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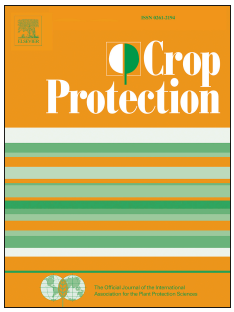

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Development and evaluation of transgenic castor (*Ricinus communis* L.) expressing the insecticidal protein Cry1Aa of *Bacillus thuringiensis* against lepidopteran insect pests

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Key message This is the first successful attempt at development and characterization of stable transgenic events expressing the *cry1Aa* gene for conferring resistance to lepidopteran foliage feeders in castor.

ABSTRACT

Castor is an industrially valued non-edible oilseed crop. Susceptibility of the crop to foliage feeders like *Achaea janata* (semilooper) and *Spodoptera litura* accounts for 30-50% of yield losses. Owing to a lack of reliable sources of resistance to these lepidopteran pests, attempts were made to develop transgenic events expressing the *Bacillus thuringiensis* (*Bt*) *cry1Aa* gene. Transformation of decotyledonated embryo axes through *Agrobacterium tumefaciens*, particle gun bombardment and *in planta* methods resulted in transformation frequencies of 2.4%, 1.1% and 2.1%, respectively. The presence and integration of the *cry1Aa* gene in the T_0 plants was confirmed by polymerase chain reaction (PCR) and Southern hybridization analysis. Based on segregation for a Mendelian ratio of 3:1, eight events (AMT-894, AMT-899, AK1304-PB-1, AK1304-PB-4, AK1304-PB-785, AK1304-PB-830, AK1304-PB-837 and DTS-43) were advanced. ELISA analysis detected protein from 0.16-2.76 ng/mg fresh leaf tissue across events and in different generations. In laboratory insect bioassays, the mortality of *S. litura* and *A. janata* ranged from 20-80% in different transgenic events and the weight reduction of surviving larvae over the control larvae after 8 days of feeding was 28.4-87.2% in the case of *S. litura* and 27.9-78.1% for *A. janata*. In field bioassays, the event AMT-894 was most promising with 43% of plants showing less than 25% leaf damage. As part of the regulatory requirement to check the toxicity of the transgenic events to beneficial insects, larval bioassays against *Samia cynthia ricini* (eri silkworm) using three transgenic events (AK1304-PB-1, AK1304-PB-4 and AMT-894) showed a 20.2 to 78.5% reduction in weight.

***Keywords*:**

Achaea janata . *Bacillus thuringiensis* . *cry1Aa* gene . Genetic transformation . Lepidoptera . *Spodoptera litura*

45 **Abbreviations**

46

47 AMT: *Agrobacterium*-mediated transformation

48 BA: N^6–Benzyladenine

49 CTAB: Cetyl trimethyl ammonium bromide

50 DTS: Direct transformed shoots

51 *Hpt*: Hygromycin phosphotransferase

52 MS: Murashige and Skoog

53 NAA: α-Naphthaleneacetic acid

54 PCR: Polymerase chain reaction

55 PGB: Particle gun bombardment

56 TDZ: 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron)

57

1. Introduction

Castor (*Ricinus communis* L.) is a non-edible oilseed crop of the family *Euphorbiaceae,* which is cultivated in the tropics and sub-tropics. It is commercially valued for its oil which has several industrial applications such as in pharmaceutical, high quality lubricants, paints and varnishes, medicines, textiles and leather (Ogunniyi, 2006). India, China and Brazil are the major castor growing countries accounting for 90% of the world's production (FAOSTAT, 2016). Castor owes its importance to the uniqueness of its oil which is rich (80–85%) in the hydroxyl fatty acid- ricinoleic acid. During the last decade the list of its applications has expanded even further as a source of biodiesel, ricinine (Zhu et al., 2018; Baskar et al., 2018), polyols, polyurethanes (Lonescu, 2016), several functional materials and biopolymers (Mubofu, 2016).

Among the major production constraints for the profitable production of this crop is the vulnerability of several castor varieties and hybrids to insect pests (Lakshminarayana and Raoof, 2005). The major pests on castor include the semilooper (*Achaea janata* Linnaeus), capsule borer (*Dichocrocis* (*Conogethes*) *puncitiferalis* (Guenée)), *Spodoptera litura* Fabricius, red hairy caterpillar (*Amsacta albistriga* Walker), jassids (*Empoasca flavescens* Fabricius) and the white fly (*Trialeurodes ricini* Misra). Larvae of *A. janata* and *S. litura* (Noctuidae: Lepidoptera) are voracious foliage feeders which totally defoliate the plants. For effective management of the defoliators, mechanical control of *S. litura* in its gregarious stage and hand picking of older *A. janata* larvae are suggested (Lakshminarayana and Raoof, 2005). Castor belongs to the monotypic genus *Ricinus;* success in castor breeding with yield stability, has subsequently been limited by a lack of exploitable genetic variability for resistance to these insect pests. Hence, development of transgenic castor through exploitation of biotechnological tools is regarded as one of the promising approaches for incorporation of desirable traits for which sources are rather limited in castor germplasm.

Alternative approaches like mutation breeding, wide (intergeneric) hybridization and use of biotechnological tools for creation of genetic variability and incorporation of desired traits have been recommended (Sujatha, 1996; Lavanya and Chandramohan, 2003). The major breakthrough in castor breeding was achieved through mutation breeding, mainly in altering the plant architecture by converting the perennial unproductive types to annual productive types and

development of new parental types (Lavanya et al., 2008). However, mutation techniques using radiation could not be exploited for the development of genotypes for resistance or tolerance to major biotic stresses. Intergeneric hybridization between castor and members of the related genera *Jatropha* and *Hevea* were unsuccessful due to variation in chromosome numbers and strong incompatibility barriers (Gedil et al., 2009; Sujatha et al., 2013). Hence, development of transgenic castor through exploitation of biotechnological tools was regarded as one of the promising approaches for incorporation of desirable traits for which sources are rather limited in castor germplasm.

The prerequisites for genetic transformation are (i) suitable candidate gene(s) for conferring insect resistance, (ii) a good tissue culture regeneration system, and (iii) a highly reproducible transformation method. Assessment of the efficacy of purified Bt crystal proteins against *S. litura* and *A. janata* showed high toxicity of Cry1Aa protein in terms of larval mortality to *A. janata* and feeding inhibition in case of *S. litura* (Lakshminarayana and Sujatha, 2005; Sujatha and Lakshminarayana, 2005).

Castor proved to be highly recalcitrant to *in vitro* manipulations and despite research efforts over the past four decades, no facile protocol of regeneration has been developed so far. Regeneration reported to date mainly has resulted from explants of meristematic tissues such as, shoot apices, embryonic axes and cotyledonary nodes (Reddy et al., 1987; Sujatha and Reddy, 1998; Ahn et al., 2007; Alam et al., 2010; Ganesh Kumari and Jayabalan, 2015). As a result, genetic transformation in castor for optimization of the protocols and incorporation of desirable genes has mostly relied on the ability of meristematic tissues to proliferate (Sujatha and Sailaja, 2005; Malathi et al., 2006; Sailaja et al., 2008; Sujatha et al., 2009; Patel et al., 2013; Li et al., 2015; Sousa et al., 2017), *in planta* transformation bypassing tissue culture (Kumar et al., 2011) and floral bud transformation (Mc Keon and Chen, 2003).

Genetic transformation studies in castor have been undertaken in India and the USA for development of transgenic castor for varied purposes. Mc Keon and Chen (2003) reported floral bud transformation in castor by employing the method of *Agrobacterium*-mediated transformation through vacuum infiltration of wounded flower buds (US Patent No 6.620.986). The first successful *Agrobacterium*-mediated transformation using embryo axes was developed by Sujatha and Sailaja (2005) which was followed by other researchers with minor modifications (Malathi et al., 2006; Patel et al., 2013; Li et al., 2015). Transformation

120 through particle gun bombardment using embryo axes was described by Sailaja et al. (2008).
121 Kumar et al. (2011) reported *in planta* transformation method in castor. *Agrobacterium*-mediated
122 and/or particle gun bombardment methods were used to develop transgenic castor for
123 introgression of genes for insect resistance (Malathi et al., 2006; Sujatha et al., 2009; Kumar et
124 al., 2011), salt tolerance (Patel et al., 2015) and ricin free castor (Sousa et al., 2017). Transgenic
125 castor plants reported to date for conferring resistance to insect pests harbor the *cry1Ab* gene
126 against *A. janata* (Malathi et al., 2006), *cry1EC* gene targeting both *S. litura* and *A. janata*
127 (Sujatha et al., 2009) and *cry1AcF* gene against *S. litura* (Kumar et al., 2011). Although the Cry
128 proteins share a common binding site, the toxicity profiles vary with the target insect. Hence in
129 the authors' laboratory, transgenic events were developed through deployment of the fusion
130 gene *cry1Ec* (Sujatha et al., 2009) and *cry1Aa*.

131 Keeping in view the toxicity of Cry1Aa protein against the major lepidopteran pests of
132 castor, the present study was undertaken to genetically transform castor through deployment
133 of the *cry1Aa* gene, characterize the events harbouring the introduced gene and assess the
134 level of protection against the two foliage feeders through insect bioassays both in laboratory
135 and field. Further, the promising transgenic lines confirmed for the presence of the *cry1Aa* gene
136 were tested against *S. cynthia* which is a beneficial insect that is reared on castor leaves.
137
138 ## 2. **Materials and methods**
139
140 ### *2.1. Plant material*
141
142 Seeds of castor *cv*. DCS-9 obtained from ICAR-Indian Institute of Oilseeds Research,
143 Hyderabad, India, were used for transformation. This variety is used as a parental line for the
144 hybrid DCH-177 and also cultivated as a variety. The seeds were decoated and surface
145 sterilized with 0.1% mercuric chloride for 8 min followed by 4 rinses with sterile distilled
146 water. The embryos with papery cotyledons were carefully excised by removing the endosperm
147 and subjected to transformation. The construct AK1304 with the *cry1Aa* gene cloned in vector
148 pCAMBIA 1304 with hygromycin resistance gene was obtained from ICAR-NRCPB, New
149 Delhi and used for the transformation experiments (Fig. S1 and the gene sequence in Fig. S2).
150

151 *2.2. Genetic transformation*

152

153 Transformations were carried out through *Agrobacterium*-mediated, particle gun
154 bombardment and *in planta* methods. *Agrobacterium*-mediated transformation was according to
155 Sujatha and Sailaja (2005) and particle gun bombardment was as described by Sailaja et al.
156 (2008). The cotyledons from embryos were removed and the embryo axes were cultured on
157 Murashige and Skoog (MS) (1962) medium fortified with 0.1 mg/l BA and incubated in dark for
158 five days. After incubation for five days, the embryo axes elongate and the meristematic center
159 become swollen. The swollen meristematic centers were injured with a surgical blade (11 No) or
160 needle and incubated in overnight grown *Agrobacterium* suspension (OD at A_{600} nm = 0.2) for
161 10 min and vacuum infiltrated for 30 min. The infected explants were placed on growth
162 regulator free MS medium and co-cultivated for 2 days. Following co-cultivation, the explants
163 were transferred to medium supplemented with 0.1 mg/l BA and cultured for 10 days and
164 then transferred to medium fortified with 0.5 mg/l TDZ for 10-12 days. The explants were
165 transferred to medium supplemented with 0.5 mg/l BA, 250 mg/l cefotaxime and hygromycin
166 for shoot proliferation and continued for three cycles with increasing levels of hygromycin (20-
167 40-60 mg/l) for selection of putative transformed shoots.

168 For particle gun bombardment, the embryo axes were cultured for five days on MS
169 medium supplemented with 0.5 mg/l TDZ. Explants were pre-plasmolysed for 2 h on
170 osmoticum medium containing equimolar (0.2 M) concentrations of mannitol and sorbitol.
171 Bombardments were done with 1.0 µ gold microcarriers at a helium pressure of 450 psi and
172 a target distance of 6 cm in a PDS He 1000 gene gun (Biorad, Hercules, CA). Following
173 bombardment, explants were subjected to post-osmoticum for 2 h. Bombarded explants were
174 transferred for shoot proliferation and selection for three cycles on medium supplemented with
175 0.5 mg/l BA and increasing concentration of hygromycin (20-40-60 mg/l). Regardless of the
176 method of transformation, the cultures following transformation were maintained at 26 ± 2 ^{0}C
177 under a 16/8-h photo period with light provided by cool white fluorescent lamps at an intensity
178 of 30 $\mu mol\, m^{-2} s^{-1}$.

179 In vector-mediated and particle gun bombardment methods, after three cycles of
180 selection of two weeks duration each, the surviving shoots were transferred to medium

supplemented with 0.5 mg/l BA to promote shoot proliferation. Multiplied shoots that were healthy were transferred to medium with 0.2 mg/l BA for shoot elongation. Elongated shoots (> 2 cm) were rooted on half-strength MS medium supplemented with 1.0 mg/l NAA. Rooted shoots were acclimatized in sterile vermiculite for 7-10 days under high humidity and then transferred to soil in pots and kept in a transgenic greenhouse.

The *in planta* transformation (DTS) method by-passing the tissue culture based regeneration was also carried out with the AK1304 construct. About 2,650 embryo axes were bombarded with 0.6 µ gold particles coated with 6 µg of plasmid DNA using 450 psi pressure, at 6 cm target distance and transferred to medium with 0.1 mg/l BA. After 15 days, the elongated embryo axes were transferred to ½ strength MS basal salt medium with 1.0 mg/l NAA + 10 mg/l hygromycin for rooting. On this media, only about 20% of the explants produced roots within 15 days after transfer. These rooted shoots were grown to maturity of which only 50% survived and produced seeds. The events were labeled as AMT, PB and DTS to represent transformants obtained through *Agrobacterium*-mediated, particle gun bombardment and *in planta* methods, respectively. The plants grown to maturity were covered with butter paper covers to obtain selfed seeds for the next generation.

2.3. Molecular analysis

2.3.1. Polymerase chain reaction

Genomic DNA was isolated from young fully expanded leaves of transformed and control plants using the CTAB method with minor modifications (Doyle and Doyle, 1990). PCR amplification of a 552 bp DNA fragment of the *cry1Aa* gene was carried out using gene specific primers (*forward*-5'-CCT CAC AGT TCT CGA CAT CG -3' and *reverse*-5'- ACG GAG TTG TCT TGT GGT GG-3') annealing at positions 710 to 1262 bp. The PCR reaction mixture contained 1X PCR buffer, 100 µM of each dNTPs, 5 pmol of each primer, 0.75 U of *Taq* DNA polymerase (Genei, Bangalore), 50 ng genomic DNA in a final volume of 10 µl. PCR amplification profile included initial denaturation at 94 ^{0}C for 5 min, followed by 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 53.5 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min with a final elongation step of 10 min at 72 ^{0}C. In case of the *hpt* gene, a 490 bp fragment was

amplified using the forward primer 5'-CAC AAT CCC ACT ATC CTT CGC-3' and reverse primer 5'-GCA GTT CGG TTT CAG GCA GGT-3'. The PCR reaction mixture contained 1X PCR buffer, 150 µM dNTPs, 2 pmol of each primer, 0.24 U of *Taq* DNA polymerase and 50 ng genomic DNA in a final volume of 10 µl. Amplification was carried out by denaturation at 94 ^{0}C for 30 s, annealing at 60 ^{0}C for 30 s and extension at 72 ^{0}C for 2 min for 30 cycles with a final elongation step of 5 min at 72 ^{0}C. The PCR products were resolved on 1.4% agarose gel containing ethidium bromide and documented in Syngene gel documentation system.

2.3.2. Southern analysis

Southern blot analysis was carried out for the primary transformants and also for plants in different generations of the promising events. The purified genomic DNA (20 µg as determined by agarose gel) of the PCR positive transformants and untransformed castor (control) were subjected to digestion with *Eco*RI restriction enzyme (8 U/µg of DNA) overnight at 37 ^{0}C. The positive control included the 552 bp PCR amplified product of the *cry1Aa* gene. The digested DNA samples were separated by electrophoresis on 0.8% (w/v) agarose gel at 60 V for 4 h. The gels were blotted on positively charged Nylon (N^+) membrane. The 552 bp PCR amplified product of *cry1Aa* gene (600 ng) was labeled using the Alkphos direct labeling and detection system as per the manufacturers' instructions (GE Health Care, UK). The membrane was exposed to the X-ray film in a cassette for 1 h and autoradiograms were developed by using the developer and fixer (Photon) for visualization of the hybridization signals.

2.4. ELISA

The Enzyme Linked Immuno Sorbent Assay (ELISA) was carried out to determine the amount of Cry1Aa protein by using Cry1Ac/Ab ELISA kit (Amar Diagnostics, India). About 180-200 mg of leaf tissue was collected from 60-90 days-old-plants and ground to a fine powder in liquid nitrogen and resuspended in 300 µl of 1X extraction buffer and processed as per the manufacturer's instructions. Observations were recorded with ELISA plate reader (Biotek ELX800, USA) at 405 nm wavelength. The experiments were repeated twice for confirmation and repeatability.

2.5. Insect bioassays

2.5.1. Laboratory

For laboratory bioassays against the neonate larvae of *A. janata*, *S. litura* and *S. cynthia*, castor leaves at 3rd or 4th from apex collected from plants in T_1 to T_4 generations were used. The test leaves of both the transgenic and control (untransformed) plants were placed in plastic specimen vials (60 ml) or in 9.0 cm petriplates containing 2.0% agar in water and overlaid with filter paper. Using a fine camel hair brush, ten neonate larvae were released on the leaves and the insect cultures were maintained at 26 + 2 ^{0}C under a 16/8 light/dark photoperiod cycle. Each treatment had three replicates and every day, larvae were transferred onto fresh leaves from the intact plants. At 2 days interval, larval mortality was recorded and cumulative mortality was computed at 8 days after treatment. Weight gain of the larvae was taken at 8 days after treatment (DAT).

2.5.2. Field

The crop growth conditions and whole plant bioassays for assessing the level of protection conferred by the transgenic events to the foliage feeders were as described earlier (Sujatha et al. 2009). The *cry1Aa* transformants from six events (AMT-799, AMT-899, AMT-936, AK1304-PB-1, DTS-43 and DTS-46) in the T_2 to T_4 generations were bioassayed against *A. janata* and *S. litura* under field conditions. The transgenic lines were sown in 6 m rows by adopting a plant to row spacing of 75 cm x 40 cm with the control (untransformed) castor (*cv.* DCS-9) after every 8 test lines. The soil type was Alfisol and the maximum temperature was 28-31 ^{0}C while minimum was 18-22 ^{0}C during the experimented period. The crop was maintained by following the recommended agronomic practices for castor. When the crop was 45-50 days old, the experimental plot was inspected for natural incidence of *A. janata* and *S. litura*. There was sporadic appearance of *A. janata* larvae in 2nd and 3rd instars while the infestation of *S. litura* was negligible. Hence, artificial releases of mature *S. litura* larvae @ 1 larva per plant were made.

The experimental field was covered with a fine nylon net to avoid the escape of artificially released *S. litura* larvae and prevent the entry of non-target pests and activity of insect parasitoids, birds and other predators (Fig. 5a). The generation cycle of *S. litura* was rapid and was allowed to complete two cycles. It was difficult to keep record of the number of larvae per leaf, dead larvae per leaf as there was heavy and mixed build up of both the foliage feeders. Hence, data were recorded on 15 plants based on the extent of defoliation due to the two defoliators on a 1-4 scale (1-resistant with <10% defoliation; 2-moderately resistant with 10-25% defoliation; 3-susceptible with >25-50% defoliation; 4-highly susceptible with >50% defoliation) for individual plants and the weighted mean average of insect damage was computed as follows.

$$\text{Average insect damage} = \frac{\Sigma\, n(1) + n(2) + n(3) + n(4)\ldots.}{\text{Total number of plants}}$$

3. Results

3.1. Transformation of embryo axes

The embryo axes derived from the variety DCS-9 were transformed with the AK1304 construct using AMT, PGB and DTS methods. Selection on hygromycin for three cycles was complete and no escapes were observed (Fig. 1a). The shoots that survived selection were multiplied and transferred to elongation medium where the selected shoots recovered and elongated with shoot lengths of at least 2.0 cm (Fig. 1b). Elongated shoots rooted with a frequency of more than 70% and were acclimatized successfully (Fig. 1c). About 4,340 embryo axes were transformed through AMT and the frequency of shoots that survived after 3 cycles of selection was 5.4%. Of the surviving putative transformed shoots, 104 plants were successfully established from which 37 plants were grown to maturity with an overall plant recovery frequency of 2.4%. About 1790 embryo axes were subjected to PGB followed by three cycles of selection from which 188 shoots survived with a frequency of 10.5% (Table 1). From these, 19 plants reached sexual maturity and the transformation efficiency was 1.1%. In the *in planta* method of transformation, of the 2650 embryo axes that were bombarded and subjected to

selection, 56 rooted shoots were recovered and grown to maturity with a frequency of 2.1%. The developmental stages, rooting and acclimatization of plants derived through *in planta* are presented in figure 1d-f.

3.2. Molecular analysis

Putative transformed plants at the primary spike stage were screened through PCR and Southern analysis for the presence of the introduced gene. Some of the leaves were tested for GUS expression as the construct harboured the *UidA* gene (data not presented). PCR amplification confirmed the presence of *cry1Aa* and *hpt* genes with amplicon lengths of 552 bp and 490 bp, respectively (Table 1). PCR screening done for 37 plants obtained through AMT showed positive amplification in 16 plants for both the genes (Fig. 2a). Out of the 19 plants obtained through PGB, 12 plants were found positive in PCR analysis. In 56 plants derived through DTS method, 14 plants were found positive in PCR analysis (Table 1). Regardless of the method of transformation used, a higher frequency (%) of plants showed the presence of *hpt* gene (54, 84, 32) as compared to the *cry1Aa* gene (43, 63, 25). Of the three transformation methods tried, frequency of PCR positive plants was low in the DTS method.

The PCR and Southern positive plants of 17 events obtained through the three methods of transformation were advanced to the successive generations in a P2 class containment facility. The progenies of six events from AMT, seven events from PGB method and four events obtained through DTS method were subjected to PCR analysis (Table 2). Segregation analysis revealed a Mendelian ratio of 3:1 with significant goodness of fit for the PCR positive and negative plants in eight events, which included two events (AMT-894, AMT-899) from plants derived through AMT (Fig. 2b), five events (AK1304-PB-1, AK1304-PB-4, AK1304-PB-785, AK1304-PB-830 and AK1304-PB-837) from PGB method and one event (DTS-43) through DTS method. These events were advanced to the subsequent generations. The event AMT-936 which did not follow Mendelian ratio was also advanced to the subsequent generations.

A total of 121 PCR positive plants from nine events (AMT-894, AMT-899, AMT-936; AK1304-PB-1, AK1304-PB-4, AK1304-PB-785, AK1304-PB-830, AK1304-PB-837 and DTS-43) were promoted to T_2 generation for progeny analysis. Out of the 121 rows of progeny, ten lines were selected for further generation advancement. These were five lines from AK1304-

PB-1, two lines from DTS-43 and three lines from AMT-894. In two of these ten lines tested, all plants of AK1304-PB-1-75 and AK1304-PB-1-73 were PCR positive (100%) while segregation for presence and absence of *cry1Aa* gene was observed in eight lines. A total of 117 progenies from three events (AMT-894, AK1304-PB-1 and DTS-43) were advanced from T_2 to T_3 generation. Out of these, one progeny row from AMT-894 (AMT-894-3-44) (Fig. 2c), two progeny rows from AK1304-PB-1 (AK1304-PB-1-73-24 and AK1304-PB-1-75-32) (Fig. 2d, e), and two lines from DTS-43 (DTS-43-20-2 and DTS-43-20-4) were selected as promising progenies based on presence of *cry1Aa* gene in all the plants as evident from the PCR analysis.

3.2.1. Southern analysis

Southern analysis of T_0 plants on digestion of genomic DNA with *Eco*RI revealed the integration of the introduced gene in the plants obtained through AMT. Southern analysis of plants showed hybridization signal in four (AK1304-PB-1, 3, 4 and 5) out of five plants obtained with PGB which confirmed the integration of the *cry1Aa* gene (Fig. 3a). There was no hybridization signal in the untransformed control (Fig. 3). The plants of three events obtained through the PGB method showed multiple inserts with the exception of AK1304-PB-1 which showed single insert (Fig. 3a).

In advanced generations, Southern analysis was done for one event each derived through AMT, PGB and DTS methods *viz.*, AMT-894, AK1304-PB-1 and DTS-43, respectively to check the inheritance and integration of the *cry1Aa* gene. The lines derived from DTS-43 (DTS 43-20-2-2, DTS 43-20-2-4, and DTS 43-20-2-10) showed multiple integration sites events (Fig. 3b) while the events AK1304-PB-1 and AMT-894 continued to show single copy integration in T2 generation (Figs. 3c, d).

3.3. ELISA

The ELISA analysis carried out for plants of seven events that were in T_4 generation showed detectable levels of Cry1Aa protein as compared to control (Fig. 4a). The protein concentration (ng/mg leaf tissue) ranged from 0.16-1.91 in AMT-894; 0.177-0.633 in AMT-

936; 0.219-2.48 in AK1304-PB-1; 0.19-1.33 in AK1304-PB-3; 0.16-1.38 in AK1304-PB-4; 0.21-0.76 in AK1304-PB-5 and 0.17-2.67 in DTS-43. The mean concentration of Cry1Aa protein across different events varied between 0.39 (AK1304-PB-5) and 0.77 (AK1304-PB-1) (Fig. 4a).

The protein level in plants of three events (AMT-894, AK1304-PB-1 and AK1304-PB-4) in T_2 to T_4 generations was compared (Fig. 4b). In the events AMT-894 and AK1304-PB-1, there was an increase in the level of Cry1Aa protein from T_2 to T_4 generations. In case of AK1304-PB-4, there was an increase in protein concentration in T_2 to T_3 generations and decreased in T_4 generation because few plants in T_3 generation had exceptionally high levels of protein (>2.0 ng/mg leaf tissue) which when carried forward to the next generation (T_4) showed the protein concentration in the same range (0.49) as the other events (Fig. 4b). In leaf samples of control (untransformed castor), colour development was occasionally observed but the maximum reading (A_{405}) was 0.001 which was far less than the lowest values detected in the transgenic plants.

3.4. Insect bioassays

3.4.1. Laboratory

Larval bioassays were conducted for *S. litura*, *A. janata* and *S. cynthia*. As the transformation is a continuous process, the putative transgenic events were recovered at regular intervals. Following confirmation for the presence of the transgene, the seeds from the primary racemes were used for rapid cycling of generations. Hence, for laboratory bioassays against the target insect pests, there was overlapping of generations of different events.

Putative transformants of AK1304-PB (five events) and seven events obtained through DTS method were tested. Mortality was observed only in the event AK1304-PB-1 which was 20% for *A. janata* and 25% for *S. litura* while no larval mortality was recorded in the other events. However, reduction in the weight of surviving larvae was recorded in all the events. Percentage weight reduction of surviving larvae on transgenic plants over control ranged from 1.1-75.7% for *A. janata* and 6.2-82.3% for *S. litura* in different transformation events. The maximum weight reduction of larvae of both the pests was high (82.3%, 75.7%) when larvae

were fed on leaves of AK1304-PB-1. The event AK1304-PB-4 had more feeding cessation of *S. litura* while events AK1304-PB-2 and AK1304-PB-3 conferred more resistance to *A. janata*.

Plants from four events obtained through AMT (AMT-685, 862, 894 and 899), five events through PGB (AK1304-PB-1, 3, 4, 5 and 785) and three events through DTS (DTS-4, 30, and 43) methods in T_1 generation were subjected to bioassays when the plants were 45-90 days old. Mortality of the *S. litura* larvae ranged from 10-50% on plants derived through AMT and PGB methods and 10-40% on plants derived through DTS method. Mean mortality ranged from 16.7-43.8, 17.1-38.6 and 0-33.3% in plants obtained through AMT, PGB and DTS methods, respectively. Reduction in weight of surviving larvae over control ranged from 17.6-64.4%, 15.8-75.0% and 6.7-71.8% on plants obtained through AMT, PGB and DTS methods, respectively (Table 3). Likewise, the mortality of *A. janata* larvae ranged from 20-40% on plants derived through AMT and DTS methods and 10-50% on those derived through PGB. The mean mortality in plants obtained through AMT and PGB methods ranged from 21.7-30 and 13.5-32.5, respectively while it was 32% in plants derived through DTS method. The weight reduction of surviving larvae over control ranged from 12.5-65.6%, 5.5-56.6% and 34.6-61.5% on plants obtained through AMT, PGB and DTS methods, respectively (Table 3). Thus, regardless of the method of gene introduction, the level of protection against the two foliage feeders was similar in all the events with no significant superiority of the method of transformation.

In T_2 generation, bioassays with *S. litura* larvae were conducted on four events derived through AMT and DTS procedures. The larval mortality ranged from 0-20% while the weight reduction of surviving larvae over control ranged from 17.8-72.9% and 16.9-86.4% on AMT and DTS plants, respectively. Bioassays with *A. janata* were conducted on one event each derived through AMT (AMT-894) and DTS (DTS-43) procedures and two events obtained through PGB (AK1304-PB-1 and 4). The mortality ranged from 0-10% only in plants derived through AMT. The weight reduction of surviving larvae over control ranged from 3.2-62.9%, 2.9-62.7% and 3.3-10.5% on plants derived through AMT, PGB and DTS methods, respectively. Bioassays were conducted on two lines of event DTS-43 in T_3 generation. Maximum mortality of *S. litura* larvae was only 10% (mean 7.0) in one line and weight reduction of larvae that survived on transgenic leaves over control ranged from 30.3-87.2%. With regard to *A. janata* reaction, the larval mortality in both the lines ranged from 20-80% (mean 34.4-37.0) and the

weight reduction of surviving larvae over control ranged from 27.9-78.1% (Table 4).

3.4.2. Field

Six events which included two events derived through DTS (DTS-43, DTS-46) one event through PGB (AK1304-PB-1) and three events through AMT (AMT- 799, 894, 936) in various generations (T_1 to T_4) were subjected to insect bioassays (Fig. 5). *Spodoptera litura* infestation was noticed after 20 days of release of mature larvae (Fig. 5b). The population of *A. janata* which was very low at the time of release of *S. litura* increased tremendously under the net. Consequently the first cycle showed predominant damage due to *S. litura* and the second cycle witnessed damage due to mixed infestation of both *S. litura* and *A. janata* (Figs. 5c-f). Close observation of the control plants showed uniform pest load of *A. janata* on leaves of control (Fig. 5c) and transgenic plants (Fig. 5d) but the foliar damage was less in the latter. Likewise in case of *S. litura*, the control plants were completely defoliated and the insects started feeding on the stems and capsules (Fig. 5e) while foliar damage was less in transgenic plants (Fig. 5f). Fig. 5g shows variation in foliar damage in different events and figure 5h shows the comparison of damage in control and transgenic plants. While the control plants were completely defoliated, foliar damage was less in transgenic plants.

In DTS-43, the frequency of plants with less than 25% damage increased in advanced generations and was stabilized by T_4 generation. In T_2 to T_4 generations of the event DTS-43, 50% of the plants were moderately resistant with less than 25% foliage damage. In events DTS-46, AMT-894 and AK1304-PB-1, 54 to 60% of the plants in T_2/T_3 generation were found promising with less than 25% damage. The event AMT-936 was found promising during the first cycle with 83% and 88% of the plants showing moderate resistance in the T_3 and T_4 generations (Table 5).

However, with the mixed infestation of both the foliage feeders, foliar damage was high which probably could be due to the overlapping cycles and the high pest load. In events, DTS-46, AK1304-PB-1 and AMT-799, none of the plants showed resistant or moderately resistant reaction. The event AMT-894 was found promising with 43% plants with less than 25% leaf damage (Table 5, Figs. 5g-h).

459 *3.4.3. Laboratory assays against S. cynthia*

460

461 Laboratory bioassays against *S. cynthia* larvae by feeding the leaves of three events
462 (AK1304-PB-1, AK1304-PB-4, AMT-894) and control (DCS-9) showed no larval mortality on
463 any of the tested events (Fig. 6b, c). However, weight reduction of 20.2-78.5% was observed
464 when compared to weight of larvae fed on untransformed castor (control) plants (Table 6).
465 Figures 6a and c show the comparison of feeding and larval growth on untransformed castor
466 leaves treated with purified *Bt* Cry1Aa protein (2.93 ng/cm^2) and the transgenic leaves with the
467 *cry1Aa* gene.

468

469 **4. Discussion**

470

471 Development of transgenic castor plants expressing insecticidal *Bt* genes *viz*., *cry1Ab*
472 (Malathi et al., 2006), *cry1EC* (Sujatha et al., 2009) and *cry1AcF* (Kumar et al., 2011) to
473 confer protection against the major foliage feeders *viz*., *A. janata* and *S. litura* has been reported.
474 The *cry1Aa* gene deployed in castor conferred protection not only to the major lepidopteran
475 foliage feeders but also to *S. cynthia* which feeds on castor leaves. Selection of *cry1Aa* gene
476 was based on the previous insect bioassays with purified *Bt* crystal proteins which proved the
477 Cry1Aa protein to be the most effective against *A. janata* in terms of larval mortality (Sujatha
478 and Lakshminarayana, 2005) while causing significant feeding cessation of *S. litura* larvae
479 (Lakshminarayana and Sujatha, 2005). This gene has been introduced to impart resistance to
480 major pests in several other crops. Duan et al. (2013) developed transgenic cassava by
481 deploying *cry1Aa* gene for resistance to *Helicoverpa armigera* (Hubner). Vinodh (2013) and
482 Visarada et al. (2014; 2016) reported development of transgenic sorghum with the *cry1Aa*
483 gene for protection against the spotted stem borer (*Chilo partellus* Swinhoe).

484 The meristem-based transformation protocol through *Agrobacterium*-mediated method
485 developed by Sujatha and Sailaja (2005) and particle gun bombardment described by Sailaja et
486 al. (2008) were followed for castor transformation using the *cry1Aa* gene. The transformation
487 frequencies were 2.4%, 1.1% and 2.1% for direct, vector mediated and *in planta* methods,
488 respectively and were similar (0.42 to 5.9%) to those reported earlier. Sujatha et al. (2009)
489 reported transformation frequencies of 0.69% and 0.82% in castor with *cry1EC* gene through

direct and vector-mediated methods, respectively. Malathi et al. (2006) reported a transformation frequency of 0.42% with *cry1Ab* gene through *Agrobacterium*-mediated method. In *planta* transformation of castor with *cry1AcF* gene was done to develop transgenics resistant to *S. litura* with a frequency of 1.4% based on plant survival and presence of the introduced gene (Kumar et al., 2011). Sousa et al. (2017) obtained four transgenic plants silenced for the ricin A chain gene with a transformation efficiency of 0.85%. Patel et al. (2015) reported enhanced transformation efficiency (2.8-5.9%) when the incubation was done with acetosyringone (200 µM) in combination with spermidine (1 mM) instead of using acetosyringone alone which could be tried in future experiments on castor.

Molecular analysis was done through PCR and Southern hybridization for confirmation of the presence, integration and stable inheritance of the introduced gene. All the plants selected on hygromycin and grown to maturity were subjected to PCR analysis, and 44%, 63% and 25% of the AMT, PGB and DTS plants had the introduced *cry1Aa* gene. Selection of untransformed shoots on hygromycin showed 100% kill of the proliferating shoots. However, only 44% and 63% of the recovered putative transformants harboured the gene based on PCR analysis, indicating the possibility of escapes despite the cultures being passed through three cycles of the selection agent. The plants derived through PGB showed more hybridization signals except in the event AK1304-PB-1 compared to those obtained through AMT. In castor transgenics with the *cry1EC* gene, events obtained through PGB showed multiple copies of the gene as against single copy insertion in plants derived through AMT (Sujatha et al., 2009).

In the present study, the concentration of Cry1Aa protein determined through ELISA ranged from 0.22-1.35 ng/mg of fresh tissue in T_2 generation, 0.18-1.69 ng/mg in T_3 generation and 0.17-1.72 ng/mg in T_4 generation of different events. In castor transgenics with the chimeric *cry1AcF* gene, differing levels of the protein were observed that varied from 1.5-6.5 µg/g fresh weight (Kumar et al., 2011). The Cry1Ab protein accumulation in different primary transformants of castor ranged from 0.23-0.47 ng/mg of fresh tissue (Malathi et al., 2006) and was similar to the protein range that was recorded in the present study with *cry1Aa* transgenics. Mehrotra et al. (2011) showed that the level of protection and mortality of *H. armigera* larvae in chickpea correlated with the level of Bt-Cry protein, and only plants with high levels of Cry1Ac protein (70-112 ng/mg of soluble protein) resulted in 100% mortality while those with moderate level of the Cry protein showed feeding inhibition and severely affected larval growth.

Transgenic sugarcane with *cry1Ac* gene expressed 1.8-50.5% protein in leaves (Weng et al., 2011). Visarada et al. (2016) reported protein levels in the range of 29.6-40.0 ng/g leaf tissue (=0.3-0.4 ng/mg) in transgenic sweet sorghum. Rai et al. (2013) also reported low levels of Cry1Aa3 protein in leaves and fruits of brinjal (20.5–44.3 mg/g) but the transgenic events conferred 100% protection against egg plant shoot and fruit borer with use of codon modified *cry1Aa3* gene. In our study, a maximum of 1.72 ng/mg of Cry1Aa protein in fresh tissue was recorded, which was many times higher than that reported for sorghum. Variations in protein level and mortality in different crops and genes could be due to plant age, physiological variability arising due to the condition under which the plants are raised, the site of integration of the transgene and its positional effect.

In castor, laboratory bioassays were carried out with leaves obtained from plants raised in the transgenic greenhouse and when the plants were about 55-60 days old. It is well known that the protein expression level varies with the age of the crop and also the conditions under which the plants are grown. Studies of Zhu et al. (2004) in Brassica indicated variations in protein levels based on the age of the crop which were 0.8-0.16 µg/g when tested at 3-5 leaf stage and 0.18-0.66 µg/g at 4 leaf stage. ELISA analysis showed that the *cry1Ac* protein levels in transgenic sugarcane ranged from 0.8-70.9 µg/g in leaves and 0.04-7.2 µg/g in stems (Gao et al., 2016). Hence, it is necessary to determine the protein levels at different developmental stages and tissues.

The level of Bt protein in total soluble protein (%) in transgenic castor plants was adequate to cause feeding cessation in the laboratory and whole plant assays in field. Modifications of *cry* gene based on codon usage pattern of castor genes could likely enhance the level of expression, as was done in the case of sugarcane (Weng et al., 2011) where a synthetic *cry1Ac* gene resulted in five-fold higher protein level than that produced by the partially modified *cry1Ac* gene.

In insect bioassays conducted in the laboratory, mortality against *S. litura* and *A. janata* ranged from 10-50% among different events. Larval weight reduction of surviving larvae vs control larvae after 8 days of feeding was 3.2-87.2% in case of *S. litura* and 1.1-78.9% for *A. janata*. Despite the same level of protein in castor transgenics with the *cry1Ab* gene (Malathi et al., 2006) and in the present study, *A. janata* mortality reached a maximum of 97%. By contrast, in the present study the maximum mortality of the two foliage feeders was 50%.

552 However, bioassay of T_1 generation castor plants with the *cry1AcF* gene, resulted in up to 97%
553 mortality of *S. litura* larvae (Kumar et al., 2011). In cassava transgenics harbouring the *cry1Aa*
554 gene, 70-100% mortality was reported for *Helicoverpa armigera* Hubner (Duan et al., 2013). In
555 sorghum, laboratory bioassays of *cry1Aa* transgenics against spotted stem borer indicated lower
556 larval mortality (21.6%), reduced leaf damage score (3.0 cm^2) and reduced feeding (41–46%) as
557 compared to 81.6% feeding in control (Visarada et al., 2016).

558 In the present study, stabilized events in T_2 to T_4 generations were subjected to larval
559 bioassays. However, in insect resistant castor transgenics harbouring the *cry1Ab* (Malathi et al.,
560 2006) and *cry1AcF* (Kumar et al., 2011) genes were bioassayed in T_0 and T_1 generations, unlike
561 in the present study where the plants were analyzed in advanced generations. Suma et al. (2009)
562 reported 0-40% mortality in T_1 plants and 0-60% mortality in T_2 generation of transgenic
563 chick pea plants with *cry1Ac* gene. Mehrotra et al. (2011) reported 12-30% mortality in T_0
564 plants and 30-100% mortality in T_1 plants. Weng et al. (2011) reported 100% mortality
565 and 50-60% weight reduction in transgenic sugarcane with *cry1Ac* gene against stem borer.
566 Bioassays with T_1 plants of transgenic cotton with *cry1Ia12* gene against *Spodoptera frugiperda*
567 (J.E. Smith) larvae, resulted in mortality up to 40% and a significant delay (up to 30-fold) in the
568 development of the target insects compared to untransformed controls (Oliveira et al., 2016).
569 *H. armigera* neonates on T_0 cotton plants recorded a mortality of 10-36.7% and showed
570 significant reduction in leaf feeding and inhibition of growth in surviving larvae (Jadhav et al.,
571 2015). The *cry1EC* gene is reported to be toxic to *S. litura* and *A. janata* and has been
572 successfully introduced in castor (Sujatha et al., 2009). Incorporation of *cry1Aa* gene also
573 proved to be effective against *S. litura* in terms of less foliar damage and feeding inhibition.
574 Probably, combining both the genes through crossing of events harbouring the *cry1Aa* and
575 *cry1EC* genes may confer better protection against the lepidopteran pests in castor.

576 Following the development of castor transgenic events with the *cry1Aa* gene, it has
577 become imperative to assess the toxicity of these events to *S. cynthia* to comply with regulatory
578 requirements. Evaluation of toxicity of the purified crystal proteins of *B. thuringiensis* against
579 *S. cynthia* indicated high toxicity of Cry1Aa protein followed by Cry1AC and Cry1Ab
580 proteins to this lepidopteran pest (Kumar et al., 2016). The Cry1Aa protein resulted in 50%
581 mortality at a very low concentration of 2.6 ng/cm^2 leaf area. Bioassays conducted against *S.*

582 *cynthia* of three events of castor harbouring the *cry1Aa* gene showed only growth retardation
583 and larval weight reduction up to 2 to 3-fold at 8 days after treatment. Despite the presence of
584 the gene, the expression is ~160 fold lower in the transgenic events as compared to the
585 concentrations tested with purified *Bt* proteins, which probably is leading to feeding cessation
586 but not causing 100% larval mortality. Since transgenics harbouring the *cry1Aa* gene are
587 showing toxicity as evident from reduced feeding, one has to be cautious while testing such
588 events in areas where castor leaves are fed to *S. cynthia*. Further, any candidate proteins have to
589 be tested for their toxicity to beneficial insects before embarking on a programme on
590 development of insect resistant transgenics in castor.

591 Although the level of expression of the Cry1Aa protein is moderate in the castor
592 transgenic events, there is significant and substantial feeding cessation of the three foliage
593 feeders. Within artificially infested field cages, larval densities of both the target foliage feeders
594 were similar but foliar damage was visibly reduced in transgenic plants as compared to the
595 control plants. Under natural infestation of the target pests, the pest load would be far lower than
596 that established in this present study through artificial releases and encaging of the crop under a
597 net. Despite low mortality of the insects under artificial assays, foliar damage in transgenic
598 events was significantly low as compared to control in both laboratory and field bioassays.
599 Based on studies on removal of leaves at spike formation stages it is evident that castor crop has
600 tolerance limit for defoliation up to 25 to 30% (Dinesh and Sundaramoorthy, 2002;
601 Lakshmamma et al., 2009). Thus, these events in tandem with additional variety development,
602 could be included as a component of future integrated pest management programmes,
603 potentially resulting in a reduction in pesticide sprays. The primary challenge going forward
604 however, will be the need to produce plants that express higher concentrations of *Bt* protein, that
605 approach a high-dose definition and cause ~100% mortality (Hutchinson et al., 2010); as the
606 high-dose refuge approach is generally viewed as the preferred strategy to minimize the
607 evolution of *Bt* resistance in target pests (Soberon et al., 2015). Also, there is a need to test these
608 castor transgenic events on a broader array of non-target insect species and natural enemies of
609 both the foliage feeders like *Trichogramma evanascens, Microplitis maculipennis, Euplectris* sp.,
610 *Apanteles prodeniae* to ensure the transgenic plants will continue to support biological control in
611 IPM modules as reported for other crops (Romeis et al., 2008; Emani, 2014).

612 In conclusion, this study reports the development of transgenic events in castor through

613 introduction of the *cry1Aa* gene and demonstrated stability of the introduced gene through the T_4
614 generation. The events displayed moderate levels of the Cry1Aa protein expression, resulting in
615 feeding cessation of the target foliage feeders, in both laboratory and field bioassays. As castor
616 can tolerate defoliation up to 25%, the gene could be transferred to other agronomically
617 superior genotypes and tested for the bioefficacy of the introduced gene in different genetic
618 backgrounds.
619
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630 **Conflict of Interest**
631
632 The authors declare that they have no conflict of interest.
633
634 **Author contribution statement**
635
636 MT assisted in the transformation work, molecular analysis and insect bioassays, PAK
637 provided the gene construct, ML helped in insect bioassays, MS conceived and designed the
638 research, analyzed the results and prepared the manuscript. All authors read and approved the
639 manuscript.
640
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782

783
784 **Table 1**

785 Putative transformants obtained with *cry1Aa* constructs through different methods of
786 transformation

Method of transformation	Number of embryo axes	Frequency of shoots after three selections	Number of plants established	Transformation efficiency (%)	Number of T_0 plants tested for PCR	PCR positive plants for *hpt**	PCR positive plants for *cry1Aa**
Agrobacterium-mediated	4340	5.4	104	2.4	37	20 (54)	16 (43)
Particle gun bombardment	1790	10.5	19	1.1	19	16 (84)	12 (63)
Direct transformed shoots (*in planta*)	2650	-	56	2.1	56	18 (32)	14 (25)

787 *Figures in parentheses indicate the frequency in percentage
788

Table 2

Segregation analysis of *cry1Aa* positive plants in T_1 progeny

Transgenic event code	No. of plants	PCR^+	PCR^-	Ratio	χ^2	P Value
Events derived through AMT						
AMT-685	16	5	11	0.45:1	16.333	<0.001
AMT-799	26	4	22	0.18:1	43.985	<0.001
AMT-889	19	1	18	0.05:1	45.871	<0.001
AMT-894	21	16	5	3.2:1	0.023	0.88**
AMT-899	14	11	3	3.6:1	0.095	0.758**
AMT-936	18	11	7	1.57:1	1.852	0.174**
Events derived through particle gun method						
AK1304-PB-1	31	23	8	2.9:1	0.185	0.668**
AK1304-PB-3	20	11	9	1.2:1	4.256	0.0398
AK1304-PB-4	14	10	4	2.5:1	0.095	0.758**
AK1304-PB-5	20	4	16	0.25:1	45	<0.001
AK1304-PB-785	14	10	4	2.5:1	0.095	0.758**
AK1304-PB-830	30	22	8	2.75:1	0.044	0.833**
AK1304-PB-837	17	12	5	2.4:1	0.176	0.074*
Events derived through DTS method						
DTS-4	10	3	7	0.42:1	10.8	0.001
DTS-30	19	10	9	1.1:1	5.897	0.015*
DTS-43	40	29	11	2.6:1	0.133	0.715**
DTS-46	30	13	17	0.76:1	16.044	0.001

* P=≥0.05

** P=≥0.10

Table 3

Insect bioassays of T_1 plants harbouring the *cry1Aa* gene against *S. litura* and *A. janata*

Transgenic event	*S. litura*					*A. janata*				
	No of plants tested	No of plants that resulted in mortality	Mortality (%) (range)	Mortality (%) (mean ± SE)	Reduction (%) in larval weight over control	No of plants tested	No of plants that resulted in mortality	Mortality (%) (range)	Mortality (%) (mean ± SE)	Reduction (%) in larval weight over control
AMT-685	17	8	10-50	43.8±3.9	17.6-64.4	4	4	20-40	30±5.8	12.5-65.6
AMT-862	20	5	10-40	25.9±6.9	21.4-53-4	2	0	0	0	29.6-52.4
AMT-894	14	6	12-47	28.5±6.5	18.7-43.7	4	0	0	0	15.6-46.8
AMT-899	8	3	10-20	16.7±3.3	27.5-35.0	3	3	20-25	21.7±1.7	34.2-43.3
AK1304-PB-785	17	8	10-25	38.6±6.8	21.8-75.0	1	1	30	30	16.8-31.3
AK1304-PB-1	18	16	10-50	23±3.9	19.6-75.0	16	15	20-50	29.8±2.7	25-50
AK1304-PB-3	NT	-	NT	-	NT	10	4	20-50	32.5±3.9	33.6-36.6
AK1304-PB-4	18	7	10-30	17.1±3.6	15.8-70.0	18	8	20-50	29.2±3.8	18.3-56.6
AK1304-PB-5	10	6	10-30	20.3±4.5	40-50	10	5	10-20	13.5±1.5	5.5-33.3
DTS-4	17	0	0	0	18.3-62.1	NT	-	NT	-	NT
DTS-30	13	3	10-40	33.3±6.6	6.7-15.8	2	0	0	0	7.8-46.1
DTS-43	10	8	20-40	30.6±6.5	53.1-71.8	8	7	20-40	32±2.7	34.6-61.5
Control	3	0	0	0	0	3	0	0	0	0

*NT=Not tested; SE= standard error of mean

800

801

802 **Table 4**

803 Bioassays of T_3 generation plants against *S. litura* and *A. janata*

Transgenic event	*S. litura*					*A. janata*				
	No of plants tested	No of plants that resulted in mortality	Mortality (%) (range)	Mortality (%) (mean ± SE)	Reduction (%) in larval weight over control	No of plants tested	No of plants that resulted in mortality	Mortality (%) (range)	Mortality (%) (mean ± SE)	Reduction (%) in larval weight over control
DTS-43-20-4	17	11	0-10	7±1.4	28.4-87.2	17	15	20-80	37±6.8	27.9-76.9
DTS-43-20-2	27	0	0	0	30.3-86.7	27	24	20-80	34.4±4.9	46.5-78.1
Control	3	0	0	0	0	3	0	0	0	0

804 SE=standard error of mean

805

Table 5

Reaction of transformants harbouring the *cry1Aa* gene to the major foliage feeders under net contained field experiment

Event code	Gene-ration	Reaction of plants to defoliators							
		S. litura damage on defoliation scale**				*S. litura* and *A. janata* damage on defoliation scale***			
		1*	**2***	**3***	**4***	**1***	**2***	**3***	**4***
DTS-43	T_2	0	0	100	0	0	0	0	100
	T_3	5.3	31.6	47.4	15.8	0	5.3	0	94.8
	T_4	18.8	31.3	25	25	2.3	5.4	23.8	68.7
DTS-46	T_2	30.8	23.1	38.5	7.7	0	0	7.6	92.4
AK1304-PB-1	T_3	13.5	46	32.4	8.1	0	0	8.1	91.9
AMT-799	T_1	0	28.6	57.1	14.3	0	0	0	100
AMT-894	T_2	4.8	52.4	19	23.8	23.8	19	19	38.2
AMT-936	T_3	55.3	27.1	16.5	1.2	1.2	21.8	22.9	54.1
	T_4	55.5	33.3	11.1	0.1	0	22.2	7.8	70

* Row three represents defoliation scale: 1. <10%-resistant; 2. 10-25%-moderately resistant; 3. >25-50%-susceptible; 4. >50%-highly susceptible

** Data scored 25 days after *S. litura* release

*** Data scored 40 days after *S. litura* release

Table 6

Bioassays against *S. cynthia* on castor events harbouring the *cry1Aa* gene

Event	Gene-ration	Concentration of Cry1Aa protein in fresh leaf as determined by ELISA (ng/mg)	Reduction (%) of weight of *S. cynthia* larvae over control
AK1304-PB-1	T_3	0.16-2.22	21.4-69.0
	T_4	0.22-2.48	35.7-78.5
AK1304-PB-4	T_3	0.21-2.76	26.5-60.3
	T_4	0.16-1.38	20.2-52.3
AMT-894	T_3	0.18-2.12	49.4-67.8
	T_4	0.16-1.91	27.7-73.4
Control (DCS-9)	-	0	0

Data was scored 8 days after treatment

Figure legends

Fig. S1 Diagram showing the restriction sites of *cry1Aa*.

Fig. S2 Sequence of *cry1Aa* gene.

Fig. 1 Genetic transformation of castor with *cry1Aa* gene construct (AK1304) through *Agrobacterium*-mediated (a-c) and *in planta* (d-f) methods.
a Selection of putative transformants on hygromycin following *Agrobacterium*-mediated transformation (bar: 17.6 mm). **b** Shoot elongation on medium supplemented with 0.2 mg/l BA (bar: 29.8 mm). **c** Acclimatized and established transgenic plants (bar: 27.6 mm). **d** Elongation of *in planta* transformed shoots following bombardment on MS medium with 0.1 mg/l BA (bar: 10 mm). **e** *In planta* transformed shoots on rooting medium with selection agent (½MS+1.0 mg/l NAA+10 mg/l hygromycin) (bar: 13.8 mm). **f** Acclimatization of *in planta* transformed shoots (bar: 27.2 mm)

Fig. 2 PCR analysis of transgenic events with different generations.
a Amplification of 552 bp fragment of the *cry1Aa* gene. Lanes denoted 1-37: DNA from putative transformed plants. **b** Amplification of 552 bp fragment of the *cry1Aa* gene. Lanes denoted 1-19: DNA from AMT 894 T_1 generation plants; **c** Amplification of 552 bp fragment of the *cry1Aa* gene. Lanes denoted 1-11: DNA from AK1304-PB-1 T_3 generation plants; **d** Amplification of 490 bp fragment of the *hpt* gene Lanes denoted M: λ DNA double digest with *Eco*RI/*Hind*III, PC: Plasmid DNA of AK1304, NC: Negative control, UT: Untransformed castor DNA and 1-11: DNA from AK1304-PB-1 T_3 generation plants; **e** Amplification of 552 bp fragment of the *cry1Aa* gene. Lanes denoted 1-20: DNA from AMT 894 T_3 generation plants. Lanes denoted M: λ DNA double digest with *Eco*RI/*Hind*III, PC: Plasmid DNA of AK1304, NC: Negative control, UT: Untransformed castor DNA

Fig. 3 Southern analysis of transgenic plants of castor harbouring the *cry1Aa* gene.
a Lane denoted 1-5 putative transformed plants (AK1304-PB-1, 2, 3, 4 and 5) DNA digested with *Eco*RI. **b** Lanes denoted 1-4: DNA from T_3 generation plants of DTS-43-20-2. **c** Lanes

denoted 1-3: DNA from T_2 generation plants of AK1304-PB-1-73. **d** , Lanes denoted 1-4: DNA from T_2 plants of AMT-894-3. Lanes denoted PC: *cry1Aa* amplified fragment from plasmid (AK1304) DNA, NC: blank, UC: DNA from untransformed castor plant (control) digested with *EcoR*1

Fig. 4 Concentration of *cry1Aa* protein through ELISA.
a Concentration of *cry1Aa* protein in T_4 generation plants of different events. **b** Concentration of *cry1Aa* protein in different generations (T_2, T_3, T_4) of different events

Fig. 5 Whole plant bioassays against *Spodoptera litura* and *A. janata* in the field.
a Net encaged transgenic plants of castor. **b** Initial damage due to release of *S. litura* and natural infestation of *A. janata*. **c** and **d** Uniform pest load of *A. janata* on control (c) and transgenic plants (d) but foliar damage was more in control as compared to transgenic plants. **e** and **f** Uniform pest load of *S. litura* on control (c) and transgenic plants (d) but larvae have completely defoliated the control plants and started feeding on the stems and capsules. **g** Variation in foliar damage in different events. **h** Reaction of control and transgenic lines to the attack of *S. litura* and *A. janata* showing complete defoliation in control plants while transgenic plants still have the foliage

Fig. 6 Bioassays against *S. cynthia* larvae on transgenic leaves harbouring the *cry1Aa* gene and coated with pure Cry1Aa protein.
a Comparison of growth and mortality of neonate larvae of *S. cynthia* fed on uncoated castor leaf and purified Cry1Aa protein coated castor leaf (2.6 ng/cm^2) (bar: 13.2 mm). **b** Growth of *S. cynthia* larvae on control castor plants eight days after treatment (bar: 14.2 mm). **c** *Samia cynthia* larvae showing feeding cessation on different events of T_3 generation plants of castor as evident from the less foliar damage and larval size in comparison with those fed on untransformed castor leaves eight days after treatment (bar: 26.5 mm)

Figure 1

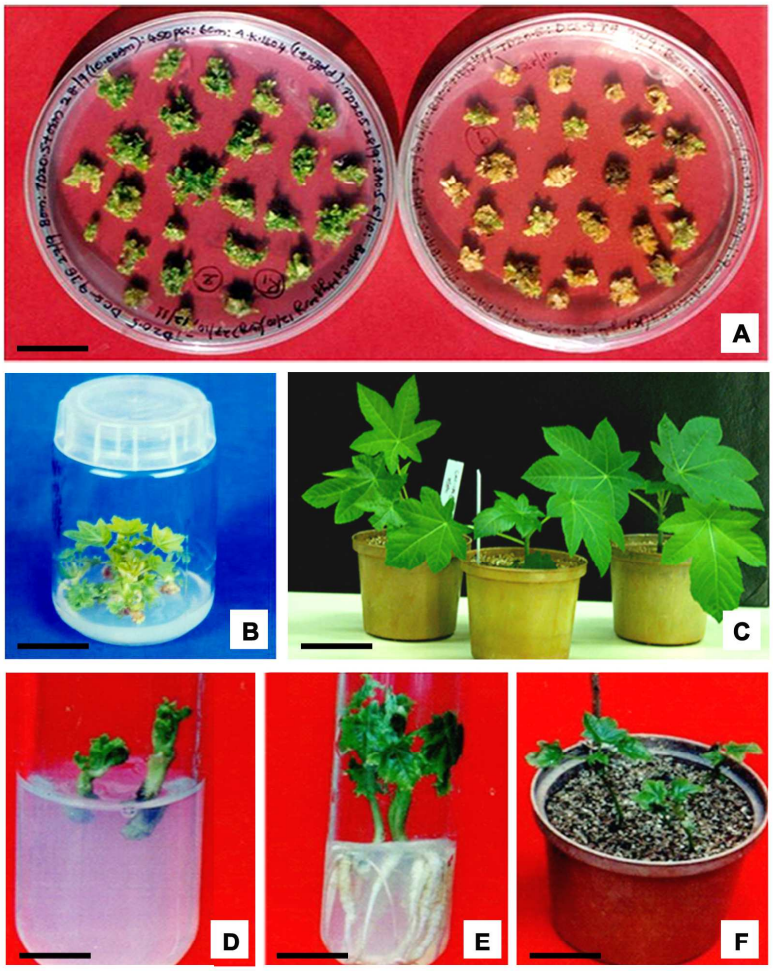
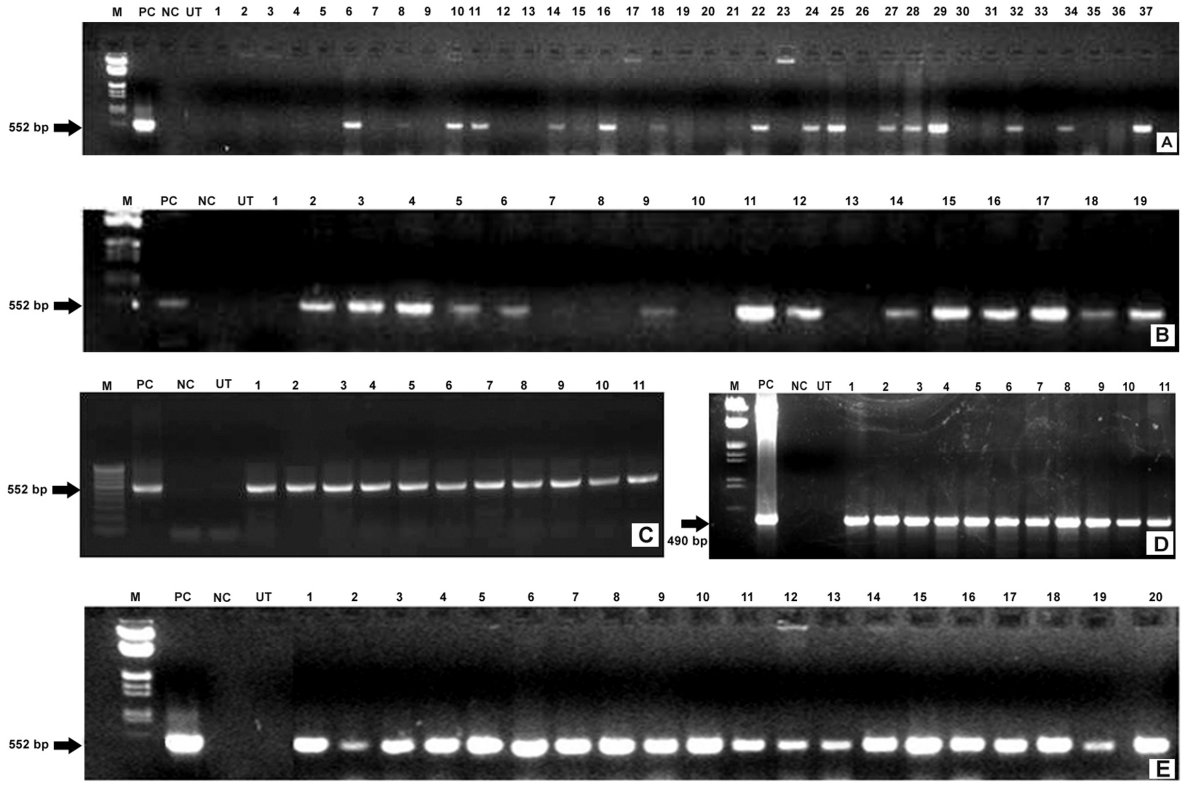


Figure 2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552 bp
A
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552 bp
B
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552 bp
C
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D
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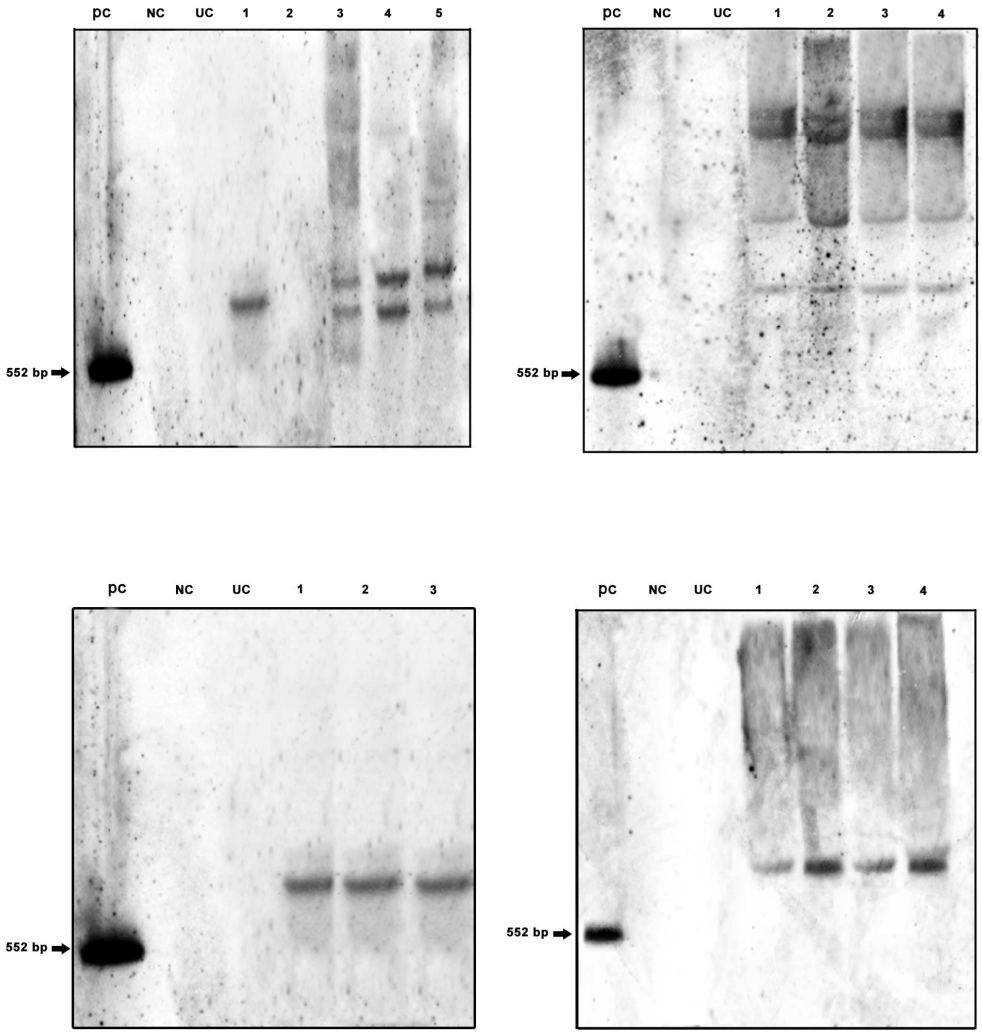
552 bp
E

Figure 3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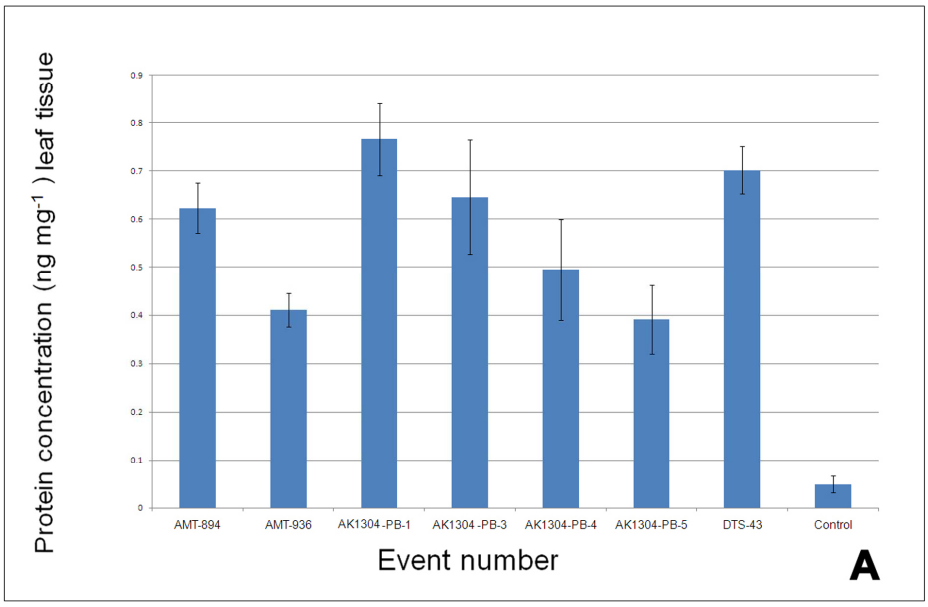
pc NC UC 1 2 3 4
552 bp

Figure 4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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
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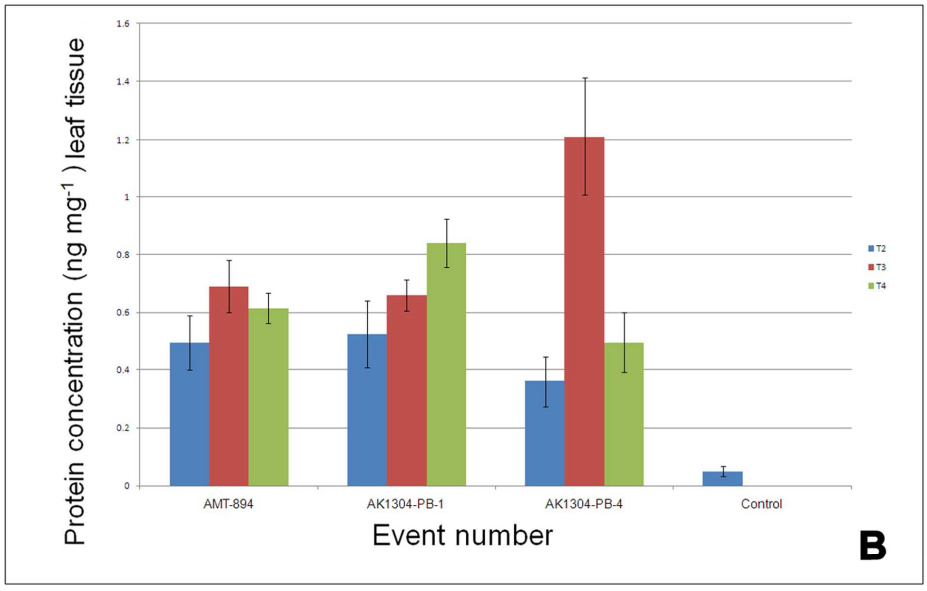
AMT-936
AK1304 -PB-1
AK1304 -PB-3
AK1304-PB-4
AK1304-PB-5
DTS-43
Control
Event number
A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1.2
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Control
Event number
T2
T3
T4
B

Figure 5

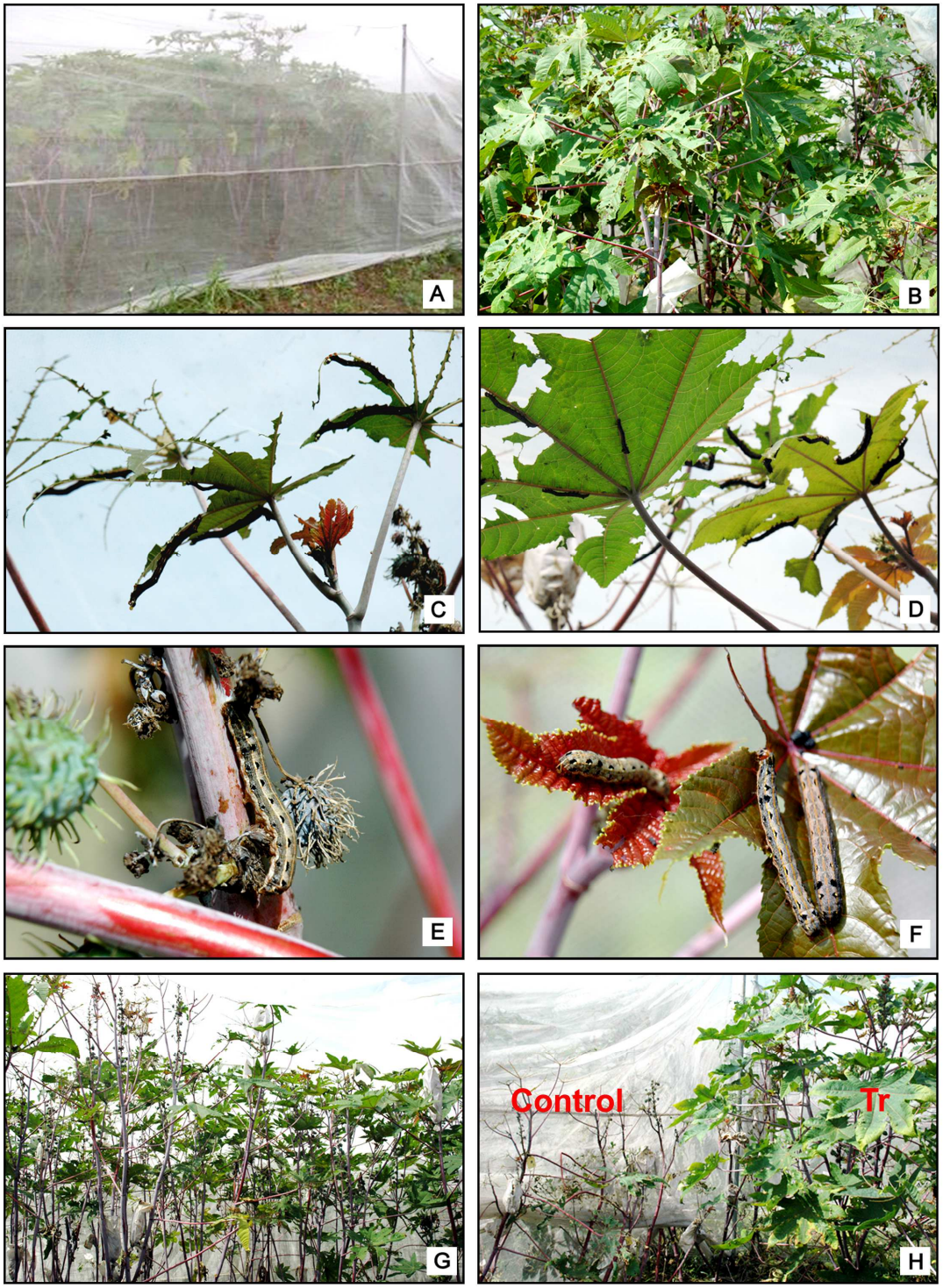
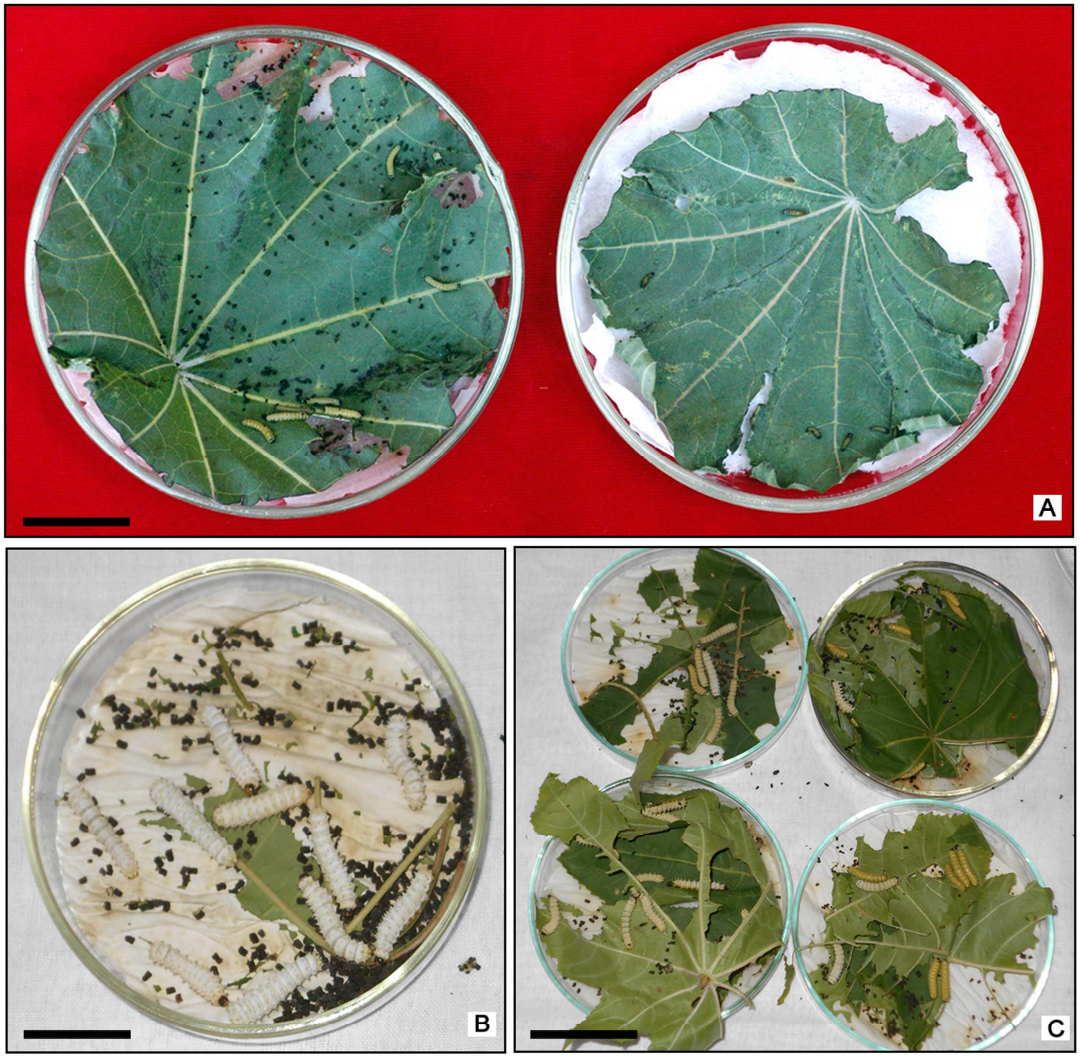


Figure 6



Highlights

- Castor crop damage due to defoliators accounted for yield losses ranging from 20 to 85% (average 31%).
- The development of transgenic castor events carrying *cry1Aa* gene through vector and direct gene transfer is reported.
- Laboratory and field bioassays against *Achaea janata* and *Spodoptera litura* indicated feeding tolerance.
- Bioassays of transgenics against *Samia cynthia ricini* were done to assess the reaction of beneficial organisms.
- The *cry1Aa* transgenic events of castor could be included as a component of future integrated pest management programmes.