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Development of transgenic *Sorghum bicolor* (L.) Moench resistant to the *Chilo partellus* (Swinhoe) through *Agrobacterium*-mediated transformation

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Abstract

An *Agrobacterium* mediated gene transformation was developed in Sorghum for insect resistance using shoot apices. Sorghum spotted stem borer (*Chilo partellus* Swinhoe) is an important insect pest of sorghum which causes severe damage in many parts of the world. Sorghum plants were transformed with fully modified synthetic *Cry1C* coding sequences along with *hpt* and *gus* genes. The calli developed from shoot apices of APK 1 cultivar were inoculated with *Agrobacterium* strain. Embryogenic calli from the shoot apices were selected on the medium containing hygromycin B. A total of 76 hygromycin resistant plants were regenerated. PCR was performed to confirm the presence of the *Cry1C* gene. All the three putative transformants were found to be positive for the amplification of 1.9 kb *Cry1C* gene. A similar band was also observed in the positive plasmid control. Southern blot analysis of transformants confirmed integration of 2.9 kb *Cry1C* coding sequences into the sorghum genome. S_1 progeny plants disclosed a monogenic pattern (3:1) of transgene segregation. The efficiency of transformation in our study was between 1.2%-3.9%. These transgenic lines were highly resistant to the spotted stem borer *Chilo partellus* as revealed by insect bioassay with 100% insect mortality rate.

Keywords: Sorghum (*Sorghum bicolor*), transgenic crops, insect resistance, GUS expression, Agrobacterium, *Cry1C*, *hpt*, *chilo partellus*

Introduction

Sorghum bicolor L. (Moench) is the fifth most important cereal crop worldwide in production and is unique since it is adapted to semi-arid environments. It is one of the main staple foods of the world's poorest and most food-insecure people, supporting more than 300 million lives in Africa and Asia. It is thought to be >200 million years old [1]. The total worldwide production of sorghum is about 60 million tonnes annually from a cultivated area of 46 millions ha. It belongs to the tribe Andropogoneae and the family Poaceae [2,3]. Sorghum grain has enhanced protein quality that could contribute significantly to nutritional value of the diets of people and livestock. The plant stem and foliage are used for green chop, hay, silage, pasture and hut making. The sorghum flour is also used as food for infants [4]. It has been used for various food products like roti (flat bread), bhakri (stiff roti) and porridge. It is also used in common foods such as traditional breakfast [5]. It also represents an ideal bio-energy crop, as grain quality is now considered less important, and concerns about the environmental cost of high-input agriculture and food security grow. The lower need for fertilizers and pesticides may make it an ecologically attractive crop, especially when combined with conservation-agriculture production systems.

Insect pest infestation acts as a serious limiting factor on crop yield and affects productivity of sorghum. The use of genes that encode insecticidal proteins in transgenic crops has the potential to benefit agricultural crop production, the environment, and the consumer. The increasing pressure to use non-hazardous, environmentally compatible pest control measures have spurred interest in the use of natural insecticides such as *Bacillus thuringiensis Bt* insecticidal crystal proteins in a number of countries [6,7]. Development of insect resistant sorghum by transferring Bt genes is one of the best ways available today to overcome insect attack and to improve the yield of sorghum. There are many reports on successful transformation of sorghum utilizing particle bombardment [8,11] or *Agrobacterium* mediated transformation [12,13]. But till date there is only one report available on sorghum transformation employing particle bombardment with *Cry1AC* gene [14]. A moderate level of tolerance was reported, which in turn conferred partial protection against neonate larvae of the spotted stem borer (*Chilo partellus*).

Cry1C toxin is effective against a wide variety of lepidopteran insect pests including stem borers and leaf folders and does not share a common binding site with *Cry1A* toxins [15-18]. Sakai et al., [19] studied the role of protein domain of *Cry1C* protein that confers its specificity to *Spodoptera* sp. by domain swapping studies on the protein and then analyzing the cytotoxicity to Sf9 insect cell lines. Transgenic crops such as alfalfa and tobacco expressed a modified *Cry1Ca* gene and the transgenic plants

showed enhanced resistance to Egyptian cotton leafworm (*Spodoptera littoralis*) and the beet armyworm. Broccoli plants were transformed with a modified *Cry1C* gene [20] and the transgenic plants showed high insect resistance to diamond backboth (*Plutella xylostella*). Synthetic *Cry1C* genes were found to be highly resistant to stem borers and leaf folders [21]. *Cry1C* toxins can be potential alternative to *Cry1AC* and *Cry1A* toxins and can also be combined with other *Cry1AC* and *Cry1A* genes. Therefore, it would appear that the *Cry1C* toxin can be a potential alternative to *Cry1A* toxins and that it can also be combined with other *Cry1A* genes to develop *Bt* crops with two-toxin [22,23].

In the present study we employed *Agrobacterium*–mediated transformation using *Cry1C* to confer resistance to *Chilo partellus*. The plant expression vector containing the reporter gene *uidA* (*GUS*), the selectable marker gene *hpt* and the *Cry1C* gene under the control of *CAMV35S* promoter was introduced via *Agrobacterium*-mediated transformation of selected sorghum genotype. Insect bioassay of the transformed plants was carried out to assess the level of resistance to *Chilo partellus*.

Materials and methods
Plant material and tissue culture
Cultivars of sorghum (*Sorghum bicolor* (L.) Moench) variety APK I was used for tissue culture and transformation studies. The seeds were obtained from the germplasm bank of Tamil Nadu Agriculture University, Coimbatore, for experimental use. This variety is being cultivated in many states of India. Seeds were washed in running tap water several times. After washing they were treated with Tween 20 detergent solution (Qualigens, Mumbai, India) for 5 minutes and rinsed in double distilled water. The rest of the sterilization process of seeds was carried out inside the laminar airflow hood. Seeds were surface-sterilized with 70% ethanol (v/v) for 30 seconds followed by treatment with 0.1% (w/v) mercuric chloride (HgCl$_2$) for 6 minutes and were washed six times with sterile double distilled water to remove the sterilant and used for further studies. Seeds were germinated on MS basal medium containing sucrose. The pH of the medium was adjusted to appropriate pH 5.6 to 5.8 using 1N KOH or 1N HCl solutions prior to adding agar; media were autoclaved at 121°C for 15 minutes. Cultures were maintained at 24±2°C under cool white fluorescent tubes of (90-150 µmol photons m^{-2} s^{-1}) light intensity.

Agrobacterium tumefaciens strains, plasmid and culture
Codon optimised *Cry1C* gene cassette (kindly provided by Dr. I. Altosaar) for expression in plants (double 35s promoter+AMV enhancer-*Cry1C*–nos terminator) was inserted into the multiple cloning site of pCAMBIA1305.1 resulting in the formation of a plasmid pCAMBIA1305.1/*Cry1C* (**Figure 1**). The plasmid was introduced into the disarmed *Agrobacterium* strain LBA 4404 by freeze thaw method [24] and confirmed by plasmid
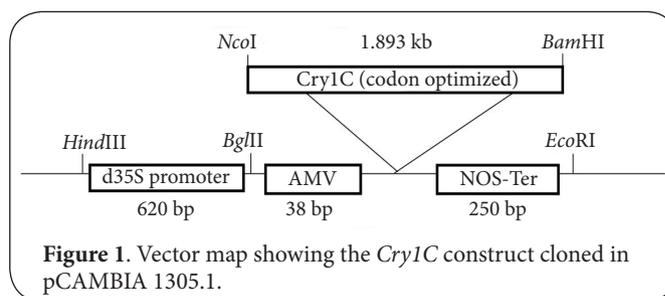

Figure 1. Vector map showing the *Cry1C* construct cloned in pCAMBIA 1305.1.

rescue and restriction analysis. Liquid YEP (10 g/L peptone, 5 g/L NaCl,10 g/L yeast extract and pH 7) medium (50 ml) containing kanamycin (50 mg/l) and 10mg/l of rifamycin was inoculated with a single colony of *Agrobacterium* and shaken at 28°C in an incubator at 175 rpm until the OD$_{600}$ reached to 0.8. At this time, 15% glycerol stocks were made and stored at -70°C. The day before *Agrobacterium* transformation, 100µl of *A. tumefaciens* glycerol stock was added to 30 ml YEP medium with kanamycin (50 mg/l) and shaken at 28°C for 16 hour at 175 rpm on a rotary shaker incubator (Orbitek, India) until the OD$_{600}$ reached to about 0.6-1.0. Before inoculation, the culture was centrifuged for 10 min at 6,000 rpm at 20°C to pellet the cells. The resulting pellet was dissolved in 10 to 15 ml (depending on the pellet size) of liquid pre-induction medium [AA medium [25], 20gm/l sucrose, 200µM acetosyringone, pH 5.5 (AA-AS)] and incubated at 28°C for 15 min for transformation studies.

Cocultivation, selection and plant regeneration
Shoot tip explants excised from 3-5days old seedlings were soaked in 15 ml of LBA 4404 bearing pCAMBIA1305.1/*Cry1C* for 30 min; after that they were blotted dry with sterile filter paper to remove excess bacteria. Cocultivation was done for 72 hr. For each experiment approximately 100 explants were used. Then they were cultured in coculture medium [(MS major salts, MS minor salts, MS vitamins, 300 mg/l casein acid hydrolysate, 500 mg/l L-proline, 2.5 mg/l 2,4-D, 0.25 mg/l kinetin, 30 g/l sucrose, 7 g/l agar, pH 5.5, 200 µM acetosyringone (CIM-AS)]. The plates were sealed with parafilm. Cocultivation was carried out in the dark at 25°C for 3 days. The calli labelled as control were not infected with *Agrobacterium*. After cocultivation, the infected calli were washed 3–5 times with sterilized deionized water containing 500 mg/l cefotaxime to kill *Agrobacterium*. The inoculated calli were first transferred to callus induction medium [MS major salts, MS minor salts, MS vitamins, 300 mg/l casein acid hydrolysate, 500 mg/l L-proline, 2.5 mg/l 2,4-D, 0.25 mg/l kinetin, 30g/l sucrose, 7g/l agar, pH 5.7 (CIM)] containing 500 mg/l cefotaxime for 1 week of callus growth without selection and then transferred to selection medium [(MS major salts, MS minor salts, MS vitamins, 300 mg/l casein acid hydrolysate, 500 mg/l L-proline, 2.5 mg/l 2,4-D, 30 g/l sucrose, 7 g/l agar, pH 5.7, plus 5 mg/l hygromycin and 500 mg/l cefotaxime (CIM-CCH)]. After three rounds of selection, each 2–3 weeks, resistant calli were transferred to regeneration

medium [MS major salts, MS minor salts, MS vitamins, 300 mg/l casein acidhydrolysate, 500 mg/l, L-proline, 30 g/l sucrose, 4mg/l BAP and 7 g/l agar, pH 5.7 plus 250 mg/l cefotaxime and 5 mg/l hygromycin (RE2-CCH)] for shoot development. The regenerated shoots were further transferred to RE2-CCH medium for full plantlet formation and then rooted on MS medium. After rooting, the transgenic plants were transferred to a glasshouse and grown to maturity. Three different experiments were done.

GUS histochemical assay

The histochemical assay for *GUS* gene expression (β-D-Glucuronidase) was performed in sorghum shoot tip calli with 5-Bromo-4-Chloro-3-Indolyl Glucuronide (X-Gluc) as a substrate by established methods [26,27]. Following cocultivation, tissues were harvested for *GUS* staining. The putative transgenic sorghum explants were incubated in Sodium Phosphate buffer (50 mM $NaPO_4$, pH 6.8) that contained 1% Triton X-100 at 37°C for 1 hour. The putative transgenic explants were incubated overnight in a solution containing 1.0 mM X-Gluc, 10 mM EDTA, 100 mM NaH_2PO_4, 0.1x Triton X-100 and 20% methanol (pH 5.8). The reaction mixture was incubated overnight at 37°C and the number of tissues that stained blue was counted. To suppress endogenous b-glucuronidase activity, we added 20% methanol to the reaction buffer. The tissue was washed twice in 99% methanol for two hours to remove the chlorophyll pigment.

PCR analysis of putative transgenic plants

PCR analysis was carried out using the following primers 5' primer, 5'-CCA TGG AGG AGA ACA ATC AGA ACC AGT G-3'; 3' primer, 5'-GGA TCC TAC TTT TGT GCT CTT TCA AGG TC-3'. These primers amplified a 1.9 kb fragment from the *Cry1C* gene respectively. PCR analysis was carried out in a reaction volume of 25 µl containing the template genomic DNA (100 ng), 2.5 µl 10 X PCR amplification buffer, 0.5 µl 10 mM dNTPS, 1.2 µl 50 mM MgCl 2, 3 µ M (2.5 µl) of each primer, 13.6 µl sterile distilled water, 1 unit (0.20 µl of Taq DNA polymerase (Genei). The samples were heated to 94ºC for 5 min and then subjected to 30 cycles of 30 s melting at 94ºC, 30 s annealing at 60ºC and 1 min synthesis at 72ºC and followed by another 10 min final extension at 72ºC. The amplified products were assayed by electrophoresis on 0.8% agarose gels, stained with ethidium bromide (EtBr; 0.5 mg/ml), visualized and photographed under ultraviolet light.

Southern hybridization

The S_1 transformants were subjected to Southern blot hybridization analysis using the coding sequence of *Cry1C* gene. Southern blot analysis was done using genomic DNA of putative (S_1) transformants positive to PCR and untransformed control plants. DNA was digested with HindIII and EcoRI; the blot analysis was carried out using ten microgram of genomic DNA. The digested DNA was resolved on 0.8%

agarose gels. DNA was transferred to negatively charged nylon membrane (as per the manufactures instructions) for Southern hybridization analysis [28]. The coding sequences of *Cry1C* genes were labelled with biotin-11-dUTP using Biotin Decalabel DNA Labelling Kits (Fermentas Life sciences) and used as a probe. The blot was subjected to detection by overnight colour development using biotin chromogenic detection kit (Fermentas Life sciences).

Bioassay of transgenic sorghum

Chilo partellus (Swinhoe) egg masses were obtained from International Crop Research Institute for Semi Arid Tropics (ICRISAT) and maintained in Entomology Research Institute (ERI). The standardized artificial diet composition for spotted stem borer (*C. partellus*) contained chickpea flour (219.2g), sorghum leaf powder (80g), sorbic acid (2g) methyl p-hydroxy benzoate (4.2g), ascorbic acid (5.2g), yeast (16g), water (1000ml), agar-agar (20.4g), water for agar (800ml), formaldehyde 10% (2.5ml) and vitamin E (400mg). Young shoots from 35 days old transformed and untransformed sorghum plants were washed in distilled water and the excess water was removed from the shoots using tissue paper. Five sorghum shoots of 5 cm sections were placed in petri dishes with moist filter paper. The larvae were starved for 4-3 hours before introducing them into the petri dishes. Five freshly hatched 2nd instar larvae of stem borer were introduced into petri-plates containing the sorghum shoots. The petri dishes were maintained at the insectary in ERI (28±1˚C/70% R.H). After 72hr insect death was recorded. Percentage of shoot weight damage was calculated by measuring the total shoot weight before and after bioassay. Each experiment was replicated five times.

Results

Transformation and regeneration of transformed sorghum plants

 The *Cry1C* gene was introduced into shoot apices derived from 3-4 days old seedling explants of the APK 1 cultivar by *Agrobacterium*-mediated gene transfer method (**Figure 2A**). The shoot apices cocultivated with LBA4404 (pCAMBIA1305.1/ *Cry1C*) produced hygromycin resistant calli (**Figure 2B**) after 42 days of co-cultivation in the selection medium (CIM-CCH) containing 5 mg/l hygromycin, 250mg/l carbencillin and 500mg/l cefotaxime. After 42 days of selection, the hygromycin resistant calli were removed and subcultured in a fresh selection medium. Growth of the calli, which were not infected with *Agrobacterium,* was effectively inhibited in a medium containing 5 mg/l hygromycin (Negative control). The positive control shoot apex (not infected with *Agrobacterium*) efficiently proliferated in the callus induction medium in the absence of hygromycin. A high frequency of hygromycin resistant calli was observed in three different experiments (**Table 1**). Out of 237 (76+79+82) shoot apex calli cocultivated in three experiments 99 (34+36+29) hygromycin resistant calli were obtained to an overall percentage of 41.7. In the present study, mature

Table 1. Summary of genetic transformation of APK 1 sorghum shoot apex calli explants by *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1305.1/*Cry1C*).

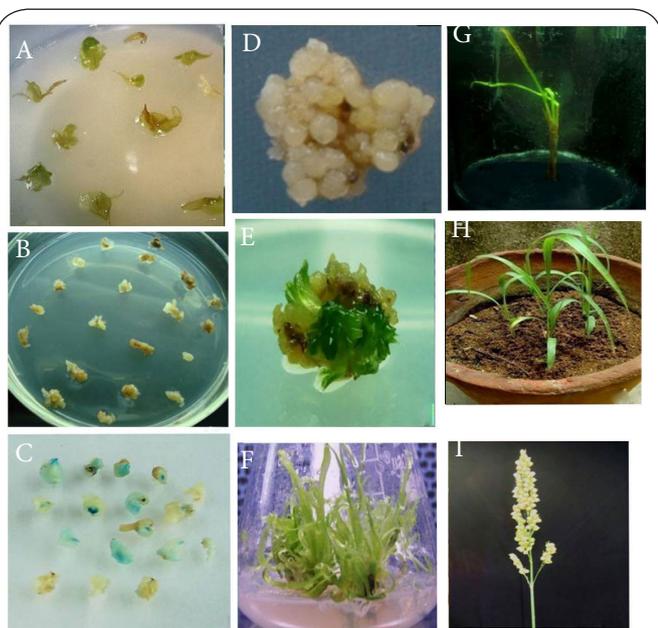| Sorghum cultivar	Number of shoot apex calli LBA4404 (pCAMBIA1305.1/*Cry1C*)					
	Experiment Number	Cocultivated (A)	Produced HyR calli	Produced HyR plants	Produced HyR and *GUS*+ plants (B)	Trans formation efficiency (%) (B/A)
APK 1	1	76	34	26	3	3.9
	2	79	36	28	1	1.2
	3	82	29	22	2	2.4

Figure 2. **Transformation and regeneration of transformed sorghum plants and GUS Histochemical assay.**
(**A**). Shoot apex explants of sorghum cultivar APK 1 (*Sorghum bicolor* L. cv. APK1) after infection with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1305.1/*Cry1C*). (**B**). Hygromycin resistant calli of the sorghum cultivar (*Sorghum bicolor* L. cv. APK1) after 42 days of cocultivation in selection medium. (**C**). The transient expression of GUS gene in the calli of sorghum cultivar APK 1 (*Sorghum bicolor* L. cv. APK1) after cocultivation with Agrobacterium tumefaciens LBA4404 (pCAMBIA1305.1) for ten days. (**D**). Hygromycin resistant embryogenic calli of sorghum (*Sorghum bicolor* L. cv. APK1) on regeneration medium. (**E**). The proliferation of hygromycin resistant shoots of the sorghum (*Sorghum bicolor* L. cv. APK1) after further subculture to selection medium. (**F**). Proliferation of hygromycin resistant plants of the sorghum cultivar (*Sorghum bicolor* L. cv. APK1) after further subculture on regeneration medium. (**G**). Rooting of hygromycin resistant plants of the sorghum cultivar (*Sorghum bicolor* L. cv. APK1) on rooting medium. (**H**). Transformed sorghum (Sorghum bicolor L. cv. APK1) plants at *in vivo* conditions. (**I**). Panicle of transformed sorghum (*Sorghum bicolor* L. cv. APK1) plants.

seedling derived shoot apices of APK I cultivars were used as explants for *Agrobacterium* mediated genetic transformation.

GUS Histochemical assay

Most of the hygromycin resistant calli that survived on the selection medium produced intense blue colour after overnight incubation with X-gluc substrate solution (**Figure 2C**). The hygromycin resistant and *GUS* positive embryogenic calli (**Figure 2D**) were transferred to regeneration medium containing 3mg/l hygromycin, 250mg/l cefotaxime (RE2-CCH). Small shoots were observed on the transformed calli after three weeks of growth (**Figure 2E**) and the regenerating calli were subcultured in a fresh regeneration medium and maintained for another three weeks. In three independent experiments, a total of 71 hygromycin resistant plants were regenerated from 99 calli (**Figure 2F**). After 6 weeks on the regeneration medium, the plantlets were rooted (**Figure 2G**) on the rooting medium (RIM-CCH). Small leaf pieces from the putatively transformed shoots were used for *GUS* histochemical assay. In these assays, 47 of the 76 regenerated shoots showed blue precipitate. These plantlets were hardened (**Figure 2H**) and transferred to field condition and set seeds (**Figure 2I**). This cultivar APK I showed a transformation frequency ranging from 1.2%-3.9% (**Table 1**).

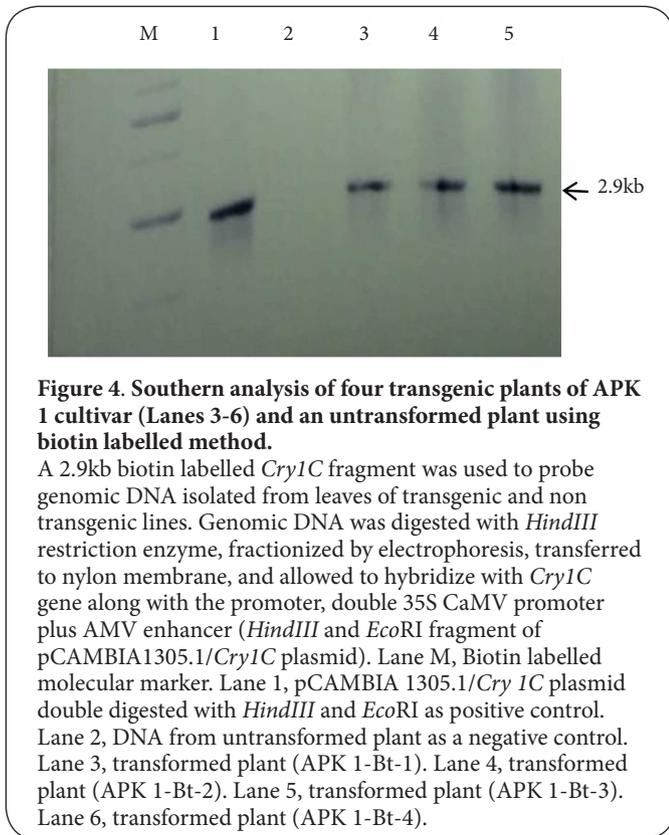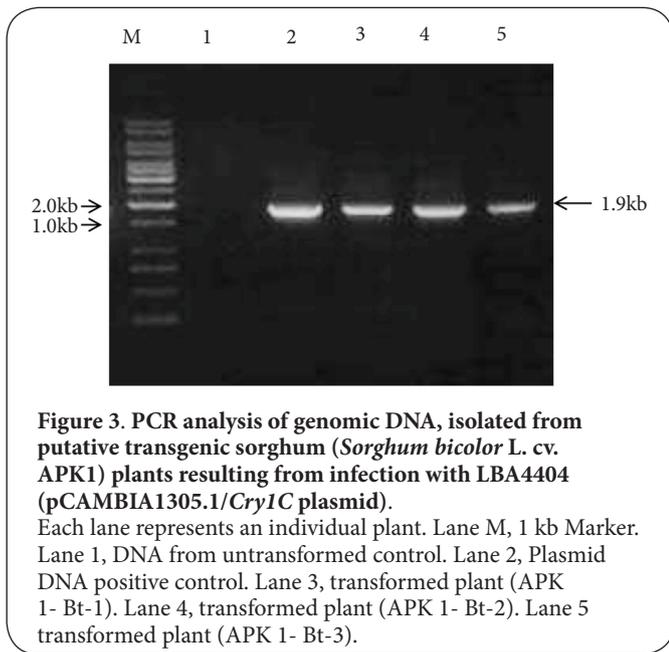## PCR analysis of hygromycin resistant transgenic sorghum plants

Total DNA from three APK I putative transformants, resulting from infection with LBA4404 (pCAMBIA1305.1/*Cry1C*) were subjected to PCR analysis with *Cry1C* genes primers. PCR was performed to confirm the presence of the *Cry1C* gene. All the three putative transformants were found to be positive for the amplification of 1.9 kb (**Figure 3**) *Cry1C* gene. A similar band was also observed in the positive plasmid control, while no such band was noticed in the untransformed control. This indicated that the tissues were completely cured of *Agrobacterium*.

Southern analysis of transgenic sorghum plants

Southern hybridization analysis was performed to confirm T-DNA integration (**Figure 4**). The transformants (S$_1$) of the cultivar APK I were subjected to genomic southern hybridization. Genomic DNA samples (10µg) from control and four S$_1$ transgenic plants were subjected to southern analysis. The coding sequences of *Cry1C* were used as probes for T-DNA integration (pCAMBIA1305.1/*Cry1C*).

Genomic DNA (10µg) from *GUS* and PCR positive sorghum

Figure 3. **PCR analysis of genomic DNA, isolated from putative transgenic sorghum (*Sorghum bicolor* L. cv. APK1) plants resulting from infection with LBA4404 (pCAMBIA1305.1/*Cry1C* plasmid).**
Each lane represents an individual plant. Lane M, 1 kb Marker. Lane 1, DNA from untransformed control. Lane 2, Plasmid DNA positive control. Lane 3, transformed plant (APK 1- Bt-1). Lane 4, transformed plant (APK 1- Bt-2). Lane 5 transformed plant (APK 1- Bt-3).

Figure 4. **Southern analysis of four transgenic plants of APK 1 cultivar (Lanes 3-6) and an untransformed plant using biotin labelled method.**
A 2.9kb biotin labelled *Cry1C* fragment was used to probe genomic DNA isolated from leaves of transgenic and non transgenic lines. Genomic DNA was digested with *HindIII* restriction enzyme, fractionized by electrophoresis, transferred to nylon membrane, and allowed to hybridize with *Cry1C* gene along with the promoter, double 35S CaMV promoter plus AMV enhancer (*HindIII* and *Eco*RI fragment of pCAMBIA1305.1/*Cry1C* plasmid). Lane M, Biotin labelled molecular marker. Lane 1, pCAMBIA 1305.1/*Cry 1C* plasmid double digested with *HindIII* and *Eco*RI as positive control. Lane 2, DNA from untransformed plant as a negative control. Lane 3, transformed plant (APK 1-Bt-1). Lane 4, transformed plant (APK 1-Bt-2). Lane 5, transformed plant (APK 1-Bt-3). Lane 6, transformed plant (APK 1-Bt-4).

plants was digested with *HindIII* and *EcoRI* enzymes, which would release an internal fragment size 2.9 kb *Cry1C* gene along with d35S promoter. The *Cry1C* gene sequence was detected as a fragment size (2.9 kb) in transformed plants (**Figure 4**). Since *Cry1C* probe hybridized to genomic DNA

from transgenic plants but not to DNA from non-transformed control plants, the result indicated that *Cry1C* DNA was incorporated into sorghum genome and Southern blot analysis of transformants confirmed integration of 2.9 kb *Cry1C* coding sequences into the sorghum genome.

Segregation analysis of the transgene in sorghum
A segregation analysis was performed to check the pattern of transgene segregation in S_1 progenies of sorghum plants transformed with selectable marker and reporter genes. The transgenic lines of APK l variety were screened on hygromycin (5 mg/l) supplemented medium to test the resistance and susceptibility of S_1 seeds. Data were validated by Chi-square (χ^2) analysis (**Table 2**). The transgenic lines (APK I) gave the S_1 segregation of Hyg^R/Hyg^S in the ratio of 3:1 confirming the normal Mendelian pattern of segregation. This study confirmed the successful introduction, integration and normal S_1 segregation of transgene in sorghum.

Bioassay of transgenic sorghum
S_1 transgenic plants expressing *Cry1C* were infested with sorghum stem borer larvae. The results are summarized in (**Table 3**). All the three transgenic lines showed high level of insect mortality. Feeding damage was estimated to be only 5% on transgenic plants. On control plants there was no mortality and 100% of the edible portion of the leaf material was consumed over a period of 3 days. In experiments using transformants expressing a single *Bt* toxin, 97–100% mortality was observed, with 10–13% leaf damage. Conversely, larvae feeding on control plants developed normally, causing massive tissue damage during the bioassay period.

Discussion
Sorghum is considered to be one of the most difficult plant species to manipulate through tissue culture and transformation. The first reports of sorghum plants transformed with agronomically important genes were the use of the *HT12* gene for higher grain lysine content [29] employing *Agrobacterium* and the *Cry1AC* gene for insect resistance [14] employing particle bombardment. Although sorghum tissue culture, including callus induction and plant regeneration, has been successful [13,30-38], genetic transformation of sorghum for commercial use was much less successful because of the lack of effective protocols [36]. Although work on transformation of sorghum began during 1990s, much less success has been achieved than with other crops. The first report of genetic transformation of sorghum described the introduction of DNA into protoplasts by electroporation and selection of transformed cells, without achieving plant regeneration [39].

Casas et al., [40] obtained the first transgenic sorghum plants with bombardment of immature embryos and later on they obtained transgenic plants using immature inflorescences. Casas et al., [40] and Zhu et al., [41] reported success in sorghum

Table 2. Segregation of hygromycin resistant gene and GUS gene in S1 generation of sorghum plants transformed with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA 1305.1/*Cry1C*).

Transformants Number	Total S_1 seeds tested	Hygromycin				GUS			
		Resistant	Sensitive	Ratio	X^2 value	Positive	Negative	Ratio	X^2 value
APK 1-Bt-5	47	38	11	3:1	0.7	40	12	3:1	0.7
APK 1-Bt-7	33	19	7	3:1	1.15	17	9	3:1	1.15
APK 1-Bt-9	26	17	5	3:1	0.52	23	7	3:1	0.52

Table 3 Percent insecticidal and shoot damage on transformed and untransformed plants expressing the *Cry1C* gene against spotted stem borer (*Chilo partellus*) after 72 hrs feeding.

Plant lines	% shoot damage	% insecticidal
APK 1 (untransformed)	99.22	-
APK 1-Bt-1	4.56	100
APK 1-Bt-2	2.73	97.7
APK 1-Bt-3	5.38	93.3

transformation with biolistic bombardment. However, the disadvantages of using biolistic bombardment are that it produces multiple copies [14] of the transgene that may lead to silencing with usually low transformation efficiency. Godwin et al., [42] reported inoculation of sorghum meristem tissue with *Agrobacterium*. Zhao's research group [12] was the first to report the successful *Agrobacterium*-mediated genetic transformation of sorghum and the production of transgenic plants that transmitted the introduced gene to progeny in a Mendelian fashion. Much of the work on sorghum transformation by earlier investigators focussed on the optimization of parameters for tissue culture and gene transfer methods, assessing the strength of promoters, and identification of efficient selectable marker and reporter genes [8,43]. Only a few reports dealt with transformation of sorghum with an agronomically important gene, rice chitinase, that confers resistance against stalk rot [41] and spotted stem borer (*Chilo partellus*) [14].

Shoot apices are used as explants in many cereals since they produce plantlets identical to their parents. Main steps involved in shoot apex culture are the separation of tissues surrounding the meristem followed by exposing the meristem by creating wounds [44]. Girijashankar et al., [14] used shoot apices of sorghum BTX623 genotype for cotransformation with a selectable marker (*bar*) and an insect resistant (*Cry1AC*) gene using plasmids pJS108 and p*mpiClcry1AC*. Our study demonstrated that the transfer of foreign genes to sorghum via *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1305.1) by the shoot apex was efficient. Aragao et al., [45] transformed bean plants with a chimeric construct containing the doubled 35S CaMV promoter plus the AMV enhancer sequence, assuming that its performance would be superior to the native 35S promoter. In the present study, a large number of sorghum

plants carrying *Cry1C* gene has been produced in APK 1 cultivar by *Agrobacterium* mediated transformation method using binary vector pCAMBIA1305.1/*Cry1C*. This study proves the efficiency and effectiveness of the *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1305.1/*Cry1C*) in transforming the sorghum plants. In genetic transformation of crops one of the most widely used selection markers is the gene encoding hygromycin phosphotransferase (*hpt*). These marker genes along with the constitutive promoters such as CaMV35S work efficiently for the selection of transformed cells. The *hpt* gene has been used as an efficient tool for selection of transformed calli [46-48]. We used hygromycin as a selection marker at 5 mg/L. This was very effective for selection. *hpt* gene has been reported to be more efficient and an acceptable marker for sorghum transformation [48].

One of the main objectives of the present study was to express the *Cry1C* gene in transgenic sorghum plants to provide resistance to the stem borer (*Chilo partellus*). The transgene's coding sequence was driven by a doubled 35S CaMV promoter and AMV enhancer sequences, which would direct and ensure the expression of recombinant protein in the whole plant. PCR amplification confirmed the presence of *Cry1C* gene in transformed sorghum plants which generated 1.9kb (*Cry1C*) genes equal to the size of the positive control and there was no amplification in the non-transformed control plants. Successful integration of *Cry1C* gene into sorghum was further confirmed by performing a Southern blot with genomic DNA (*Hind* III and EcoRI digest) of PCR positive plants. All PCR positive plants produced signals at 2.9 kb which is the expected band size of *Cry1C* expression cassette indicating the integration of the *Cry1C* gene into the genome of sorghum and proved that they are derived from independent transformants. Southern hybridization analysis offers many advantages in the analysis of transgenic plants [49] and is equally informative as real-time PCR analysis [50]. Segregation of the *Cry1C* gene in the next generation was examined by hygromycin resistance and *GUS* assay experiments. Segregation analysis of these three independent S_1 lines demonstrated that the transgenes were stably inherited in S_2 progeny. The transformation efficiency in our study was found to be ranging between 1.2%-3.9%; only 1.5% was reported by [14].

Chilo partellus Swinhoe (Lepidoptera: Pyralidae) which is an important pest of sorghum is found in India [51] and South and eastern Africa [52]. Brietler et al., [53] reported that transgenic

rice plants carrying *Cry1B* under control of the *mpiC1* promoter expressed the δ-endotoxin to levels of up to 0.2% of total soluble protein, and these plants exhibited 100% resistance to second-instar larvae of striped stem borer (*Chilo suppressalis* Walker). Datta et al., [54] reported that, in transgenic rice plants, 10–200 ng *Cry1Ab* protein per gram of leaf tissue was capable of conferring 100% larval mortality on the yellow stem borer in cut-stem bioassays. The results of our bioassay study revealed significant larval mortality and reduction in tissue damage by the stem borer larvae in transformed plants, where as larvae feeding on control plants developed normally, and caused massive tissue damage during the bioassay period, which indirectly indicated the presence of the *Cry1C* gene. Bioassay studies proved the expression pattern of *Cry1C* gene in all four transgenic lines, which also revealed *Cry1C* protein activity. The insect mortality observed in the present study was coinciding with the previous studies [14,55,56]. Our study clearly established that transgenic plants which were resistant to *Chilo partellus* were produced with stable integration of *Cry1C* gene with high larval mortality.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SI	AP
Research concept and design	✓	✓
Collection and/or assembly of data	✓	✓
Data analysis and interpretation	✓	✓
Writing the article	✓	✓
Critical revision of the article	✓	✓
Final approval of article	✓	✓
Statistical analysis	✓	✓

Acknowledgement

Publication history

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