25 E event the same phenomena seems to be applicable where stable integration accompanied by functional stability of the transgene made the event a successful line.

The commercially released Bt-cotton was developed with full-length cry1Ac-like gene whose nucleotide alignment study revealed that ‘Monsanto 531’ cry gene sequence is a hybrid gene where the sequence 1–1398 bp is that of cry1Ab gene. We can easily summarize that it was done in order to provide a blend of binding as well as pore formation characteristics in this successful cry gene for raising transgenic cotton and its commercialization. The Bt-cry1Ab gene offers higher transformation frequency (24.98 ± 3.56), optimal expression (0.02–0.13% of TSP) and good receptor-binding characteristics and is a promising candidate gene for gene pyramiding strategy to delay insect resistance.

Conclusions
The Cry1Ac toxin has been reputedly effective against lepidopteron insects, especially H. armigera (Mandaokar
et al. 2000). In the present study, although, the expression of Tcry1Ac gene was 100 folds higher than the Flcry1Ac gene the latter gave full-protection from H. armigera at very low concentration as evident from promising Tg plants (plant ID FlAc7 and FlAc11) and their Tg transgenic population study. The functional role of protoxin segments in Cry-pore forming toxin activity is yet to be studied in detail. The full-length cry1Ac gene can be redesigned for higher expression and stability in crop plants and can be pyramided with other cry gene(s) or a hybrid gene can be designed to broaden its toxicity spectrum and efficacy, as a remedy to cope with the problem of insect resistance.

Methods

Agrobacterium strain and gene constructs

Agrobacterium tumefaciens strain LBA4404 harbouring binary vector pRD400, with modified and truncated 1,845 bp cry1Ac gene having 47.74% GC content (Sardana et al. 1996; courtesy provided by Prof. I. Altosaar, University of Ottawa, Ottawa, Canada) and pNBRI-1 with modified full-length 3,510 bp cry1Ac gene 47.89% GC content (Koul 2013) respectively, driven by double enhancer DECaMV35S promoter and neomycin phosphotransferase gene (nptII) for kanamycin resistance in pBIN 20 backbone (Hennegan and Danna 1998) has been used for tomato transformation, as shown in Figure 1A and B. Cultures of A. tumefaciens were grown at 28°C in YEB medium containing 20 mg l⁻¹ rifampicin, 50 mg l⁻¹ kanamycin and 50 mg l⁻¹ streptomycin for 24 h at 200 rpm and utilized for transformation of tomato leaf-discs.

Plant material

Breeder seeds of Solanum lycopersicum cv. Pusa early dwarf (PED) were obtained from National Seeds Corporation, New Delhi, India and used for Agrobacterium-mediated leaf-disc transformation of tomato.

Tomato transformation and plantlet regeneration

Tomato seeds were surface sterilized and placed on semi-solid MS medium (Murashige and Skoog 1962), containing B5 vitamins (Gamborg et al. 1968) 3% (w/v) sucrose (HiMedia Labs, Mumbai, India) and 8 g l⁻¹ agar (Sigma, USA) followed by incubation at 24 ± 2°C in dark and shifted after three days of 16:8 h light–dark cycle in culture room maintained at 22 ± 2°C, illuminated with light intensity of 100 μmol m⁻² s⁻¹ and 78 ± 4% relative humidity. Vegetative leaves from axenic tomato seedlings of 16–18 days, were excised and initially precultured on MS medium supplemented with 2.5 mg l⁻¹ 6-benzyladenine (BAP) + 0.5 mg l⁻¹ indole-3-acetic acid (IAA) for three days prior to Agrobacterium co-cultivation (Koul 2013).

Agrobacterium-mediated transformation of tomato was performed by the method described by Koul et al. 2014a, 2014b. Tomato leaf discs were dipped in Agrobacterium suspension OD₆₀₀ = 0.25–0.3 (2 x 10⁸ cfu ml⁻¹), in MS liquid co-cultivation medium supplemented with 100 μM acetosyringone (As) for 20 min. The leaf disc explants were dried on sterilized blotting paper and transferred onto semi-solid co-cultivation medium comprising of MS salts + 3% (w/v) maltose + 100 μM As + 2.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA and co-cultivated in dark, for two days in the culture room. The explants thereafter were incubated on medium consisting of MS salts + 3% (w/v) maltose + 500 mg l⁻¹ cefotaxime + 2.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA for 5–7 days and transferred to shoot induction medium one (SIM-1) containing MS salts + 3% (w/v) maltose + 2.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA + 250 mg l⁻¹ cefotaxime + 50 mg l⁻¹ kanamycin and incubated for 30 days for the first screening of putative transgenic plants (I¹ selection). The independent regenerated shoots with a pair of vegetative leaves developed on first cycle of kanamycin screening were identified. These first pair of vegetative leaves were excised and subcultured on the shoot induction medium supplemented with kanamycin (SIM–2) having the same constituents as SIM–1 and incubated for 30 days for subsequent direct second screening and selection. The independent shoots that regenerated after successive I² selection cycles were subcultured on shoot elongation medium (SEM) containing MS medium + 1.0 mg l⁻¹ gibberellic acid (GA₃) + 3% (w/v) sucrose and 50 mg l⁻¹ kanamycin. The transformation frequency percentage for each experiment with respective vector-construct was calculated as total putative transgenic shoots developed after second selection divided by total number of leaf disc explants used, multiplied by 100. The shoots recovered from SEM medium having 2–3 leaves were transferred to root induction medium (RIM) containing half-strength MS medium + 0.5 mg l⁻¹ indole-3-butyric acid (IBA) + 50 mg l⁻¹ kanamycin + 2% (w/v) sucrose and 0.8% (w/v) agar for 14 days. The rooted plantlets were transferred to plastic pots containing sterilized soilrite (Keltech Energies Ltd. Bengaluru, India) and irrigated with half-strength liquid MS medium devoid of sucrose. The pots were kept in a plant growth chamber (Conviron Adaptis 1000 PG, Canada) set at desired relative humidity starting from 90 to 70% for 14 days of hardening step and the hardened plantlets were potted in earthen pots filled with soil:sand:farmyard manure (in 3:1:1 ratio) and transferred to glasshouse maintained at 24 ± 1°C under natural light for normal development, flowering and seed setting.

The seeds of promising T₉ transgenic plants of tomato were graded on the basis of quantitative Cry1Ac toxin expression, processed, air dried and kept under vacuum at 24 ± 2°C. The segregation and selection of promising events in subsequent generations from each transgenic plant were screened on kanamycin-supplemented semi-
solid medium and Cry1Ac toxin level was estimated by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The selected promising transgenic plants showing higher expression of Cry1Ac toxin, normal growth and flowering were analyzed in subsequent generations.

Screening of transgenic plants by PCR
Genomic DNA from leaves of T₀ putative transformed plants as well as control plants was isolated using GenElute plant genomic DNA miniprep kit, according to the manufacturer’s instructions (Sigma, USA). PCR amplification of cry1Ac and nptII genes from plant genomic DNA (100 ng) was achieved by using set of primers (Additional file 5: Table S4) designed to amplify 995 bp, 768 bp and 678 bp amplicons of Trcry1Ac and Flcry1Ac and nptII genes respectively, in the GeneAmp® PCR system 9700 (PE Biosystems, USA). The 25 μl PCR reaction mixture was prepared containing 100 ng plant genomic DNA, 100 μM dNTPs mix, 25 ng of each primer, 2 mM MgSO₄ and 1 U Taq DNA polymerase (NEB, USA). Amplification was performed with initial denaturation at 95°C for 5 min followed by 30 cycles, each comprising of denaturation at 94°C for 90 sec, annealing at 58°C (Trcry1Ac)/67°C (Flcry1Ac)/58°C (nptII) for 1 min and extension at 72°C for 3 min followed by final extension for 5 min at 72°C for 5 min. In all PCR experiments pRD400 plasmid was taken as positive control for cry1Ac gene and pNBRI–1 plasmid was taken as positive control for Flcry1Ac gene. Amplified DNA fragments of PCR assays were electrophoresed on 1% agarose (w/v) gels, visualized, documented and analyzed on Gel Doc XR (Bio-Rad, USA).

Southern blot hybridization analysis
Southern blot hybridization analysis was performed to confirm the integration of T-DNA into transformants according to Sambrook and Russell (2001), with few modifications (Koul et al. 2012). Aliquot of 10 μg genomic DNA purified from untransformed and transgenic plants was digested overnight with EcoRI (pRD400 transformants) and SalI (pNBRI–1 transformants) cutting at single site within the T-DNA. The digested genomic DNA was separated by gel electrophoresis and transferred onto Bio Bond Plus nylon membrane (Sigma, USA). The blots were hybridized at 58°C for 24 h with 1,845 bp and 3,510 bp fragment of cry1Ac and Flcry1Ac genes respectively, radio labeled with αP³²DCTP (BRIT, Mumbai India), washed under stringent conditions, exposed to Fuji screen for 48 h followed by scanning and documentation on Typhoon Trio Plus phosphorimager (GE Healthcare Life Sciences AB, Sweden).

RT-PCR and quantitative real-time PCR
RT-PCR analysis of T₀ and T₁ transgenic plants was done by synthesis of first-strand of cDNA with enhanced Avian RT-PCR kit using 5 μg of total RNA purified from the transgenic plant according to manufacturer’s instructions (Sigma, USA). The relative quantity of cry1Ac transcripts in transgenic tomato plants was analyzed by quantitative PCR performed in StepOne real-time PCR system (Applied Biosystems, USA) using Quantifast SYBR green PCR kit (Qiagen, Germany). Tomato β-actin gene (GenBank accession no. U60482) was used as an endogenous control in all real-time PCR assays. The nucleotide sequences of the set of primers for Trcry1Ac gene were, forward; 5'-ACACAGTTTTCGTCAGCGAGTT-3' and reverse; 5'- ACCAAAGATACCCCAGATGATGCTCGTGCAGTA-3' and reverse; 5'-CA GCCCTTTTGCCCCTTTC-3', while for β-actin gene forward; 5'-GCTGGTTTGGGTGTGGAGATGATA-3' and reverse; 5'-TCCATGTCATTCCAATGTGCA-3' giving an amplicon of 99, 101 and 194 bp respectively.

Total RNA extracted from 100 mg of leaf tissues was reverse transcribed into cDNA and used as template in real-time PCR assays with cry1Ab and β-actin gene-specific primers. Reverse transcription reaction was performed at 50°C for 10 min with initial denaturation at 95°C for 5 min (for activation of Hot-start Taq polymerase) followed by 40 amplification-cycles comprising of 10 s denaturation at 95°C and combined annealing and extension for 30 s at 60°C in 25 μl reaction mixture, according to manufacturer’s instructions (Qiagen, Germany). The relative values obtained from the quantitation of mRNA were expressed as 2^ΔΔCt where ΔCt represents the difference between Ct (cycle threshold) values of a target and the endogenous control (β-actin) in the same sample and ΔΔCt is the difference between the ΔCt value of a particular sample and that of the reference sample. The quantitative data of real-time PCR represent mean values with standard error of three independent experiments with three replicates of the transgenic plant samples.

Western immunoassay
Western immunoblotting of the transgenic plants expressing Cry1Ac toxin was performed using the cell-free extract of leaf tissues. Aliquots of the cell-free extracts were boiled for 10 min with 2× sample loading dye (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and electrophoresed on 10% denaturing SDS-PAGE (Laemmli 1970). Protein bands were visualized following Coomassie blue staining and the other set of SDS-PAGE was transferred onto immunoblot™ PVDF membrane (Bio-Rad, USA) using trans-blot SD semi-dry transfer cell (Bio-Rad, USA) in transfer buffer (25 mM Tris base, 192 mM glycine,
pH 8.3 and 0.1% SDS). The membrane was blocked for 2 h at 25°C in blocking buffer and incubated with primary antibody (rabbit polyclonal to Bt-Cry1Ac toxin, Amar Diagnostics, India) diluted to 1:1000 ratio in blocking buffer, for 2 h at 25°C. The membranes were washed four times with PBST, incubated in blocking buffer for 1 h followed by incubation with secondary antibody (goat polyclonal to rabbit IgG alkaline phosphatase conjugated antibody) at 1:5000 dilutions for 2 h at 25°C and developed with BCIP-NBT substrate solution (Sigma, USA).

Quantitative estimation of Bt-toxin
Vegetative leaves from 12 weeks old transgenic tomato plants of T₀ and T₁ generations were used for protein extraction by grinding in 1:10 (w/v) ratio of plant tissue to PBST buffer (pH 7.4), in liquid nitrogen. The total soluble protein (TSP) concentration in cell-free extracts was determined by Bradford dye-binding procedure with bovine serum albumin (BSA) as standard protein (Bradford 1976). The quantitative estimation of expressed recombinant Cry1Ac (toxin) in cell-free extracts of transgenic plants was determined by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using peroxidase labeled PathoScreen kit for Bt-Cry1Ab/1Ac protein (Agdia, USA). Cell-free extracts (100 ng TSP) from leaves of transgenic plants were dispensed into wells of ELISA plate, pre-coated with primary antibody followed by reaction with secondary antibody conjugated with alkaline phosphatase to develop colour and detection of Cry1Ac toxin was monitored at 650 nm using SpectraMax 340PC spectrophotometer (Molecular Devices, USA).

Insect bioassay
The larval populations of H. armigera were reared in the insectary on an artificial diet (Gupta et al. 2004) at 26 ± 2°C, 70% relative humidity on 14 h light and 10 h dark regime. The detached leaves from fourth to sixth nodes of untransformed control, T₀ and T₁ transgenic plants were washed thoroughly with distilled water, blotted dry and placed in a plastic container with 10 second instar larvae of H. armigera per leaf, in three replicates. Feeding was allowed for 48–96 h and the data on larval weight and percent mortality were analyzed statistically (SPSS Inc., USA). Each experiment was repeated thrice with three replicates and results were co-related to the quantitative expression of Cry1Ac toxin.

Statistical analysis
Each experiment was performed with three replicates, unless otherwise mentioned and repeated at least three times. T₁ seeds were germinated on MS basal medium supplemented with 50 mg l⁻¹ kanamycin and subjected to χ² fitness test for progeny segregation to compare the expected and observed data. All graphs were prepared using Sigma Plot software (Sigma Plot, USA).

Additional files

Additional file 1: Table S1. Tomato transformation using pRD400 and pNBRI−1 for regeneration of transgenic plants.
Additional file 2: Table S2. Insect mortality data of T₀ transgenic plants.
Additional file 3: Table S3. Segregation analysis of nptII gene in T₁ seeds developed with vector pRD400 and pNBRI−1.
Additional file 4: Figure S1. Quantitative assessment of Bt-Cry1Ab protein in different T₀ plants and selected T₁ population by A & B Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as percentage of total soluble protein (TSP) (striped bar) and corresponding insect mortality of Helicoverpa armigera (open square) and Spodoptera litura (open circle). Average quantity of Bt-Cry1Ab protein in transgenic plants is shown as % TSP ± standard deviation on the top of histogram bars and also indicated by horizontal mark for individual transgenic plant. ‘n’ is the number of T₁ transgenic population from each parent.
Additional file 5: Table S4. Sets of forward and reverse primers used in the experiments.

Abbreviations
As: Acetosyringone; BAP: 6-Benzyladenine; BBMV: Brush bordered membrane vesicle; bp: Base pairs; cfs: Colony forming units; Cry: Crystal protein; DAS-ELISA: Double antibody sandwich enzyme-linked immunosorbent assay; DECAmV/3SS: CaMV/3SS promoter with duplicated enhancer; GA3: Gibberellic acid IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; MS: Murashige and Skoog’s medium; nptII: Neomycin phosphotransferase; PBST: Phosphate buffered saline with Tween-20; PCR: Polymerase chain reaction; RIM: Root induction medium; SIM: Shoot induction medium; SEM: Shoot elongation medium; TSP: Total soluble protein.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
DVA and IS conceived the project. BK performed tomato transformation, raised the transgenic plants, carried out molecular analysis, insect bioassays of the transgenic tomato plants and analysis of the results. RY performed quantitative estimation of BT-content in transgenic lines. BK wrote the manuscript. All the authors have read and approved the manuscript.

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